Estudi de la neurotransmissió a l'esfínter esofàgic inferior

Ricard Farré i Martí

Memòria presentada per optar al grau de Doctor en Biologia

Fundació de Gastroenterologia
Dr. Francisco Vilardell i Departament de Biologia Cel·lular, Fisiologia i Immunologia. Unitat de Fisiologia Animal
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Fan constar que:

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Director de la tesi	Tutor de la tesi

Dibuix de la coberta procedent del llibre *Textura del sistema nervioso del hombre y de los vertebrados* de Santiago Ramón y Cajal (1904). Gangli del plexe d'Auerbach de l'intestí del conill. Mètode de Ehrlich.

“Ensenya més la necessitat que la universitat”

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Abreviacions

AC: adenilat ciclasa

ACh : acetilcolina

AChT: colinacetiltransferasa

ADP: adenosina difosfat

AMP: adenosina monofosfat

ATP: adenosina 5-trifosfat

ATR: atropina

APA: apamina

cAMP: adenosina monofosfat cíclic

cGMP: guanosina monofosfat cíclic

CGRP: pèptid relacionat amb el gen de la calcitonina

CNS: sistema nerviós central

CO: monòxid de carboni

DAG: diacilglicerol

DMN: nucli motor dorsal

DRG: gangli de l'arrel dorsal

EB: cos esofàgic

EFS: estimulació elèctrica de camp

ENS: sistema nerviós entèric

GERD: malaltia per reflux gastroesofàgic

GI: gastrointestinal

HEX: hexametoni

HO-2: heme oxigenasa del tipus 2

IAS: esfínter anal intern

ICC: cèl·lules intersticials de Cajal

ICC-AP: ICC del plexe mientèric

ICC-IM: ICC intramuscular

ICC-DMP: ICC del plexe muscular profund

ICC-SMP: ICC del plexe submucós

IP_3: inositol trifosfat

LES: esfínter esofàgic inferior

LMNs: neurones motores inferiors

L-NNA: N-nitro-L-arginina

L-NAME: N-nitro-L-arginina metil ester

nAChR: receptor nicotínic d'acetilcolina

mAChR: receptor muscarínic d'acetilcolina

NANC: no adrenèrgic no colinèrgic

MP: plexe mientèric

MRS2179: N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate

NA: nucli ambigu

NF279: 8,8'-[Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt

NIC: nicotina

nNOS: sintetasa de l'òxid nítric neuronal

NO: òxid nítric

NOS: sintetasa de l'òxid nítric

ODQ: 1H-[1,2,4]oxadiazolo-[4,3-α]quinoxalin-1-one

PACAP: pèptid activador de l'adenil ciclasa pituïtària

sGC: guanilat ciclasa soluble

SKCa: canals de potassi de baixa conductància activats per calci

SNP: nitroprussiat de sodi

SP: substància P

tLESRs: relaxacions transitòries de l'esfínter esofàgic inferior

TTX: tetrodotoxina

VIP: pèptid intestinal vasoactiu

Llista de publicacions

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Introducció

1. Innervació del tracte gastrointestinal

1.1 General

El tracte gastrointestinal (GI) dels humans està format per un tub de 6-9 m que comença a la boca i finalitza a l'anus, en el qual buiden el contingut una sèrie de glàndules annexes. De més oral a més caudal, el tracte GI està format per: la boca, l'esòfag, l'estómac, l'intestí prim (dividit en duodè, jejú i íleum), el còlon (dividit en ascendent, transvers, descendent i sigma) i el recte. L'aliment entra per la boca, on se'n redueix la grandària i on s'inicia la digestió abans d'anar a l'estómac, via esòfag. A l'estómac s'emmagatzema l'aliment, es tritura i es barreja junt amb secrecions que contenen pepsina, ions i aigua, fins obtenir partícules suficientment petites com per passar pel pílor i entrar a l'intestí prim. A l'intestí prim, els enzims converteixen les macromolècules en material absorbible durant el procés anomenat digestió; els productes de la digestió s'absorbeixen a través de l'epiteli fins entrar als vasos sanguinis i a la limfa. L'aigua dels aliments no digerits es reabsorbeix al còlon i la resta del contingut s'evacua. Els processos motors són molt importants per assegurar la trituració, la barreja, la propagació del contingut a través del tracte digestiu i, finalment, per a l'eliminació dels aliments no digerits.

Encara que el tracte GI està format per diferents regions, la seva estructura histològica és similar i consisteix en una sèrie de capes superposades, de l'interior a l'exterior de la llum:

-Mucosa (especialitzada en cada tram del tracte GI).

-Submucosa: conté les glàndules de secreció i el plexe submucós o de Meissner amb les neurones que controlen la secreció.

-Capes musculars circular i longitudinal: formades per múscul llis, encara que a part l'esòfag són també de musculatura estriada. En algunes regions, com l'esfínter esofàgic inferior (LES), les fibres musculars llises estan dividides per septes, teixit conjuntiu que contenen vasos i axons que les embolcallen.

-Plexe mientèric (MP) o de Auerbach, situat entremig de les capes musculars, format per estructures nervioses organitzades amb forma de ganglis i feixos nerviosos interconnectats. És el lloc d'integració del control intrínsec de la motilitat.

-La serosa forma la capa més externa del tracte digestiu. L'esòfag no està

envoltat per serosa, la capa més externa s'anomena adventícia i consisteix en teixit conjuntiu laxe que l'uneix als teixits del mediastí del voltant. Només petites àrees del segment toràcic i un petit segment abdominal estan coberts per serosa.

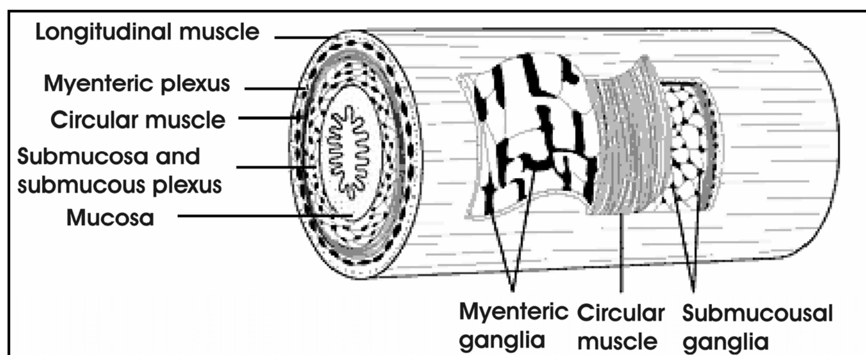

Figura 1. Anatomia de les diferents capes que formen el tracte gastrointestinal (Christensen, 2001)

El control de la funció motora digestiva es produeix a dos nivells fonamentals: un control intrínsec, que s'integra a nivell del sistema nerviós entèric (ENS) format pels plexes submucós i mientèric, i una altre d'extrínsec constituït pel sistema nerviós autònom (simpàtic i parasimpàtic). El sistema nerviós central (CNS) regula l'activitat de l'autònom i, a més a més, participa en el control de les funcions digestives que impliquen un control voluntari – la deglució i la defecació – (Aziz & Thompson, 1998;Bennett, 1997;Costa & Brookes, 1994;Furness et al., 1999;Goyal & Hirano, 1996;Kunze & Furness, 1999;Grundy, 2002).

1.2 Innervació extrínseca de l'esòfag i de l'esfínter esofàgic inferior

Tant el sistema nerviós parasimpàtic com el simpàtic contenen fibres aferents (branca sensitiva) i eferents (branca motora) que innerven l'esòfag i el LES.

1.2.1. Aferents sensitives

Les neurones aferents sensitives de l'esòfag i del LES viatgen amb els nervis simpàtics, i s'anomenen *aferents simpàtiques* o *aferents espinals*, o amb els nervis parasimpàtics vagals, i s'anomenen *aferents vagals*. Les primeres van cap al gangli de l'arrel dorsal (DRG) i les segones cap al gangli nodós (Figura 2). Del total de les fibres vagals, un 70-90% són fibres aferents. Les aferents espinals són neurones que transmeten informació dolorosa mentre que les aferents vagals transmeten

principalment informació fisiològica (no dolorosa). Estudis en el gat (Collman et al., 1992), realitzats usant marcadors retrògrads, demostren que les neurones espinals es distribueixen majoritàriament en C1-T8 per al múscul estriat; C5-L2 per al múscul llis i T1-L3 per al LES. També demostren que les aferents vagals viatgen cap al gangli nodós, que el nombre total de neurones aferents vagals duplica la quantitat d'aferents espinals i que les aferents de totes les parts de l'esòfag convergeixen amb les aferents cardíaques, fet que explica la dificultat en discriminar en moltes ocasions el dolor d'origen esofàgic de dolor d'origen cardíac. Estudis en el gat amb marcadors anterògrads injectats en el gangli nodós i al DRG toràcic als nivells T8-T13 demostren que la informació sensorial vagal i espinal del LES s'origina en terminals nerviosos probablement situats en diferents estructures de la paret de l'esòfag. Així, els receptors de distensió mecànica estan situats en terminals nerviosos propers a la musculatura llisa, els de tensió a nivell del plexe mientèric i els terminals localitzats a la mucosa, els quals detecten detecten la consistència del bol alimentari (Clerc & Condamin, 1987;Mazzia & Clerc, 2000;Clerc & Mei, 1983;Clerc, 1984;Clerc & Mazzia, 1994). En el CNS, les neurones sensitives connecten amb les neurones eferents vagals, que són les responsables de la resposta motora.

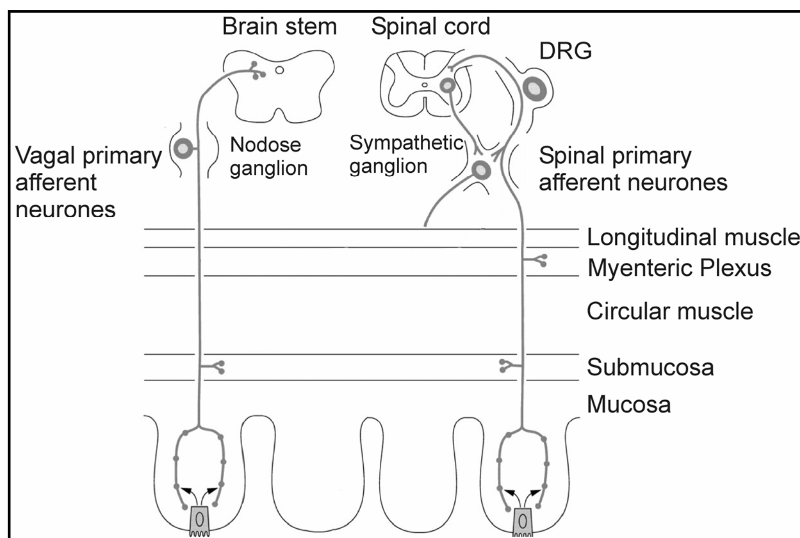

Figura 2. Neurones aferents en l'intestí (adaptat de Grundy, Gut 2002)

1.2.2. Eferents motores vagals

El sistema nerviós parasimpàtic eferent consta de dos nervis: 1) el nervi vague, que innerva la major part del tracte GI fins la meitat del còlon transvers; i 2) el nervis pèlvics (parasimpàtic sacre), que s'origina a la regió sacra de la medul·la espinal (S2-S4) i innerva la part baixa del còlon i el recte (Figura 3). Un petit nombre de fibres

eferents vagals (10-20%) innerven un gran nombre de ganglis entèrics, fet que explica la forta influència del vague sobre la motilitat (Wood J.D., 1989). Les fibres motores vagals s'originen en nuclis diferents en funció del tipus de musculatura que innerven.

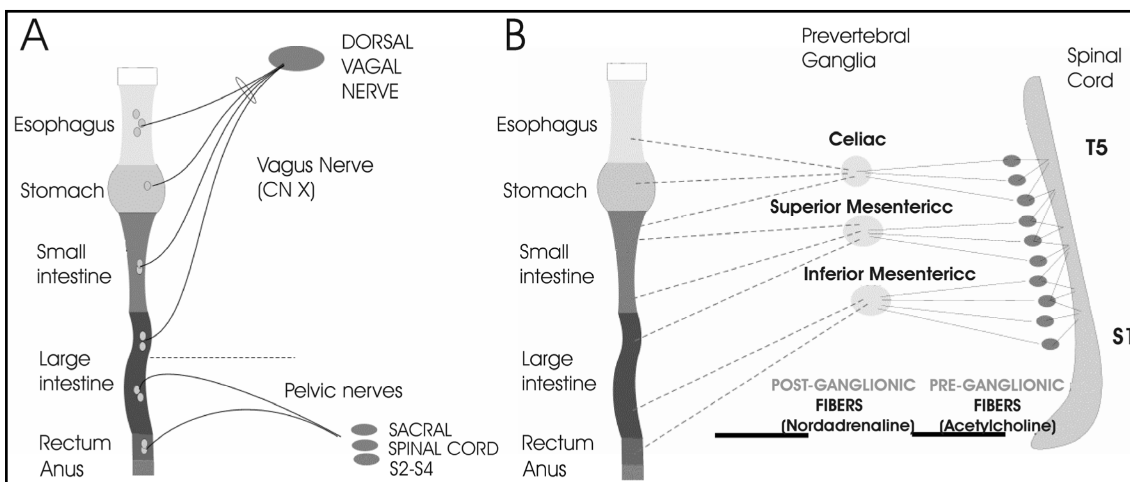

Figura 3. Innervació eferent extrínseca del tracte GI. A) Eferents motores vagals i B) Eferents motores simpàtiques

Esòfag estriat

En algunes espècies com el ratolí, la rata i el gos, l'esòfag sencer està format per múscul estriat, excepte la part del LES, que està constituït per musculatura llisa. En canvi, el gat, la fura, l'opòssum i el porc tenen múscul estriat en la regió més proximal de l'esòfag, mentre que una porció de la part distal està constituïda per musculatura llisa. En humans, la porció cervical de l'esòfag està formada per múscul estriat; mentre que el segment toràcic està constituït per fibres llises. Tot el múscul estriat de l'esòfag està innervat majoritàriament per neurones localitzades en el nucli ambigu (NA). Així doncs, estudis amb rata, en què s'han fet servir traçadors retrògrads, demostren que la zona compacta del NA conté totes les neurones motores inferiors (LMN) vagals que controlen la funció motora de la faringe i de l'esòfag (Broussard & Altschuler, 2000). Per altra banda, estudis amb gat en què s'ha injectat traçadors retrògrads fluorescents han demostrat que principalment les fibres eferents que innerven la musculatura estriada de l'esòfag es localitzen a la part rostral del NA, però que existeix un 8% de neurones localitzades al nucli motor dorsal (DMN) del vague (Collman et al., 1993).

Mitjançant estudis immunohistoquímics amb rata, s'ha vist que aquestes neurones contenen el transportador vesicular d'ACh, però també contenen CGRP (Lee et al., 1992;Sang & Young, 1998), fet que suggereix que aquest últim actua com a co-

transmissor amb l'ACh. Els seus terminals nerviosos contacten les fibres musculars estriades mitjançant plaques motores (Zhou et al., 1996). Estudis in vitro amb estimulació vagal demostren que l'ACh alliberada a la placa motora activa receptors colinèrgics nicotínics en el múscul estriat, per iniciar així la contracció muscular (Kerr et al., 1995). La porció de múscul estriat de l'esòfag té un plexe mientèric poc dens i irregular (Izumi et al., 2002), que per estudis immunohistològics s'ha vist que conté neurones que expressen l'enzim òxid nítric sintetasa (NOS), galanina, CGRP (Kuramoto et al., 1999) i VIP (Sang & Young, 1997), i que innerven el terminal nerviós de la placa motora. Les eferents vagals colinèrgiques estan inhibides per aquestes neurones nitrèrgiques, les quals modulen les contraccions peristàltiques a l'esòfag estriat (Izumi et al., 2003). En canvi, les eferents vagals no semblen actuar sobre les neurones inhibidores nitrèrgiques (Neuhuber et al., 1998).

Esòfag llis i LES

Estudis amb gat, en què s'han injectat traçadors retrògrads a la regió toràcica de múscul llis de l'esòfag i també del LES, han demostrat que principalment les fibres eferents que innerven la musculatura llisa de l'esòfag es localitzen en el DMN en dos grups, un de rostral i un de caudal. Un grup addicional es troba en el nucli retroambigu i aproximadament un 8% són presents en el NA rostral, lateralment a les eferents del múscul estriat. En el LES les aferents tenen una distribució similar que les aferents de l'esòfag, però els cossos neuronals del DMN estan desplaçats més caudalment (Collman et al., 1993). En un estudi similar en els que es va emprar la fura, es veu també una distribució rostrocaudal dels cossos de les neurones eferents en el DMN (Hyland et al., 2001).

Durant molt de temps es va creure que el nervi vague només tenia influències excitadores en el tracte digestiu. Un dels majors avenços en el nostre coneixement de com el nervi vague controla la motilitat gastrointestinal va ser demostrar que la via motora vagal estava formada per vies paral·leles que provoquen accions inhibidores i excitadores sobre el múscul. A diferència de la musculatura estriada, les neurones vagals eferents no innerven directament la musculatura llisa, sinó que fan sinapsi amb les motoneurones inhibidores i excitadores del plexe mientèric. En el LES existeix una diferent distribució dels cossos de les neurones inhibidores i excitadores en el DMN, les neurones de la via excitadora es localitzen en el DMN rostral, mentre les de la via

inhibidora estan presents en la part caudal del DMN (Rossiter et al., 1990). Estudis *in vivo* amb opòssum (Goyal & Rattan, 1975) i estudis *ex vivo* amb fura (Smid & Blackshaw, 2000) fent servir estimulació vagal han demostrat que la transmissió sinàptica entre les neurones eferents i les neurones inhibidores del plexe està mitjançada tant per receptors nicotínics com per muscarínics de tipus M1. Estudis en opòssum suggereixen, a més, la participació de la 5-hidroxitriptamina (5-HT o serotonina) en la transmissió sinàptica a aquest nivell (Rattan & Goyal, 1978;Paterson et al., 1992).

1.2.3. Eferents motores simpàtiques

A l'esòfag, la innervació simpàtica parteix de cossos neuronals situats en els segments espinals T5 i T6, envia els seus axons cap als ganglis cervicals i ganglis simpàtics toràcics paravertebrals. En el LES, els cossos neuronals del component simpàtic es troben en els segments espinals T6-T10, envien els axons cap al gangli celíac a través del nervi esplàncnic major (Figura 3). Les neurones preganglionars són colinèrgiques i activen els receptors nicotínics de les neurones noradrenèrgiques postganglionars; aquestes últimes innerven l'esòfag o el LES acompanyant els vasos sanguinis o, en menor quantitat, junt amb el nervi vague. Les fibres adrenèrgiques postganglionars poden innervar tant les motoneurones entèriques com directament les cèl·lules musculars del LES (Papasova M, 1989). La resposta simpàtica del LES difereix en funció de l'espècie. Estudis d'estimulació del nervi esplàncnic en la fura activa neurones adrenèrgiques i produeix relaxació a través de receptors de tipus β (Blackshaw et al., 1997). En canvi, s'ha descrit en el gat (Gonella et al., 1979), que la resposta simpàtica del LES és excitadora i és el resultat de l'estimulació tant de receptors adrenèrgics situats en la musculatura llisa com de receptors situats en les neurones del plexe que provoquen la posterior alliberació d'acetilcolina per part de les motoneurones.

1.3 Innervació intrínseca: plexe mientèric i plexe submucós

1.3.1. General

El sistema nerviós entèric està format per uns 100 milions de neurones i és conegut també com "el cervell de l'intestí" perquè pot funcionar independentment del CNS com van suggerir William M. Bayliss and Ernest H. Starling el 1899. Aquests dos investigadors van demostrar, en gossos anestesiats, que l'aplicació d'una distensió

intraluminal produïa una contracció oral i una relaxació anal, seguida d'una ona propulsiva de suficient magnitud com per propulsar l'aliment a través del tracte digestiu (ells van anomenar aquest fenomen com a "llei de l'intestí" i equival actualment al reflex peristàltic). Com que aquest reflex persistia sense la innervació extrínseca, van deduir correctament que l'ENS pot operar independent dels *inputs* del CNS. Divuit anys després, Paul Trendelenburg va confirmar aquest fet demostrant que el reflex peristàltic es pot produir in vitro en l'intestí de conill porquí aïllat, sense participació del cervell, de la medul·la espinal, de l'arrel de ganglis dorsal o de la cranials. Tot i això, com ja s'ha vist, l'ENS comunica amb el CNS a través de fibres aferents i eferents simpàtiques i parasimpàtiques.

La funció de l'ENS és controlar la motilitat, la secreció exocrina i l'endocrina i la microcirculació del tracte digestiu, i està també implicat en la regulació immune i en processos inflamatoris. Els cossos cel·lulars de les neurones s'agrupen en ganglis, que estan connectats per processos intergangliònics formant dos plexes neuronals, el plexe mientèric o de Auerbach i plexe submucós o de Meissner. El plexe submucós conté menys neurones en termes globals, menys neurones per gangli i connexions interganglièniques més primes que el plexe mientèric. El plexe mientèric s'estén per tot el tracte gastrointestinal, des de l'esòfag fins a l'esfínter anal intern (IAS). El plexe submucós està molt desenvolupat a l'intestí prim, on juga un paper important en el control de la secreció i l'absorció, encara que també innerva la muscularis mucosa, algunes cèl·lules endocrines intestinals i vasos submucosos. A l'intestí també hi ha un altre plexe, el plexe muscular profund, situat en la profunditat de la musculatura circular (Furness et al., 1987) (Figura 4).

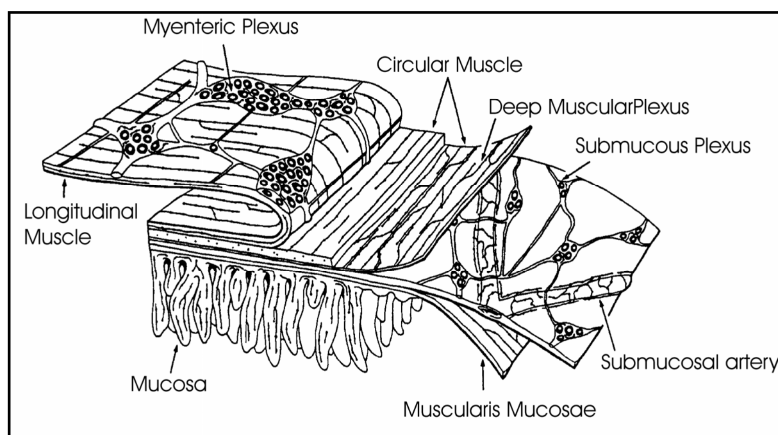

Figura 4. Representació esquemàtica de les diferents capes del tracte gastrointestinal i de la localització dels plexes entèrics (adaptat de Furness i Costa, 1987)

L'ENS és estructuralment i funcionalment molt semblant al cervell. Igual que a la resta del sistema nerviós perifèric, els seus elements neuronals no estan suportats per col·lagen ni per les cèl·lules de Schwann, però si per cèl·lules de la glia, que recorden els astròcits del CNS.

1.3.2. Glia

Existeixen evidències que fan pensar que la funció de les cèl·lules de la glia és donar suport metabòlic a les neurones del MP, i que també poden jugar un paper en la inflamació d'aquest (Cabarrocas et al., 2003;Ruhl et al., 2001). L'ENS es pot afectar en malalties neurodegeneratives, apareixen en l'intestí de malalts de Parkinson cossos de Lewy (Kupsky et al., 1987) i plaques amiloides en malalts d'Alzheimer (Joachim et al., 1989).

1.3.3. Tipus de neurones entèriques

S'han descrit quatre tipus funcionals de neurones entèriques: les neurones secretores, les neurones motores, les neurones sensitives, també conegudes per IPAN (*intrinsic primary afferent neurons*) i les interneurones. Les neurones motores, IPAN i les interneurones estan implicades en els reflexos, inclosa la peristalsi intestinal. Els seus cossos neuronals estan localitzats en el plexe mientèric i els terminals sensitius estan situats a la mucosa; són sensibles a diferents estímuls com ara a tensió, a canvis en el contingut luminal i a la serotonina. Algunes d'aquestes IPAN tenen els seus cossos cel·lulars al plexe submucós; la seva estimulació pot controlar el transport d'aigua i d'electròlits a través de la mucosa i el flux sanguini local (Kunze & Furness, 1999).

1.3.4. Neurones motores i neurotransmissors

Estudis immunohistoquímics posen en evidència la presència de l'enzim de síntesi de l'ACh (acetilcolintransferasa, AChT) en les motoneurones excitadores del plexe mientèric de tot el tracte digestiu (Konomi et al., 2002;Porter et al., 2002;Ratcliffe et al., 1998) i també de taquiquinines (SP, neurokinin A i B) (Sandler et al., 1991;Shuttleworth et al., 1991;Yip et al., 2003;Yunker et al., 1999). Les motoneurones inhibidores contenen els enzims de síntesi d'NO (sintetasa de l'òxid nítric, NOS) i del monòxid de carboni (CO) (heme oxigenasa del tipus 2 o constitutiva, HO-2), els pèptids VIP, PACAP, CGRP i també vesícules que contenen ATP (visualitzades usant la

tècnica fluorescent de quinacrina) (Belai & Burnstock, 1994;Clague et al., 1985;Miller et al., 2001;Ny et al., 1997;Sann et al., 1998;Sundler et al., 1992). Per altra banda, estudis mecànics i electrofisiològics realitzats usant antagonistes i inhibidors, han evidenciat la implicació de l'NO com a principal neurotransmissor en l'esòfag (Preiksaitis et al., 1994a), l'estómac (Todorov et al., 2003), l'intestí (Vanneste et al., 2004), el còlon (Pluja et al., 1999) i l'IAS (Tomita et al., 2001). Existeix un paper secundari per als altres neurotransmissors, les purines actuen en paral·lel amb l'NO a l'estómac, a l'intestí i al còlon (de Man et al., 2003;Mule & Serio, 2003;Pluja et al., 1999), de manera semblant a com ho fa el VIP també a l'estómac (Bayguinov et al., 1999;Mule & Serio, 2003). Sembla que aquest pèptid no està implicat en la neurotransmissió ni en l'intestí (Matsuda et al., 2004) ni en el còlon (Pluja et al., 2000). Usant ratolins amb deleció de l'HO-2 (Xue et al., 2000a) s'ha vist que el CO podria jugar un paper com a neurotransmissor en l'intestí, com també ho podria fer en l'IAS, degut possiblement a la síntesi de CO induïda pel VIP (Watkins et al., 2004).

Pel que fa als neurotransmissors excitadors, s'ha vist que l'ACh és el principal neurotransmissor en el tracte GI i que les taquiquinines poden tenir un paper més o menys important en la contracció depenent de la porció del budell estudiada (Cao et al., 2000;El-Mahmoudy et al., 2003;Krysiak & Preiksaitis, 2001).

1.3.5. Receptors dels diferents neurotransmissors inhibidors

Guanilat ciclasa soluble

L'NO i el CO són dos gasos que produeixen el seu efecte atesa l'activació de la guanilat ciclasa soluble (sGC). Aquest enzim està format per una subunitat α i una subunitat β, el seu acoblament és limitant perquè l'activació pels seus lligands produeixi cGMP (Lucas et al., 2000). La producció de cGMP i la posterior fosforilació de proteïnes tradueixen el senyal de l'NO per produir la relaxació del múscul llis (Lincoln & Cornwell, 1991). Tot i això, s'ha vist en diferents teixits que l'NO pot tenir altres dianes: l'NO pot actuar a través de canals iònics (Bolotina et al., 1994), incrementant la conductància al K^+ i que indueix així la hiperpolarització de la membrana, o bé activant tirosina-cinases, fosfatases (Stamler, 1994), GTPases (Lander et al., 1995), ADPribosiltransferases citosòliques (Brune & Lapetina, 1989) o factors de transcripció (Lander et al., 1995). A diferència d'aquestes dos gasos, la resta de neurotransmissors del tracte digestiu actuen sobre receptors.

Receptors purinèrgics

Als anys 80, Burnstock (Burnstock, 1980) va proposar una classificació formal dels receptors per l'adenosina i per l'ATP. Els receptors selectius per adenosina i adenosina monofosfat (AMP) es van anomenar purinoceptors P1 i els selectius per ATP i adenosina difosfat (ADP) es van anomenar purinoceptors P2. Aquesta classificació es va ampliar en subdivisions addicionals, els receptors P1 es van dividir en A_1, A_{2A}, A_{2B} i A_3 i els receptors P2 en les famílies P2X i P2Y. Actualment hi ha pocs agonistes i antagonistes selectius per discriminar clarament entre receptors P2X i P2Y, o entre diferents subtipus de receptors dins de cada família, encara que el 2-methylthio ADP (2-MeSADP) i el MRS2179 sembla que siguin un agonista i un antagonista selectius, respectivament, per als receptors $P2Y_1$ (Camaioni et al., 1998).

Els receptors per l'adenosina com per l'ATP juguen un paper important en la modulació de la motilitat en el tracte GI. L'adenosina pot activar directament receptors localitzats en la musculatura llisa i produir tant relaxació com contracció (Kadowaki et al., 2000;Nicholls et al., 1996) o actuar presinàpticament reduint l'alliberació de neurotransmissors excitadors com l'ACh i la SP (Kadowaki et al., 2000;Moneta et al., 1997). En moltes espècies, l'ATP activa receptors P2 en el múscul llis per produir relaxació de la musculatura llisa en el tracte GI, encara que hi ha algunes evidències en què l'ATP també produeix contracció del múscul llis en algunes regions. En un estudi en el qual s'usen diferents parts del tracte digestiu del ratolí (fundus gàstric, duodè, íleum i còlon) s'observa que receptors $P2Y_1$ neurals estan implicats en la relaxació a totes les regions del tracte digestiu, en gran part alliberant NO; la relaxació també és deguda a receptors $P2Y_1$, $P2Y_2$ i $P2Y_n$ localitzats en la musculatura llisa. Els receptors $P2X_2$ estan implicats en la contracció només en el còlon (Giaroni et al., 2002). En estudis més recents s'ha vist que l'estimulació elèctrica de camp (EFS) allibera NO i un segon neurotransmissor sensible a l'MRS2179 en el fundus del ratolí (de Man et al., 2003) i en el còlon humà (Auli et al., 2005;Gallego et al., 2005) fet que suggereix, la participació d'una purina actuant a receptors $P2Y_1$.

Els receptors P1 estan acoblats a la proteïna G, ja sigui disminuint-ne la producció de cAMP o bé augmentant-la. També s'ha descrit que poden activar la via de la fosfolipasa C (PLC) i produir la formació del trifosfat d'inositol (IP_3) i diacilglicerol (DAG). L'IP_3 estimula l'alliberament de Ca^{++} intracel·lular per la interacció amb receptors específics localitzats al reticle sarcoplasmàtic. L'augment del Ca^{++} citosòlic

per l'IP_3 pot estimular diferents vies com la de la proteïna cinasa C (PKC), fosfolipasa A2 (PLA2), canals de K^+ dependents de Ca^{++} i òxid nítric sintetasa. Els receptors P2X són canals iònics que uneixen ATP (ATP-gated), la seva activació produeix una ràpida (entre 10 ms) i selectiva permeabilitat a cations (Na^+, K^+ i Ca^{++}). Això contrasta amb la resposta lenta (menys de 100 ms) de l'ATP quan actua sobre receptors metabotròpics P2Y, que impliquen acoblament a proteïna G i sistemes de segons missatgers. La majoria dels receptors P2Y actuen via proteïna G acoblada a l'activació de PLC, i produeixen la formació d'IP_3 i la mobilització de calci intracel·lular. S'ha descrit també l'acoblament a l'AC en alguns receptors P2Y (Ralevic & Burnstock, 1998).

Receptors de VIP/PACAP

Els receptors per VIP i per PACAP formen part d'una família de receptors formada per set receptors transmembrana acoblats a la proteïna G, en la qual també s'inclouen els receptors per CGRP. El PACAP té elevada afinitat per tres receptors diferents (receptors PAC1, VPAC1 i VPAC2). Dos d'aquests, el VPAC1 i el VPAC2, són també receptors d'alta afinitat pel VIP. Els tres receptors estan majoritàriament acoblats a la proteïna Gs, que és la que activa l'adenilat ciclasa (AC); els PAC1 i VPAC1 poden estar també acoblats a les proteïnes Gq i Go respectivament, les quals són responsables de la mobilització de calci (Harmar et al., 1998).

Receptors de CGRP

Històricament, els receptors de CGRP han estat classificats en dues classes: CGRP1 i CGRP2. Els receptors CGRP1 són més sensibles que els CGRP2 a l'antagonista peptídic CGRP8-37. Per altra banda, els anàlegs lineals de CGRP, [Cys(ACM)2,7]-CGRP i [Cys(Et)2,7]hαCGRP, són agonistes més potents per receptors CGRP2 que per CGRP1. El CGRP augmenta el cAMP intracel·lular en molts sistemes però no en tots, i pot activar també canals de K^+ i de Ca^{++} via proteïna G (Poyner et al., 2002).

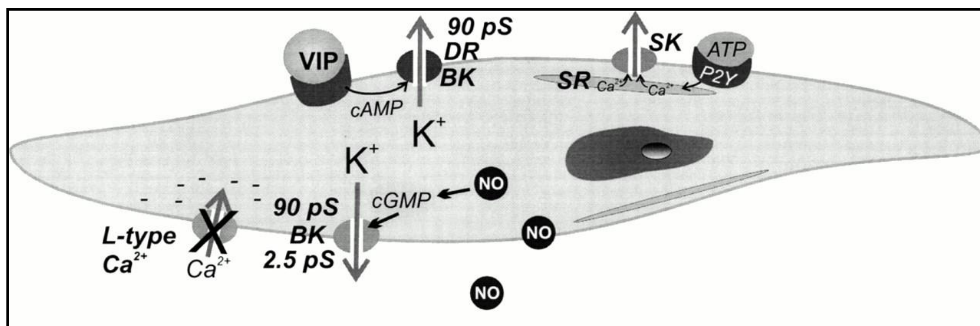

Figura 5. **Esquema resum dels principals neurotransmissors inhibidors amb els seus canals de K^+ associats (Sanders, 2000)**

1.3.6. Interaccions entre els neurotransmissors inhibidors

S'han descrit diferents mecanismes d'interacció entre els neurotransmissors inhibidors. S'ha proposat que l'NO es pot alliberar en paral·lel i tenir accions independents (cotransmissió) junt amb l'ATP (Soediono & Burnstock, 1994) i el VIP (Bayguinov et al., 1999). L'NO també pot ser alliberat per altres neurotransmissors com el VIP a nivell presinàptic (Kumano et al., 2001;Grider et al., 1992) i també és possible que el VIP indueixi la síntesi d'NO a nivell postsinàptic (Ergun & Ogulener, 2001), de la mateixa manera que ho poden fer l'ATP (Xue et al., 2000b), el PACAP (Zizzo et al., 2004) i el CO (Lim et al., 2005). Per altra banda, també s'ha descrit que l'NO pot alliberar VIP i PACAP a nivell del terminal nerviós (Grider et al., 1992;Zizzo et al., 2004) i que el VIP pot induir la síntesi de CO (Watkins et al., 2004).

1.3.7. Cèl·lules intersticials de Cajal

A més de les neurones i la glia de l'ENS, el tracte digestiu conté cèl·lules intersticials de Cajal (ICC). Aquestes van ser descrites per primera vegada per Santiago Ramón y Cajal cap a l'any 1890 usant tincions de blau de metilè. Les va descriure com: *"células fusiformes o triangulares de pequeña talla, pobres en protoplasma, del cual parten varias expansiones varicosas, larguísimas y de ordinario ramificadas en ángulo recto. Habitan especialmente entre los haces de fibro-células, adosándose a los manojos del plexo intersticial o secundario; pero se las ve también en el contorno del plexo de Auerbach y en la inmediación de los vasos. Algunas de sus expansiones abandonan los hacecillos nerviosos y marchan independientemente sobre la trama contráctil, siguiendo de preferencia a los intersticios musculares y constituyendo un plexo de malla angosta, desigual y frecuentemente incompleta. Las últimas ramillas,*

pálidas y granulosas, parecen conexionarse con las células musculares." (Ramón y Cajal, 1904). Cajal creia que aquestes cèl·lules eren neurones simpàtiques intersticials, no vas ser fins als anys 90 que es va veure amb estudis immunohistoquímics que no tenien marcadors clàssics de neurones mientèriques, ni de glia, ni de fibroblasts, ni de múscul llis, però sí d'enolasa específica de neurones, resultats que suggerien una relació amb algun tipus neuronal (Prosser et al., 1989). Posteriorment Huizinga et al. (Huizinga et al., 1995), van demostrar que aquestes cèl·lules expressaven el receptor c-kit. Actualment la presència d'aquest receptor és usada com a *gold standard* per marcar aquestes cèl·lules.

Estudis morfològics on s'observa una disminució de les ICC, han portat a la conclusió que aquestes cèl·lules poden tenir un paper important en la patofisiologia de diferents malalties com ara l'estenosi pilòrica hipertròfica infantil, alguns síndromes de pseudoobstrucció intestinal crònica (Kenny et al., 1998;Vanderwinden et al., 1996a), la constipació de trànsit lent, la malaltia de Chagas, les alteracions de la motilitat relacionades amb la diabetis , i l'hipoganglionosi (Vanderwinden et al., 1996b;Hagger et al., 2000;He et al., 2000;Nakahara et al., 2002;Rolle et al., 2002;Tong et al., 2004;Yu et al., 2002).

Localització de les ICC

Les ICC es localitzen en les diferents capes que formen el tracte digestiu (Sanders et al., 2002).

- ICC del plexe mientèric (ICC-AP)

En el plexe mientèric, les ICC estan estretament associades amb els ganglis i amb les cèl·lules musculars de les capes circulars i longitudinals adjacents.

- ICC intramusculars (ICC-IM)

Amb tècniques d'immunohistoquímica, usant anticossos contra aquest receptor, s'ha evidenciat la presència d'ICC-IM al llarg de tot el tracte digestiu, des de l'esòfag fins a l'IAS (Hagger et al., 1998;Romert & Mikkelsen, 1998;Ward et al., 1998;Battish et al., 2000;Burns et al., 1996). Usant tècniques de microscòpia electrònica, s'ha vist que a l'esòfag, al LES, a l'estómac, a l'intestí prim i al còlon, aquestes cèl·lules estan en contacte molt estret amb les varicositats terminals de les neurones motores entèriques i formen unions gap amb les cèl·lules musculars llises (Daniel & Posey-Daniel,

1984;Faussone-Pellegrini & Cortesini, 1985;Faussone-Pellegrini, 1987) fet que fa suggerir que aquestes cèl·lules puguin participar en la neurotransmissió (Figura 6).

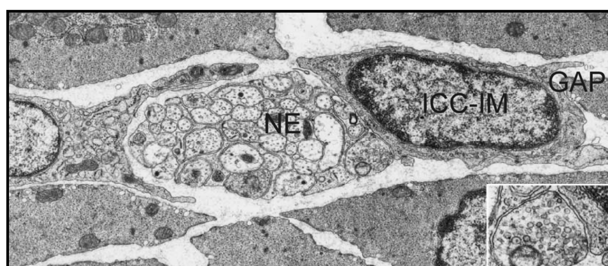

Figura 6. Fotografia de microscòpia electrònica en la musculatura circular de l'estómac de la rata. Es Pot veure el contacte estret entre el terminal nerviós (NE) i una ICC-IM, aquesta està en contacte amb una cèl·lula muscular llisa a través d'una unió gap (Mitsui & Komuro, 2002) .

Altres estudis en microscòpia electrònica, en canvi, han descrit que la varicositat terminal pot contactar amb el múscul directament, sense la presència de les ICC (Mitsui & Komuro, 2002). Aquestes observacions suggereixen que hi ha dos tipus d'interaccions varicositat-múscul: una innervació directa, on hi ha un contacte directe entre el terminal i la cèl·lula muscular llisa; i una innervació indirecta, on el terminal interacciona amb la cèl·lula muscular llisa a través d'una ICC (Figura 7).

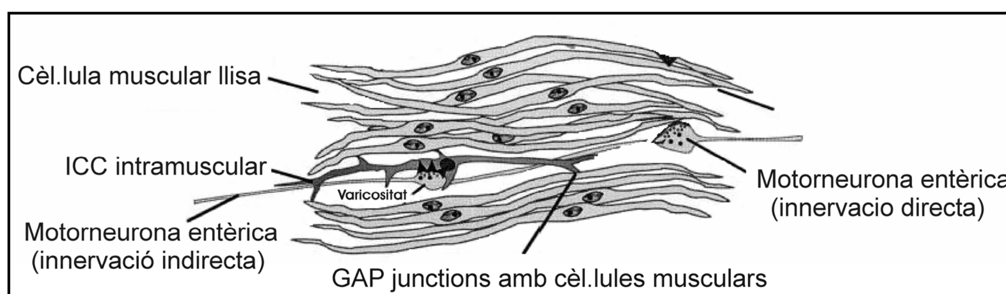

Figura 7. Models d'interacció motoneurona-cèl·lula muscular llisa. Modificat de Sanders et al. News Physiol. Sci. 2000

- ICC del plexe muscular profund (ICC-DMP)

A l'intestí, les cèl·lules semblants a les ICCs-IM estan restringides a la regió del plexe muscular profund i reben el nom d'ICC-DMP (Wang et al., 2003).

- ICC del plexe submucós (ICC-SMP)

A l'estómac i al còlon, una població d'ICC també es troba a la musculatura circular tocant el plexe submucós (Alberti et al., 2005;Mitsui & Komuro, 2003).

Funció de les ICCs

- ICC-AP i ICC-SMP

En estudis de microscòpi electrònic s'ha vist que les ICC formen unions gap amb les cèl·lules musculars llises i amb altres ICC del seu voltant. En estudis amb

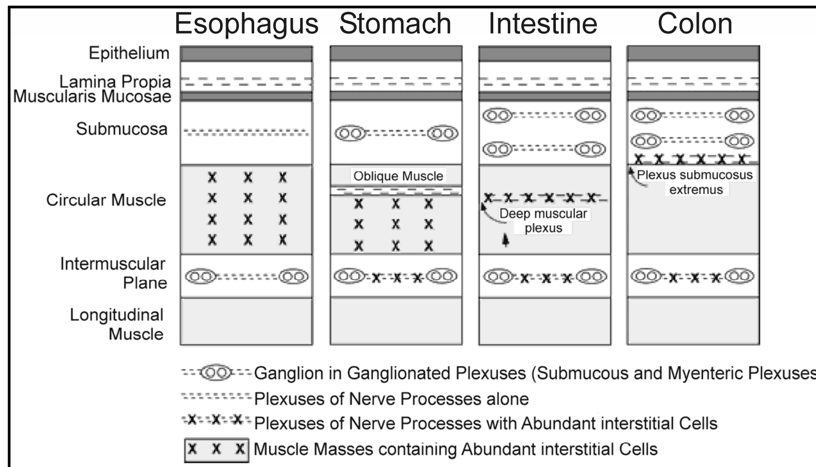

Figura. 8. Esquema resum de la relació de les cèl·lules intersticial de Cajal amb altres estructures del tracte GI (Christensen, 2001)

ratolins mutants W/Wv (no tenen ICCs al plexe mientèric) i en estudis de *patch-clamp* posaria: s'ha suggerit que les ICC-AP de l'estómac, de l'intestí prim i del còlon, i les ICC-SMP en el còlon, poden tenir funcions duals com a marcapassos i com a vies de conducció de les ones lentes (Huizinga et al., 1995;Langton et al., 1989;Ordog et al., 1999).

- ICC-IM i ICC-DMP

En estudis funcionals in vitro, s'ha vist que en ratolins mutants Ws/Ws, que no tenen ICC-IM, la innervació inhibidora nitrèrgica no és funcional ni en l'estómac (Burns et al., 1996), ni en el LES ni en el pílor (Ward et al., 1998). En canvi, en estudis *in vivo* en el LES (Sivarao et al., 2001) i en estudis *in vivo* i in vitro en l'IAS (Terauchi et al., 2005;de Lorijn F. et al., 2005), s'ha vist que la neurotransmissió nitrèrgica inhibidora està preservada en els animals mutants, cosa que suggereix que les ICC-IM no tenen un paper important en aquest tipus de neurotransmissió. Pel que fa al component excitador, s'ha descrit en el fundus gàstric del ratolí que aquestes cèl·lules juguen un paper molt important en la neurotransmissió colinèrgica (Ward et al., 2000).

S'ha descrit que les ICC-DMP formen contactes semblants amb sinapsi amb nervis nitrèrgics i colinèrgics i contactes amb cèl·lules musculars mitjançant unions gap, fet que fa pensar que les ICC-DMP poden estar relacionades amb la neurotransmissió del múscul llis en l'intestí (Wang et al., 2003). També s'ha descrit que les ICC-IM poden funcionar com a sensors d'estirament (*streach sensors*) (de Lorijn F. *et al.*, 2005).

2. Anatomia muscular i fisiologia de l'esòfag

La funció de l'esòfag és transportar el material engolit des de la faringe fins a l'estómac. L'esòfag es diferencia de la resta del tracte digestiu perquè no té funcions secretores o d'absorció importants, i també pel tipus d'epiteli de la mucosa. Per altra banda, la funció de l'esfínter esofàgic inferior és crear una zona d'alta pressió que impedeixi el reflux del contingut gàstric cap a l'esòfag.

2.1 Anatomia muscular de l'esfínter esofàgic inferior

En humans, l'esòfag està format per una porció de múscul estriat d'uns 12 cm que comprèn la faringe i part de l'esòfag cervical, i, per una altra d'uns 15 cm formada per múscul llis, que s'estén del terç del mig fins a la unió gastroesofàgica, localitzada al nivell del diafragma. En aquest nivell, la musculatura de l'esòfag forma el LES. Les principals funcions del LES són permetre el pas del bol durant la ingestió d'aliments de l'esòfag fins a l'estómac i prevenir el reflux d'una quantitat significativa del contingut gàstric cap a l'esòfag. La funció de la barrera antireflux depèn de la competència tant del LES com del diafragma crural (Figura 9) (Hill et al., 1996;Pandolfino et al., 2003).
Estudis amb teixit fixat i usant tècniques d'imatge de manometria tridimensional *in vivo* demostren que en humans el LES no és un anell muscular, sinó que està format per les fibres semicirculars de clasp adjacents a la curvatura menor (que forma un anell incomplet en forma de U) i per bandes musculars a la banda de la curvatura major, formades per fibres gàstriques obliqües (fibres de sling) (Liebermann-Meffert et al., 1979;Stein et al., 1995). En altres espècies, com en el gos (Friedland et al., 1971), l'opòssum (Christensen & Torres, 1975) i el gat (Friedland et al., 1971;Preiksaitis et al., 1994b) el múscul circular del LES forma un anell complet. Ambdós músculs mantenen l'esfínter tancat i contribueixen a la pressió del LES. En l'única espècie on s'ha observat que la distribució de les fibres de clasp i de sling és idèntica a la de l'home ha estat en el porc (Vicente et al., 2001).

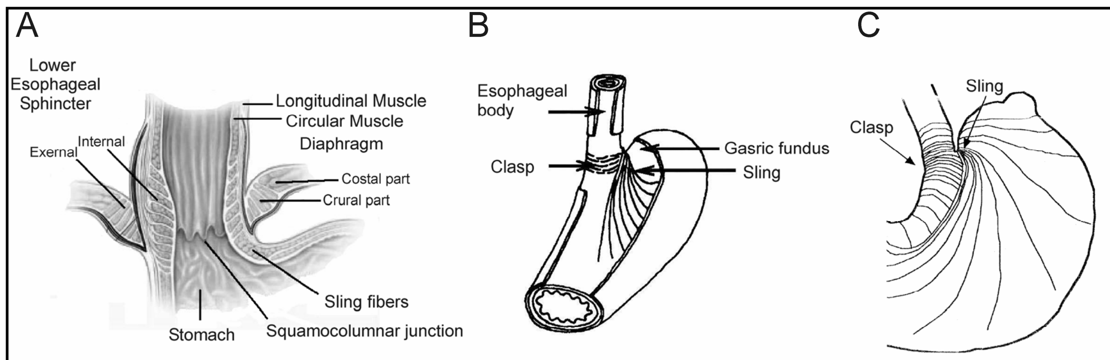

Figura 9. Anatomia de la unió gastroesofàgica en l'home. A) L'esfínter esofàgic inferior representa la part interna (esfínter de múscul llis) mentre que el diafragma representa la part externa (esfínter de múscul estriat). Podem veure també a la part dreta de la figura les fibres de sling. Adaptat de (Mittal & Balaban, 1997). B) Esquema de la distribució de les fibres de clasp i sling en el LES humà (Preiksaitis & Diamant, 1997) i C) en el porc (Vicente et al., 2001)

2.2 Fisiologia de l'esòfag i del LES. Control de l'activitat motora

En la porció del múscul llis de l'esòfag, la deglució genera un reflex peristàltic que és generat centralment i que està mediat per neurones eferents vagals procedents del DMN. La peristalsi induïda per la deglució voluntària s'anomena *peristalsi primària* per distingir-la de la peristalsi secundària, la qual és produïda per reflexes locals com la distensió. La peristalsi primària és abolida per vagotomia o refredament vagal bilateral (Roman & Gonella, 1981) (Ryan et al., 1977). Els eferents vagals estimulen la peristalsi per activació i modificació del reflex peristàltic intrínsec. Per altra banda, la peristalsi secundària en el múscul llis està generada per un reflex local i no es modifica per vagotomia (Kravitz et al., 1978;Roman & Gonella, 1981); en canvi, la peristalsi secundària en el múscul estriat està controlada centralment (Roman & Gonella, 1981).

2.2.1. Peristalsi secundària en el múscul estriat

Contràriament al que ocorre en el múscul llis, en el múscul estriat, la peristalsi secundària sí que s'aboleix per vagotomia. Aquest fet indica que el control de la peristalsi és d'origen central (Roman & Gonella, 1981). L'estimulació elèctrica d'aquestes neurones eferents generen només contraccions simultànies a tots els nivells del múscul estriat, fet que fa pensar que es necessita aquesta integració central per organitzar la natura seqüencial de les contraccions peristàltiques (Grundy, 1988). La peristalsi en la porció de múscul estriat està generada pel nucli ambigu i mitjançada per

LMNs vagals. El nucli ambigu genera una activació seqüencial d'aquestes neurones que produeixen contraccions successives en l'esòfag estriat (Figura 10).

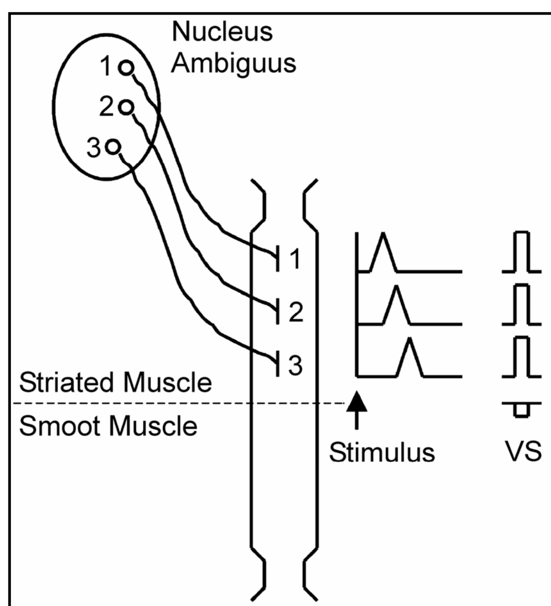

Figura 10. Mecanisme de la peristalsi evocada vagalment en el múscul estriat de l'esòfag. vs, Estimulació vagal. (Chang et al., 2003)

2.2.2. Peristalsi secundària en el múscul llis

La peristalsi esofàgica intrínseca està mitjançada per neurones mientèriques inhibidores no adrenèrgiques no colinèrgiques (NANC) i motoneurones excitadores actuant coordinadament. Encara que s'anomenen inhibidores, l'estimulació de les motoneurones inhibidores en tires de múscul llis indueix una resposta bifàsica que consisteix en una inhibició seguida d'una contracció rebot. En el múscul llis que no té to basal, l'inhibició es veu només com un període de latència durant el període d'estimulació nerviosa. La contracció del múscul llis després d'aquest període d'estímul prolongat s'anomena contracció rebot o contracció en "off" (Knudsen et al., 1991) . Weisbrodt et al. (Weisbrodt & Christensen, 1972) van descriure en tires de múscul circular esofàgic d'opòssum que la duració del període de latència de la contracció en "off" s' incrementa progressivament en direcció craneocaudal. A partir d'aquests resultats, aquests autors van proposar que el gradient de latència al llarg de l'esòfag està generat per nervis NANC. Estudis posteriors van demostrar que l'NO era el neurotransmissor NANC responsable d'aquesta resposta bifàsica (Preiksaitis et al., 1994a;Murray et al., 1991;Knudsen et al., 1991). Estudis in vitro i *in vivo* amb opòssums demostren que l'L-NAME (inhibidor NOS) aboleix la latència i el gradient de latència de les contraccions esofàgiques produïdes per l'estimulació de nervis NANC a diferents nivells de l'esòfag (Yamato et al., 1992b;Murray et al., 1991).

Les neurones excitadores del plexe mientèric esofàgic utilitzen majoritàriament l'ACh com a neurotransmissor i freqüentment són anomenades colinèrgiques. In vitro, l'estimulació nerviosa de curta durada, indueix una resposta inhibidora NANC que apareix primer i que va seguida per una contracció colinèrgica que pot sobreposar-se amb la contracció rebot NANC. Quan l'estímul nerviós és perllongat, la contracció colinèrgica i la contracció rebot NANC poden aparèixer separades. Les contraccions que ocorren durant l'estímul s'anomenen contraccions en "on"; les contraccions que ocorren al finalitzar l'estímul s'anomenen contraccions en "off" (Crist et al., 1984a). De manera similar a les neurones NANC, les neurones colinèrgiques formen un gradient colinèrgic, però en aquest cas decreix distalment al llarg de l'esòfag (Crist et al., 1984b). Aquest model incorpora el gradient d'influències NANC incrementades i un d'influències colinèrgiques disminuïdes al llarg de l'esòfag per explicar l'amplitud i la latència de la peristalsi esofàgica (Figura 11). Així es pot explicar tant els mecanismes perifèrics pel comportament *peristalsi-like* de les tires circulars de l'esòfag in vitro, com la peristalsi esofàgica provocada per estimulació vagal aferent *in vivo*.

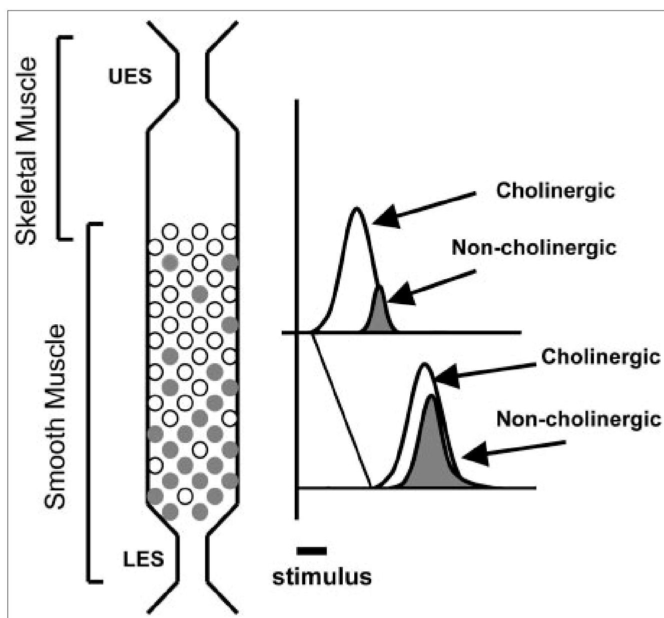

Figura 11. Mecanisme perifèric de la peristalsi. En aquest model proposat per Crist et al., es descriuen gradients de neurones colinèrgiques i no colinèrgiques al llarg de la porció de múscul llis de l'esòfag. L'influència colinèrgica predomina a l'esòfag proximal i decreix distalment. Contràriament, la influència no colinèrgica és mínima proximalment i incrementa en l'esòfag distal. UES, esfínter esofàgic superior (Chang et al., 2003)

2.2.3. Peristalsi primària

El reflex deglutori, que evoca peristalsi primària, està organitzat en el centre de deglució (localitzat al tronc cerebral), aquest centre es comunica amb el plexe mientèric esofàgic mitjançant neurones aferents vagals. Estudis amb opòssums anestesiats (Gidda & Goyal, 1984) en els quals es van provocar deglucions i es van enregistrar potencials a

les fibres eferents, han suggerit que després de la deglució s'activen primer les neurones NANC del plexe, i causen una ràpida inhibició del múscul llis de l'esòfag, fet que ocorre en menys d'1 s. Aquest fenomen es coneix com a inhibició deglutòria. El grau i la durada de la inhibició augmenta a mesura que ens apropem a la part distal de l'esòfag, per raó de l'increment del gradient NANC, descrit anteriorment. La resposta inhibidora està seguida per una contracció amb latències progressivament incrementades al llarg de l'esòfag. La deglució també inicia l'activació seqüencial d'un segon grup de fibres eferents amb períodes de latència compresos entre 1 i 5 s. Aquestes fibres amb latències de major durada s'especula que projecten cap a les neurones excitadores del plexe mientèric de l'esòfag. Durant la deglució la seqüència d'excitació colinèrgica se superposa a la NANC modulant l'amplitud i el gradient de latència de la contracció peristàltica primària (Chang et al., 2003) (Figura 12).

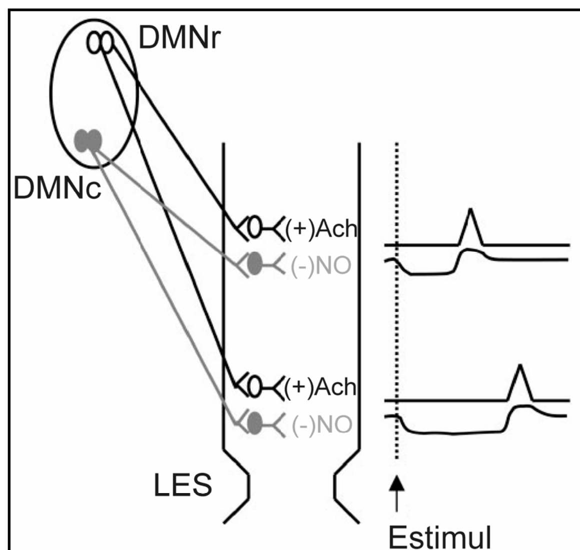

Figura 12. Paper dels nervis eferents vagals en la peristalsi primària. L'activació seqüencial de les neurones NANC i colinèrgiques del plexe que innerven la porció de múscul llis de l'esòfag explica la inhibició deglutòria seguida de peristalsi. Les fibres de latència curta activen la NANC del plexe per produir inicialment una inhibició del múscul llis, per altra banda, les fibres de latència llarga estimulen subseqüentment neurones colinèrgiques per produir contraccions que estan programades per coincidir amb la contracció NANC rebot (Chang et al., 2003).

En humans, la inhibició deglutòria que precedeix la peristalsi primària s'evidencia quan deglucions repetides i successives es produeixen en intervals propers. Cada deglució successiva inhibeix la contracció peristàltica de la deglució precedent, i només l'última deglució està associada amb la contracció peristàltica (Sifrim et al., 1992).

2.2.4. Esfínter esofàgic inferior

El LES està format per múscul llis amb propietats miogèniques intrínseques que manté l'esfínter tancat en condicions basals. Aquesta contracció tònica fa que manomètricament el LES s'evidenciï com una zona d'alta pressió a l'extrem inferior de l'esòfag. Aquest to miogènic està modulat tant per les vies vagals inhibidores com per les excitadores. En estudis *in vivo* s'ha vist que quan les influències excitadores i inhibidores són iguals, ni la vagotomia bilateral (Rattan & Goyal, 1974) ni la neurotoxina tetrodotoxina (TTX) canvien la pressió del LES (Goyal & Rattan, 1976). Per altra banda, antagonistes selectius de només una via produeixen efectes oposats de l'altra via. Això vol dir que el bloqueig de la via excitadora colinèrgica usant anticolinèrgics (Dodds et al., 1981) o toxina botulínica (Pasricha et al., 1993) disminueix la pressió del LES a causa d'una acció oposada dels nervis inhibidors, tot i que en algunes espècies l'atropina no produeix cap canvi en la pressió del LES (Dodds et al., 1981) . En canvi, el bloqueig de la via nitrèrgica inhibidora amb inhibidors de la NOS produeix un increment de la pressió del LES (Xue et al., 1996;Yamato et al., 1992a).

L'activació selectiva de la via vagal inhibidora pot generar la relaxació del LES. La relaxació del LES és una part integral de la peristalsi primària induïda per la deglució. Amb l'estímul de la deglució, el LES es relaxa durant 8-10 s i posteriorment es produeix una contracció a la part proximal del LES. Aquesta sèrie d'esdeveniments permeten al bol engolit passar a través de la zona d'alta pressió. La relaxació del LES és el component més sensible de la peristalsi primària perquè la relaxació pot ocórrer sense una peristalsi faríngia o esofàgica associada. La relaxació del LES produïda per distensió de la porció llisa de l'esòfag, amb o sense peristalsi secundària associada, és conseqüència de reflexos locals, i no s'afecta per vagotomia. Les vies vagals inhibidores i excitadores s'activen selectivament en un nombre de reflexos motors que impliquen l'esfínter esofàgic inferior. L'estimulació de les aferents vagals abdominals i la distensió de l'estómac proximal disparen les vies inhibidores que produeixen la relaxació del LES. Reflexos vagovagals com eructar i les relaxacions transitòries de l'esfínter (tLESRs) també depenen de l'activació de la via inhibidora del LES (Chang et al., 2003).

3. Trastorns de la motilitat esofàgica

Els trastorns de la motilitat de l'esòfag comprenen diferents símptomes, especialment disfàgia i dolor toràcic. Aquests trastorns es diagnostiquen per manometria esofàgica, on es mesura la pressió i la relaxació del LES i la presència de peristalsi en el cos esofàgic. Els paràmetres de les contraccions peristàltiques que es tenen en compte per al diagnòstic són l'amplitud (alta o baixa), la duració, la naturalesa repetitiva i la presencia d'ones no transmeses o parcialment transmeses. A continuació s'expliquen breument les anomalies primàries com són l'acalàsia, l'espasme esofàgic difús, l'esòfag hipercontràctil, l'hipocontràctil i també algunes anomalies secundàries, com els trastorns de la motilitats produïts en l'escleroderma, malaltia de Chagas i diabetis (Richter, 2001). Després es parla també de la malaltia per reflux gastroesofàgic (GERD), que, com es veurà, es pot deure a una alteració de la motilitat i, secundàriament, pot ocasionar alteracions en la motilitat esofàgica a causa de l'exposició àcida (Castell et al., 2004).

3.1. Anomalies primàries de la motilitat esofàgica

Acalàsia

La simptomatologia es caracteritza per una obstrucció funcional amb disfàgia, malestar toràcic i regurgitació. El símptoma més freqüent és la disfàgia a sòlids i líquids, la gravetat de la qual fluctua, però que pot ser important fins a produir una pèrdua de pes significativa.

Els estudis manomètrics es caracteritzen per (Clave & Castellvi, 2004):

- absència d'ones peristàltiques en l'esòfag llis (criteri diagnòstic obligat)

-absència o relaxació incompleta de l'esfínter durant la deglució. Aquest fet el distingeix d'altres trastorns amb aperistalsi

-pot produir-se també un increment de la pressió del LES (>45 mmHg)

L'origen de l'acalàsia és desconeguda, s'han suggerit factors hereditaris, degeneratius, autoimmunes i infecciosos com a possible causa. Aquests dos últims factors són els dos més comunament acceptats (Castagliuolo et al., 2004;Ruiz-de-Leon et al., 2002;Storch et al., 1995;Storch et al., 1995). S'han identificat canvis patològics en espècimens procedents de necròpsies o d'intervencions quirúrgiques com són una resposta inflamatòria prominent però irregular, consistent en l'aparició de limfòcits T i

un nombre variable d'eosinòfils i mastòcits, i algun grau de fibrosis neural mientèrica (Raymond et al., 1999). El resultat final d'aquests canvis inflamatoris és una pèrdua selectiva de motoneurones inhibidores nitrèrgiques (Lui et al., 1997;Mearin et al., 1993) i neurones que contenen VIP (Aggestrup et al., 1983;Sigala et al., 1995). En estudis de microscòpia electrònica, en teixit de pacients amb acalàsia, es mostra la presència de vacuoles en les ICCs (Zarate et al., 2004) fet que fa pensar en una possible lesió també d'aquestes cèl·lules. Aquesta pèrdua de neurones inhibidores provoca una relaxació incomplerta del LES i una aperistalsi causada per la pèrdua del gradient de latència que permet contraccions seqüencials al llarg de l'esòfag.

Espasme esofàgic difús

L'espasme esofàgic difús es caracteritza per un peristalsi normal intermitentment interrompuda per contraccions simultànies. Aquest malaltia és poc freqüent, ocorre al voltant d'un 3-5% dels pacients estudiats per anomalies de la motilitat esofàgica. Es desconeix l'etiopatologia d'aquest desordre. En estudis en què s'usen tests de provocació amb metacolina, betanecol, carbacol i pentagastrina s'observen unes contraccions augmentades en aquests malalts (Mellow, 1977). En un estudi on es correlaciona el gruix de la musculatura en estat basal i l'amplitud de la contracció peristàltica (Pehlivanov et al., 2002), s'observa que hi ha una associació que explicaria per què aquests malalts tenen les contraccions augmentades i responen amb contraccions majors a l'estimulació d'agonistes colinèrgics. També s'ha suggerit que aquesta sensitivitat augmentada pot ser deguda a un defecte en la inhibició neural al llarg el cos esofàgic, possiblement en relació amb una disfunció en la síntesi d'NO i/o en la seva degradació (Konturek et al., 1995). L'espasme esofàgic difús es diagnostica per manometria esofàgica, s'observen unes contraccions simultànies no peristàltiques intermitents barrejades amb peristalsi normal. Altres troballes manomètriques menys consistents inclouen contraccions de llarga durada, ones repetitives (tres pics o més), contraccions espontànies no induïdes per la deglució, i anomalies de la pressió del LES o de la seva relaxació. En estudis de pH-metria ambulatòria de 24 h s'ha vist que entre un 20% i un 50% dels malalts tenen GERD.

Esòfag hipercontràctil

Els pacients amb peristalsi esofàgica simptomàtica (esòfag en trencanous, nutcracker esophagus) presenten contraccions no peristàltiques d'elevada amplitud i poden presentar també contraccions peristaltiques de duració major. Existeix un subgrup de pacients que presenta també un LES hipertens. Aquests dos resultats manomètrics sovint coexisteixen, i suggereixen que poden representar una síndrome d'un esòfag hipercontràctil. La causa de l'esòfag hipercontràctil és incerta; tot i això, algunes contraccions d'elevada pressió poden ser secundàries a factors exògens com reflux gastroesofàgic o l'estrès. El principal símptoma dels pacients amb esòfag hipercontràctil és dolor toràcic.

Esòfag hipocontràctil

La majoria del pacients que són diagnosticats, com que tenen una alteració de la motilitat no especifica, tenen patrons de motilitat caracteritzats per qualsevol de les dues alteracions següents: contraccions peristàltiques de baixa amplitud o contraccions simultànies en l'esòfag distal, o una peristalsi fallida en la qual les ones no travessen per complet tot l'esòfag distal (anomenada *motilitat esofàgica inefectiva*). El concepte d'ones de baixa amplitud que són inefectives està suportat per estudis que usen simultàniament manometria i videoradiografia de bari per mostrar que les ones contràctils de menys de 30 mmHg d'amplitud, encara que són peristàltiques, no transporten eficaçment i no netegen un bol de bari de l'esòfag. La majoria dels malalts amb motilitat esofàgica inefectiva tenen GERD. Una pressió baixa del LES és vista comunament amb pacients amb GERD, fet que suggereix que l'esòfag hipocontràctil pot ser secundari a un dany àcid crònic de l'esòfag distal. El LES hipotònic pot no ser sever; tot i això, hi ha estudis que suggereixen que una exposició àcida anormal en aquests malalts es correlaciona millor amb una dèbil bomba esofàgica que amb la pressió del LES disminuïda.

3.2. Anomalies secundàries de la motilitat esofàgica

Els malalts amb alteracions secundàries de la motilitat esofàgica tenen patrons de motilitat anormals secundaris a una malaltia sistèmica. En l'escleroderma, per exemple, les anomalies de la motilitat es troben en un 80% dels malalts. El procés de la malaltia subjacent està causat per l'obliteració vascular i la fibrosis secundària que

afecta el múscul llis esofàgic i la seva innervació. Aquest procés produeix una disminució de la pressió en el LES (<10 mmHg) i una motilitat distal dèbil i inefectiva. Aquests malalts normalment tenen GERD, fins un 60% tenen esofagitis, i alguns tenen esòfag de Barrett.

La malaltia de Chagas, que està causada per la infecció amb al paràsit *Trypanosoma cruzi*, és una malaltia endèmica del Brasil. En l'esòfag produeix una síndrome molt semblant a l'acalàsia. Tot i això, a diferència dels malalts amb acalàsia clàssica, els malalts amb Chagas tenen altres evidències de malaltia sistèmica que inclouen cardiomiopatia, megacòlon i megaurèter.

La neuropatia autonòmica pot conduir a una motilitat anormal en la majoria de pacients amb diabetis insulinodepenent, i algunes vegades pot assemblar-se a l'espasme esofàgic difús. S'ha vist en malalts diabètics asimptomàtics, en què s'han usat estudis radiogràfics, que mostren una reducció o una absència de contraccions peristàltiques. D'una manera semblant, estudis manomètrics mostren una fallida de les seqüències de contraccions i múltiples ones desorganitzades junt amb una descoordinació de la funció del LES. L'amiloïdosi, l'alcoholisme i l'esclerosi múltiple poden estar també acompanyades per hipocontraccions en l'esòfag distal. La pseudoobstrucció intestinal idiopàtica crònica està gairebé sempre associada amb anomalies de la motilitat, particularment pèrdua de peristalsi en l'esòfag distal, la qual de vegades pot mimetitzar l'acalàsia (Richter, 2001).

3.3. Malaltia per reflux gastroesofàgic (GERD)

El reflux gastroesofàgic és un fenomen tant fisiològic, que ocorre en la població general, com patològic que pot produir símptomes des de suaus a severs. La pirosi, una sensació de cremor o desconfort darrera de l'estèrnum, és el símptoma més comú del GERD. La majoria de malalts amb GERD tenen un esòfag endoscòpicament normal. Aquests malalts que tot i no tenir signes d'inflamació ni d'erosió, però que tenen els símptomes de GERD així com sensacions de cremor darrera de l'estèrnum, s'inclouen en el que s'anomena malaltia per reflux no erosiva (NERD). S'estima que un 10% d'individus de la població general tenen episodis de pirosi un cop al dia i que més d'un 40% experimenten aquest símptoma al menys un cop al mes. S'utilitzen bàsicament dos grups de fàrmacs per al tractament del reflux: els que redueixen la producció d'àcid o

inhibidors de la bomba de protons (PPIs) i els que protegeixen la mucosa (antiàcids), que actuen neutralitzant l'àcid.

La patogènesi del GERD és multifactorial; implica l'aparició de diferents canvis en la fisiologia normal que augmenten la presència del reflux àcid a l'esòfag. Aquests canvis inclouen alteracions en les relaxacions transitòries de l'esfínter (tLESRs), disminució de la pressió del LES, disminució del buidament gàstric, aclariment esofàgic inefectiu, disminució de la salivació i alteracions en la resistència de la mucosa (Castell et al., 2004).

Relaxacions transitòries de LES (tLESRs)

Després de menjar, el LES està normalment tancat. Quan relaxa, pot permetre el pas d'aliments i àcid cap a l'esòfag. En malalts amb GERD, la distensió gàstrica indueix un augment del nombre de tLESRs, les quals són la principal causa dels episodis de reflux. Com que el nombre de tLESRs augmenta, la freqüència dels episodis de reflux augmenta, i llavors també augmenta el temps de pas d'àcid a l'esòfag.

Les tLESRs és el mecanisme pel qual ocorre el reflux en la població sana (reflux fisiològic). La majoria dels malalts amb GERD tenen una pressió normal del LES, i són les tLESRs la causa principal de reflux en aquests malalts (80% dels episodis de reflux). Les tLESRs poden ser induïdes per estimulació gàstrica o faríngia, la qual inicia un reflex inhibidor vagovagal (Figura 13). S'activen les vies sensitives que van cap al nucli del tracte solitari, s'activen després les fibres motores eferents del nucli motor dorsal del vague, que innerven el MP, on fan sinapsi amb les motoneurones inhibidores i excitadores. Normalment les tLESRs duren menys de 30 s. S'ha vist en voluntaris sans que l'hormona gastrointestinal colecistoquinina augmenta el reflux gastroesofàgic després de la ingesta per raó de l'increment de l'índex de tLESRs i per reducció del to del LES (Clave et al., 1998). Aquestes accions estan mitjançades per receptors CCK-A situats fora del LES. L'intens efecte de l'activació d'aquests receptors extraesfintèrics (probablement situats als aferents vagals) s'imposa a la resposta contràctil directa induïda per la CCK sobre el múscul del LES, resposta mitjançada també per receptors CCK-A (Gonzalez et al., 2000). Així doncs, les tLESRs poden ser també induïdes per l'augment dels nivells de CCK postprandials. Actualment no hi ha fàrmacs al mercat per tractar el GERD que disminueixin la freqüència de les tLESRs, encara que s'han

provat en animals i en humans fàrmacs com el baclofèn (agonista GABA B) amb un bon resultat (Wise & Conklin, 2004).

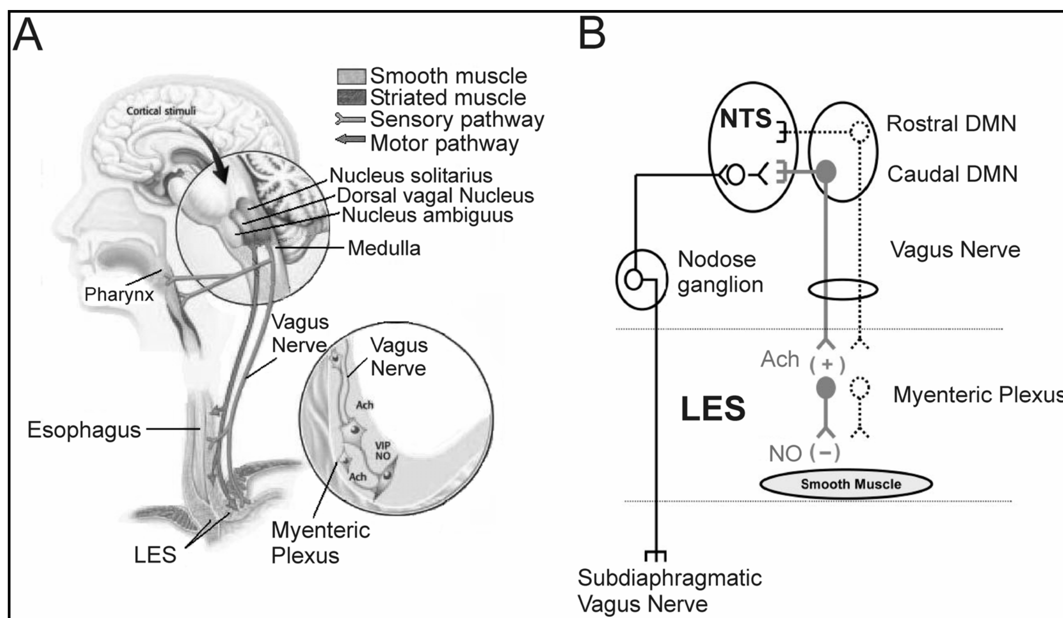

Figura 13. Vies nervioses que coordinen les relaxacions transitòries de l'esfínter esofàgic inferior. A) Adaptat de (Mittal & Balaban, 1997) i B) adaptat de (Chang et al., 2003)

Disminució del to del LES

El LES és la barrera primària antireflux. Una pressió disminuïda permet el reflux cada cop que la pressió de l'estómac sobrepassa la del LES. Aquesta condició està present en una minoria dels casos de GERD, i està normalment associada a esofagitis severa.

Buidament gàstric endarrerit

Si el buidament gàstric està endarrerit, el volum del fluid gàstric està augmentat. Es creu que un buidament gàstric endarrerit contribueix en una petita proporció de casos de GERD per un increment del fluid disponible per refluir.

Aclariment esofàgic inefectiu

L'aclariment àcid de l'esòfag consta d'un procés de dos passos, anomenat mecanisme antireflux. La peristalsi elimina el contingut gàstric de l'esòfag, i la saliva engolida neutralitza l'àcid residual. S'ha vist en alguns pacients amb GERD una disminució en l'amplitud de la peristalsi secundària i contraccions segmentàries.

Alteracions en l'aclariment esofàgic poden ser causades per un augment en el volum del refluent i/o un efecte irritant durant un episodi de reflux precedent.

Disminució de la salivació

La saliva – que té un pH de 7,8-8,0 –, és rica en bicarbonat i pot neutralitzar normalment l'àcid residual que recobreix l'esòfag després de la peristalsi secundària. Així doncs, una disminució en la salivació pot contribuir a la duració de l'exposició àcida a l'esòfag.

Alteracions en la resistència de la mucosa

La resistència tissular en l'esòfag consisteix en unes membranes de cèl·lules i unes complexes unions intercel·lulars, que protegeixen contra l'àcid limitant que els hidrogenions (H+) difonguin dins l'epiteli. L'esòfag també produeix bicarbonat, que tampona l'àcid, i moc, que forma una barrera protectora en la superfície de l'epiteli. La resistència de la mucosa esofàgica a l'àcid és menor que la de l'estómac. Quan ocorre un dany esofàgic, hi ha massa àcid i massa pepsina presents perquè la mucosa estigui protegida. Llavors la pepsina del refluent pot danyar l'esòfag digerint les proteïnes de l'epiteli.

4. Objectius

L'objectiu d'aquesta tesi ha estat estudiar in vitro, usant la tècnica de bany d'òrgans, els neurotransmissors implicats en la relaxació i la contracció i en el control del to en l'esfínter esofàgic inferior. Per això s'ha usat teixit procedent de tres espècies: l'home, el porc i la rata. També s'ha estudiat el paper de les cèl·lules intersticials de Cajal (ICCs) en la neurotransmissió excitadora i inhibidora, usant un model mutant en rata (Ws/Ws) que no presenta algun dels tipus d'ICCs. Els objectius concrets de cada estudi es descriuen a continuació.

Objectiu 1. Caracteritzar l'efecte de l'estimulació de les motoneurones excitadores i inhibidores i les influències neurals sobre el to en el LES humà.

1.1. Caracteritzar els neurotransmissors alliberats per les motoneurones excitadores i inhibidores
1.2. Comparar la resposta a l'estimulació de les motoneurones excitadores i inhibidores usant nicotina i estimulació elèctrica de camp (EFS)
1.3. Avaluar les influències neurals sobre el to

Objectiu 2. Caracterització dels mecanismes neuromiogènics i dels neurotransmissors que controlen el to i la relaxació de LES del porc.

2.1. Avaluar les influències neurals sobre el to
2.2. Caracteritzar la resposta dels possibles neurotransmissors
2.3. Caracteritzar farmacològicament els neurotransmissors alliberats per les motoneurones usant EFS i estimulació dels receptors nicotínics

Objectiu 3. Avaluar les diferències existents entre les regions de clasp i sling en el LES del porc pel que fa al control del to i a la contribució dels diferents components implicats en la relaxació i en la contracció.

3.1. Avaluar les influències neurals sobre el to
3.2. Caracteritzar i avaluar la contribució dels components implicats en la relaxació i en la contracció
3.3. Avaluar l'efecte de l'addició exògena del donador d'NO nitroprussiat de sodi (SNP), de l'ATP i del carbacol

Objectiu 4. Caracterització i implicació de les cèl·lules intersticials de Cajal (ICCs) en la neurotransmissió inhibidora i excitadora en el LES de la rata.

4.1. Estudiar de la distribució de les ICC en rates Sprague-Dawley, en rates mutants Ws/Ws i en les seves germanes no mutants +/+

4.2. Caracterització dels neurotransmissors inhibidors i excitadors en rates Sprague-Dawley, rates +/+ i rates mutants Ws/Ws

Referències

Aggestrup S, Uddman R, Sundler F, Fahrenkrug J, Hakanson R, Sorensen HR, & Hambraeus G (1983). Lack of vasoactive intestinal polypeptide nerves in esophageal achalasia. *Gastroenterology* **84**, 924-927.

Alberti E, Mikkelsen HB, Larsen JO, & Jimenez M (2005). Motility patterns and distribution of interstitial cells of Cajal and nitrergic neurons in the proximal, mid- and distal-colon of the rat. *Neurogastroenterol Motil* **17**, 133-147.

Auli M, Farre R, Hernandez P, Gallego D, Martinez E, Marti-Rague J, Suñol X, Jimenez M, & Clave P. Nitrergic and purinergic co-neurotransmission in human sigmoid colon. Neurogastroenterology and Motility 17[Supplement 2], 27. 2005.
Ref Type: Abstract

Aziz Q & Thompson DG (1998). Brain-gut axis in health and disease. *Gastroenterology* **114**, 559-578.

Battish R, Cao GY, Lynn RB, Chakder S, & Rattan S (2000). Heme oxygenase-2 distribution in anorectum: colocalization with neuronal nitric oxide synthase. *Am J Physiol Gastrointest Liver Physiol* **278**, G148-G155.

Bayguinov O, Keef KD, Hagen B, & Sanders KM (1999). Parallel pathways mediate inhibitory effects of vasoactive intestinal polypeptide and nitric oxide in canine fundus. *Br J Pharmacol* **126**, 1543-1552.

Belai A & Burnstock G (1994). Evidence for coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurones in the rat ileum, colon and anococcygeus muscle. *Cell Tissue Res* **278**, 197-200.

Bennett MR (1997). Non-adrenergic non-cholinergic (NANC) transmission to smooth muscle: 35 years on. *Prog Neurobiol* **52**, 159-195.

Blackshaw LA, Haupt JA, Omari T, & Dent J (1997). Vagal and sympathetic influences on the ferret lower oesophageal sphincter. *J Auton Nerv Syst* **66**, 179-188.

Bolotina VM, Najibi S, Palacino JJ, Pagano PJ, & Cohen RA (1994). Nitric oxide directly activates calcium-dependent potassium channels in vascular smooth muscle. *Nature* **368**, 850-853.

Broussard DL & Altschuler SM (2000). Brainstem viscerotopic organization of afferents and efferents involved in the control of swallowing. *Am J Med* **108 Suppl 4a**, 79S-86S.

Brune B & Lapetina EG (1989). Activation of a cytosolic ADP-ribosyltransferase by nitric oxide-generating agents. *J Biol Chem* **264**, 8455-8458.

Burns AJ, Lomax AE, Torihashi S, Sanders KM, & Ward SM (1996). Interstitial cells of Cajal mediate inhibitory neurotransmission in the stomach. *Proc Natl Acad Sci U S A* **93**, 12008-12013.

Burnstock G (1980). Purinergic nerves and receptors. *Prog Biochem Pharmacol* **16**, 141-154.

Cabarrocas J, Savidge TC, & Liblau RS (2003). Role of enteric glial cells in inflammatory bowel disease. *Glia* **41**, 81-93.

Camaioni E, Boyer JL, Mohanram A, Harden TK, & Jacobson KA (1998). Deoxyadenosine bisphosphate derivatives as potent antagonists at P2Y1 receptors. *J Med Chem* **41**, 183-190.

Cao W, Pricolo VE, Zhang L, Behar J, Biancani P, & Kirber MT (2000). Gq-linked NK(2) receptors mediate neurally induced contraction of human sigmoid circular smooth muscle. *Gastroenterology* **119**, 51-61.

Castagliuolo I, Brun P, Costantini M, Rizzetto C, Palu G, Costantino M, Baldan N, & Zaninotto G (2004). Esophageal achalasia: is the herpes simplex virus really innocent? *J Gastrointest Surg* **8**, 24-30.

Castell DO, Murray JA, Tutuian R, Orlando RC, & Arnold R (2004). Review article: the pathophysiology of gastro-oesophageal reflux disease - oesophageal manifestations. *Aliment Pharmacol Ther* **20 Suppl 9**, 14-25.

Chang HY, Mashimo H, & Goyal RK (2003). Musings on the wanderer: what's new in our understanding of vago-vagal reflex? IV. Current concepts of vagal efferent projections to the gut. *Am J Physiol Gastrointest Liver Physiol* **284**, G357-G366.

Christensen J (2001). Structure of the Motor Apparatus of the Gut. In *The Motility of the Gastrointestinal Tract* Peer Review Status.

Christensen J & Torres EI (1975). Three layers of the opossum stomach: responses to nerve stimulation. *Gastroenterology* **69**, 641-648.

Clague JR, Sternini C, & Brecha NC (1985). Localization of calcitonin gene-related peptide-like immunoreactivity in neurons of the rat gastrointestinal tract. *Neurosci Lett* **56**, 63-68.

Clave P & Castellvi JM (2004). Acalasia. Estrategia Diagnóstica. *Gastroenterología y Hepatología Continuada* **3**, 52-58.

Clave P, Gonzalez A, Moreno A, Lopez R, Farre A, Cusso X, D'Amato M, Azpiroz F, & Lluis F (1998). Endogenous cholecystokinin enhances postprandial gastroesophageal reflux in humans through extrasphincteric receptors. *Gastroenterology* **115**, 597-604.

Clerc N (1984). Afferent innervation of the lower esophageal sphincter of the cat. Pathways and functional characteristics. *J Auton Nerv Syst* **10**, 213-216.

Clerc N & Condamin M (1987). Selective labeling of vagal sensory nerve fibers in the lower esophageal sphincter with anterogradely transported WGA-HRP. *Brain Res* **424**, 216-224.

Clerc N & Mazzia C (1994). Morphological relationships of choleragenoid horseradish peroxidase-labeled spinal primary afferents with myenteric ganglia and mucosal associated lymphoid tissue in the cat esophagogastric junction. *J Comp Neurol* **347**, 171-186.

Clerc N & Mei N (1983). Vagal mechanoreceptors located in the lower oesophageal sphincter of the cat. *J Physiol* **336**, 487-498.

Collman PI, Tremblay L, & Diamant NE (1992). The distribution of spinal and vagal sensory neurons that innervate the esophagus of the cat. *Gastroenterology* **103**, 817-822.

Collman PI, Tremblay L, & Diamant NE (1993). The central vagal efferent supply to the esophagus and lower esophageal sphincter of the cat. *Gastroenterology* **104**, 1430-1438.

Costa M & Brookes SJ (1994). The enteric nervous system. *Am J Gastroenterol* **89**, S129-S137.

Crist J, Gidda JS, & Goyal RK (1984a). Characteristics of "on" and "off" contractions in esophageal circular muscle in vitro. *Am J Physiol* **246**, G137-G144.

Crist J, Gidda JS, & Goyal RK (1984b). Intramural mechanism of esophageal peristalsis: roles of cholinergic and noncholinergic nerves. *Proc Natl Acad Sci U S A* **81**, 3595-3599.

Daniel EE & Posey-Daniel V (1984). Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal. *Am J Physiol* **246**, G305-G315.

de Lorijn F., de Jonge WJ, Wedel T, Vanderwinden JM, Benninga MA, & Boeckxstaens GE (2005). Interstitial cells of Cajal are involved in the afferent limb of the rectoanal inhibitory reflex. *Gut* **54**, 1107-1113.

de Man JG, de Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* **140**, 1108-1116.

Dodds WJ, Dent J, Hogan WJ, & Arndorfer RC (1981). Effect of atropine on esophageal motor function in humans. *Am J Physiol* **240**, G290-G296.

El-Mahmoudy A, Matsuyama H, Khalifa M, Shimizu Y, & Takewaki T (2003). Tachykinins mediate non-adrenergic, non-cholinergic excitatory neurotransmission to the hamster ileum via NK1 and NK2 receptors. *Life Sci* **73**, 1939-1951.

Ergun Y & Ogulener N (2001). Evidence for the interaction between nitric oxide and vasoactive intestinal polypeptide in the mouse gastric fundus. *J Pharmacol Exp Ther* **299**, 945-950.

Faussone-Pellegrini MS (1987). Comparative study of interstitial cells of Cajal. *Acta Anat (Basel)* **130**, 109-126.

Faussone-Pellegrini MS & Cortesini C (1985). Ultrastructural features and localization of the interstitial cells of Cajal in the smooth muscle coat of human esophagus. *J Submicrosc Cytol* **17**, 187-197.

Friedland GW, Kohatsu S, & Lewin K (1971). Comparative anatomy of feline and canine gastric sling fibers. Analogy to human anatomy. *Am J Dig Dis* **16**, 493-507.

Furness JB, Bornstein JC, Kunze WA, & Clerc N. (1999). The enteric nervous system and its extrinsic connections. In *Textbook of Gastroenterology*, ed. Yamada T, pp. 11-35. Lippincott-Raven, Philadelphia.

Furness JB, Costa M, & . (1987). *The enteric nervous system*, 3 ed., pp. 6-25. Churchil Living-stone, Edinburg,London, Melbourne, New YorK.

Gallego D, Hernandez P, Marti-Rague J, Clave P, & Jimenez M. P2YI receptor mediates inhibitory purinergic neuromuscular transmission in the human sigmoid colon. Gastroenterology 128[4], A13. 2005.
Ref Type: Abstract

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, & Burnstock G (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43**, 1313-1323.

Gidda JS & Goyal RK (1984). Swallow-evoked action potentials in vagal preganglionic efferents. *J Neurophysiol* **52**, 1169-1180.

Gonella J, Niel JP, & Roman C (1979). Sympathetic control of lower oesophageal sphincter motility in the cat. *J Physiol* **287**, 177-190.

Gonzalez AA, Farre R, Mones J, Capella G, & Clave P (2000). Pharmacological and molecular characterization of muscular cholecystokinin receptors in the human lower oesophageal sphincter. *Neurogastroenterol Motil* **12**, 539-546.

Goyal RK & Hirano I (1996). The enteric nervous system. *N Engl J Med* **334**, 1106-1115.

Goyal RK & Rattan S (1976). Genesis of basal sphincter pressure: effect of tetrodotoxin on lower esophageal sphincter pressure in opossum in vivo. *Gastroenterology* **71**, 62-67.

Goyal RK & Rattan S (1975). Nature of the vagal inhibitory innervation to the lower esophageal sphincter. *J Clin Invest* **55**, 1119-1126.

Grider JR, Murthy KS, Jin JG, & Makhlouf GM (1992). Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am J Physiol* **262**, G774-G778.

Grundy D (2002). Neuroanatomy of visceral nociception: vagal and splanchnic afferent. *Gut* **51 Suppl 1**, i2-i5.

Grundy D (1988). Vagal control of gastrointestinal function. *Baillieres Clin Gastroenterol* **2**, 23-43.

Hagger R, Finlayson C, Kahn F, De OR, Chimelli L, & Kumar D (2000). A deficiency of interstitial cells of Cajal in Chagasic megacolon. *J Auton Nerv Syst* **80**, 108-111.

Hagger R, Gharaie S, Finlayson C, & Kumar D (1998). Regional and transmural density of interstitial cells of Cajal in human colon and rectum. *Am J Physiol* **275**, G1309-G1316.

Harmar AJ, Arimura A, Gozes I, Journot L, Laburthe M, Pisegna JR, Rawlings SR, Robberecht P, Said SI, Sreedharan SP, Wank SA, & Waschek JA (1998). International Union of Pharmacology. XVIII. Nomenclature of receptors for vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide. *Pharmacol Rev* **50**, 265-270.

He CL, Burgart L, Wang L, Pemberton J, Young-Fadok T, Szurszewski J, & Farrugia G (2000). Decreased interstitial cell of cajal volume in patients with slow-transit constipation. *Gastroenterology* **118**, 14-21.

Hill LD, Kozarek RA, Kraemer SJ, Aye RW, Mercer CD, Low DE, & Pope CE (1996). The gastroesophageal flap valve: in vitro and in vivo observations. *Gastrointest Endosc* **44**, 541-547.

Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, & Bernstein A (1995). W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* **373**, 347-349.

Hyland NP, Abrahams TP, Fuchs K, Burmeister MA, & Hornby PJ (2001). Organization and neurochemistry of vagal preganglionic neurons innervating the lower esophageal sphincter in ferrets. *J Comp Neurol* **430**, 222-234.

Izumi N, Matsuyama H, Ko M, Shimizu Y, & Takewaki T (2003). Role of intrinsic nitrergic neurones on vagally mediated striated muscle contractions in the hamster oesophagus. *J Physiol* **551**, 287-294.

Izumi N, Matsuyama H, Yamamoto Y, Atoji Y, Suzuki Y, Unno T, & Takewaki T (2002). Morphological and morphometrical characteristics of the esophageal intrinsic nervous system in the golden hamster. *Eur J Morphol* **40**, 137-144.

Joachim CL, Mori H, & Selkoe DJ (1989). Amyloid beta-protein deposition in tissues other than brain in Alzheimer's disease. *Nature* **341**, 226-230.

Kadowaki M, Takeda M, Tokita K, Hanaoka K, & Tomoi M (2000). Molecular identification and pharmacological characterization of adenosine receptors in the guinea-pig colon. *Br J Pharmacol* **129**, 871-876.

Kenny SE, Connell MG, Rintala RJ, Vaillant C, Edgar DH, & Lloyd DA (1998). Abnormal colonic interstitial cells of Cajal in children with anorectal malformations. *J Pediatr Surg* **33**, 130-132.

Kerr KP, Stevenson JE, & Mitchelson F (1995). Simultaneous comparison of nicotinic receptor antagonists on three nicotinic acetylcholine receptors. *J Pharm Pharmacol* **47**, 1002-1006.

Knudsen MA, Svane D, & Tottrup A (1991). Importance of the L-arginine-nitric oxide pathway in NANC nerve function of the opossum esophageal body. *Dig Dis* **9**, 365-370.

Konomi H, Meedeniya AC, Simula ME, Toouli J, & Saccone GT (2002). Characterization of circular muscle motor neurons of the duodenum and distal colon in the Australian brush-tailed possum. *J Comp Neurol* **443**, 15-26.

Konturek JW, Gillessen A, & Domschke W (1995). Diffuse esophageal spasm: a malfunction that involves nitric oxide? *Scand J Gastroenterol* **30**, 1041-1045.

Kravitz JJ, Snape WJ, Jr., & Cohen S (1978). Effect of thoracic vagotomy and vagal stimulation on esophageal function. *Am J Physiol* **234**, E359-E364.

Krysiak PS & Preiksaitis HG (2001). Tachykinins contribute to nerve-mediated contractions in the human esophagus. *Gastroenterology* **120**, 39-48.

Kumano K, Fujimura M, Oshima S, Yamamoto H, Hayashi N, Nakamura T, & Fujimiya M (2001). Effects of VIP and NO on the motor activity of vascularly perfused rat proximal colon. *Peptides* **22**, 91-98.

Kunze WA & Furness JB (1999). The enteric nervous system and regulation of intestinal motility. *Annu Rev Physiol* **61**, 117-142.

Kupsky WJ, Grimes MM, Sweeting J, Bertsch R, & Cote LJ (1987). Parkinson's disease and megacolon: concentric hyaline inclusions (Lewy bodies) in enteric ganglion cells. *Neurology* **37**, 1253-1255.

Kuramoto H, Kawano H, Sakamoto H, & Furness JB (1999). Motor innervation by enteric nerve fibers containing both nitric oxide synthase and galanin immunoreactivities in the striated muscle of the rat esophagus. *Cell Tissue Res* **295**, 241-245.

Lander HM, Ogiste JS, Pearce SF, Levi R, & Novogrodsky A (1995). Nitric oxide-stimulated guanine nucleotide exchange on p21ras. *J Biol Chem* **270**, 7017-7020.

Langton P, Ward SM, Carl A, Norell MA, & Sanders KM (1989). Spontaneous electrical activity of interstitial cells of Cajal isolated from canine proximal colon. *Proc Natl Acad Sci U S A* **86**, 7280-7284.

Lee BH, Lynn RB, Lee HS, Miselis RR, & Altschuler SM (1992). Calcitonin gene-related peptide in nucleus ambiguus motoneurons in rat: viscerotopic organization. *J Comp Neurol* **320**, 531-543.

Liebermann-Meffert D, Allgower M, Schmid P, & Blum AL (1979). Muscular equivalent of the lower esophageal sphincter. *Gastroenterology* **76**, 31-38.

Lim I, Gibbons SJ, Lyford GL, Miller SM, Strege PR, Sarr MG, Chatterjee S, Szurszewski JH, Shah VH, & Farrugia G (2005). Carbon monoxide activates human intestinal smooth muscle L-type Ca2+ channels through a nitric oxide-dependent mechanism. *Am J Physiol Gastrointest Liver Physiol* **288**, G7-14.

Lincoln TM & Cornwell TL (1991). Towards An Understanding of the Mechanism of Action of Cyclic-Amp and Cyclic-Gmp in Smooth-Muscle Relaxation. *Blood Vessels* **28**, 129-137.

Lucas KA, Pitari GM, Kazerounian S, Ruiz-Stewart I, Park J, Schulz S, Chepenik KP, & Waldman SA (2000). Guanylyl cyclases and signaling by cyclic GMP. *Pharmacol Rev* **52**, 375-414.

Lui H, Vanderwinden JM, Ji P, & De Laet MH (1997). Nitric oxide synthase distribution in the enteric nervous system of children with cardiac achalasia. *Chin Med J (Engl)* **110**, 358-361.

Matsuda NM, Miller SM, Sha L, Farrugia G, & Szurszewski JH (2004). Mediators of non-adrenergic non-cholinergic inhibitory neurotransmission in porcine jejunum. *Neurogastroenterol Motil* **16**, 605-612.

Mazzia C & Clerc N (2000). Ultrastructural analysis of spinal primary afferent fibers within the circular muscle of the cat lower esophageal sphincter. *Histochem Cell Biol* **113**, 235-239.

Mearin F, Mourelle M, Guarner F, Salas A, Riveros-Moreno V, Moncada S, & Malagelada JR (1993). Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction. *Eur J Clin Invest* **23**, 724-728.

Mellow M (1977). Symptomatic diffuse esophageal spasm. Manometric follow-up and response to cholinergic stimulation and cholinesterase inhibition. *Gastroenterology* **73**, 237-240.

Miller SM, Reed D, Sarr MG, Farrugia G, & Szurszewski JH (2001). Haem oxygenase in enteric nervous system of human stomach and jejunum and co-localization with nitric oxide synthase. *Neurogastroenterol Motil* **13**, 121-131.

Mitsui R & Komuro T (2003). Distribution and ultrastructure of interstitial cells of Cajal in the gastric antrum of wild-type and Ws/Ws rats. *Anat Embryol (Berl)* **206**, 453-460.

Mitsui R & Komuro T (2002). Direct and indirect innervation of smooth muscle cells of rat stomach, with special reference to the interstitial cells of Cajal. *Cell Tissue Res* **309**, 219-227.

Mittal RK & Balaban DH (1997). The esophagogastric junction. *N Engl J Med* **336**, 924-932.

Moneta NA, McDonald TJ, & Cook MA (1997). Endogenous adenosine inhibits evoked substance P release from perifused networks of myenteric ganglia. *Am J Physiol* **272**, G38-G45.

Mule F & Serio R (2003). NANC inhibitory neurotransmission in mouse isolated stomach: involvement of nitric oxide, ATP and vasoactive intestinal polypeptide. *Br J Pharmacol* **140**, 431-437.

Murray J, Du C, Ledlow A, Bates JN, & Conklin JL (1991). Nitric oxide: mediator of nonadrenergic noncholinergic responses of opossum esophageal muscle. *Am J Physiol* **261**, G401-G406.

Nakahara M, Isozaki K, Hirota S, Vanderwinden JM, Takakura R, Kinoshita K, Miyagawa J, Chen H, Miyazaki Y, Kiyohara T, Shinomura Y, & Matsuzawa Y (2002). Deficiency of KIT-positive cells in the colon of patients with diabetes mellitus. *J Gastroenterol Hepatol* **17**, 666-670.

Neuhuber WL, Kressel M, Stark A, & Berthoud HR (1998). Vagal efferent and afferent innervation of the rat esophagus as demonstrated by anterograde DiI and DiA tracing: focus on myenteric ganglia. *J Auton Nerv Syst* **70**, 92-102.

Nicholls J, Brownhill VR, & Hourani SM (1996). Characterization of P1-purinoceptors on rat isolated duodenum longitudinal muscle and muscularis mucosae. *Br J Pharmacol* **117**, 170-174.

Ny L, Alm P, Larsson B, & Andersson KE (1997). Morphological relations between haem oxygenases, NO-synthase and VIP in the canine and feline gastrointestinal tracts. *J Auton Nerv Syst* **65**, 49-56.

Ordog T, Ward SM, & Sanders KM (1999). Interstitial cells of cajal generate electrical slow waves in the murine stomach. *J Physiol* **518 (Pt 1)**, 257-269.

Pandolfino JE, Shi G, Trueworthy B, & Kahrilas PJ (2003). Esophagogastric junction opening during relaxation distinguishes nonhernia reflux patients, hernia patients, and normal subjects. *Gastroenterology* **125**, 1018-1024.

Papasova M (1989). Sphincteric function. In *Handbook of physiology, The gastrointestinal system*, ed. Wood J.D., pp. 987-2023. American Physiological Society, Washington, DC.

Pasricha PJ, Ravich WJ, & Kalloo AN (1993). Effects of intrasphincteric botulinum toxin on the lower esophageal sphincter in piglets. *Gastroenterology* **105**, 1045-1049.

Paterson WG, Anderson MA, & Anand N (1992). Pharmacological characterization of lower esophageal sphincter relaxation induced by swallowing, vagal efferent nerve stimulation, and esophageal distention. *Can J Physiol Pharmacol* **70**, 1011-1015.

Pehlivanov N, Liu J, Kassab GS, Beaumont C, & Mittal RK (2002). Relationship between esophageal muscle thickness and intraluminal pressure in patients with esophageal spasm. *Am J Physiol Gastrointest Liver Physiol* **282**, G1016-G1023.

Pluja L, Fernandez E, & Jimenez M (1999). Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. *Br J Pharmacol* **126**, 883-892.

Pluja L, Fernandez E, & Jimenez M (2000). Electrical and mechanical effects of vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide in the rat colon involve different mechanisms. *Eur J Pharmacol* **389**, 217-224.

Porter AJ, Wattchow DA, Brookes SJ, & Costa M (2002). Cholinergic and nitrergic interneurones in the myenteric plexus of the human colon. *Gut* **51**, 70-75.

Poyner DR, Sexton PM, Marshall I, Smith DM, Quirion R, Born W, Muff R, Fischer JA, & Foord SM (2002). International Union of Pharmacology. XXXII. The mammalian calcitonin gene-related peptides, adrenomedullin, amylin, and calcitonin receptors. *Pharmacol Rev* **54**, 233-246.

Preiksaitis HG & Diamant NE (1997). Regional differences in cholinergic activity of muscle fibers from the human gastroesophageal junction. *Am J Physiol* **272**, G1321-G1327.

Preiksaitis HG, Tremblay L, & Diamant NE (1994b). Cholinergic responses in the cat lower esophageal sphincter show regional variation. *Gastroenterology* **106**, 381-388.

Preiksaitis HG, Tremblay L, & Diamant NE (1994a). Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. *Dig Dis Sci* **39**, 770-775.

Prosser CL, Holzwarth MA, & Barr L (1989). Immunocytochemistry of the interstitial cells of Cajal in the rat intestine. *J Auton Nerv Syst* **27**, 17-25.

Ralevic V & Burnstock G (1998). Receptors for purines and pyrimidines. *Pharmacol Rev* **50**, 413-492.

Ramón y Cajal R (1904). *Textura del sistema nervioso del hombre y de los vertebrados* Madrid.

Ratcliffe EM, deSa DJ, Dixon MF, & Stead RH (1998). Choline acetyltransferase (ChAT) immunoreactivity in paraffin sections of normal and diseased intestines. *J Histochem Cytochem* **46**, 1223-1231.

Rattan S & Goyal RK (1978). Evidence of 5-HT participation in vagal inhibitory pathway to opossum LES. *Am J Physiol* **234**, E273-E276.

Rattan S & Goyal RK (1974). Neural control of the lower esophageal sphincter: influence of the vagus nerves. *J Clin Invest* **54**, 899-906.

Raymond L, Lach B, & Shamji FM (1999). Inflammatory aetiology of primary oesophageal achalasia: an immunohistochemical and ultrastructural study of Auerbach's plexus. *Histopathology* **35**, 445-453.

Richter JE (2001). Oesophageal motility disorders. *Lancet* **358**, 823-828.

Rolle U, Yoneda A, Solari V, Nemeth L, & Puri P (2002). Abnormalities of C-Kit-positive cellular network in isolated hypoganglionosis. *J Pediatr Surg* **37**, 709-714.

Roman C & Gonella J (1981). Extrinsec Control of Digestive Tract Motility. In *Physiology of the Gastrointestinal Tract*, ed. Johnson RL, pp. 289-333. Raven Press, New York.

Romert P & Mikkelsen HB (1998). c-kit immunoreactive interstitial cells of Cajal in the human small and large intestine. *Histochem Cell Biol* **109**, 195-202.

Rossiter CD, Norman WP, Jain M, Hornby PJ, Benjamin S, & Gillis RA (1990). Control of lower esophageal sphincter pressure by two sites in dorsal motor nucleus of the vagus. *Am J Physiol* **259**, G899-G906.

Ruhl A, Franzke S, Collins SM, & Stremmel W (2001). Interleukin-6 expression and regulation in rat enteric glial cells. *Am J Physiol Gastrointest Liver Physiol* **280**, G1163-G1171.

Ruiz-de-Leon A, Mendoza J, Sevilla-Mantilla C, Fernandez AM, Perez-de-la-Serna J, Gonzalez VA, Rey E, Figueredo A, az-Rubio M, & De-la-Concha EG (2002). Myenteric antiplexus antibodies and class II HLA in achalasia. *Dig Dis Sci* **47**, 15-19.

Ryan JP, Snape WJ, Jr., & Cohen S (1977). Influence of vagal cooling on esophageal function. *Am J Physiol* **232**, E159-E164.

Sanders KM (2000). Postjunctional electrical mechanisms of enteric neurotransmission. *Gut* **47 Suppl 4**, iv23-iv25.

Sanders KM, Ordog T, & Ward SM (2002). Physiology and pathophysiology of the interstitial cells of Cajal: from bench to bedside. IV. Genetic and animal models of GI motility disorders caused by loss of interstitial cells of Cajal. *Am J Physiol Gastrointest Liver Physiol* **282**, G747-G756.

Sandler AD, Maher JW, Weinstock JV, Schmidt CD, Schlegel JF, Jew JY, & Williams TH (1991). Tachykinins in the canine gastroesophageal junction. *Am J Surg* **161**, 165-170.

Sang Q & Young HM (1997). Development of nicotinic receptor clusters and innervation accompanying the change in muscle phenotype in the mouse esophagus. *J Comp Neurol* **386**, 119-136.

Sang Q & Young HM (1998). The origin and development of the vagal and spinal innervation of the external muscle of the mouse esophagus. *Brain Res* **809**, 253-268.

Sann H, Hoppe S, Baldwin L, Grundy D, & Schemann M (1998). Presence of putative neurotransmitters in the myenteric plexus of the gastrointestinal tract and in the musculature of the urinary bladder of the ferret. *Neurogastroenterol Motil* **10**, 35-47.

Shuttleworth CW, Murphy R, Furness JB, & Pompolo S (1991). Comparison of the presence and actions of substance P and neurokinin A in guinea-pig taenia coli. *Neuropeptides* **19**, 23-34.

Sifrim D, Janssens J, & Vantrappen G (1992). A wave of inhibition precedes primary peristaltic contractions in the human esophagus. *Gastroenterology* **103**, 876-882.

Sigala S, Missale G, Missale C, Villanacci V, Cestari R, Grigolato PG, Lojacono L, & Spano PF (1995). Different neurotransmitter systems are involved in the development of esophageal achalasia. *Life Sci* **56**, 1311-1320.

Sivarao DV, Mashimo HL, Thatte HS, & Goyal RK (2001). Lower esophageal sphincter is achalasic in nNOS(-/-) and hypotensive in W/W(v) mutant mice. *Gastroenterology* **121**, 34-42.

Smid SD & Blackshaw LA (2000). Vagal ganglionic and nonadrenergic noncholinergic neurotransmission to the ferret lower oesophageal sphincter. *Auton Neurosci* **86**, 30-36.

Soediono P & Burnstock G (1994). Contribution of ATP and nitric oxide to NANC inhibitory transmission in rat pyloric sphincter. *Br J Pharmacol* **113**, 681-686.

Stamler JS (1994). Redox signaling: nitrosylation and related target interactions of nitric oxide. *Cell* **78**, 931-936.

Stein HJ, Liebermann-Meffert D, DeMeester TR, & Siewert JR (1995). Three-dimensional pressure image and muscular structure of the human lower esophageal sphincter. *Surgery* **117**, 692-698.

Storch WB, Eckardt VF, Wienbeck M, Eberl T, Auer PG, Hecker A, Junginger T, & Bosseckert H (1995). Autoantibodies to Auerbach's plexus in achalasia. *Cell Mol Biol (Noisy -le-grand)* **41**, 1033-1038.

Sundler F, Ekblad E, Absood A, Hakanson R, Koves K, & Arimura A (1992). Pituitary adenylate cyclase activating peptide: a novel vasoactive intestinal peptide-like neuropeptide in the gut. *Neuroscience* **46**, 439-454.

Terauchi A, Kobayashi D, & Mashimo H (2005). Distinct Roles of Nitric Oxide Synthases and Interstitial Cells of Cajal in Rectoanal Relaxation. *Am J Physiol Gastrointest Liver Physiol*.

Todorov S, Pozzoli C, Zamfirova R, & Poli E (2003). Prejunctional modulation of non-adrenergic non-cholinergic (NANC) inhibitory responses in the isolated guinea-pig gastric fundus. *Neurogastroenterol Motil* **15**, 299-306.

Tomita R, Tanjoh K, Fujisaki S, & Fukuzawa M (2001). The role of nitric oxide (NO) in the human internal anal sphincter. *J Gastroenterol* **36**, 386-391.

Tong WD, Liu BH, Zhang LY, Zhang SB, & Lei Y (2004). Decreased interstitial cells of Cajal in the sigmoid colon of patients with slow transit constipation. *Int J Colorectal Dis* **19**, 467-473.

Vanderwinden JM, Liu H, De Laet MH, & Vanderhaeghen JJ (1996a). Study of the interstitial cells of Cajal in infantile hypertrophic pyloric stenosis. *Gastroenterology* **111**, 279-288.

Vanderwinden JM, Rumessen JJ, Liu H, Descamps D, De Laet MH, & Vanderhaeghen JJ (1996b). Interstitial cells of Cajal in human colon and in Hirschsprung's disease. *Gastroenterology* **111**, 901-910.

Vanneste G, Robberecht P, & Lefebvre RA (2004). Inhibitory pathways in the circular muscle of rat jejunum. *Br J Pharmacol* **143**, 107-118.

Vicente Y, Da RC, Yu J, Hernandez-Peredo G, Martinez L, Perez-Mies B, & Tovar JA (2001). Architecture and function of the gastroesophageal barrier in the piglet. *Dig Dis Sci* **46**, 1899-1908.

Wang XY, Paterson C, & Huizinga JD (2003). Cholinergic and nitrergic innervation of ICC-DMP and ICC-IM in the human small intestine. *Neurogastroenterol Motil* **15**, 531-543.

Ward SM, Beckett EA, Wang X, Baker F, Khoyi M, & Sanders KM (2000). Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. *J Neurosci* **20**, 1393-1403.

Ward SM, Morris G, Reese L, Wang XY, & Sanders KM (1998). Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. *Gastroenterology* **115**, 314-329.

Watkins CC, Boehning D, Kaplin AI, Rao M, Ferris CD, & Snyder SH (2004). Carbon monoxide mediates vasoactive intestinal polypeptide-associated nonadrenergic/noncholinergic neurotransmission. *Proc Natl Acad Sci U S A* **101**, 2631-2635.

Weisbrodt NW & Christensen J (1972). Gradients of contractions in the opossum esophagus. *Gastroenterology* **62**, 1159-1166.

Wise J & Conklin JL (2004). Gastroesophageal reflux disease and baclofen: is there a light at the end of the tunnel? *Curr Gastroenterol Rep* **6**, 213-219.

Wood J.D. (1989). Electrical and synaptic behavior of enteric neurons. In *Handbook of physiology*, ed. Wood J.D., pp. 465-517. American Physiological Society, Bethesda.

Xue L, Farrugia G, Miller SM, Ferris CD, Snyder SH, & Szurszewski JH (2000a). Carbon monoxide and nitric oxide as coneurotransmitters in the enteric nervous system: evidence from genomic deletion of biosynthetic enzymes. *Proc Natl Acad Sci U S A* **97**, 1851-1855.

Xue L, Farrugia G, & Szurszewski JH (2000b). Effect of exogenous ATP on canine jejunal smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **278**, G725-G733.

Xue S, Valdez D, Collman PI, & Diamant NE (1996). Effects of nitric oxide synthase blockade on esophageal peristalsis and the lower esophageal sphincter in the cat. *Can J Physiol Pharmacol* **74**, 1249-1257.

Yamato S, Saha JK, & Goyal RK (1992a). Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci* **50**, 1263-1272.

Yamato S, Spechler SJ, & Goyal RK (1992b). Role of nitric oxide in esophageal peristalsis in the opossum. *Gastroenterology* **103**, 197-204.

Yip L, Kwok YN, & Buchan AM (2003). Cellular localization and distribution of neurokinin-1 receptors in the rat stomach. *Auton Neurosci* **104**, 95-108.

Yu CS, Kim HC, Hong HK, Chung DH, Kim HJ, Kang GH, & Kim JC (2002). Evaluation of myenteric ganglion cells and interstitial cells of Cajal in patients with chronic idiopathic constipation. *Int J Colorectal Dis* **17**, 253-258.

Yunker AM, Krause JE, & Roth KA (1999). Neurokinin B- and substance P-like immunoreactivity are co-localized in enteric nerves of rat ileum. *Regul Pept* **80**, 67-74.

Zarate N, Wang XY, & Huizinga JD. Ultrastructural injury to ICC is evident in the early stages of achalasia and is associated with mast cell infiltration. Gastroenterology 126[4], A111. 2004.
Ref Type: Abstract

Zhou DS, Desaki J, & Komuro T (1996). Neuro-muscular junctions of longitudinal and circular muscle fibers of the guinea-pig esophagus and their relation to myenteric plexus. *J Auton Nerv Syst* **58**, 63-68.

Zizzo MG, Mule F, & Serio R (2004). Interplay between PACAP and NO in mouse ileum. *Neuropharmacology* **46**, 449-455.

Capítol 1

Different responsiveness of excitatory and inhibitory enteric motor neurons in the human esophagus to electrical field stimulation and nicotine

1.1. Abstract

To compare electrical field stimulation (EFS) with nicotine in the stimulation of excitatory and inhibitory enteric motor neurones (EMN) in the human oesophagus. Circular lower oesophageal sphincter (LES) and circular and longitudinal esophageal body (EB) strips from 20 humans were studied in organ baths. Responses to EFS or nicotine 100 μM were compared in basal conditions, following L-NNA 100μM, and following L-NNA and apamin 1 μM. LES strips developed myogenic tone enhanced by tetrodotoxin 5 μM (TTX) or L-NNA. EFS-LES relaxation was abolished by TTX, unaffected by hexamethonium 100 μM, and enhanced by atropine 3 μM. Nicotine-LES relaxation was higher than EFS-relaxation, reduced by TTX or atropine, and blocked by hexamethonium. Following L-NNA, EFS elicited a strong cholinergic contraction in circular LES and EB, and nicotine a small relaxation in LES and no contractile effect in EB. Following L-NNA and apamin, EFS elicited a strong cholinergic contraction in LES and EB, and nicotine a weak contraction amounting to 6.64±3.19% and 9.20±5.51% of that induced by EFS. EFS elicited a contraction in longitudinal strips; following L-NNA and apamin, nicotine did not induce any response.

Inhibitory EMN tonically inhibit myogenic LES tone, and are efficiently stimulated both by EFS and nicotinic acetylcholine receptors (nAChRs) located in somatodendritic regions and nerve terminals, releasing NO and an apamin-sensitive neurotransmitter. In

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contrast, although oesophageal excitatory EMN are efficiently stimulated by EFS, their stimulation through nAChRs is difficult and causes a weak response, suggesting the participation of non-nicotinic mechanisms in neurotransmission to excitatory EMN in human oesophagus.

1.2. Introduction

The two main physiologic motor events in the human oesophagus are the swallowing-induced primary peristalsis and LES relaxation, and the transient LES relaxation that causes physiological gastro-oesophageal reflux and allows belching. Stimuli triggering both motor events are integrated in the CNS, conveyed to the dorsal motor nucleus of the vagus which contains the preganglionic neurones, and travel along a few vagal efferent fibres to a huge number of EMN located in the myenteric plexus (MP) (Christensen J, 1994;Hornby & Abrahams, 2000). The oesophageal MP is a network of interconnected ganglia that also have integrative functions independent of the CNS that determine the peristaltic nature of the oesophageal body contraction and LES relaxation, and modulate LES resting tone (Christensen J, 1994;Paterson *et al.*, 1992). The oesophageal EMN have their cell bodies in the myenteric ganglia, and their motor axons travel in small nerve bundles, penetrating the longitudinal and circular smooth muscle layers (Christensen J, 1994;Hornby & Abrahams, 2000). Efforts during recent years have been directed at characterising the final neurotransmitters involved in the terminal motor pathways of EMN to the oesophageal smooth muscle, but little is known about the physiology of the neuronal circuitry that directly controls oesophageal peristalsis, LES tone, and LES relaxation. *In vitro* studies on human oesophageal strips using direct activation of EMN by electrical field stimulation (EFS) have shown that LES relaxation is mainly caused by nitric oxide (NO) released from inhibitory EMN, while oesophageal contraction is mainly caused by acetylcholine (ACh) released from excitatory EMN acting on muscarinic receptors located in the smooth muscle (Krysiak & Preiksaitis, 2001;Preiksaitis *et al.*, 1994).

The vagal efferent fibres synapse with EMN in myenteric ganglia, and the overall consensus is that vagal fibres can stimulate both inhibitory and excitatory EMN (Hornby & Abrahams, 2000). Acetylcholine released by vagal fibres in the LES of guinea pigs acts upon inhibitory motor neurones through nicotinic and muscarinic receptors (Goyal & Rattan, 1975), although in most animals a serotoninergic antagonist is also required to completely antagonise ganglionic transmission in this pathway

(Rattan & Goyal, 1978). Nevertheless, there is some controversy over the existence and nature of the vagal input to the excitatory EMN, as they appear to lack direct vagal nicotinic inputs in the rat (Coruzzi *et al.*, 1985) and guinea pig (Yuan *et al.*, 1998) LES. On the other hand, excitatory and inhibitory enteric motor reflexes caused by distention or mucosal stimulation are also inhibited by nAChR antagonists (Schneider *et al.*, 2000). There is little data available on the receptors and interneurones involved in neural transmission from vagal pathways and interneurones to EMN in the smooth muscle of the human oesophagus.

The aim of the present study was to characterise, *in vitro,* the effect of stimulation with nicotine upon intrinsic excitatory and inhibitory EMN in the smooth muscle of the human oesophagus, and to compare it with the effect of direct stimulation by EFS.

1.3. Methods

1.3.1. Preparations

Twenty specimens including part of the gastric fundus, the gastro-oesophageal junction (GOJ), and the EB were obtained from eight patients with oesophageal cancer and twelve organ donors (female/male=1.2, mean age=56.3±4.5 years). The study protocol had been previously approved by the Institutional Review Board of the Hospital de la Santa Creu i Sant Pau, that of the Hospital de Mataró, and that of the Hospital Clínic i Provincial, Barcelona, Spain. The specimens were opened following the lesser curvature of the stomach, the transitional line was marked with sutures and the mucosa was resected. Consecutive 3 mm-wide strips, including the circular muscle, the longitudinal muscle and the MP, were cut parallel to the circular muscle fibres. Four to six consecutive strips were obtained from each specimen, from both the right (clasp) and/or the left (sling) side of the GOJ starting 3 mm distal to the zone of the transitional line (Preiksaitis & Diamant, 1997), and four to six circular strips were obtained from the EB, 6-9 cm above the transitional line. In five specimens, full-thickness longitudinal strips were also obtained from the EB, 6-9 cm above the transitional line. Strips were always obtained from macroscopically non-invaded regions.

1.3.2. Procedures

Studies started within the 18 h-period following resection. Final strips measuring 10 mm in length were placed in 25 ml-organ baths containing Krebs solution constantly bubbling with 5% CO_2 in O_2 (Clave *et al.*, 1998;Tottrup *et al.*, 1990a). Changes in tension of the strips were measured using isometric force transducers and recorded on a chart recorder (model 03 Force Transducer and model 7 Series Polygraph, Grass Instruments Co, Quincy, MA). In each experiment, up to six strips from the same specimen were simultaneously studied. After an equilibration period of one hour, strips were stretched to 150% of their initial length and positioned between two parallel platinum wire electrodes 10 mm apart (Tottrup *et al.*, 1990a;Tottrup *et al.*, 1990c). Thereafter, the tension of most strips taken from the GOJ progressively increased over the following 1-2 hours. This increase in tension was defined as the active resting tone (Tottrup *et al.*, 1990c).

1.3.3. Experimental design

The influence of EMN on active LES resting tone was studied by measuring the changes in tension of LES strips after exposure to tetrodotoxin 5 µM (TTX, Sigma, St. Louis, MO, USA), to hexamethonium 100 µM (HEX, Sigma), to N^G-nitro-L-arginine 100 µM (L-NNA, Sigma), to atropine 3 µM (ATR, Braun Medical S.A., Barcelona, Spain); and the myogenic contribution was evaluated after 10 min of exposure to a Krebs calcium-free buffer [14]. The concentrations of TTX and L-NNA had previously been shown to abolish maximal EFS-induced relaxation [12-14]. The effect of these antagonists was evaluated 30 minutes after their being added to the tissue bath. Responses of circular LES and circular and longitudinal EB strips to EFS and nicotine were assessed: a) at basal conditions, b) during nitrergic blockade by L-NNA 100 µM, and c) during simultaneous blockade by L-NNA and apamin (APA, Sigma) 1 µM. The concentration of apamin was the same as that which abolished the relaxation induced by exogenous ATP 10 mM or PACAP 10µM in porcine LES in our previous studies (Farré *et al.*, 2002).

1.3.4 Electrical field stimulation (EFS)

EFS was applied by means of an electrical stimulator (Model S88, Grass Instruments Co) and a power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO) in order to obtain four identical and undistorted signals. Pulses of 0.4 ms duration and 0.3-70 Hz frequency were delivered in 5 s-trains using supramaximally

effective voltage which had been previously set at 26 V (Clave *et al.*, 1998;Tottrup *et al.*, 1990a). LES and EB responses to EFS were characterised using TTX 5 μM, hexamethonium 100 μM, or atropine 3 μM. The concentration of atropine was the same as that which had abolished the response of LES strips to carbachol 10 μM (Sigma) in our previous studies on human LES (Gonzalez *et al.*, 2000).

1.3.5. Stimulation of nicotinic acetylcholine receptors (nAChRs)

The effect of nicotine (NIC, Sigma) was evaluated by exposing the strips to the drug (2-100 μM) for 2 minutes. After washing the strips with 100 ml of fresh buffer there was a 30-minute period before the next exposure. Repetitive additions of nicotine (20 μM) did not result in desensitisation of nAChRs (not shown). LES and EB response to nicotine (100 μM) were characterised by HEX, TTX 10 μM, or ATR. The effect of nicotine was studied on circular LES and EB strips at rest, on circular EB strips precontracted with carbachol 2 μM, and on longitudinal EB strips.

1.3.6. Data analysis

The effect of EFS or pharmacological agents was determined in terms of changes in tone. Relaxation was expressed in g and/or in the percentage of active LES resting tone or carbachol-induced tone in EB strips. Contraction was expressed in g. The number of experiments was represented by n (number of strips) and N (number of specimens). Values were expressed as mean ± SEM. Student-t test was selected for comparisons, using the paired mode when appropriate. Statistical significance was accepted if $P<0.05$.

1.4. Results

1.4.1. Control of LES resting tone and effect of EFS and nicotine on LES strips

75 strips taken from the GEJ of 17 specimens that developed active tone and relaxed upon EFS were considered as pertaining to the LES. Mean resting tone was 3.6±0.36 g. Both TTX 5 μM and L-NNA 100 μM significantly increased ($P<0.01$) LES resting tone by 13.43±2.47 (n=11, N=6) and 21.54±2.9% (n=20, N=9), respectively. Neither hexamethonium 100 μM (n=6, N=4) nor atropine 3 μM (n=5, N=4) significantly affected resting tone**.** Atropine also failed to significantly modify LES resting tone following TTX (-2.20±0.35% n=4, N=2) or L-NNA, (-0.76±0.23%, n=4, N=2). Exposition

for 10 min of the LES strips to a Krebs calcium-free buffer significantly decreased resting tone by 47.5±4.8% (n=8, N=4).

At basal conditions, EFS induced an isolated "on" relaxation during stimulus in 35/75 LES strips, and an "on" relaxation followed by an "off" contraction at the end of the stimulus in 40/75 (fig 1A).

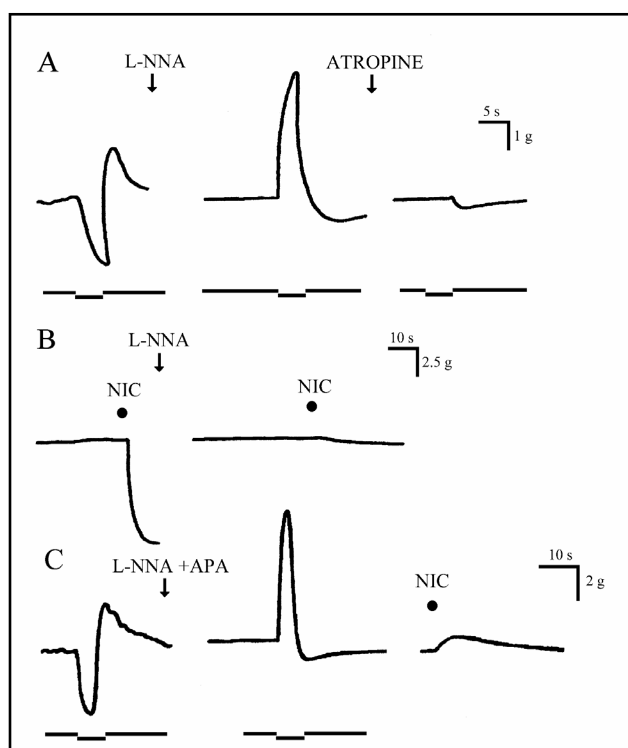

Figure 1. Tracings showing the effect of EFS (10 Hz, pulses indicated below tracings) or nicotine (100 µM, dots above tracings) on LES strips. A. EFS-induced responses ("on" relaxation, "off" contraction). L-NNA transformed this response into an "on" contraction (central panel) that was fully blocked by atropine. Following L-NNA and atropine, a slight "off" relaxation appeared. B. Nicotine-induced LES relaxation (left panel) was inhibited but not blocked by L-NNA. C. EFS induced a strong cholinergic contraction during simultaneous blockade by L-NNA and APA; in this experimental setting, NIC induced a slight cholinergic contraction

When strips from the same specimen were compared, LES strips that showed biphasic responses to EFS were always obtained from higher parts of the LES than the strips that showed only an isolated "on" relaxation (n=16, N=8). The amplitude of both "on" and "off" responses was frequency-dependent. Maximal relaxation in lower LES strips was observed at 10 Hz (3.54±0.45 g). Maximal relaxation in upper LES strips was observed at 3 Hz (3.35±0.41 g, NS vs. lower, fig 2A), and maximal "off" contraction (1.60±0.25 g) at 3 Hz (fig. 2B, n=22, N=11). EFS-induced relaxation was unaffected by HEX 100 µM, completely blocked by TTX 5 µM and significantly enhanced by ATR (fig.3). In all LES strips, L-NNA 100 µM transformed the "on" relaxation into an "on" contraction during EFS (fig 1A) which was also frequency-dependent and of higher amplitude than the "off" contraction, (fig 2B), and fully abolished by atropine 3 µM (fig. 2B, n=7, N=3). A slight

"off" relaxation following L-NNA and atropine appeared in 5/7 of these strips (fig 1A); this "off" relaxation was fully blocked by TTX 5 µM.

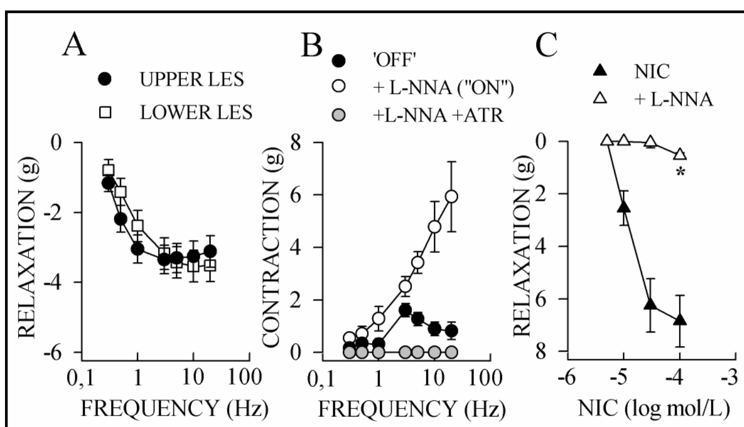

Figure 2. A. Frequency-dependent relaxation curves induced by EFS in upper (n=12, N=4) and lower (n=11, n=5) LES strips showing similar profiles. B. L-NNA changed the "on" relaxation to a frequency-dependent "on" contraction during EFS which was fully blocked by atropine (n=12, N=6). C. Nicotine induced a dose-dependent relaxation (n=5, N=3). Following NO blockade by L-NNA, a residual but significant relaxation was still induced by 100 µM nicotine (n=4, N=3, * = p<0.05)

Nicotine relaxed LES strips in a concentration-dependent manner (fig 2C). Mean relaxation induced by the highest concentration tested (100 µM), was 4.44±0.68 g (n=14, N=7), significantly higher than that obtained by EFS 10 Hz (2.32±0.41, n=15, N=7, p<0.01). HEX (100 µM) completely blocked the maximal response to nicotine. In contrast, TTX (10µM) significantly decreased by 38±7.6%, but did not block, the relaxation induced by nicotine, and ATR significantly inhibited the NIC induced relaxation by 88.45±11.55% (n=5, N=2, fig 3). L-NNA 100 µM abolished the relaxation induced by nicotine at concentrations lower than 30 µM; at 100 µM, nicotine induced a slow relaxation accounting for 0.54±0.19 g (p<0.05, fig. 1B and fig. 2C). Nicotine 100 µM did not elicit any contraction in circular LES strips in the presence of L-NNA 100 µM. Following simultaneous blockade with L-NNA and Apamin, EFS induced a strong cholinergic "on" contraction (6.28±1.65 g at 10 Hz, n=8, N=3) in all LES strips; in contrast, in the same experimental setting, NIC only elicited a slight contraction (0.27±0.15 g, n=8, N=3) amounting to 6.64±3.19% of that induced by EFS on the same strips (fig 4).

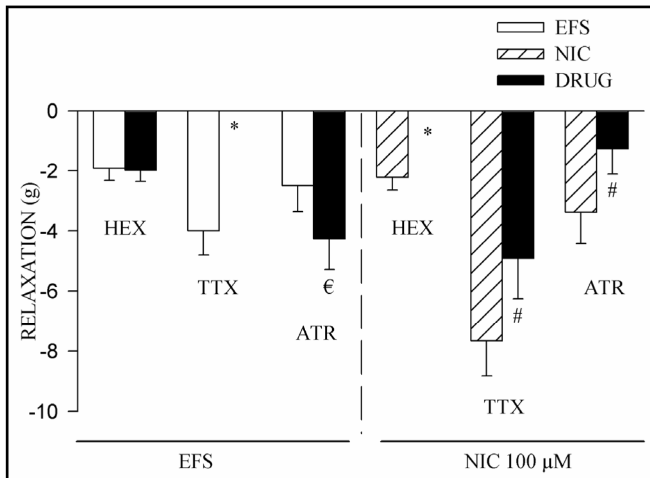

Figure 3. Effect of the neurotoxin TTX, the ganglionic blocker HEX, and ATR on EFS (5 Hz) or nicotine-induced LES relaxation (#,*,€ = p<0.05 vs control; * = blockade, # = inhibition, € = increase). HEX did not affect EFS-relaxation (n=5, N=3) and fully blocked nicotine-induced relaxation (n=3, N=2). TTX fully blocked EFS-induced relaxation (n=5, N=3); in contrast, relaxation induced by nicotine was significantly reduced but not abolished by TTX (n=4, N=2) suggesting the presence of somatodendritic as well as nerve terminal nAChRs in nitrergic EMN. ATR significantly enhanced EFS-induced relaxation (10 Hz, n=5, N=3) and, in contrast, significantly inhibited nicotine-induced relaxation (n=5, N=3)

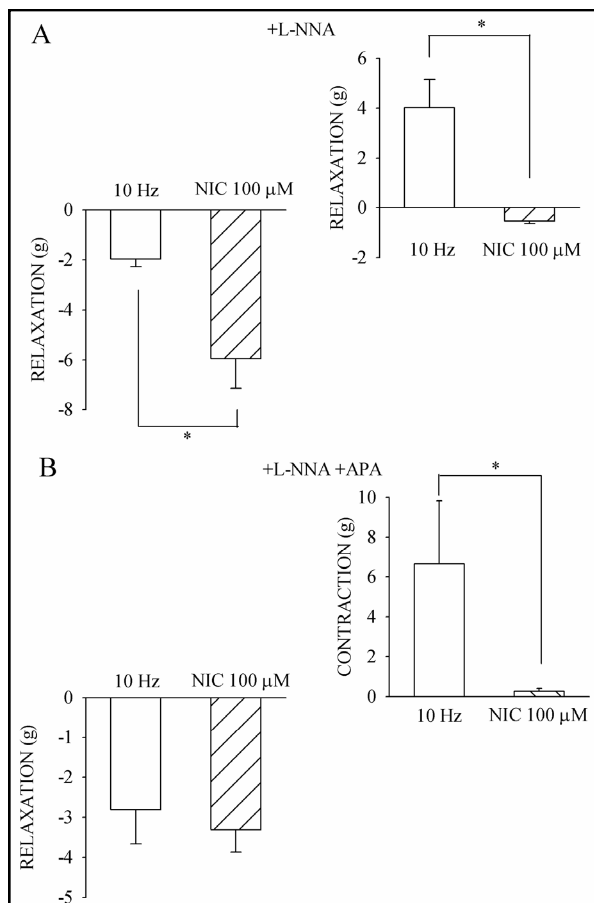

Figure 4. Quantitative effects of stimulation of LES EMN by EFS or nicotine. At basal conditions (A and B left panels) inhibitory LES EMN were efficiently stimulated by both EFS and nAChRs (A: n=7, N=3; B: n=8, N=3). A. Following Nitrergic blockade, EFS induced a strong cholinergic contraction showing intense stimulation of excitatory EMN, and, in the same experimental conditions, stimulation of nAChRs still induced a slight relaxation. B. During simultaneous blockade with L-NNA and apamin, EFS also induced a strong contraction and nicotine induced a slight cholinergic contraction amounting to 6.64% of that induced by EFS and showing less efficient stimulation of excitatory LES EMN through nAChRs. (* = p<0.05)

1.4.2. Effect of EFS and nicotine on circular and longitudinal EB strips

Thirty-eight circular strips taken from the EB of 11 specimens did not develop active resting tone. EFS induced two types of contractions on circular EB strips. During the application of the stimulus, 50% strips responded with slight mono- or multiphasic "on" contractions and, briefly, after cessation of the stimulus, a more prominent "off" contraction emerged (fig 5A) in all strips. The amplitude of both contractions was frequency-dependent and "off" contractions were higher than "on" contractions ($p<0.05$, fig 6A). Atropine 3 µM fully abolished "on" contractions; in contrast it only partially antagonised "off" contractions (fig. 6A). L-NNA 100 µM transformed the response to EFS into a frequency-dependent monophasic "on" contraction (fig 5A) of higher amplitude than the "off" contraction (17.02±1.48 g vs. 4.73±4.04 g at 20 Hz, $p<0.05$) which was totally blocked by atropine 3 µM at 0.5-20 Hz (fig 6B).

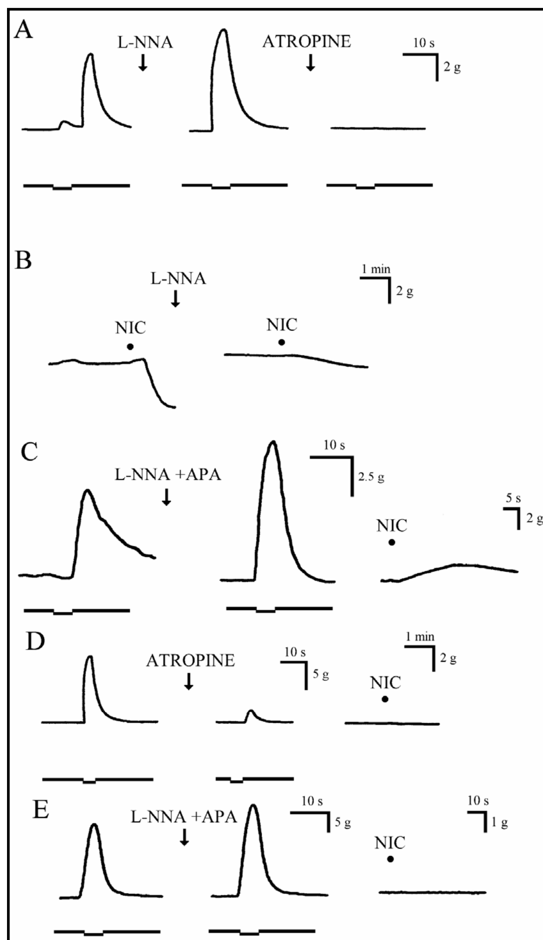

Figure 5. Representative tracings showing the response of human EB strips to EFS (20 Hz, pulses indicated below tracings) or to nicotine (100 µM, dots above tracings). A. Left panel: control response including "on" contraction, latency and "off" contraction. Central panel: L-NNA transformed the response to EFS into a monophasic "on" contraction that was totally blocked by atropine (right). B. Nicotine induced a relaxation in circular EB strips precontracted with carbachol. L-NNA antagonised, but did not block, the relaxation induced by nicotine (central). C. During simultaneous nitrergic and purinergic blockade, EFS induced a strong "on" contraction and nicotine elicited a slight cholinergic contraction in circular EB strips (right). D. Longitudinal EB strips responded to EFS with an "on" contraction that was antagonised by atropine, and a minor "off" contraction appeared (center). Nicotine did not induce any effect. E. In longitudinal strips pre-treated by L-NNA and APA, EFS induced an "on" contraction (center) and nicotine did not induce any effect (right)

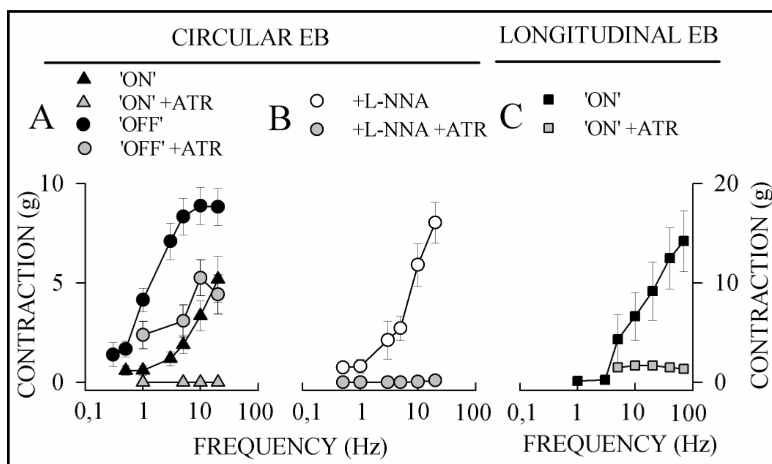

Figure 6. Frequency-response curves of EB strips. <u>Circular EB strips:</u> A. EFS-induced "off" contractions (n=24, N=9) were of higher amplitude than "on" contractions (n=19, N=9). "On" contractions were fully cholinergic (n=5, N=4) whereas "off" contractions were only partly inhibited by atropine (n=5, N=4). B. EFS induced only "on" contractions in the presence of L-NNA that were fully blocked by atropine (n=6, N=4). <u>Longitudinal EB strips:</u> C. EFS-induced "on" contractions that were antagonised but not blocked by atropine (n=6, N=3)

Nicotine (20-100 µM) did not elicit any mechanical response in EB circular strips at rest (n=4, N=2). In EB strips precontracted at 5.3±0.6 g by 2 µM carbachol, nicotine 100 µM induced an immediate relaxation (fig 5B), decreasing the carbachol-induced tone by 82.2±5.8% (n=6, N=2). The relaxation induced by nicotine was completely blocked by HEX 100 µM, and antagonised by pretreatment with 100 µM L-NNA, but a remaining relaxation of 0.83±0.3 g ($p<0.05$) on the carbachol-induced tone was still elicited (fig. 5B). Following simultaneous blockade with L-NNA and apamin, EFS induced a strong cholinergic "on" contraction (16.56±1.57g at 20Hz) and NIC elicited a slight cholinergic contraction (1.49±1.02 g, n=9, N=3), 9.20±5.51% of that induced by EFS (fig 7B).

Longitudinal EB strips did not develop active resting tone. EFS induced a frequency-dependent "on" contraction on longitudinal EB strips (fig 5D) reaching 14.03±2.52 g at 70 Hz (fig 6C). Atropine 3 µM significantly reduced (from 90.2±9.8% at 10Hz to 94.39±3.21% at 70 Hz) the amplitude of longitudinal contractions (fig 7C) and evoked the appearance of a residual low-amplitude "off" contraction following EFS (fig. 6C). Nicotine (20 µM -100 µM) did not elicit any mechanical response in longitudinal EB strips at rest (n=5, N=3). Following L-NNA and apamin, EFS also induced a strong cholinergic "on" contraction and nicotine did not induce any response (n=6, N=3, fig 7C).

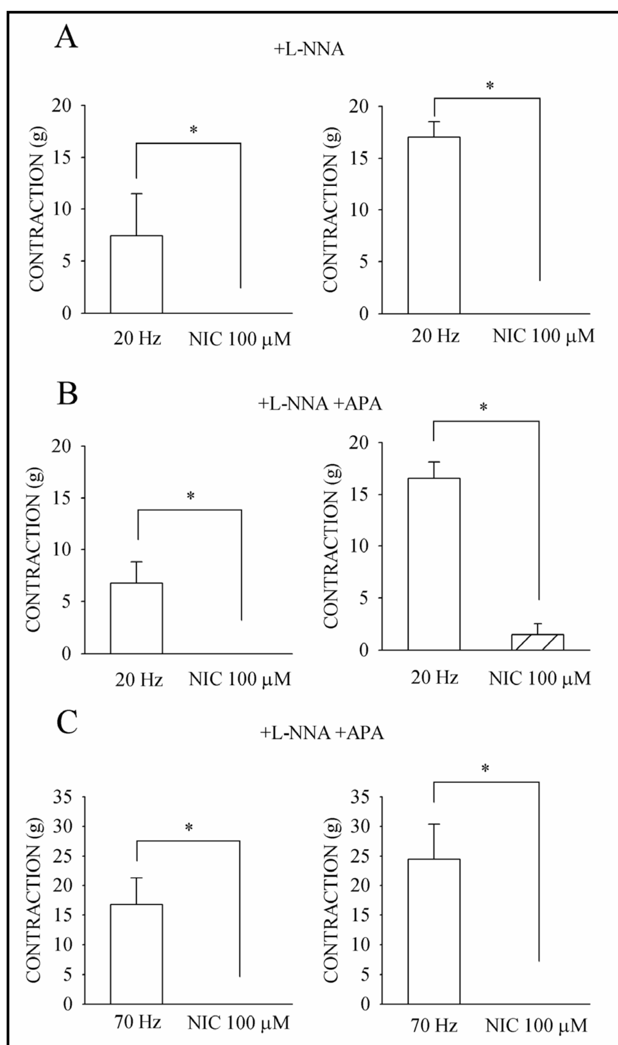

Figure 7. Quantitative effects of stimulation of LES EMN by EFS or nicotine: At basal conditions (A and B left panels) stimulation of inhibitory LES by EFS caused an "off" contraction, in contrast nicotine did not induce any effect. A. Following Nitrergic blockade, EFS induced a strong cholinergic "on" contraction showing intense stimulation of excitatory EMN, and, in the same experimental conditions, stimulation of nAChRs did not induce any effect. B. During simultaneous blockade with L-NNA and apamin, EFS also induced a strong contraction and stimulation of nAChRs a weak cholinergic contraction amounting to 9.20% of that induced by EFS and suggesting a less efficient stimulation of excitatory LES EMN. C. EFS also induced an "on" contraction in longitudinal EB strips at rest and also in those pre-treated by L-NNA and apamin. Nicotine did not induce any affect on longitudinal strips in both experimental settings

1.5. Discussion

We have found that inhibitory EMN in the human oesophagus are easily and efficiently stimulated both by EFS and by nAChRs located in somatodendritic regions as well as in nerve terminals. In contrast, although oesophageal excitatory EMN can be efficiently stimulated by EFS even at basal conditions, its stimulation through nAChRs is very difficult, requiring a full blockade of inhibitory motor pathways and causing a weak response, suggesting the participation of non-nicotinic mechanisms in ganglionic neurotransmission to excitatory oesophageal EMN in humans. We have also shown that inhibitory EMN tonically inhibit LES resting tone, and that NO is the main but not the only inhibitory neurotransmitter released by inhibitory EMN, suggesting a minor role for an apamin-sensitive neurotransmitter in human oesophagus.

The human LES is constituted by the sling muscle on the angle of Hiss and the clasp component in lesser curvature, which develop greater active tone. This explains the asymmetry of LES pressure *in vivo* (Preiksaitis & Diamant, 1997). We studied a mixture of strips coming from both LES sides although, due to our method of dissection, we probably obtained more sling strips than clasp-like fibres, thus influencing our resting LES tone values. Our results show a sharp reduction in LES resting tone following 10 min exposure to the clacium-free buffer, further confirming that human LES tone is mainly myogenic and depends on extracellular calcium (Tottrup *et al.*, 1990c). There is controversy over the relative contribution of extra- and intracellular calcium sources on myogenic LES tone (Biancani *et al.*, 1987;Salapatek *et al.*, 1998a), and we cannot exclude a further reduction or even abolition of active tone by prolongation of exposition of LES strips to a calcium-free medium (Biancani *et al.*, 1987). On the other hand, the relative neurogenic contribution to LES tone may vary according to the species of animal. Previous studies on human LES strips failed to demonstrate any effect of TTX (Preiksaitis & Diamant, 1997); however i*n vivo,* NO synthase inhibitors increased LES resting pressure in animal (Boulant *et al.*, 1994;Xue *et al.*, 1996;Yamato *et al.*, 1992), and human (Konturek *et al.*, 1997) studies. A muscular as well as a neural constitutive NO synthase could be responsible for this NO-mediated inhibitory tonic influence on the LES muscle (Salapatek *et al.*, 1998b). In our study, the similarity to the increase in LES tone induced by TTX and L-NNA suggests that, in humans, NO tonically released from inhibitory EMN continuously inhibits the myogenic LES resting tone. The lack of effect of atropine suggests the absence of an intrinsic cholinergic contribution to LES tone *in vitro,* agreeing with previous *in vitro* studies on the human LES (Preiksaitis & Diamant, 1997). *In vivo*, atropine reduced LES pressure in healthy humans (Dodds *et al.*, 1981;Mittal *et al.*, 1995), and hyoscine butylbromide, a muscarinic antagonist which does not cross the blood-brain barrier, did not alter LES pressure (Lidums *et al.*, 1997). Altogether, these findings strongly suggest that the atropine-induced fall in LES pressure in humans occurs at the CNS rather than at peripheral sites.

In our study, EFS exclusively activates EMN during stimulus without direct activation of muscle (Clave *et al.*, 1998;Richards *et al.*, 1995;Tottrup *et al.*, 1990a;Tottrup *et al.*, 1990c) or preganglionic fibres because the neurotoxin TTX blocks and the ganglionic blocker hexamethonium did not affect EFS-induced responses. The simultaneous activation of both excitatory and inhibitory EMN by EFS is shown in our

LES experiments by the selective blockade of inhibitory neurotransmitters (the relaxation disappears and is replaced by a contraction induced by activation of excitatory EMN) or blockade of the excitatory neurotransmitters (the relaxation increases). We used full thickness oesophageal preparations that included the MP in order to compare these well-characterised neural responses induced by EFS with those induced by nicotinic stimulation of EMN. Most oesophageal EMN have single process Dogiel type I morphology and the soma located in myenteric ganglia. In the LES of opossums, inhibitory EMN are larger and with longer projections than the excitatory EMN, and the proportion of local excitatory and inhibitory EMN is similar and about 50% (Brookes *et al.*, 1996). In the human intestine, circular inhibitory EMN also have larger cell bodies and larger projections (3.2 mm) than the excitatory EMN (2 mm) (Porter *et al.*, 1997), and EMN to longitudinal muscle were smaller than those to the circular muscle (Porter *et al.*, 1996;Wattchow *et al.*, 1995). Based on these morphological data, we inferred that our human oesophageal circular preparations also contain a significant number and a similar proportion of both local excitatory and inhibitory EMN. Immunohistochemical and functional studies have shown that nAChRs are located in somatodendritic regions of EMN in myenteric ganglia (Schneider *et al.*, 2000), and nAChRs that cause transmitter release by action potential-independent mechanisms have also recently been described in nerve terminals of excitatory EMN (Galligan, 1999;Schneider *et al.*, 2000). In our study, nicotine-induced LES relaxation was partly antagonised by higher concentrations of TTX than those that fully blocked EFS-relaxation and more than 30 times higher than those used to block nerve-mediated effects in previous studies (Galligan, 1999;Schneider *et al.*, 2000). Our data suggests the presence of nAChRs in the soma as well as in the nerve terminals of human LES inhibitory EMN, providing strong evidence for functional integrity of myenteric ganglia in our preparation. It also allows an adequate experimental model for the comparison of the responses elicited by stimulation of both intact and undamaged excitatory and inhibitory EMN. Our preparation is transmural and contains the circular as well as the longitudinal muscular layer and all the components of MP including myenteric ganglia, excitatory and inhibitory EMN, and is not merely a muscle-axon preparation as nicotine did not induce any effect on human muscle-axon LES preparations without MP (Tottrup *et al.*, 1990a;Tottrup *et al.*, 1990c).

Integrated neuromuscular responses to EFS in our study were similar to those previously reported in the human oesophagus (Burleigh, 1979;Preiksaitis & Diamant, 1997). Our results further confirm that NO is the main mediator for EFS-relaxation in the

human LES (Preiksaitis *et al.*, 1994) and suggest the release of one or more minor inhibitory neurotransmitters, as an “off” relaxation sensitive to TTX appeared in our study following nitrergic blockade (Uc *et al.*, 1999). Our study also agrees with previous studies that showed a relaxant effect through stimulation of nAChRs by nicotine or DMPP in human LES strips (Burleigh, 1979;Smid & Blackshaw, 2000) also mainly mediated by NO. Immunomorphological studies in the human oesophagus found that most nitrergic EMN also contain VIP, galanin and PACAP (Ny *et al.*, 1995;Singaram *et al.*, 1994;Uddman *et al.*, 1991); and VIP, PACAP and adenosine relaxed human LES strips in functional studies (Ny *et al.*, 1995;Tottrup *et al.*, 1990b). We recently found nitrergic and apamin-sensitive inhibitory co-transmission on porcine LES (Farré *et al.*, 2002), very similar to that found by Yuan on the opossum (Yuan *et al.*, 1998). In our previous study on porcine LES, apamin inhibited the relaxation induced by ATP or PACAP (Farré *et al.*, 2002), and ATP has been proposed as the mediator for the apamin-sensitive component in the opossum LES (Yuan *et al.*, 1998). What we found in the present study on human LES is indirect evidence for an apamin-sensitive inhibitory neuromuscular co-neurotransmitter, as nicotine induced a relaxation following nitrergic blockade and a small contraction following simultaneous blockade with L-NNA and apamin. Further studies will ascertain the specific co-neurotransmitter involved in human LES relaxation and explore its interaction with NO.

We compared the responses induced by direct stimulation of EMN by EFS with those induced through stimulation of nAChRs. In our human study, LES stimulation by nicotine in basal conditions caused a more intense relaxation than that induced by EFS, both being LES inhibitory responses of comparable magnitude and suggesting high efficiency of stimulation of inhibitory EMN. Simultaneous blockade of inhibitory neurotransmitters by L-NNA and APA was required in opossum LES in order to show focal EFS-induced stimulation of excitatory EMN (Yuan *et al.*, 1998). This was not the case in our study as a significant number of circular LES strips responded in basal conditions to EFS with “off” contractions and circular EB strips responded with both "on" and "off" frequency-dependent contractions (Richards *et al.*, 1995;Tottrup *et al.*, 1990a;Tottrup *et al.*, 1990c). The "on" contraction was abolished by atropine, clearly showing its origin in cholinergic EMN. The latency depended on activation of inhibitory EMN and it was fully blocked by NO synthesis inhibitors, as shown in other studies (Christensen J, 1994;Preiksaitis *et al.*, 1994). In contrast, the "off" contraction was only partly antagonised by atropine (Christensen J, 1994;Krysiak & Preiksaitis, 2001;Richards

et al., 1995;Tottrup *et al.*, 1990a) suggesting the participation of a second non-cholinergic mechanism such as rebound depolarisation (Paterson *et al.*, 1991), or a second excitatory neurotransmitter, such as tachykinins (Krysiak & Preiksaitis, 2001). We explored the effect of nicotine on circular LES and EB circular strips at basal conditions and following blockade of NO synthesis representing the majority of inhibitory innervation. We expected nicotine would strongly stimulate nAChRs on cholinergic EMN and make the strips contract, but it had no effect. However, EFS easily induced a strong stimulation of excitatory EMN in both human LES and EB under the same experimental conditions during nitrergic blockade. It could be argued that the experiment must prove that inhibitory EMNs are completely suppressed prior to stimulation of excitatory EMNs with nicotine in order to explain why nicotine does not contract the muscle strips. Residual effect of the inhibitory EMNs after blockade with L-NNA may be strong enough to negate the effects of nicotine on excitatory EMNs; however this is not the case for EFS that acts as our “control” of a strong direct activation of excitatory EMN in identical experimental conditions. In addition, our experimental design included the comparison between EFS and nAChRs stimulation of EMN during simultaneous blockade by L-NNA and Apamin in a third set of experiments. In order to enhance our observations, the comparisons between EFS and nAChRs-mediated responses were always performed in pairs, each strip acting as its own control. In this experimental setting, stimulation of nAChRs induced a slight contraction in LES and EB accounting for 6.64% and 9.20% respectively, of that obtained by EFS, and showing that nAChRs stimulate excitatory EMN much less efficiently than EFS. Two additional observations further confirmed the low sensitivity of excitatory oesophageal EMN to nAChRs stimulation. First, as an isolated but significant observation, we found -as others have (Misiewicz *et al.*, 1969)- a strong direct stimulation of excitatory EMN by EFS and no responses to nicotine in patients with achalasia and fully impaired inhibitory neurotransmission (not shown). Second, that atropine inhibits nicotine-LES relaxation (the opposite effect than on EFS relaxation) in our study, an effect very similar to that caused by atropine during vagal efferent stimulation (Goyal & Rattan, 1975). As we cannot attribute this effect to direct pharmacological antagonism (Heller J, 2001), we speculate that the effect caused by atropine during LES stimulation by nAChRs could be exerted on an interneuronal synapse, overriding the effect of atropine on excitatory EMN at a neuromuscular level, and further showing a strong stimulation by nAChRs of a polysynaptic inhibitory motor pathway and a weak stimulation of the excitatory one. In

addition, the differences between the responsiveness of excitatory EMN to EFS and to nicotine are also clearly observed in our experiments with longitudinal strips. As few inhibitory EMN were found in the outer longitudinal smooth muscle layer of human oesophagi in morphological studies (Singaram *et al.*, 1994), no relaxant responses to EFS could be evoked in longitudinal strips from human LES and EB strips (Tottrup *et al.*, 1990c), and EFS induced an "on" contraction in our basal studies. Stimulation of nAChRs in these conditions did not induce any contraction, nor were any responses obtained by nicotine following simultaneous blockade by L-NNA and apamin in longitudinal studies. Thus, the low responsiveness of oesophageal excitatory EMN to stimulation through nAChRs was observed in our study of circular LES and EB circular strips as well as in the longitudinal EB layer.

Classical *in vivo* studies by Rattan and Goyal on the opossum suggested that the vagi do not mediate any influence upon excitatory LES EMN and the vagal influence on the sphincter is entirely inhibitory (Goyal & Rattan, 1975;Rattan & Goyal, 1974). These authors also found that nicotine only stimulated inhibitory EMN (Rattan & Goyal, 1975) and suggested that excitatory EMN may lie in an extravagal sympathetic pathway to the LES (Rattan & Goyal, 1974). Recent studies on the same animal also found that vagal stimulation did not evoke measurable excitatory responses even following complete blockade of inhibitory LES pathways by L-NNA and apamin, suggesting that preganglionic vagal efferent pathways probably activate LES inhibitory EMN more efficiently than excitatory, or the excitatory lack direct vagal inputs (Yuan *et al.*, 1998). These results also agreed with those of our study but did not show how cholinergic EMN in LES and EB were stimulated during primary and secondary peristalsis. I*n vivo* studies found hexamethonium-sensitive transmission to excitatory EMN in rat LES (Kawahara *et al.*, 1997), contrasting with *in vitro* studies also in the LES of the same animal showing the absence of direct nicotinic inputs to excitatory EMN (Coruzzi *et al.*, 1985), and suggesting a polysynaptic pathway. In addition, recent studies in mice found completely separate sets of preganglionic neurones located in different parts of dorsal motor nucleus of the vagus projecting onto nitrergic or cholinergic LES EMN (Goyal *et al.*, 2001), providing an anatomical base for the physiological differences observed. Otherwise, synaptic transmission in the ganglia of the autonomic nervous system is classically described as mediated by acetylcholine acting on nAChRs; however, ganglionic neurotransmission in the oesophagus can include important non-nicotinic and also non-cholinergic synaptic responses. On the one hand, classical studies

characterized the vagal efferent pathway to the opossum LES by means of both vagal efferent stimulation and intramural EFS, and found that muscarinic (M1) and serotoninergic (5HT3) receptors also mediate vagal inputs to inhibitory EMN (Gilbert *et al.*, 1984;Paterson *et al.*, 1992) probably representing a mechanism of reserve or redundancy of ganglionic transmission in this pathway [6]. We failed to show this pathway in the present study, as the muscarinic ganglionic stimulant McN-A-343 (Gilbert *et al.*, 1984) induced a contraction very similar to that induced by carbachol and was also unaffected by TTX in our LES preparations (not shown) suggesting an effect on muscular receptors. On the other hand, recent studies on animal models found up to three subsets of EMNs in the MP that could be distinguished on the basis of the neurotransmitters producing fast excitatory postsynaptic potentials (fEPSPs) in each subset. In one subset (representing only 25%-36% of EMNs), fEPSPs was mediated solely by Ach acting at nAChRs. In the other subsets, fEPSPs were mixed with a slight contribution of Ach acting on nAChRs and a strong contribution of ATP acting on purine P2X receptors, or 5-HT acting on 5-HT(3) receptors (Galligan *et al.*, 2000;Galligan, 2002). The relevance of each of these alternative mechanisms of ganglionic neurotransmission can vary along the gastrointestinal tract and its role on neurotransmission to excitatory oesophageal EMN needs further investigation.

Our results suggest that while vagal fibres or interneurones could easily and efficiently stimulate inhibitory EMN through nAChRs in human LES and EB, full stimulation of intrinsic excitatory EMN requires other neurotransmitters or other circuits. Little is known about the anatomy and physiology of the neuronal circuitry that directly controls the smooth muscle of the oesophagus. Understanding these circuits could improve our current pharmacological approach of transient LES relaxations and gastro-oesophageal reflux disease, and also our current treatments for primary and secondary oesophageal motor disorders. Further studies are needed to describe these neuronal circuits and to assess its relevance in health and disease.

References

Biancani P, Hillemeier C, Bitar KN, & Makhlouf GM (1987). Contraction mediated by Ca2+ influx in esophageal muscle and by Ca2+ release in the LES. *Am J Physiol* **253**, G760-G766.

Boulant J, Fioramonti J, Dapoigny M, Bommelaer G, & Bueno L (1994). Cholecystokinin and nitric oxide in transient lower esophageal sphincter relaxation to gastric distention in dogs. *Gastroenterology* **107**, 1059-1066.

Brookes SJ, Chen BN, Hodgson WM, & Costa M (1996). Characterization of excitatory and inhibitory motor neurons to the guinea pig lower esophageal sphincter. *Gastroenterology* **111**, 108-117.

Burleigh DE (1979). The effects of drugs and electrical field stimulation on the human lower oesophageal sphincter. *Arch Int Pharmacodyn Ther* **240**, 169-176.

Christensen J CJ (1994). Motor functions of the pharynx and esophagus. In *Physiology of the Gastrointestinal Tract*, ed. Johnson LR, pp. 903-928. New York.

Clave P, Gonzalez A, Moreno A, Lopez R, Farre A, Cusso X, D'mato M, Azpiroz F, & Lluis F (1998). Endogenous cholecystokinin enhances postprandial gastroesophageal reflux in humans through extrasphincteric receptors. *Gastroenterology* **115**, 597-604.

Coruzzi G, Poli E, & Bertaccini G (1985). Action of different agonists and antagonists of the cholinergic system on the rat lower esophageal sphincter. *Gen Pharmacol* **16**, 561-566.

Dodds WJ, Dent J, Hogan WJ, & Arndorfer RC (1981). Effect of atropine on esophageal motor function in humans. *Am J Physiol* **240**, G290-G296.

Farré R, Martinez E, Aulí M, Jimenez M, & Clave P. Nitric oxide, adenosine triphosphate and vasoactive intestinal peptide interactions in lower oesophageal sphincter relaxation. Neurogastroentrol Mot 14, 588. 2002.
Ref Type: Abstract

Galligan JJ (1999). Nerve terminal nicotinic cholinergic receptors on excitatory motoneurons in the myenteric plexus of guinea pig intestine. *J Pharmacol Exp Ther* **291**, 92-98.

Galligan JJ (2002). Ligand-gated ion channels in the enteric nervous system. *Neurogastroenterol Motil* **14**, 611-623.

Galligan JJ, LePard KJ, Schneider DA, & Zhou X (2000). Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* **81**, 97-103.

Gilbert R, Rattan S, & Goyal RK (1984). Pharmacologic identification, activation and antagonism of two muscarine receptor subtypes in the lower esophageal sphincter. *J Pharmacol Exp Ther* **230**, 284-291.

Gonzalez AA, Farre R, Mones J, Capella G, & Clave P (2000). Pharmacological and molecular characterization of muscular cholecystokinin receptors in the human lower oesophageal sphincter. *Neurogastroenterol Motil* **12**, 539-546.

Goyal RK, Padmanabhan R, & Sang Q (2001). Neural circuits in swallowing and abdominal vagal afferent-mediated lower esophageal sphincter relaxation. *Am J Med* **111 Suppl 8A**, 95S-105S.

Goyal RK & Rattan S (1975). Nature of the vagal inhibitory innervation to the lower esophageal sphincter. *J Clin Invest* **55**, 1119-1126.

Heller J TP (2001). Muscarinic receptor agonists and antagonists. In *The pharmacological basis of therapeutics*, ed. Hardman JG LJaGGA, pp. 155-175.

Hornby PJ & Abrahams TP (2000). Central control of lower esophageal sphincter relaxation. *Am J Med* **108 Suppl 4a**, 90S-98S.

Kawahara H, Blackshaw LA, Lehmann A, & Dent J (1997). Responses of the rat lower oesophageal sphincter (LOS) to vagal efferent activation. *Neurogastroenterol Motil* **9**, 85-97.

Konturek JW, Thor P, Lukaszyk A, Gabryelewicz A, Konturek SJ, & Domschke W (1997). Endogenous nitric oxide in the control of esophageal motility in humans. *J Physiol Pharmacol* **48**, 201-209.

Krysiak PS & Preiksaitis HG (2001). Tachykinins contribute to nerve-mediated contractions in the human esophagus. *Gastroenterology* **120**, 39-48.

Lidums I, Bermingham H, & Holloway RH (1997). Effect of hyoscine butylbromide on gastroesophageal reflux and lower esophageal sphincter function in healthy volunteers. *Gastroenterology* **112**, A776.

Misiewicz JJ, Waller SL, Anthony PP, & Gummer JW (1969). Achalasia of the cardia: pharmacology and histopathology of isolated cardiac sphincteric muscle from patients with and without achalasia. *Q J Med* **38**, 17-30.

Mittal RK, Holloway R, & Dent J (1995). Effect of atropine on the frequency of reflux and transient lower esophageal sphincter relaxation in normal subjects. *Gastroenterology* **109**, 1547-1554.

Ny L, Larsson B, Alm P, Ekstrom P, Fahrenkrug J, Hannibal J, & Andersson KE (1995). Distribution and effects of pituitary adenylate cyclase activating peptide in cat and human lower oesophageal sphincter. *Br J Pharmacol* **116**, 2873-2880.

Paterson WG, Anderson MA, & Anand N (1992). Pharmacological characterization of lower esophageal sphincter relaxation induced by swallowing, vagal efferent nerve stimulation, and esophageal distention. *Can J Physiol Pharmacol* **70**, 1011-1015.

Paterson WG, Hynna-Liepert TT, & Selucky M (1991). Comparison of primary and secondary esophageal peristalsis in humans: effect of atropine. *Am J Physiol* **260**, G52-G57.

Porter AJ, Wattchow DA, Brookes SJ, & Costa M (1997). The neurochemical coding and projections of circular muscle motor neurons in the human colon. *Gastroenterology* **113**, 1916-1923.

Porter AJ, Wattchow DA, Brookes SJ, Schemann M, & Costa M (1996). Choline acetyltransferase immunoreactivity in the human small and large intestine. *Gastroenterology* **111**, 401-408.

Preiksaitis HG & Diamant NE (1997). Regional differences in cholinergic activity of muscle fibers from the human gastroesophageal junction. *Am J Physiol* **272**, G1321-G1327.

Preiksaitis HG, Tremblay L, & Diamant NE (1994). Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. *Dig Dis Sci* **39**, 770-775.

Rattan S & Goyal RK (1978). Evidence of 5-HT participation in vagal inhibitory pathway to opossum LES. *Am J Physiol* **234**, E273-E276.

Rattan S & Goyal RK (1975). Effect of nicotine on the lower esophageal sphincter. Studies on the mechanism of action. *Gastroenterology* **69**, 154-159.

Rattan S & Goyal RK (1974). Neural control of the lower esophageal sphincter: influence of the vagus nerves. *J Clin Invest* **54**, 899-906.

Richards WG, Stamler JS, Kobzik L, & Sugarbaker DJ (1995). Role of nitric oxide in human esophageal circular smooth muscle in vitro. *J Thorac Cardiovasc Surg* **110**, 157-164.

Salapatek AM, Lam A, & Daniel EE (1998a). Calcium source diversity in canine lower esophageal sphincter muscle. *J Pharmacol Exp Ther* **287**, 98-106.

Salapatek AM, Wang YF, Mao YK, Mori M, & Daniel EE (1998b). Myogenic NOS in canine lower esophageal sphincter: enzyme activation, substrate recycling, and product actions. *Am J Physiol* **274**, C1145-C1157.

Schneider DA, Perrone M, & Galligan JJ (2000). Nicotinic acetylcholine receptors at sites of neurotransmitter release to the guinea pig intestinal circular muscle. *J Pharmacol Exp Ther* **294**, 363-369.

Singaram C, Sengupta A, Sweet MA, Sugarbaker DJ, & Goyal RK (1994). Nitrinergic and peptidergic innervation of the human oesophagus. *Gut* **35**, 1690-1696.

Smid SD & Blackshaw LA (2000). Neuromuscular function of the human lower oesophageal sphincter in reflux disease and Barrett's oesophagus. *Gut* **46**, 756-761.

Tottrup A, Forman A, Funch-Jensen P, Raundahl U, & Andersson KE (1990a). Effects of transmural field stimulation in isolated muscle strips from human esophagus. *Am J Physiol* **258**, G344-G351.

Tottrup A, Forman A, Madsen G, & Andersson KE (1990b). The actions of some beta-receptor agonists and xanthines on isolated muscle strips from the human oesophago-gastric junction. *Pharmacol Toxicol* **67**, 340-343.

Tottrup A, Forman A, Uldbjerg N, Funch-Jensen P, & Andersson KE (1990c). Mechanical properties of isolated human esophageal smooth muscle. *Am J Physiol* **258**, G338-G343.

Uc A, Oh ST, Murray JA, Clark E, & Conklin JL (1999). Biphasic relaxation of the opossum lower esophageal sphincter: roles of NO., VIP, and CGRP. *Am J Physiol* **277**, G548-G554.

Uddman R, Luts A, Absood A, Arimura A, Ekelund M, Desai H, Hakanson R, Hambreaus G, & Sundler F (1991). PACAP, a VIP-like peptide, in neurons of the esophagus. *Regul Pept* **36**, 415-422.

Wattchow DA, Brookes SJ, & Costa M (1995). The morphology and projections of retrogradely labeled myenteric neurons in the human intestine. *Gastroenterology* **109**, 866-875.

Xue S, Valdez D, Collman PI, & Diamant NE (1996). Effects of nitric oxide synthase blockade on esophageal peristalsis and the lower esophageal sphincter in the cat. *Can J Physiol Pharmacol* **74**, 1249-1257.

Yamato S, Saha JK, & Goyal RK (1992). Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci* **50**, 1263-1272.

Yuan S, Costa M, & Brookes SJ (1998). Neuronal pathways and transmission to the lower esophageal sphincter of the guinea Pig. *Gastroenterology* **115**, 661-671.

Capítol 2

Pharmacologic characterization of intrinsic mechanisms controlling tone and relaxation of porcine lower esophageal sphincter

2.1. Abstract

The neurotransmitters mediating relaxation of lower esophageal sphincter (LES) were studied by using circular LES strips from adult pigs in organ baths. LES relaxation by sodium nitroprusside (SNP, 1 nM-3 μM), VIP and PACAP (1 nM-1 μM), ATP (10 μM-30 mM), and tricarbonyldichlororuthenum dimer (CORM-1 1 μM-1 mM) was unaffected by tetrodotoxin (TTX 1 μM) or L-NAME (100 μM). Calcitonin gene-related peptide (CGRP 1 nM-1 μM) unaffected LES tone. ATP-relaxation was blocked by 1 μM apamin and the $P2Y_1$ antagonist N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate (MRS2179 10 μM). Apamin inhibited PACAP-relaxation. VIP- and PACAP-relaxation was blocked by α-chymotrypsin 10 U/ml. L-NAME (-62.52±13.13%) and ODQ 10 μM (-67.67±6.80%) similarly inhibited EFS-relaxation, and apamin blocked non-nitrergic relaxation. Nicotine-relaxation (100 μM) was inhibited by L-NAME (-60.37±10.8%) and ODQ (-41.90±7.89%); and apamin also blocked non-nitergic relaxation. Non-nitrergic and apamin-sensitive LES relaxation caused by EFS or nicotine was strongly inhibited by MRS2179, slightly inhibited by α-chymotrypsin and the $P2X_{1,2,3}$ receptor antagonist NF279 (10 μM), and unaffected by selective heme oxygenase inhibitor tin protoporphyrin IX (100 μM). Porcine LES relaxation following stimulation of intrinsic inhibitory motor neurons is mediated by two main neuromuscular pathways: a) NO through guanylate cyclase signaling and apamin-insensitive mechanisms, and b) by nonintrergic apamin-sensitive neurotransmission mainly mediated by ATP or a related purine acting on $P2Y_1$

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receptors but also with a minor contribution of purinergic P2X receptors and PACAP on LES relaxation. Nitrergic and purinergic co-transmitters show parallel effects of similar magnitude without major interplay. Our study shows no role for CGRP, and only a minor one for VIP and CO in porcine LES relaxation.

2.2. Introduction

The lower esophageal sphincter (LES) acts as a barrier at the gastroesophageal junction (GEJ). Basal tone of LES is primarily myogenic in origin and is modulated by a combination of hormonal factors and neurogenic mechanisms that are not yet fully understood and involve local enteric motor neurons (EMN) and extrinsic nerves. Non-adrenergic, non-cholinergic (NANC) EMN are the final step in the inhibitory vagal pathway to LES, allowing swallowing-induced and transient LES relaxation that cause physiologic gastroesophageal reflux and belching (Chang *et al.*, 2003). Vasoactive intestinal peptide (VIP), NO, ATP, pituitary adenylate cyclase-activating peptide (PACAP), carbon monoxide (CO), and calcitonin gene-related peptide (CGRP) have been proposed as putative neurotransmitters for these inhibitory EMN in LES on the basis of morphological and physiological studies. Colocalization of these neurotransmitters and/or their synthesizing enzymes on inhibitory EMN of the upper gastrointestinal tract including LES has been described by immunohistological and other morphological studies (Ny *et al.*, 1994;Ny *et al.*, 1995a;Uc *et al.*, 1997;Werkstrom *et al.*, 1997). Physiological and pharmacological studies have demonstrated the direct effects or characterized the actions of these neurotransmitters following stimulation of inhibitory LES EMNs (Imaeda & Cunnane, 2003;Ny *et al.*, 1995b;Ny *et al.*, 1997;Uc *et al.*, 1997;Yuan *et al.*, 1998). However, evidence suggests that NO from neural sources is the major contributor to LES relaxation (Gonzalez *et al.*, 2004;Murray *et al.*, 1991;Tottrup *et al.*, 1991), and the relative physiological contribution of other neurotransmitters remains unclear. On the other hand, several mechanisms of interaction between these neurotransmitters have been proposed: a) release "in parallel" and independent actions on specific postjunctional sites (cotransmission) (Burnstock, 2004), b) effects coupled "in series" with NO-mediated release of other neurotransmitters (Grider *et al.*, 1992), or pre- and/or postjunctional modulation of NO synthesis by other neurotransmitters (Ergun & Ogulener, 2001;Mashimo *et al.*, 1996). The underlying mechanisms and physiological relevance of these interactions on LES have not been discovered. Most of these studies focused on the mechanisms of LES relaxation during simultaneous and direct electrical

stimulation of excitatory and inhibitory EMN and little data is available on the effects of these inhibitory neurotransmitters following other more specific stimuli for inhibitory EMN. In a recent *in vitro* study on human LES, we found that inhibitory EMNs are efficiently stimulated both by EFS and nicotinic acetylcholine receptors (AChRs) located in somatodendritic regions and nerve terminals, whereas esophageal excitatory EMNs are also efficiently stimulated by EFS but their stimulation through nicotinic AChRs is difficult and causes a weak response (Gonzalez *et al.*, 2004).

The porcine gastrointestinal tract possesses anatomic and pathological similarities to that of humans and similar organization of the enteric nervous system, differing from small laboratory animals, and has been used as a homologous animal model for the development of new pharmacologic strategies to treat human neurogastrointestinal disorders (Brown & Timmermans, 2004;Pasricha *et al.*, 1993). The size, histology (smooth muscle cells) and neurochemical code of porcine LES EMNs are similar to that of humans (Aggestrup *et al.*, 1986;Pasricha *et al.*, 1993). The aim of the present study was to characterize the neuromyogenic mechanisms and neurotransmitters (NTs) controlling tone and relaxation of porcine lower esophageal sphincter (LES) following stimulation of inhibitory EMNs by EFS and through nicotinic AChRs.

2.3. Methods

2.3.1. Preparations

Specimens including part of the gastric fundus, the gastro-esophageal junction (GEJ), and the esophageal body were obtained from 53 adult pigs (age, 6 months; weight, 75-80 Kg). Animals were stunned and killed by exsanguination in a slaughterhouse in compliance with specific national laws following the guidelines of the European Union (Pérez Rubalcaba A., 1995) . Specimens were immediately collected, placed in carbogenated Krebs solution at 4ºC and transported to the laboratory within 1 hour. The GEJ was opened along the greater curvature, the mucosa and submucosa were resected at the squamocolumnar union, and clasp and sling fibers composing LES were identified (Preiksaitis & Diamant, 1997). Full thickness preparations including the circular and longitudinal muscle layers as well as the myenteric plexus were obtained by cutting 3mm-wide strips parallel to circular muscle fibers from the clasp region of the LES of each specimen.

2.3.2. Procedures

Studies started within 18 hours of sacrifice. Strips measuring 10 mm in length were placed in 15 mL organ baths containing Krebs' solution constantly bubbling with 5% CO_2 in O_2. Changes in tension of the strips were measured using isometric force transducers, recorded on a chart recorder (model 03 Force Transducer and model 7 Series Polygraph, respectively, Grass Instruments Co, Quincy, MA, USA), and digitized (AcqKnowledge MP100, Biopac Systems Inc, CA, USA). After an equilibration period of 30 min, strips were stretched up to 150% of their initial length and positioned between two parallel platinum wire electrodes 10 mm apart. Most strips taken from the GEJ progressively increased their tension during the following 1-2 hours. This increase in tension was defined as the active resting tone (Tottrup *et al.*, 1990). EFS was applied by means of an electrical stimulator (Model S88, Grass Instruments Co) and a power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO, USA) in order to obtain six identical and undistorted signals. Only the strips that developed active tension during the equilibration period and relaxed with EFS and/or nicotine were considered as pertaining to the LES and included in the study.

2.3.3. Solutions and Drugs

The Krebs solution used in these experiments contained (in mM) 138.5 Na^+, 4.6 K^+, 2.5 Ca^{2+}, 1.2 Mg^+, 125 Cl^-, 21.9 HCO_3^- ,1.2 $H_2PO_4^-$, 1.2 SO_4^- and 11.5 glucose. Sodium nitroprusside (SNP), VIP, ATP, CGRP, apamin, nicotine, hexamethonium, L-NAME, suramin, VIP 6-28, 1H-[1,2,4]oxadiazolo-[4,3-α]quinoxalin-1-one (ODQ), α-chymotrypsin and CORM-1 (tricarbonyl dichlororuthenum dimer) were obtained from Sigma–Aldrich Co (Madrid, Spain). Tetrodotoxin (TTX) was purchased from Latoxan (Valence, France), PACAP 28 from Peptides Institute (Osaka, Japan), and the competitive antagonist for $P2Y_1$ receptors MRS 2179 (2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetraammonium salt), the antagonist for the $P2X_{1,2,3}$ receptors NF 279 (8,8'-[Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt) and the selective heme oxygenase inhibitor tin protoporphyrin IX (SnPP-IX) from Tocris Cookson Ltd (Bristol, UK). All drugs were dissolved in distilled water, with the exception of ODQ which was dissolved in ethanol <5% vv^{-1}, and CORM-1 and SnPP-IX that were freshly prepared before each experiment and dissolved in DMSO. Final concentration of DMSO in the organ bath did not exceed 0.1% vv^{-1} . Experiments using SnPP-IX were conduced in the dark. In pilot studies we found that at these concentrations neither ethanol and DMSO did not significantly alter resting tone or EFS-induced responses (data not shown).

2.3.4. Experimental Design

In order to ascertain the physiological relevance of each putative neurotransmitter on LES physiology, our experimental design included: a) the effect of neurotransmitters on LES tone, the selection and characterization of specific antagonists for each neurotransmitter, and the evaluation of their possible interactions, particularly the possible induction of NO-synthesis by any other neurotransmitter; b) the study of neuromyogenic mechanisms controlling active LES tone; and c) the characterization of the relaxatory responses following stimulation of LES enteric inhibitory motor neurons by EFS or through nicotinic AChRs, the effects of the neurotransmitters released, and the assessment of the possible NO-mediated release of any other putative neurotransmitters.

2.3.4.1. The effect of neurotransmitters on LES tone and the selection of specific antagonists

Concentration-related curves of the effects of SNP, VIP, PACAP, ATP and CGRP were obtained by exposing LES strips to single doses of agonists for up to 3 min. After washing the strips with 45 mL of fresh buffer there was a 30-minute period before the next exposure. Concentration-response curves for CORM-1 were obtained in a cumulative fashion. Concentrations of neurotransmitters were selected according to previous studies, most on LES (Ny *et al.*, 1995b;Rattan *et al.*, 2004;Uc *et al.*, 1997;Uc *et al.*, 1999;Yamato *et al.*, 1992). We used 1 nM-3 μM for SNP; 1 nM-1 μM for VIP, PACAP, and CGRP; 10 μM-30 mM for ATP, and 1 μM-1 mM for CORM-1. Reported concentrations are final bath concentrations. Submaximal LES responses to SNP, ATP, VIP, PACAP, and CORM-1 were studied in the presence of the neurotoxin TTX (1 μM) and the following substances: ODQ (10 μM), inhibitor of soluble GC; apamin (1 μM), blocker of small conductance Ca^{2+}-activated K^+ (SK) channels; MRS 2179 (10 μM), competitive antagonist for $P2Y_1$ receptors and NF279 (10 μM) (De Man *et al.*, 2003) for $P2X_{1,2,3}$ receptors; the peptidase α-chymotrypsin (10 U/mL), and the VIP antagonist, VIP 6-28 (100 nM); and also following the NO-synthase inhibitor L-NAME (100 μM) to explore the possible involvement of NO in the response of other neurotransmitters. Antagonists were added to the baths 30 min before addition of neurotransmitters with the exception of α-chymotrypsin which was added 10 min before. In preliminary experiments we found that, at the dose and time of exposition used, α-chymotrypsin did not affect responses to repetitive doses of VIP or PACAP (not shown).

2.3.4.2. Control of active LES resting tone

The neurogenic vs myogenic contribution to active LES resting tone was assessed by measuring changes in tone following exposure to the neurotoxin TTX 1 µM and to a Ca^{2+}-free medium for 30 min. The influence of tonic activity of inhibitory EMN upon LES resting tone was studied by measuring maximal changes in tone following exposure to L-NAME 100 µM, ODQ 10 µM, apamin 1 µM, and MRS 2179 10 µM for 30 min; or α-chymotrypsin 10 U/mL for 10 min. The influence of cholinergic EMN upon LES tone was assessed by atropine 1 µM for 30 min.

2.3.4.3. Stimulation of EMN by EFS, the effects of the neurotransmitters released, and characterization of NO-mediated effects.

Transmural EFS (pulses of 0.4 mS duration, frequency 0.3-20 HZ) were applied to LES preparations in 5 s trains at 26 V (Gonzalez *et al.*, 2004). The amplitude of EFS-induced responses did not decay during control experiments lasting up to four hours (not shown). The neural origin of EFS-induced responses was assessed by TTX 1 µM and hexamethonium 100 µM. First, two experiments were conducted during direct stimulation of inhibitory EMN by EFS and sequential addition of antagonists in order to assess the nature and effects of the neurotransmitters released. In the fist experiment (Experiment E-1) the nitrergic and cholinergic components of EFS responses were sequentially blocked by L-NAME and atropine 1 µM, and apamin was assessed on non-nitergic relaxation. Results were compared with experiment E-2 during blockade by ODQ 10 µM of the target (guanylate cyclase) of the synthesized and released NO, allowing the effect of any neurotransmitter coupled in series with NO, or released by NO. In experiments E-3 to E-6 the effect of MRS 2179 (experiment E-3), NF 279 (experiment E-4), α-chymotrypsin (experiment E-5), and SnPP-IX 100 µM (Experiment E-6) on L-NAME resistant relaxation was assessed. Drugs were added to the baths 30 min prior to EFS with the exception of α-chymotrypsin which was added 10 min before.

2.3.4.4. Stimulation of EMN with nicotine, identification of the neurotransmitters released and characterization of NO-mediated effects.

A concentration-related curve of the effect of nicotine (1-300 µM) on LES strips was obtained by exposing the strips for 2 min to single doses of the drug in order to assess the LES relaxation induced by stimulation of EMN through nicotinic AChRs. Strips were washed with 45 mL of fresh buffer and left for 30 min before exposure. Repeated additions of nicotine (100 µM) did not desensitize nicotinic receptors (not shown). The specificity of the effects of nicotine was assessed by the ganglionic blocker hexamethonium 100 µM, and the

site of effect by TTX 1 μM (Galligan, 1999;Gonzalez *et al.*, 2004). Nicotine dose which caused a submaximal LES relaxation was first selected for the studies with antagonists in order to characterize the neurotransmitters released by stimulation of inhibitory EMN through nicotinic AChRs. In the fist experiment (Experiment N-1) the nitrergic component of nicotine response was assessed by L-NAME, and apamin was assessed on non-nitergic relaxation. Results were compared with experiment N-2 during blockade by ODQ 10 μM of the target (guanylate cyclase) of the synthesized and released NO allowing the effect of any neurotransmitter coupled in series with NO, or released by NO. In experiments N-3 to N-6 the relative effect of MRS 2179 (experiment N-3), NF 279 (experiment N-4), α-chymotrypsin (experiment N-5), and SnPP-IX (Experiment N-6) on L-NAME resistant relaxation was assessed. Drugs were added to the baths 30 min before the stimulation of inhibitory EMN with nicotine with the exception of α-chymotrypsin which was added 10 min before. Experiments were conduced at basal conditions and not in NANC conditions in order to compare neural stimulation by EFS or with that of nicotinic AChRs (Gonzalez *et al.*, 2004).

2.3.5. Data Analysis

The effect of EFS and pharmacological agents were determined in terms of changes in tone. Relaxation was expressed in g and/or in the percentage of active LES resting tone. The dose-response curve was computer-fitted using nonlinear regression and the maximal response elicited by the agonist and the EC_{50} were calculated (GraphPad prism, Version 2.1, U.S.A.). Contraction was expressed in g and/or in percentage of active LES resting tone. Data are expressed as mean ± mean standard error. Student-t test was selected for comparisons, using the paired mode when appropriate, and the effect of pharmacological agents on frequency-response curves was performed using two-way repeated measure ANOVA. When the t-test was significant, the Bonferroni test was carried out to determine the frequencies of statistically different responses. A p value <0.05 was considered statistically significant.

2.4. Results

2.4.1. Effect of neurotransmitters on LES tone and selection of specific antagonists

LES strips developed an active resting tone of 4.90±0.30g (n=34). SNP, VIP, PACAP, ATP, and CORM-1 relaxed LES strips; in contrast CGRP had no effect at any dose tested (Figure 1 and 2). The NO-donor SNP, the CO donor CORM-1 and VIP (Figure 1) induced a monophasic LES relaxation. PACAP induced a biphasic response with an initial fast followed

by a slow and sustained relaxation (Figure 1). ATP 1 mM evoked a triphasic mechanical response including an initial fast relaxation, which was followed by a fast contraction, and a slow sustained relaxation (Figure 1). Relaxation induced by SNP, CORM-1 and VIP was slower than initial fast relaxation induced by PACAP and ATP (Table 1). SNP, VIP, PACAP, CORM-1 and ATP relaxed LES strips in a concentration-dependent manner (Figure 2). Table 1 summarizes the dynamics of the effects of proposed neurotransmitters on LES. PACAP and NO induced a complete LES relaxation at μM concentrations and showed EC_{50} in the 10 nM range. ATP induced a complete relaxation at mM concentrations. VIP relaxed the active tone of strips by only 47±12.2% at μM concentrations and CORM-1 by 30.94±6.7% at mM concentrations.

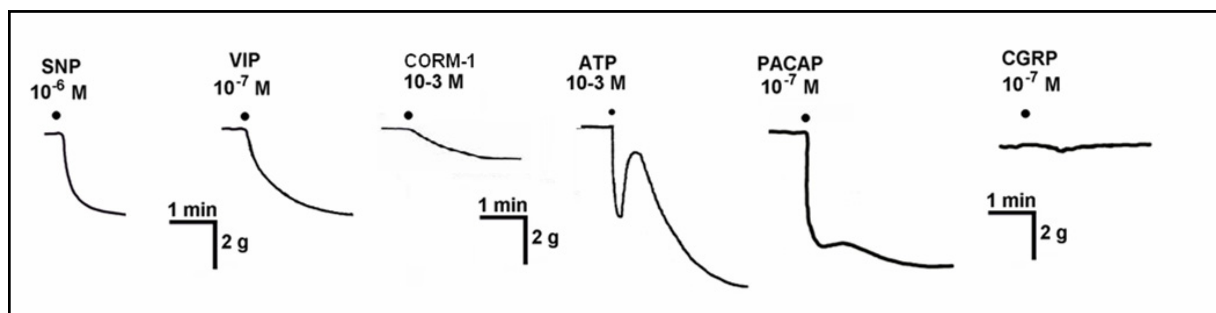

Figure 1. Representative tracings showing the morphology of LES relaxation induced by each proposed neurotransmitter. The NO donor SNP, VIP, and the CO donor CORM-1 induced a monophasic relaxation. PACAP induced a biphasic relaxation. ATP induced an initial fast relaxation, followed by a fast contraction and a sustained relaxation and CGRP did not induce any effect on LES tone

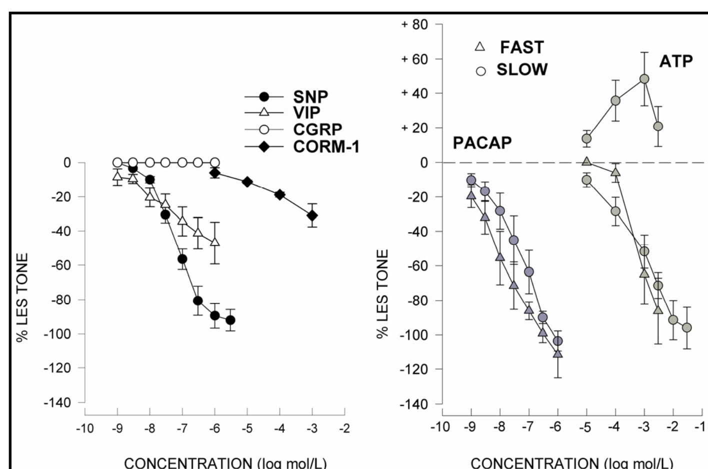

Figure 2. Log concentration-response relaxation and contraction curves to the proposed neurotransmitters in porcine LES strips. Left panel: Neurotransmitters with monophasic responses (SNP, CORM-1, VIP, CGRP). Right panel: Neurotransmitters with polyphasic responses (PACAP and ATP). Effects are expressed in terms of percentage changes of active LES tone (n=3 at each point)

The doses of neurotransmitters causing submaximal LES relaxation selected for studies with antagonists were SNP (1 μM), VIP (100 nM), CORM-1 (500 μM), ATP (1 mM), and PACAP (100 nM), the effects of antagonists having been evaluated in individual experiments using 3-7 specimens. Inhibition of NO synthesis by L-NAME did not

significantly modify any of the responses induced by SNP (+0.50±4.02%, ns), VIP (-8.56±2.91%, ns), ATP (FR +0.30±4.86%, FC -13.24±4.47%, SR +4.01±11.27%, ns), or PACAP (FR -11.64±10.92%, SR -13.40± 5.28%, ns). NO-induced relaxation by SNP was specifically blocked by ODQ (-98.35±1.15%, $p<0.05$) and unaffected by the other antagonists. Fast relaxation induced by ATP was unaffected by ODQ (+4.00±3.13%, ns), L-NAME (+0.30±4.86%, ns) or α-chymotrypsin (+0.26±8.04%, ns). In contrast, apamin fully blocked (-98.84±1.16%, $p<0.01$) the initial fast relaxation and switched the LES response to ATP into a biphasic response with an initial contraction of 1.68±0.78g and a slow sustained relaxation. The ATP-induced contraction was not affected either by TTX (-0.44±16.18%, ns), or ODQ (-12.18±6.05%, ns). MRS 2179 10 μM strongly inhibited fast ATP-induced relaxation (-76.3±4.88%, $p<0.05$) and fast contraction (-80.38±0.75%, $p<0.05$). Apamin inhibited fast component (-76.78±6.06%, $p<0.05$) and had no effect on the slow component (-31.75±9.49, ns) of PACAP-induced LES relaxation. Unexpectedly, CORM-1 relaxation was unaffected by either ODQ or apamin. VIP 6-28 100 nM did not significantly affect VIP relaxation (+1.78±7.02%, ns); and α-chymotrypsin fully blocked VIP and PACAP-induced relaxation (-100%, $p<0.05$). TTX did not significantly affect the relaxation induced by SNP (–3.04 ±2.17%, ns), VIP (-5.62±5.49%, ns), ATP (FC +27.31± 9.21% and SR +21.30± 9.57%, ns), and PACAP (FR -20.26±16.89%, ns and SR -30.57± 8.93%, ns).

2.4.2. Control of active LES resting tone

Figure 3 illustrates the myogenic and neurogenic factors contributing to LES tone. Exposure of LES strips to a Krebs Ca^{2+}-free buffer fully abolished active resting tone by 96.40±5.82% (n=9). Simultaneous blockade of intrinsic excitatory and inhibitory neural inputs by TTX did not significantly affect active LES tone (-6.93±8.84% n=7, ns). In contrast, atropine significantly reduced active LES resting tone by -24.3±3.1% (n=17) ($p<0.05$); and L-NAME, ODQ and apamin significantly enhanced active LES resting tone by 46.45±3.31% (n=17), 28.22±2.83% (n=19), and 20.3±2.1% (n=12), respectively ($p<0.05$); MRS 2179 enhanced LES tone by only 2.87±2.16% (n=6, ns). The enhancement caused by L-NAME on LES tone was higher than that caused by ODQ or apamin ($p<0.001$). α-chymotrypsin exerted a variable effect on LES, as active tone was either enhanced by 41±47% (n=8), or reduced by 36.81±47% (n=7) following 10 min exposure to the drug. These experiments show that porcine LES tone is mainly myogenic, depends on extracellular Ca^{2+}, and is modulated by tonic input from cholinergic EMNs as well as by continuous influence of inhibitory EMNs.

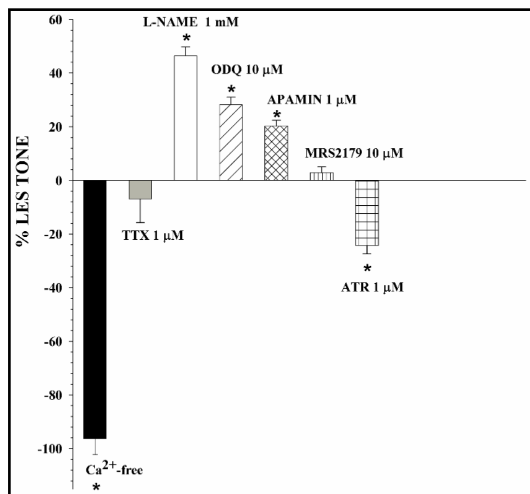

Figure 3. Neuromyogenic control of LES tone. Effect on active LES tone induced by: Ca^{2+}-free medium, TTX, L-NAME, ODQ, apamin, MRS 2179 and atropine

2.4.3. Inhibitory NTs in EFS-induced relaxation

LES strips responded to EFS with a sharp relaxation during electrical stimulus ("on" relaxation) followed by a phasic contraction at the end of the stimulus ("off" contraction) (Figure 4). The amplitude of both responses was frequency-dependent and maximal relaxation (4.21±0.25g or 90.4±3.1% of resting tone) was reached at 3 Hz (n=34). EFS-induced relaxation was fully blocked by TTX at all frequencies tested (-100%, n=5, p<0.001), and unaffected by hexamethonium (+6.78±14.65% at 3 Hz, n=3, ns).

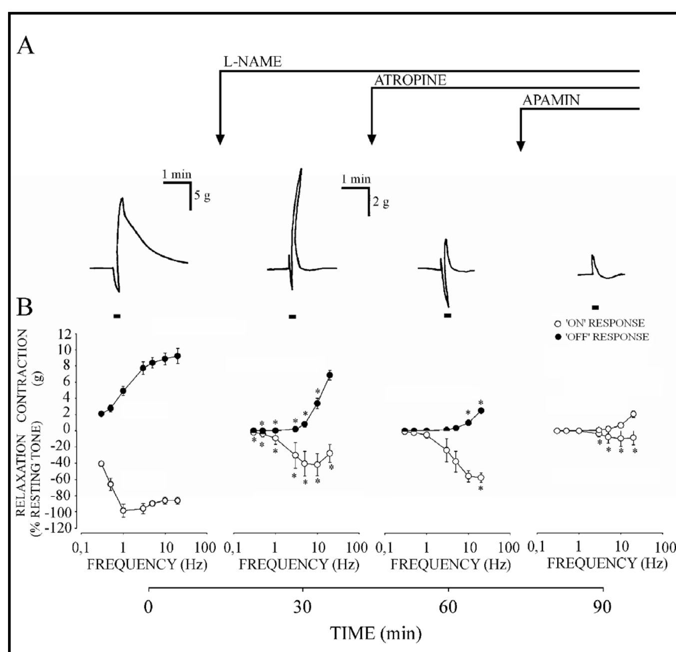

Figure 4. Characterization of NTs released during stimulation of LES EMN by EFS. A) Representative tracings and B) quantitative effects on frequency-dependent (0.3-20 Hz) EFS-induced responses (n=5 in each experiment). Horizontal axis depicts the time schedule of the experiment during sequential addition of antagonists. * = p<0.05 vs the same frequency in the previous experimental condition. Experiment E-1. Blockade of NO synthesis by L-NAME significantly reduced the amplitude of both "on" relaxation and "off" contraction

Subsequent addition of atropine reduced "off" contraction and enhanced "on" relaxation. Apamin blocked L-NAME resistant EFS-induced relaxation and switched "off" contraction to an "on" contraction during EFS without changes in amplitude. In experiment E-1 (Figure 4),

L-NAME significantly inhibited EFS relaxation with significant effects at all frequencies tested and an average effect of -62.52±13.13% (n=5). Subsequent addition of atropine enhanced relaxation at 20 Hz ($p<0.05$).

Apamin fully blocked the L-NAME resistant "on" relaxation in 4 out of 5 experiments ($p<0.05$). In the same experiment, L-NAME reduced the amplitude of basal EFS-"off" contraction with significant effects at 0.5 to 20 Hz ($P<0.05$). The subsequent addition of atropine further reduced "off" contraction at 10 and 20 Hz and apamin switched the "off" contraction to an "on" contraction during EFS without affecting the amplitude of the response. These results clearly show that EFS- induced LES relaxation is mainly mediated by both NO synthesis and other neurotransmitters acting through apamin-sensitive K^+ channels.

In the second experiment (Experiment E-2, Figure 5) we explored the possible effect of any inhibitory neurotransmitter released by NO. In this experiment ODQ reduced EFS relaxation with significant effects at all frequencies tested (n=5, $p<0.05$). Subsequent addition of atropine increased the relaxation at 10 and 20 Hz, and apamin fully blocked this non-nitrergic EFS "on" relaxation in 4 out of 5 experiments ($p<0.05$). Also in experiment E-5, ODQ reduced the amplitude of the "off" contraction at frequencies above 1 Hz ($p<0.05$); subsequent addition of atropine further reduced the amplitude of the contraction at 10 and 20 Hz ($p<0.05$), and similarly, apamin switched the "off" response into an "on" contraction. In these experiments with stimulation of EMNs by EFS, the average of inhibitory effect of ODQ on relaxation (-67.67±6.80%) was similar to that caused by L-NAME arguing against the possibility of a relaxatory effect of any NTs released by NO. Apamin also caused similar and comparable effects in both experiments.

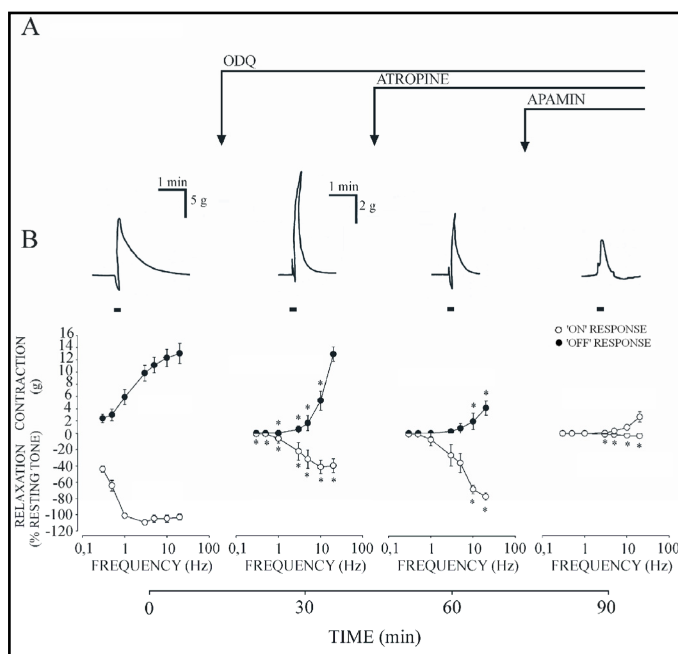

Figure 5. Experiment E-2. Blockade of NO effects by ODQ significantly reduced the amplitude of both "on" relaxation and "off" contraction. Subsequent addition of atropine reduced "off" contraction and enhanced "on" relaxation. Apamin blocked ODQ resistant EFS-induced relaxation and switched "off" contraction to an "on" contraction during EFS without changes in amplitude

The nature of the non-nitrergic neurotransmitter released by EFS was explored in experiments E-3 to E.6. In experiment E-3 (Figure 6), MRS 2179 strongly inhibited the non-nitrergic EFS relaxation with significant effects at 3-20 Hz (n=5, $p<0.001$). In experiment E-4 NF 279 slightly but consistently reduced non-nitrergic EFS relaxation, also with significant effects at 3-20 Hz (n=5, $p<0.001$). In experiment E-5 (Figure 6), α-chymotrypsin only slightly inhibited the L-NAME resistant “on” relaxation with significant effects at 3 and 20 Hz (n=5, $p<0.05$), and in experiment E-6, SnPP-IX did not affect EFS relaxation (n=5, ns).

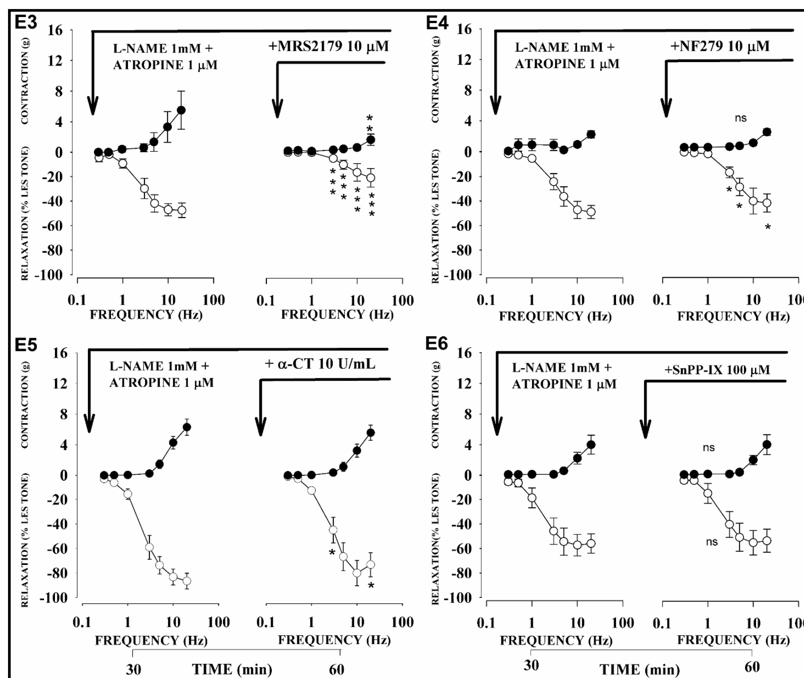

Figure 6. Characterization of NTs involved in non-nitrergic and apamin-sensitive EFS-induced LES relaxation following L-NNA and atropine. The purinergic antagonist MRS 2179 strongly inhibited EFS-induced “on” relaxation at frequencies above 3Hz in experiment E-3. The P2X antagonist NF279 (experiment E-4) and chymotrypsin (experiment E-5) slightly but significantly antagonized the EFS-relaxation, and, in contrast, Tin-protoporphyrin IX did not induce any effect on EFS-relaxation in experiment E-6

Results from experiments E-1 suggest that EFS relaxation is mainly mediated by NO and an apamin-sensitive neurotransmitter, and results from experiments E-3 and E-4 show that the non-nitrergic and apamin-sensitive component is strongly inhibited by a purinergic $P2Y_1$ receptor antagonist and only slightly reduced by a $P2X_{1,2,3}$ purinergic receptor antagonist. This clearly indicates the effect of a purinergic neurotransmitter mainly acting at $P2Y_1$ receptors through apamin-sensitive small conductance Ca^{2+}-activated K^+ channels. In addition the non-nitrergic and apamin-sensitive component was only slightly reduced by α-chymotrypsin, and unaffected by heme oxygenase inhibition, suggesting only a minor role for a peptidic neurotransmitter agreeing with the pharmacological profile of PACAP as the direct

effect of PACAP on LES is inhibited by both apamin and α-chymotrypsin, and no role for CO during EFS-relaxation.

2.4.4. Inhibitory neurotransmitters in nicotine-induced relaxation

Nicotine (1-300 µM) relaxed LES strips in a concentration-dependent manner. EC_{50} was 20.2 µM and maximal relaxation (3.93±1.03g or 100.36±3.37% of resting tone) was reached at 100 µM (n=5). The amplitude of the relaxation induced by stimulation of nicotinic AChRs was similar to that obtained by EFS (ns). Hexamethonium fully blocked nicotine-induced LES relaxation (-98.27±3.51%, n=3, p<0.05), and TTX reduced by 28.08±12% but did not block maximal relaxation induced by nicotine (n=5, p<0.05).

Nicotine-induced relaxation (100 µM) was also significantly reduced by L-NAME by 60.37±10.80% (n=5, p<0.05, Experiment N-1 in Figure 7).

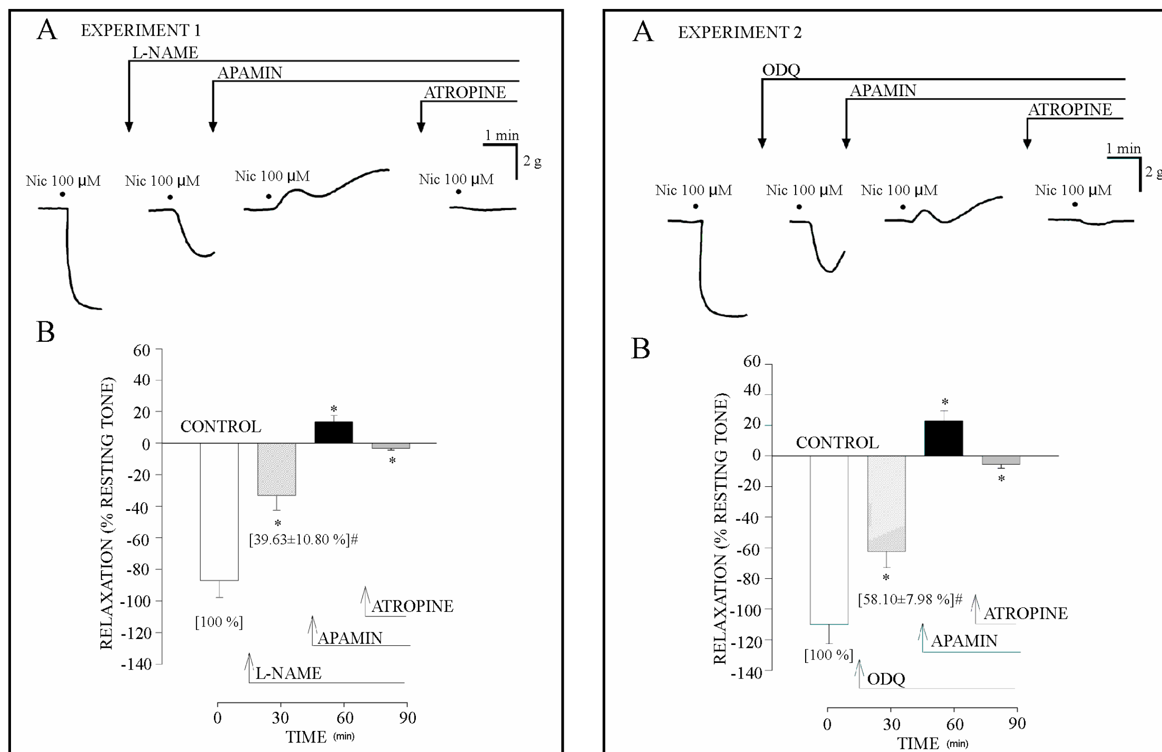

Figure 7. Characterization of NTs released during stimulation of LES EMN by nicotine 100µM. A) Representative tracings showing the morphology of nicotine-induced responses. B) Quantitative effects (n=5 in each experiment). Horizontal axis depicts the time schedule of the experiment during sequential addition of antagonists. * = p<0.05 vs previous experimental condition. Experiment N-1. Blockade of NO synthesis by L-NAME significantly reduced the amplitude of the nicotine-induced relaxation. Subsequent addition of apamin fully blocked the non-nitrergic relaxation and induced the appearance of a contraction that was fully blocked by atropine. Experiment N-2. Blockade of NO effects by ODQ reduced the amplitude of the relaxation. Subsequent addition of apamin blocked the residual relaxation and induced the appearance of a contraction that was fully blocked by atropine

Subsequent addition of apamin blocked nonitrergic relaxation and transformed the nicotine-response into a biphasic contraction with an amplitude of 0.65±0.14 g and 1.76±0.19 g, respectively. This double-peaked contraction was fully blocked by atropine ($p<0.05$). In parallel experiments (Experiment N-2, Figure 7), ODQ significantly reduced the nicotine-induced relaxation by 41.90±7.98% ($n=5$, $p<0.05$), and apamin further blocked the nicotine-relaxation which also led to a cholinergic double-peaked contraction (0.77±0.13 g and 2.10 ±0.41 g, respectively). In these experiments with stimulation of EMN trough nicotine AChRs, the effect of ODQ was similar to that induced by L-NAME (ns), and comparable to that caused by apamin in both parallel studies. Relative inhibition caused by L-NAME, ODQ, and apamin did not differ in both EFS and nicotine experiments (ns).

Experiments N-3 to N-6 (Figure 8) were performed to characterize the nature of the non-nitrergic neurotransmitters released following stimulation of inhibitory LES EMN through nicotinic AChRs. Nicotine-induced relaxation resistant to L-NAME was strongly inhibited by MRS 2179 by 76.6±9.27% ($n=5$, $p<0.01$) in experiment N-3, and also significantly inhibited by NF 279 by 48.04±16.64% ($n=5$, $p<0.05$) in experiment N-4 and by 18.88±1.00% in experiment N-5 by α-chymotrypsin ($n=5$, $p<0.001$) in experiment N-5, in experiment N-6 SnPP-IX did not significantly affect nicotine relaxation. Experiment N-1 shows that LES relaxation following stimulation of inhibitory EMNs through nicotinic AChRs is mainly caused by NO and one or more apamin-sensitive neurotransmitters. The nonitrergic and apamin-sensitive relaxation was strongly antagonized by the purinergic antagonist MRS 2179 in experiment N-3, further confirming the effect of a purinergic neurotransmitter mainly acting at $P2Y_1$ receptors through apamin-sensitive small conductance Ca^{2+}-activated K^+ channels. In addition, nonitrergic and apamin-sensitive nicotine-induced relaxation is also moderately antagonized through purinergic $P2X_{1,2,3}$ receptor antagonists and by α-chymotrypsin also suggesting a minor role for a peptidic neurotransmitter agreeing with the pharmacological profile of PACAP, and no role for CO in LES relaxation following stimulation of inhibitory EMN through nicotinic AChRs.

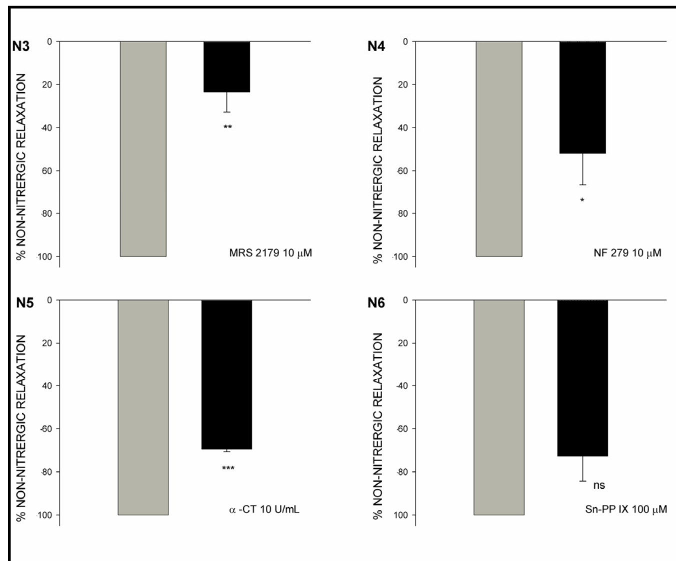

Figure 8. Characterization of NTs involved in L-NAME resistant and apamin-sensitive nicotine-induced LES relaxation. Results are expressed in percentage of inhibition of L-NAME resistant relaxation. MRS 2179 strongly inhibited nicotine relaxation in experiment N-3. NF 279 in experiment N-4 and chymotrypsin in experiment N-6 also partly antagonized nicotine relaxation and Tin-protoporphyrin IX did not induce any significant effect on nicotine-relaxation

2.5. Discussion

Our study shows that porcine LES tone is mainly myogenic and is modulated by tonic input from excitatory and inhibitory EMNs. We found that relaxation of porcine LES following stimulation of inhibitory EMNs is caused to a similar extent by two main pathways: a) a nitrergic one mediated by NO through guanylate cyclase signaling and apamin-insensitive pathways, and b) a nonintrergic pathway coupled to apamin-sensitive small conductance Ca^{2+}-activated K^{+} channels mainly mediated by ATP or a related purine acting through $P2Y_1$ receptors and a minor contribution of purinergic P2X receptors and PACAP on LES relaxation. Our results suggest parallel release of nitrergic, purinergic, and peptidergic neurotransmitters, independent effects, and no major interplay on their release or postjunctional effects (Figure 9). Our study did not find any role for CGRP in porcine LES. In addition although CO and VIP caused a direct LES relaxation in our study, we did not find any major role for them in neuroeffector LES relaxation.

Porcine, like human, LES is formed by the gastric sling muscle on the angle of Hiss and the clasp component in lesser curvature (transverse esophageal fibers) (Preiksaitis & Diamant, 1997). We only included clasp strips that developed active tension and relaxed during stimulation of inhibitory EMN, ensuring that we had selected LES strips with intact innervation. Our preparation was transmural and contained all the components of MP, including myenteric ganglia and excitatory and inhibitory EMN, and was not merely a muscle-axon preparation. We tested the effect of a NO-donor, VIP, ATP, PACAP, a CO-

donor and CGRP because these substances colocalize in esophageal inhibitory EMN (Ny *et al.*, 1995a;Uc *et al.*, 1997). Colocalized substances can participate as neuromodulators, neurotransmitters or with neurotrophic effects (Burnstock, 2004). We found that the NO-donor, VIP, ATP, the CO-donor, and PACAP, but not CGRP, relaxed LES strips through a TTX-insensitive mechanism. NO, ATP, and PACAP induced a complete LES relaxation, and CO and VIP were clearly less efficient, as observed in porcine gastric fundus (Colpaert *et al.*, 2002). In contrast, the same dose of CGRP that relaxed LES strips in opossums failed to relax our preparation despite the similarity between both species in the EFS-induced responses (Uc *et al.*, 1997). These discrepancies could be explained by species differences and/or CGRP acting through more complex neural circuits in “in vivo” studies (Rattan *et al.*, 1988). As expected, relaxation induced by SNP was specifically blocked by ODQ in our study showing a direct NO effect through guanylate cyclase pathways. The relaxation caused by VIP was unaffected by L-NAME or by ODQ arguing against the theory of a “serial cascade” involving NO production in nerve terminals and/or smooth muscle cells by VIP (Ergun & Ogulener, 2001;Grider *et al.*, 1992). Apamin inhibited ATP and PACAP but not NO, CO or VIP relaxation in our study; and α-chymotrypsin that cleaves VIP and PACAP at the level of tyrosine residues (Mule & Serio, 2003) antagonized both VIP and fast-PACAP relaxation without affecting the NO or ATP response. ATP induced a complex LES response similar to that observed on vascular smooth muscle (Ralevic, 2002). Smooth muscle cells along the gastrointestinal tract can simultaneously express G-protein coupled P2Y receptors mediating the relaxatory effects of purines, and ion-gated P2X receptors mediating either contractions (Giaroni *et al.*, 2002) or relaxations (Storr *et al.*, 2000). Activation of P2X receptors could initially induce an increase in intracellular Ca^{++} leading a contraction, and a relaxation as a result of secondary activation of apamin-sensitive Ca^{++}-dependent K^{+}-channels (Ishiguchi *et al.*, 2000). MRS 2179 is a specific antagonist for $P2Y_1$ purinoreceptor subtype (De Man *et al.*, 2003) that in our study strongly antagonized the ATP relaxation. ATP can also induce NO production in smooth muscle cells (Xue *et al.*, 2000), however in our study neither L-NAME or α-chymotrypsin affected ATP-induced relaxation, also arguing against an “in series” relation between NO or VIP and ATP.

We assessed the physiological relevance of NO, ATP, PACAP, CO and VIP by testing the effect of their antagonists on LES resting tone and during stimulation of inhibitory EMNs by EFS or through nicotinic AChRs. The most important component of LES tone is myogenic and the relative neurogenic contribution to LES tone varies with the animal species. Our results showed an intense reduction in LES resting tone by the Ca^{2+}-free buffer, further confirming that

porcine LES tone is mainly myogenic and depends on extracellular Ca^{2+} (Tottrup *et al.*, 1990). Our study also showed that atropine decreased LES less intensively tone than the removal of extracellular Ca^{2+} showing a moderate tonic input from cholinergic EMNs; and L-NAME, ODQ, and apamin enhanced LES tone also showing a tonic influence of inhibitory EMNs. NO synthase inhibitors also increased LES resting tone in humans (Gonzalez *et al.*, 2004). The higher effect of L-NAME compared with ODQ in our study could be explained by a finding in canine LES showing ODQ-dependent mechanisms mediating only the actions of NO from nerves, whereas NO from muscles utilized ODQ independent mechanisms to modulate LES tone (Daniel *et al.*, 2002). Interestingly, we failed to show any effect of atropine on LES tone in an earlier study on human LES (Gonzalez *et al.*, 2004), suggesting a stronger influence of cholinergic EMNs in porcine LES that should be taken into account when considering the effects of some new pharmacological treatments for achalasia in humans based on the reduction of cholinergic inputs such as botulin toxin (Pasricha *et al.*, 1993).

Porcine LES responses to EFS and nicotine in this study are similar to those we found in earlier studies on human LES (Gonzalez *et al.*, 2004). The effects of EFS were fully blocked by the neurotoxin TTX and unaffected by the ganglionic blocker hexamethonium, showing the origin of these responses in the activation of EMNs. Our results on pigs show that maximal EFS relaxation was inhibited to the same degree by L-NAME and ODQ (by 60%), and that apamin fully blocked the EFS induced relaxation resistant to NO inhibition in both series of experiments. Our results clearly show that the neural relaxation induced by EFS has two main components, one mediated by a direct effect of NO on smooth muscle and a second one of comparable magnitude mediated by an apamin-sensitive neurotransmitter that has been indirectly attributed to ATP in previous studies (Imaeda & Cunnane, 2003;Yuan *et al.*, 1998). We found that the apamin-sensitive component of EFS-relaxation has three components: it is strongly inhibited by MRS 2179 showing that a purine mediates this component by acting on $P2Y_1$ receptors; it is slightly but consistently inhibited by NF 279 also suggesting the involvement of P2X receptors; and it is slightly inhibited by the peptidase chymotrypsin suggesting that PACAP (an apamin-sensitive peptidergic neurotransmitters) also has a minor role in EFS-induced relaxation. Similar results on the involvement of P2Y1 and also P2X receptors on purinergic inhibitory neurotransmission were found by De Man in the mouse jejunum (De Man *et al.*, 2003) and ATP-induced relaxation of rat pylorus has been attributed to P2X purinoceptors located on smooth muscle cells (Ishiguchi *et al.*, 2000). Finally, heme oxygenase inhibitors did not modify the EFS-inhibitory responses in our preparation or in porcine stomach (Colpaert *et al.*, 2002) or porcine ileum (Matsuda *et al.*,

2004) precluding a major role for CO during EFS relaxation. In addition, the similarity of the responses in experiments with ODQ (where NO is synthesized and released and could induce the release of any other NT) and L-NAME (where NO is not synthesized) argues against release of VIP or any other neurotransmitter by NO (Grider *et al.*, 1992) and also against NO inhibition of the release of ATP (Ishiguchi *et al.*, 2000). Our results suggest mechanisms of simultaneous release and independent actions (co-transmission) for the nitrergic, purinergic, and peptidergic inhibitory neurotransmitters released during EFS.

We also explored the neurotransmitters released on stimulation of EMN with nicotine. Immunohistological and functional studies have shown that nicotinic AChRs are located in somatodendritic regions of EMN and participate in the transmission of vagal inputs (Chang *et al.*, 2003;Galligan, 1999). In earlier studies we found functional evidence of the presence of nicotinic AChRs at pre-junctional sites of inhibitory EMN in human LES (Gonzalez *et al.*, 2004). In the present study, the effects of EFS were fully blocked by TTX, but in contrast, nicotine-induced LES relaxations were only partly antagonized by TTX, further suggesting the presence of nAChRs in nerve terminals of the inhibitory EMN of porcine LES. Previous studies on cats (Kortezova *et al.*, 1994) and our previous studies on human LES also found a residual relaxation induced by nicotine following NO blockade. However, the relative magnitude of the non-nitregic component varies between these species as NO synthesis inhibitors reduced relaxation induced by stimulation of nAChRs by more than 85% in humans (Gonzalez *et al.*, 2004), 70-80% in cats (Kortezova *et al.*, 1994), and by 40-60% in the present study on pigs. On the other hand, stimulation of myenteric plexus ganglia of guinea-pig ileum with the nicotinic agonist DMPP induced both VIP and NO release, VIP release being further facilitated by NO production (Grider *et al.*, 1992). In our present study, the relaxation resistant to NO blockade (L-NAME or ODQ) was fully antagonized by apamin and a weak contraction induced by stimulation of cholinergic EMNs appeared. Similarly to the findings on EFS-relaxation, we also found three components on apamin-sensitive nicotine-LES relaxation the main component being antagonized by MRS 2179 and mediated by a purine through P2Y1 receptors, a second minor component antagonized by NF279 and mediated also by a purine but through P2X receptors, and a minor third component antagonized by α-chymotrypsin and also probably mediated by PACAP (Smid & Blackshaw, 2000) and unaffected by inhibitors of heme oxygenase. Our results also showed that LES relaxation induced by nicotine in basal conditions was similar to that induced by EFS, suggesting high efficiency of stimulation of inhibitory EMN by nicotinic AChRs. The "off" contraction induced by EFS in this study was partly antagonized by atropine, partly caused by a rebound

depolarization —because an important part of the "off" contraction is inhibited by NO blockers— and partly caused by the probable participation of a minor secondary non-cholinergic excitatory neurotransmitter such as tachykinins (Chang *et al.*, 2003). In this experimental setting, stimulation of nicotinic AChRs during simultaneous blockade of nitrergic and apamin-sensitive effects induced only a slight contraction in LES, suggesting that nicotinic AChRs stimulate excitatory EMN much less efficiently than inhibitory EMN, as we found in human LES in an earlier study (Gonzalez *et al.*, 2004). These results further suggest that whereas vagal fibers or interneurons could easily and efficiently stimulate inhibitory EMNs in porcine LES, full stimulation of intrinsic excitatory EMNs requires non-nicotinic neurotransmitters (Galligan *et al.*, 2000) or other circuits that need further investigation.

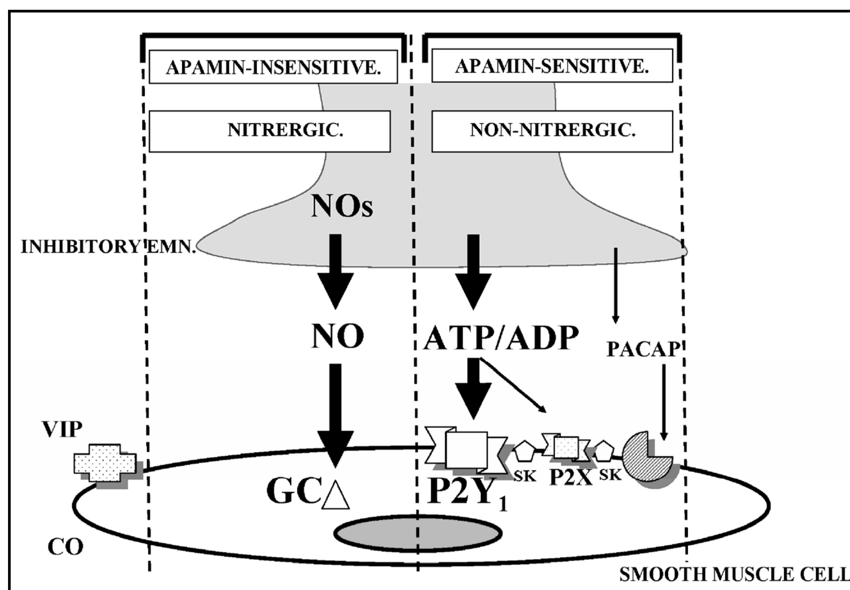

Figure 9. Schematic representation of two main parallel pathways of inhibitory neurotransmission in porcine LES: a) NO through guanylate cyclase signaling and apamin-insensitive mechanisms, and b) nonintrergic apamin-sensitive neurotransmission mainly mediated by ATP or a related purine acting on $P2Y_1$ receptors but also with a minor contribution of purinergic P2X receptors and PACAP. Our study shows no role for CGRP, and, although CO and VIP caused a direct LES relaxation in our study, we did not found any major role for these substances as neuroeffector neurotransmitters in LES relaxation. small conductance. GC: guanylate cyclase ; NOs: Nitric oxide synthase; SK: small conductance Ca^{2+}-activated K^+ channels

References

Aggestrup S, Uddman R, Jensen SL, Hakanson R, Sundler F, Schaffalitzky dM, & Emson P (1986). Regulatory peptides in lower esophageal sphincter of pig and man. *Dig Dis Sci* **31**, 1370-1375.

Brown DR & Timmermans JP (2004). Lessons from the porcine enteric nervous system. *Neurogastroenterol Motil* **16 Suppl 1**, 50-54.

Burnstock G (2004). Cotransmission. *Curr Opin Pharmacol* **4**, 47-52.

Chang HY, Mashimo H, & Goyal RK (2003). Musings on the wanderer: what's new in our understanding of vago-vagal reflex? IV. Current concepts of vagal efferent projections to the gut. *Am J Physiol Gastrointest Liver Physiol* **284**, G357-G366.

Colpaert EE, Timmermans JP, & Lefevbre RA (2002). Investigation of the potential modulatory effect of biliverdin, carbon monoxide and bilirubin on nitrergic neurotransmission in the pig gastric fundus. *European Journal of Pharmacology* **457**, 177-186.

Daniel EE, Bowes TJ, & Jury J (2002). Roles of guanylate cyclase in responses to myogenic and neural nitric oxide in canine lower esophageal sphincter. *J Pharmacol Exp Ther* **301**, 1111-1118.

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *British Journal of Pharmacology* **140**, 1108-1116.

Ergun Y & Ogulener N (2001). Evidence for the interaction between nitric oxide and vasoactive intestinal polypeptide in the mouse gastric fundus. *J Pharmacol Exp Ther* **299**, 945-950.

Galligan JJ (1999). Nerve terminal nicotinic cholinergic receptors on excitatory motoneurons in the myenteric plexus of guinea pig intestine. *J Pharmacol Exp Ther* **291**, 92-98.

Galligan JJ, LePard KJ, Schneider DA, & Zhou X (2000). Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* **81**, 97-103.

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, & Burnstock G (2002). P2 receptors in the murine gastrointestinal tract. *Neuropharmacology* **43**, 1313-1323.

Gonzalez AA, Farre R, & Clave P (2004). Different responsiveness of excitatory and inhibitory enteric motor neurons in the human esophagus to electrical field stimulation and to nicotine. *Am J Physiol Gastrointest Liver Physiol* **287**, G299-G306.

Grider JR, Murthy KS, Jin JG, & Makhlouf GM (1992). Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am J Physiol* **262**, G774-G778.

Imaeda K & Cunnane TC (2003). Electrophysiological properties of inhibitory junction potential in murine lower oesophageal sphincter. *J Smooth Muscle Res* **39**, 119-133.

Ishiguchi T, Takahashi T, Itoh H, & Owyang C (2000). Nitrergic and purinergic regulation of the rat pylorus 3. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **279**, G740-G747.

Kortezova N, Velkova V, Mizhorkova Z, Bredy-Dobreva G, Vizi ES, & Papasova M (1994). Participation of nitric oxide in the nicotine-induced relaxation of the cat lower esophageal sphincter. *J Auton Nerv Syst* **50**, 73-78.

Mashimo H, He XD, Huang PL, Fishman MC, & Goyal RK (1996). Neuronal constitutive nitric oxide synthase is involved in murine enteric inhibitory neurotransmission. *J Clin Invest* **98**, 8-13.

Matsuda NM, Miller SM, Sha L, Farrugia G, & Szurszewski JH (2004). Mediators of non-adrenergic non-cholinergic inhibitory neurotransmission in porcine jejunum
1. *Neurogastroenterol Motil* **16**, 605-612.

Mule F & Serio R (2003). NANC inhibitory neurotransmission in mouse isolated stomach: involvement of nitric oxide, ATP and vasoactive intestinal polypeptide
2. *British Journal of Pharmacology* **140**, 431-437.

Murray J, Du C, Ledlow A, Bates JN, & Conklin JL (1991). Nitric oxide: mediator of nonadrenergic noncholinergic responses of opossum esophageal muscle. *Am J Physiol* **261**, G401-G406.

Ny L, Alm P, Ekstrom P, Hannibal J, Larsson B, & Andersson KE (1994). Nitric oxide synthase-containing, peptide-containing, and acetylcholinesterase-positive nerves in the cat lower oesophagus. *Histochem J* **26**, 721-733.

Ny L, Alm P, Larsson B, Ekstrom P, & Andersson KE (1995a). Nitric oxide pathway in cat esophagus: localization of nitric oxide synthase and functional effects. *Am J Physiol* **268**, G59-G70.

Ny L, Larsson B, Alm P, Ekstrom P, Fahrenkrug J, Hannibal J, & Andersson KE (1995b). Distribution and effects of pituitary adenylate cyclase activating peptide in cat and human lower oesophageal sphincter. *Br J Pharmacol* **116**, 2873-2880.

Ny L, Waldeck K, Carlemalm E, & Andersson KE (1997). Alpha-latrotoxin-induced transmitter release in feline oesophageal smooth muscle: focus on nitric oxide and vasoactive intestinal peptide. *Br J Pharmacol* **120**, 31-38.

Pasricha PJ, Ravich WJ, & Kalloo AN (1993). Effects of intrasphincteric botulinum toxin on the lower esophageal sphincter in piglets. *Gastroenterology* **105**, 1045-1049.

Pérez Rubalcaba A. Real decreto sobre proteccion de los animales en el momento de su sacrificio o matanza. Boletin Oficial del Estado 39, 5146-5153. 1995.
Ref Type: Journal (Full)

Preiksaitis HG & Diamant NE (1997). Regional differences in cholinergic activity of muscle fibers from the human gastroesophageal junction. *Am J Physiol* **272**, G1321-G1327.

Ralevic V (2002). The involvement of smooth muscle P2X receptors in the prolonged vasorelaxation response to purine nucleotides in the rat mesenteric arterial bed. *British Journal of Pharmacology* **135**, 1988-1994.

Rattan S, Gonnella P, & Goyal RK (1988). Inhibitory effect of calcitonin gene-related peptide and calcitonin on opossum esophageal smooth muscle. *Gastroenterology* **94**, 284-293.

Rattan S, Haj RA, & De Godoy MAF (2004). Mechanism of internal anal sphincter relaxation by CORM-1, authentic CO, and NANC nerve stimulation
1. *American Journal of Physiology-Gastrointestinal and Liver Physiology* **287**, G605-G611.

Smid SD & Blackshaw LA (2000). Vagal ganglionic and nonadrenergic noncholinergic neurotransmission to the ferret lower oesophageal sphincter. *Auton Neurosci* **86**, 30-36.

Storr M, Franck H, Saur D, Schusdziarra V, & Allescher HD (2000). Mechanisms of alpha,beta-methylene atp-induced inhibition in rat ileal smooth muscle: Involvement of intracellular Ca2+ stores in purinergic inhibition. *Clinical and Experimental Pharmacology and Physiology* **27**, 771-779.

Tottrup A, Forman A, Uldbjerg N, Funch-Jensen P, & Andersson KE (1990). Mechanical properties of isolated human esophageal smooth muscle. *Am J Physiol* **258**, G338-G343.

Tottrup A, Svane D, & Forman A (1991). Nitric oxide mediating NANC inhibition in opossum lower esophageal sphincter. *Am J Physiol* **260**, G385-G389.

Uc A, Murray JA, & Conklin JL (1997). Effects of calcitonin gene-related peptide on opossum esophageal smooth muscle. *Gastroenterology* **113**, 514-520.

Uc A, Oh ST, Murray JA, Clark E, & Conklin JL (1999). Biphasic relaxation of the opossum lower esophageal sphincter: roles of NO., VIP, and CGRP. *Am J Physiol* **277**, G548-G554.

Werkstrom V, Ny L, Persson K, & Andersson KE (1997). Carbon monoxide-induced relaxation and distribution of haem oxygenase isoenzymes in the pig urethra and lower oesophagogastric junction
3. *British Journal of Pharmacology* **120**, 312-318.

Xue L, Farrugia G, & Szurszewski JH (2000). Effect of exogenous ATP on canine jejunal smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **278**, G725-G733.

Yamato S, Saha JK, & Goyal RK (1992). Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci* **50**, 1263-1272.

Yuan S, Costa M, & Brookes SJ (1998). Neuronal pathways and transmission to the lower esophageal sphincter of the guinea Pig. *Gastroenterology* **115**, 661-671.

Capítol 3

Asymmetrical mechanisms controlling resting tone, relaxation and contraction in clasp and sling regions of porcine LES

3.1. Abstract

To compare in vitro the functional properties of the clasp and sling muscles of the Lower Esophageal Sphincter (LES). Circular LES strips (clasp/sling pairs) from 25 adult pigs were studied in organ baths to assess the mechanisms controlling LES tone and the responses following neural stimulation by electrical field stimulation (EFS) or through nicotinic receptors. Tension developed by sling strips was higher than that developed by clasp fibers (7.59±0.89 g versus 4.72±0.67 g, $p<0.05$). LES tone is more sensitive to extracellular calcium depletion in clasp strips and to nitrergic and cholinergic blockade in sling strips. TTX did not affect LES tone and blocked responses to EFS in both LES regions. Amplitude of EFS "on" relaxation and "off" contraction was higher in clasp strips. ODQ reduced the "on" relaxation and "off" contraction in clasp; and blocked the "on" relaxation and enhanced the "on" contraction in sling fibers. Amplitude of nicotine relaxation was similar in clasp and sling regions. In the clasp region, nicotine relaxation was reduced by ODQ or TTX. In sling fibers, nicotine induced a relaxation at rest and, in contrast, a cholinergic contraction following ODQ or TTX.

The porcine LES shows a strong transversal intrinsic functional asymmetry suggesting a specialization at the neuronal level. Tonic input from EMNs has a greater influence on tone developed by sling fibers. Neural stimulation induced strong nitrergic responses and

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weak cholinergic responses in clasp fibers; and, in contrast, weak nitrergic responses and strong cholinergic responses in the sling region.

3.2. Introduction

The main physiological function of the lower esophageal sphincter (LES) is to generate a high pressure barrier to prevent reflux of gastric acid contents into the esophagus. The second is to open during swallowing to allow bolus passage, and also during transient LES relaxations that causes physiological gastro-esophageal reflux and allows belching. In vivo studies on humans, piglets, opossums, and cats using manometry have shown a significant radial asymmetry in the distribution of the high pressure barrier as the highest pressures were recorded in the left lateral direction of the LES (Preiksaitis *et al.*, 1994a;Richardson & Welch, 1981;Schulze *et al.*, 1977;Vicente *et al.*, 2001;Welch & Drake, 1980). Moreover, in both human and animal in vivo studies, atropine produces a greater drop in LES pressure on the left side of the LES (Preiksaitis *et al.*, 1994a;Richardson & Welch, 1981). There is little data available on the origin and physiological role of this transversal functional LES asymmetry and its relevance to the pathophysiology of esophageal diseases such as achalasia or gastroesophageal reflux disease.

The origin of the asymmetry in LES motility is uncertain and has been attributed to anatomical and physiological factors. The macroscopic arrangement of LES circular smooth fibers of the inner muscle in humans and pigs is very similar, with transverse muscle clasps on the lesser curve side (“clasp fibers”), and long oblique gastric “sling” fibers in the greater curvature on the left side of LES (Liebermann-Meffert *et al.*, 1979;Vicente *et al.*, 2001). In addition, clasp and sling fibers differ in function: in vitro studies have shown that LES pressure is due to a myogenic mechanism that is concentrated in the clasp muscle fibers and a neurogenic cholinergic contribution primarily due to the action of sling muscle fibers (Preiksaitis *et al.*, 1994a;Preiksaitis & Diamant, 1997). This transversal functional asymmetry has been attributed to specialization at both muscular (Muinuddin *et al.*, 2003;Muinuddin *et al.*, 2004a;Preiksaitis *et al.*, 1994a) and neuronal level (Brookes *et al.*, 1996;Yuan *et al.*, 1998;Yuan & Brookes, 1999).

The porcine and human LES possess anatomical and pathological similarities and similar organization of the enteric nervous system, differing from small laboratory animal models. Porcine LES has been used as a homologous animal model for the development of

new pharmacologic strategies to treat human neurogastrointestinal disorders (Brown & Timmermans, 2004;Pasricha *et al.*, 1993). The size, the transversal anatomical asymmetry (clasp and sling fibers), the histology (smooth muscle cells) and the neurochemical code of porcine LES EMNs is similar to that of humans (Aggestrup *et al.*, 1986;Pasricha *et al.*, 1993). We have recently explored the intrinsic mechanisms that control human and porcine LES motility in vitro (Gonzalez *et al.*, 2004;Farré *et al.*, 2005) . In humans we found that inhibitory EMN releases NO as the main neurotransmitter and also a minor apamin-sensitive one (Gonzalez *et al.*, 2004). Relaxation of porcine LES following stimulation of inhibitory EMNs is caused to a similar extent by two parallel pathways: a) a nitrergic one mediated by NO through guanylate cyclase signaling pathways, and b) an apamin-sensitive pathway mainly mediated by ATP or a related purine acting through P2Y1 receptors (Farré *et al.*, 2005). In both species, effects of excitatory EMNs were mainly mediated by acetylcholine (ACh).

The present study was undertaken to compare the in vitro functional properties of LES clasp and sling muscles in a porcine model. We explored the myogenic and neurogenic mechanisms of control of resting tone and characterized the inhibitory and excitatory responses following stimulation of intrinsic nerves with electrical field stimulation (EFS) or through nicotinic ACh receptors.

3.3. Methods

3.3.1. Preparations

Specimens were obtained from 25 adult pigs (age: 6 months, weight: 75-80 Kg) and included part of the gastric fundus, the gastro-esophageal junction (GEJ), and the esophageal body. Animals were stunned and killed by exsanguination in a slaughterhouse in compliance with specific national laws that follow the guidelines of the European Union (Perez Rubalcaba A., 1995). Specimens were immediately collected, placed in carbogenated Krebs solution at 4ºC and transported to the laboratory within 1 hour. The GEJ was opened along the anterior side, the gastric mucosa was stripped and the clasp and sling fiber bundles of LES were readily identified as a thickened band of semicircular smooth muscle adjacent to the greater and lesser curvature of the stomach, respectively (Figure 1 A and B) (Preiksaitis & Diamant, 1997). Full thickness preparations including the circular and longitudinal muscle layers as well as the myenteric plexus were obtained from each LES side by cutting 3 mm-wide strips parallel to circular muscle fibers. Up to

six consecutive strips were obtained from either the clasp or sling region of the LES of each specimen, starting 3 mm distal to the transitional line.

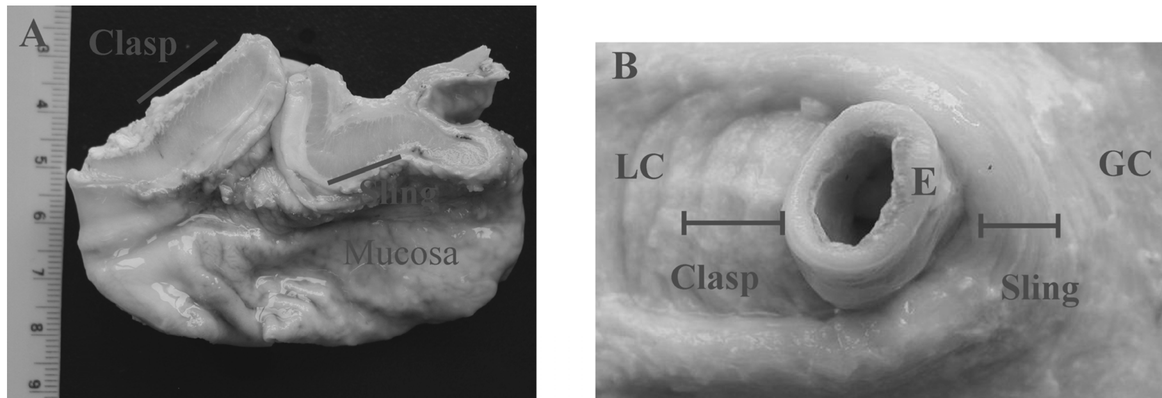

Figure 1. Anatomical disposition of clasp and sling fibers at the porcine gastroesophageal junction. A) Orientation of the fibers in a frontal plane incised along the greater and lesser curvature. B) Arrangement of clasp and sling fibers in a transversal plane observed from the gastric side. Esophageal lumen (E), greater curvature side (GC) and lesser curvature side (LC)

3.3.2. Procedures

Studies started within 18 hours of sacrifice. Final strips measuring 10 mm in length were placed in 15 mL-organ baths containing Krebs' solution constantly bubbling with 5% CO_2 in O_2. Changes in tension of the strips were measured using isometric force transducers (model 03 Force Transducer and model 7 Series Polygraph, respectively, Grass Instruments Co, Quincy, MA), and recorded in a computer using the data acquisition software Acqknowledge 3.7.2 (Biopac Systems, Inc. Ca, USA). In each experiment, up to three pairs of clasp/sling strips were simultaneously studied. After an equilibration period of 30 min, strips were stretched up to 150% of their initial length and positioned between two parallel platinum wire electrodes 10 mm apart. Thereafter, most strips taken from the GEJ progressively increased their tension over the following 2-3 hours. This increase in tension was defined as the spontaneous tension (Christensen *et al.*, 1973) and the total tone developed by each strip was measured. EFS was applied by means of an electrical stimulator (Model S88, Grass Instruments Co) and a power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO) in order to obtain six identical and undistorted signals. Only the strips that developed spontaneous tension during the equilibration period and relaxed during EFS were considered as pertaining to the LES and studied thereafter. At the end of each experiment, the strips were blotted on filter paper for 30 minutes and

the weight of each strip was determined. Weight of the strips was similar in both regions (0.31±0.02 g in clasp vs 0.30±0.02 g, n=13 pairs, N=5 pigs, ns).

3.3.3. Experimental design

3.3.3.1. Origin and mechanisms controlling LES tone. The myogenic contribution to LES resting tone was assessed by measuring changes in total tone following exposure to a Ca^{2+}-free medium for 30 minutes (Gonzalez *et al.*, 2004). The influence of EMN tonic activity upon resting tone on each side of the LES was studied by measuring changes in tone following exposure to the neurotoxin TTX 1 μM, to ODQ 10 μM, and atropine 1 μM for 30 minutes.

3.3.3.2. Effect of agonists for putative excitatory and inhibitory neurotransmitters. The dose-related effects and maximum responses on LES tone of the NO-donor SNP, carbachol (CCh) or ATP were assessed in the presence of the neurotoxin TTX 1 μM in order to compare the responsiveness of clasp and sling strips to the putative neurotransmitters. Concentration-related curves of the effects of agonists were obtained by exposing LES strips to single doses of agonists for up to 3 minutes (reported concentrations are final bath concentrations). After washing the strips with 45 mL of fresh buffer there was a 30-minute period before the next exposure.

3.3.3.3. Characterization of motor responses following stimulation of EMNs by EFS or through nAChRs. The neural responses in clasp and sling regions were compared in experiments with stimulation LES EMNs by EFS or through nAChRs. Transmural EFS (pulses of 0.4 ms duration, frequency 0.3-20 Hz) were applied to LES preparations in 5 s trains at 26 V (Gonzalez et al., 2004). Experiments were conducted during stimulation of inhibitory EMN by EFS with sequential addition of antagonists in order to assess the nature and effects of the neurotransmitters released. The nitrergic and cholinergic components of EFS responses were sequentially blocked by ODQ and atropine and the effect of apamin on ODQ resistant relaxation was assessed . The effect of single dosis of nicotine (100 μM) on LES strips was also assessed (Farré et al., 2005). After washing the strips with 45 mL of fresh buffer there was a 30-minute period before the following exposure. Repetitive additions of nicotine did not result in desensitization of nAChRs (not shown). The NTs released by stimulation of EMN through nicotinic AChRs were characterized by sequential addition of ODQ and atropine; and the site of effect by TTX 1 μM (Galligan, 1999). Drugs were added to the baths 30 minutes before the stimulation of inhibitory EMN with nicotine.

3.3.4. Data analysis

Studies were designed to study and compare pairs of clasp and sling strips obtained from the same specimen. The effect of EFS or pharmacological agents was determined in terms of changes in total tone. Relaxation was expressed in g and/or as a percentage of total LES tone. The dose-response curve was computer-fitted using nonlinear regression and the maximal response elicited by the agonist and the EC50 were calculated (GraphPad prism, Version 2.1, U.S.A.). Contraction was expressed in g. The number of experiments was represented by n (number of strips) and N (number of specimens). Data are expressed as means ± mean standard error. Student-t test was selected for comparisons, using the paired mode when appropriate. In order to further characterize EFS responses, the effect of pharmacological agents on frequency-response curves was performed using two-way repeated-measures ANOVA. When the t-test was significant, the Bonferroni test was carried out to determine the frequencies of statistically different responses. A P value <0.05 was considered statistically significant.

3.3.5. Solutions and drugs

The Krebs solution used in these experiments contained (in mM) 138.5 Na^+, 4.6 K^+, 2.5 Ca^{2+}, 1.2 Mg^+, 125 Cl^-, 21.9 HCO_3^- ,1.2 $H_2PO_4^-$, 1.2 SO_4^- and 11.5 glucose. Sodium nitroprusside (SNP), apamin, nicotine, atropine and ODQ were obtained from Sigma –Aldrich Co (Madrid, Spain). Tetrodotoxin (TTX) was purchased from Latoxan (Valence, France). All drugs were dissolved with distilled water, with the exception of ODQ which was dissolved with ethanol <5% vol/vol. In preliminary studies we observed that, at these concentrations, ethanol did not alter resting tone or EFS or SNP 100 µM -induced responses (data not shown).

3.4. Results

3.4.1. Control of LES tone, effect of antagonists

All LES strips from both clasp and sling fibers spontaneously developed active tone. Total tension after the equilibration period was higher in sling strips than in clasp fibers (4.72±0.67 g versus 7.59±0.89 g, $p<0.05$). SNP 10 µM, reduced total tension to a similar extent in clasp and sling muscle strips, by 88.83±1.96 % in clasp versus 85.05±1.44 % (ns) in sling fibers, suggesting that total LES tone was mainly caused by muscular contraction and not by fibroelastic properties of the tissue. Residual LES tone

after exposition of LES strips to SNP 10µM was also higher in sling fibers, 1.22±0.24 g, than in clasp strips, 0.56±0.11 g, $p<0.05$. Figure 2 illustrates the dynamics of neuromyogenic factors contributing to LES tone in each region of the LES. Strips from both regions of LES demonstrate a time-dependent decrease in tone when perfused in Ca^{2+}-free Krebs buffer, the decrease being greater and faster in clasp than in sling fibers. At T=30 minutes, total tone was reduced by 69.31±5.87% in clasp and 39.18±3.88% in sling fibers ($p<0.05$ N=5 pairs). Subsequent perfusion with calcium-containing Krebs resulted in a return of tone almost to control levels in both LES sides (not shown). Neural blockade by TTX did not significantly affect total tension either in clasp or sling muscle (ns, N=5 pairs). In contrast, ODQ induced a rapid increase in tone in both LES sides with a greater increase in sling (2.46±0.41 g) than in clasp fibers (1.63±0.82 g), $p<0.05$. Blockade of tonic effect of excitatory EMNs by atropine induced a decrease in tone in both LES regions, the effect being stronger in sling than clasp fibers ($p<0.05$). At T=30 minutes, total tension was reduced by atropine by 15.18±5.62% in clasp versus 43.38±3.07% in sling strips ($p<0.05$, N=5 pairs).

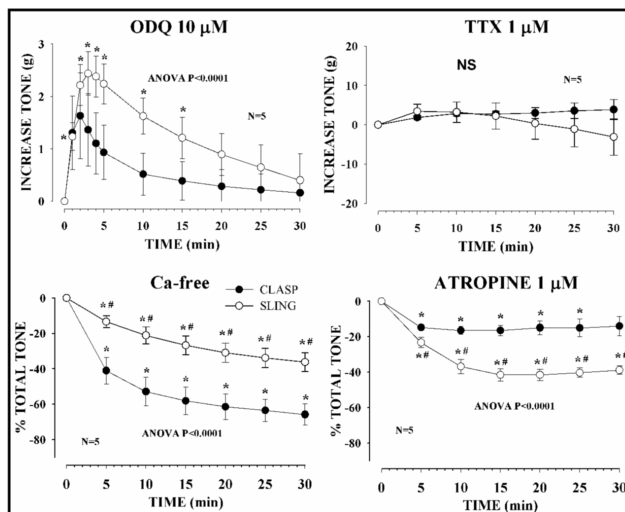

Figure 2. Mechanisms of control of LES tone in clasp and sling fibers. LES tone is more sensitive to extracellular calcium depletion in clasp strips and to nitrergic and cholinergic blockade in sling strips. *p<0.05 vs T=0; #p<0.05 vs clasp fibers

These results suggest that in clasp fibers LES tone is myogenic in origin and extremely dependent on extracellular Ca^{2+} sources and is moderately affected by tonic neural inputs from either excitatory or inhibitory EMN; in contrast, tone in sling fibers is less dependent on extracellular Ca^{2+} sources and much more influenced by tonic neural inputs from both local nitrergic and cholinergic EMNs.

3.4.2. Effects of stimulation of EMNs by EFS

LES strips from both clasp and sling regions responded to EFS with a sharp relaxation during electrical stimulus ("on" relaxation) followed by a phasic contraction at the end of the stimulus ("off" contraction) (Figure 3A). The amplitude of both responses was frequency-dependent (Figure 3B) and TTX 1 μM abolished both EFS-induced responses in clasp and sling muscles at all frequencies tested (-100%, n=5, N=5). Amplitude of 'on' relaxation' and 'off' contraction was higher in clasp strips than in sling fibers (n=16, N=7, $p<0.05$).

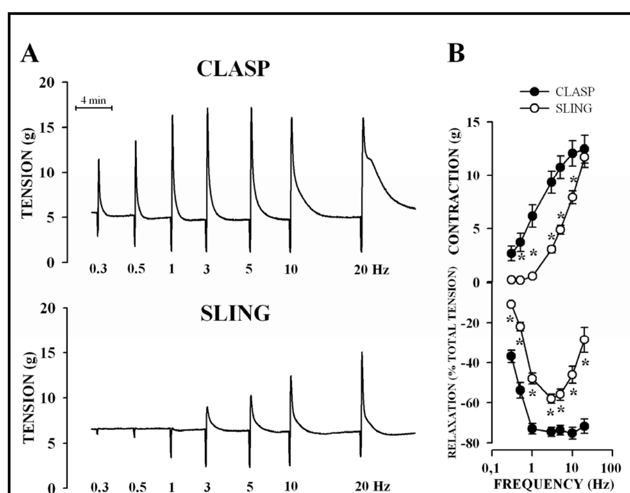

Figure 3. A. Morphology of EFS-responses was similar in clasp and sling regions. B. Relaxation and "off" contraction was stronger in clasp strips than in sling fibers (*p<0.05 paired data, n=10 pairs, N=5)

Figure 4 show the results of experiments assessing the nature of the neurotransmitters released following stimulation of inhibitory EMN by EFS in each region of the porcine LES. In clasp muscle (Figure 4A), ODQ strongly inhibited EFS 'on' relaxation with significant effects at all frequencies tested ($p<0.05$). Subsequent addition of atropine increased relaxation at high frequencies ($p<0.05$), and apamin blocked the non-nitrergic EFS "on" relaxation in 4 out of 5 experiments ($p<0.05$). ODQ reduced the amplitude of the "off" contraction at 1-10 Hz ($P<0.05$); atropine further reduced the amplitude of the contraction at 10 and 20 Hz ($P<0.05$) showing a moderate contribution of cholinergic EMNs in the contractile response, and apamin switched the "off" response into an "on" contraction during EFS. In contrast, in sling muscle, ODQ fully abolished the 'on' relaxation at all frequencies (Figure 4B), sequential addition of atropine induced an 'on' relaxation at 10-20 Hz ($p<0.05$), and apamin further reduced the ODQ resistant 'on' relaxation ($p<0.05$). ODQ switched the 'off' contraction to an 'on' contraction of increased amplitude that was almost blocked by atropine ($p<0.05$).

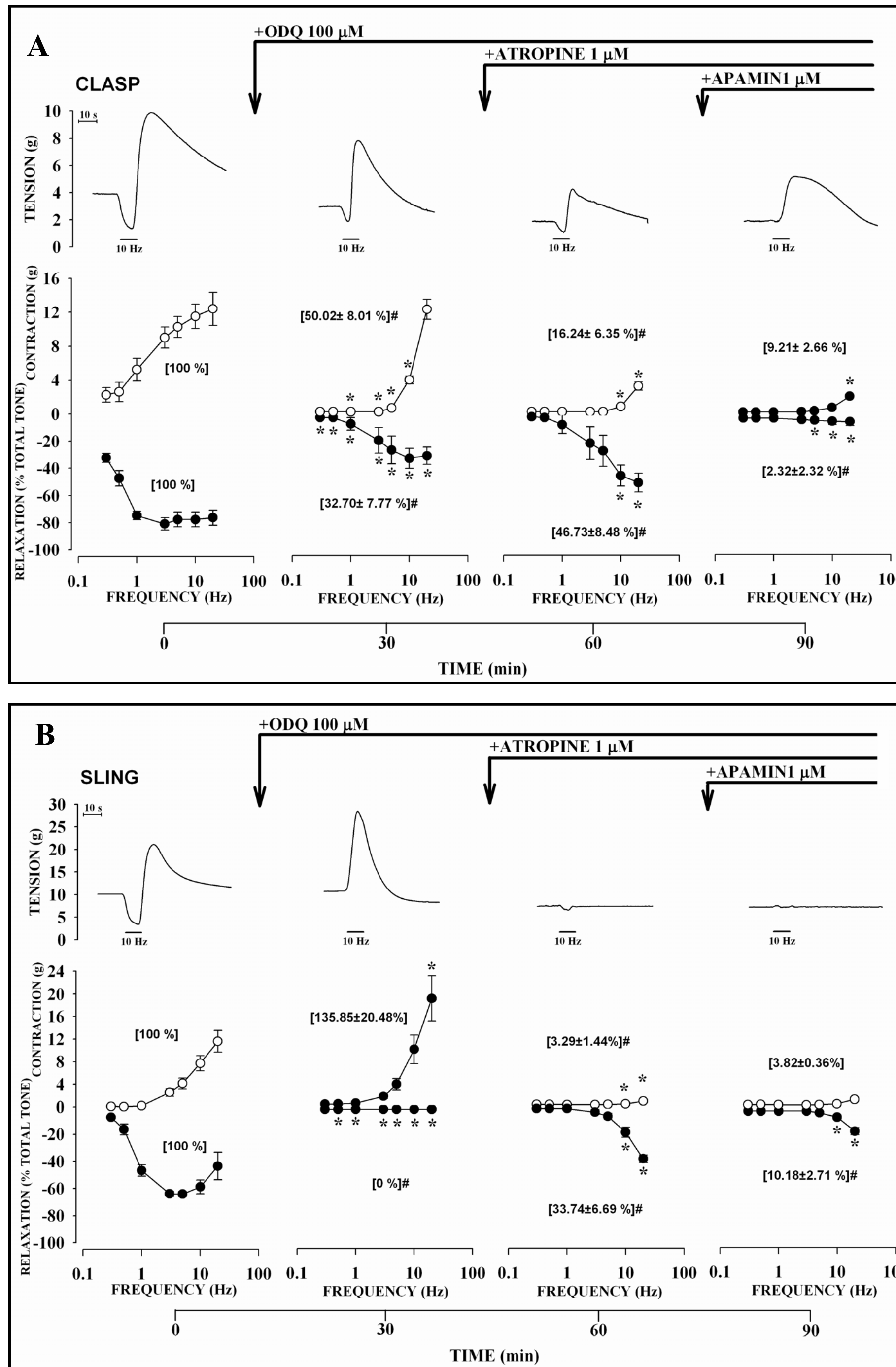

Figure 4. Characterization of EFS-responses to sequential addition of antagonists in clasp (A) and sling (B) fibers. ODQ reduced both EFS-relaxation and contraction in clasp but blocked EFS-relaxation and increased the contraction in sling region. The effect of atropine was higher on the sling side. The APA-sensitive component of EFS relaxation was similar on both LES sides

The effect of atropine was more intense in sling than in clasp fibers. Both clasp and sling regions show the APA-sensitive component significantly contributes to EFS relaxation, more so in the clasp region. Comparison by substraction of the frequency

response curves obtained before and after the addition of each specific antagonist show that the inhibitory nitrergic and purinergic contribution was higher in clasp than in sling regions and the cholinergic response was higher in the sling region (Figure 5A, B and C).

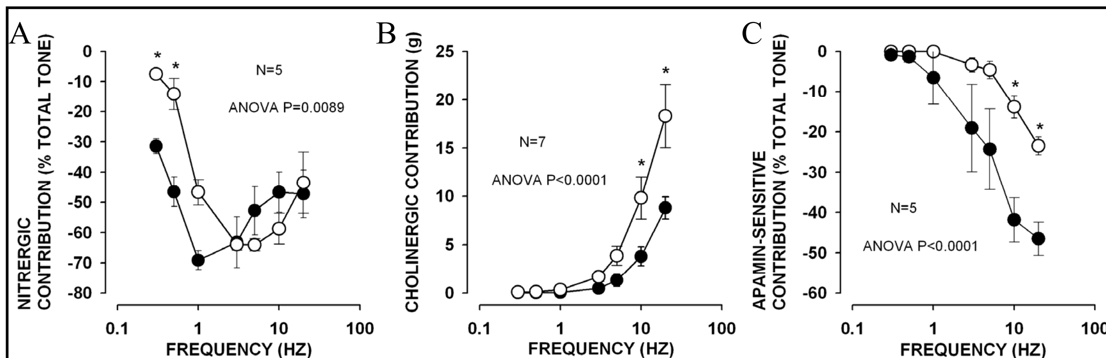

Figure 5. A) Nitrergic, B) cholinergic and C) apamin-sensitive contribution to EFS-induced responses in clasp and sling regions

3.4.3. Effects of stimulation of EMNs through nAChRs

Nicotine 100 µM induced a relaxation of similar magnitude in both clasp and sling muscles (-86.61±6.19% versus –79.58±2.53% of total tone, N=8 pairs, ns). In clasp strips, ODQ reduced the amplitude of nicotine-induced relaxation by 57.17±3.26% (N=7 pairs, $p<0.05$), and in sling fibers ODQ fully blocked relaxation and switched the nicotine response in a contraction that was blocked by atropine (Figure 6A) and unaffected by TTX (Figure 6B). In clasp strips, TTX reduced the amplitude of nicotine-induced relaxation by 33.59±7.44% (N=7 pairs, $p<0.05$) and, in contrast, in sling strips TTX switched the nicotine-induced relaxation to a contraction (Figure 6C) that was also blocked by atropine (N=6 pairs, $p<0.05$).

3.4.4. Effect of agonists on putative excitatory and inhibitory neurotransmitters

Strips from each region of the LES also differed in the concentration-dependent profiles induced by agonists (SNP, ATP, CCh) on the proposed main putative inhibitory and excitatory and neurotransmitters in porcine LES (Farré *et al.*, 2005). The EC50 of the relaxation induced by SNP was higher in clasp fibers (Table 1), with maximal effects of similar magnitude (Figure 7). In contrast, maximal effects induced by CCh and ATP differed. Sling strips showed a higher contraction in response to carbachol and

a lower relaxatory response to ATP. The EC50 for carbachol and ATP responses did not differ between clasp and sling fibers (Table 1).

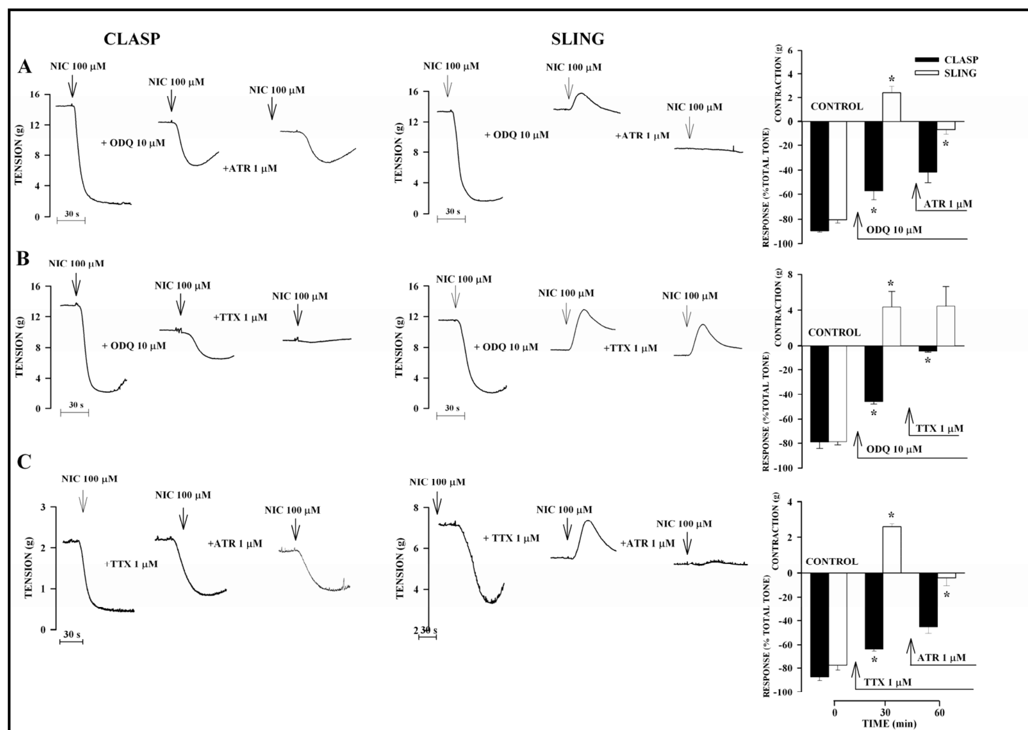

Figure 6. Regional characteristics of responses induced by stimulation of nAChRs. ODQ (A,B) or TTX (C) reduced NIC-relaxation in clasp strips and switched this response to a cholinergic contraction in sling strips (A,B,C)

Table 1. EC50 of responses to NO donor SNP, carbachol and ATP.

	EC50 (mol/l)	SLOPE
SNP clasp	7.27×10^{-8}	-1.02±0.14
sling	3.02×10^{-7}***	-0.97±0.13
CCh clasp	3.13×10^{-7}	0.60±0.44
sling	2.80×10^{-7}	0.64±0.45
ATP clasp	1.46×10^{-4}	-0.81±0.70
sling	3.65×10^{-4}	-0.99±1.42

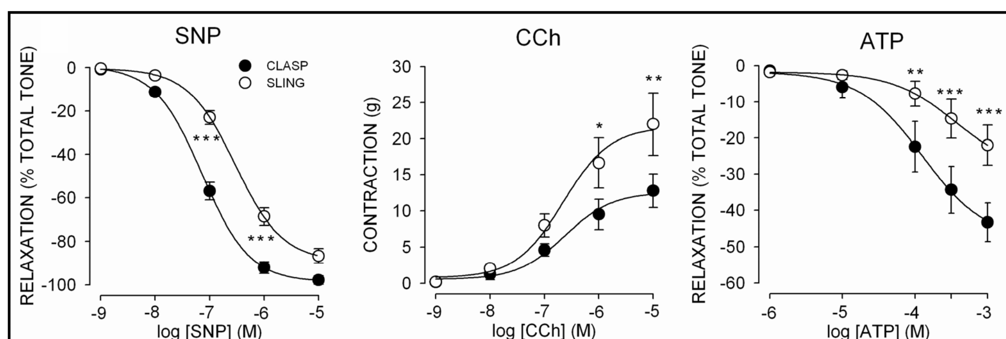

Figure 7. Effect of agonists. Comparison of the C-R curve in response to SNP, carbachol and ATP in the clasp and sling muscles of the porcine LES

3.5. Discussion

In this study we found that porcine LES shows a strong transversal functional asymmetry in the intrinsic mechanisms that control resting tone and the relaxatory and contractile responses that follow stimulation of myenteric EMNs. Tone developed by sling fibers is higher, less dependent on extracellular calcium sources, and more dependent on tonic input of excitatory and inhibitory EMNs than that developed by clasp fibers. Neural stimulation induced stronger nitrergic responses and weaker cholinergic responses in clasp fibers; and in contrast, weaker nitrergic responses and stronger cholinergic responses in the sling region.

Like humans, the porcine LES is formed by the sling muscle on the angle of Hiss (oblique gastric fibers) and the clasp component in lesser curvature (transverse esophageal fibers) (Liebermann-Meffert *et al.*, 1979;Vicente *et al.*, 2001). In this study we studied pairs of clasp/sling LES strips that developed active tension during the equilibration period and relaxed during stimulation of inhibitory EMN by EFS and/or nicotine, ensuring that we had selected LES strips with intact intrinsic innervation. Most esophageal EMN have single process Dogiel type 1 morphology and the soma located in myenteric ganglia (Brookes *et al.*, 1996). Our preparation was transmural and contained the circular as well as the longitudinal muscular layer and all the components of MP, including myenteric ganglia and excitatory and inhibitory EMN, and was not merely a muscle-axon preparation (Gonzalez *et al.*, 2004). The size and weight of the strips coming from both LES sides was identical, ensuring an adequate experimental setting for functional comparisons.

The first result coming from our study was that LES tone was actively caused by a muscular contraction in both LES regions as tone progressively developed during the equilibration period and was largely abolished by the muscular relaxation induced by SNP. In our study, total tone developed by sling fibers was higher than that developed by claps fibers, a result consistent with the fact that in vivo human and animal studies show higher contribution of the left side in LES pressure (Preiksaitis *et al.*, 1994a;Welch & Drake, 1980). In contrast to our results and the functional in vivo asymmetry, initial in vitro studies in cats (Preiksaitis *et al.*, 1994a) found no differences or showed that clasp fibers developed more tone than the sling region in feline and human LES (Muinuddin *et al.*, 2004b;Preiksaitis *et al.*, 1994b). Minor differences in the methodology used to establish LES tone and probably major differences in the anatomic

region providing LES strips could explain some of the differences. In our study both groups of clasp/sling fibers fulfilled our strict definition of LES response: to develop spontaneous tone and to relax during neural stimulation. In our study, muscular relaxation caused by SNP was above 85% of total tone on both LES sides, thus intrinsic myogenic and/or neurogenic factors inducing muscular LES contraction should account for the differences in total tone. There is disagreement over the relative contribution of extra- and intracellular calcium sources on myogenic LES tone. Initial studies on feline LES strips showed only a partial reduction in LES tone after the removal of extracellular Ca++ or the blocking of extracellular Ca++ influx suggesting an important role for intracellular Ca sources in maintaining tone (Biancani *et al.*, 1987). In contrast, other studies on canine LES clearly demonstrate that LES spontaneous tone is mainly dependent on Ca^{++} entry from the extracellular solution (Salapatek *et al.*, 1998). In an earlier study we demonstrated the strong relevance of extracellular calcium sources in maintaining LES tone in human LES (Gonzalez *et al.*, 2004) and in this study we also found that removing calcium from the buffer induced a decrease in tone on both LES sides, the LES tone in the clasp fibers being more intensely and rapidly affected by calcium depletion. Muinuddin et al (Muinuddin *et al.*, 2004b) also described transversal differences of extracellular or intracellular calcium sources in maintaining LES tone in feline LES very similar to that observed in the present study. Regional differences in calcium handling were attributed to different pathways for calcium entrance and to a different contribution of intracellular calcium sources from SR, both being mechanisms of regional specialization at a muscular level (Muinuddin *et al.*, 2004b). We have also described in an earlier study how muscular contraction is locally modulated by tonic neural inputs from local EMNs in both human and porcine LES (Gonzalez *et al.*, 2004;Farré *et al.*, 2005). NO synthase inhibitors increased LES resting pressure in opossums (Yamato *et al.*, 1992), dogs (Boulant *et al.*, 1994), cats (Xue *et al.*, 1996) and humans (Konturek *et al.*, 1997), also indicating the presence of a NO-mediated inhibitory tonic influence on the LES muscle in these species. Otherwise, it has been shown in the canine LES that ODQ mediated only the actions of NO from nerves, whereas NO from muscles utilized ODQ independent mechanisms to modulate LES tone (Daniel *et al.*, 2000;Daniel *et al.*, 2002). In the present study ODQ induced an increase in LES tone on both LES sides, the tone increasing more in the sling region. This result could be caused by a higher tonic influence of inhibitory EMNs on the sling region and/or to a higher contribution of cholinergic EMNs to LES tone during blockade of inhibitory neural

influences. The higher contribution of cholinergic EMNs to LES tone in sling strips was made apparent in our experiments with atropine and agrees with the observation that atropine decreases left LES pressure in vivo in humans and cats (Preiksaitis *et al.*, 1994a;Welch & Drake, 1980). In order to interpret the effect of blockade of enteric EMNs it should be noted that in our study the clasp region responded more to agonists mimicking putative inhibitory neurotransmitters (the NO donor SNP and ATP), and in contrast, sling fibers showed a stronger cholinergic sensitivity. Similar differences between clasp and sling fibers to cholinergic stimulation have been described in cats and humans (Preiksaitis *et al.*, 1994a;Preiksaitis & Diamant, 1997). Patients with achalasia lack inhibitory EMNs in the gastro-esophageal junction (Mearin *et al.*, 1993) and usually show increased LES pressures; an enhanced loss of influence of inhibitory EMNs in the sling region with the maintenance of cholinergic EMNs could explain the observation that tone in the sling region is strongly increased in achalasic patients (Mattioli *et al.*, 1993).

The esophagus presents a pattern of motility based on mechanisms of regional specialization which have been investigated for many years. It is well known that the esophageal body presents a regional gradient of functional specialization at the neuronal level that allows for esophageal peristalsis (Chang *et al.*, 2003). The influence of inhibitory EMNs increases distally enhancing latency and amplitude of “off” contractions, and the influence of cholinergic excitatory EMNs decreases distally reducing “on” contractions (Chang *et al.*, 2003). Immunohistochemical studies on animal models did not find any morphological differences in the distribution of excitatory or inhibitory EMNs to account for this regional specialization. There is much less information on possible “transversal” specialization and regional characteristics of the relaxation in clasp and sling LES regions. Initial studies show that EFS-relaxation was more “complete” in feline strips from the right (Preiksaitis *et al.*, 1994a), and found a similar pattern of relaxation between clasp/sling fibers in human LES (Preiksaitis & Diamant, 1997). In addition, studies mapping the regional distribution of EMNs in guinea pig LES found marked differences in the density, cell body location, and chemical code of EMNs (Brookes *et al.*, 1996). In the clasp region, the density of EMNs was low and the location of their cell bodies was equally distributed within the sphincter and the EB. In contrast, in the sling region, the density of EMNs was four times higher, being one third local EMNs, only 10% of cell bodies of EMNs were located in the EB, and up to 57% were located in the MP of the stomach fundus

(Brookes *et al.*, 1996). This population of "gastric" EMNs reaching the LES was specific for the sling side, and whereas most descending EMNs from the EB were inhibitory, the proportion of local EMNs were similarly excitatory or inhibitory, and the majority of "gastric" EMNs were excitatory (Yuan & Brookes, 1999). Based on these morphological data, clasp LES fibers probably received more inhibitory influences than excitatory, and sling fibers received more terminals coming from excitatory EMNs than from inhibitory. In the present study we characterized the regional LES responses to stimulation of intrinsic EMNs by EFS or through nAChRs and we found important differences in the responses induced by both neural stimuli that match the described morphological neuronal asymmetry. During EFS in the clasp region we found mechanical responses caused by strong stimulation of inhibitory EMNs causing greater relaxation than in the sling region, and there was a clear and strong contribution of nitergic EMNs to "off" contraction, probably by a mechanism of "rebound" contraction (Chang *et al.*, 2003). In contrast, EFS in the sling region induced a less efficient relaxatory response, no contribution of nitrergic EMNs to contraction (as ODQ switched the "off" contraction to an "on" contraction of increased magnitude), and a more intense stimulation of cholinergic EMNs as the increased "on" contraction following nitrergic blockade is fully cholinergic. This transversal functional asymmetry in porcine LES was also evident in our studies with nicotine. The vagal efferent fibers synapse with EMN in myenteric ganglia and transmission is classically described as mediated by ACh acting on nAChRs. However, ganglionic neurotransmission in the esophagus can also include muscarinic (M_1) and serotoninergic (5-HT_3) receptors to mediate vagal inputs to inhibitory EMN (Gilbert *et al.*, 1984;Paterson *et al.*, 1992). We have recently described that inhibitory EMN in the human LES are easily and efficiently stimulated both by EFS and by nAChRs located in somatodendritic regions as well as in nerve terminals. In contrast, although LES excitatory EMN can be efficiently stimulated by EFS, stimulation through nAChRs is very difficult, requiring full blockade of inhibitory motor pathways and causing a weak response (Gonzalez *et al.*, 2004). Ganglionic transmission to excitatory EMNs could also be mediated by a contribution of ATP acting on purine P2X receptors, or 5-HT acting on 5-HT_3 receptors (Galligan *et al.*, 2000;Galligan, 2002). What we found in the present study on pigs further confirms our earlier functional observations on humans and matches the transversal asymmetry of morphological distribution of neural elements described in the guinea pig LES (Brookes *et al.*, 1996;Yuan & Brookes, 1999). In the clasp region we

found that nicotine induced a relaxant response, similar to that obtained by maximal EFS and caused by strong stimulation of nitrergic EMNs through nACRs located in cell bodies and nerve terminals, and there were no effects caused by stimulation of excitatory EMNs. In the sling region, inhibitory EMNs were also efficiently stimulated through nAChRs, and, unlike the clasp region, a contractile response was obtained, induced by activation of excitatory cholinergic EMNs ¼ of the strength of the response obtained by stimulation by EFS. Studies on guinea pig LES have shown a similar marked functional neural asymmetry as vagal stimulation causes activation of excitatory and inhibitory EMNs on the sling LES side (Yuan & Brookes, 1999) and only inhibitory responses in the clasp (Yuan *et al.*, 1998). Our results could be explained by a model with different populations of motor neurons innervating each region of the porcine LES with few EMNs in the clasp region (mainly inhibitory) and a denser population of both excitatory and inhibitory EMNs in the sling region. This distribution could account for the asymmetrical effects of antagonists and agonists (as more EMNs means more nerve endings and more postjunctional receptors) and for neural responses.

In this study we have provided evidence of asymmetrical mechanisms controlling resting tone, relaxation and contraction in clasp and sling regions of porcine LES that are largely based on regional specialization at neuronal levels. These mechanisms could be involved in the pathophysiology of diseases affecting LES motility. Understanding the regional characteristics of denervation and reinnervation processes in the LES of achalasic patients might help select more regionally specific surgical or medical treatments. And understanding the regional characteristics of LES motility during reflux events caused by swallow-induced and transient LES relaxations, or slow downward LES pressure drifts (Clave *et al.*, 1998) might help to improve our knowledge of the pathophysiology of gastroesophageal reflux disease. The relevance of these issues awaits further investigation.

References

Aggestrup S, Uddman R, Jensen SL, Hakanson R, Sundler F, Schaffalitzky dM, & Emson P (1986). Regulatory peptides in lower esophageal sphincter of pig and man. *Dig Dis Sci* **31**, 1370-1375.

Biancani P, Hillemeier C, Bitar KN, & Makhlouf GM (1987). Contraction mediated by Ca2+ influx in esophageal muscle and by Ca2+ release in the LES. *Am J Physiol* **253**, G760-G766.

Boulant J, Fioramonti J, Dapoigny M, Bommelaer G, & Bueno L (1994). Cholecystokinin and nitric oxide in transient lower esophageal sphincter relaxation to gastric distention in dogs. *Gastroenterology* **107**, 1059-1066.

Brookes SJ, Chen BN, Hodgson WM, & Costa M (1996). Characterization of excitatory and inhibitory motor neurons to the guinea pig lower esophageal sphincter. *Gastroenterology* **111**, 108-117.

Brown DR & Timmermans JP (2004). Lessons from the porcine enteric nervous system. *Neurogastroenterol Motil* **16 Suppl 1**, 50-54.

Chang HY, Mashimo H, & Goyal RK (2003). Musings on the wanderer: what's new in our understanding of vago-vagal reflex? IV. Current concepts of vagal efferent projections to the gut. *Am J Physiol Gastrointest Liver Physiol* **284**, G357-G366.

Christensen J, Freeman BW, & Miller JK (1973). Some physiological characteristics of the esophagogastric junction in the opossum. *Gastroenterology* **64**, 1119-1125.

Clave P, Gonzalez A, Moreno A, Lopez R, Farre A, Cusso X, D'mato M, Azpiroz F, & Lluis F (1998). Endogenous cholecystokinin enhances postprandial gastroesophageal reflux in humans through extrasphincteric receptors. *Gastroenterology* **115**, 597-604.

Daniel EE, Bowes TJ, & Jury J (2002). Roles of guanylate cyclase in responses to myogenic and neural nitric oxide in canine lower esophageal sphincter. *J Pharmacol Exp Ther* **301**, 1111-1118.

Daniel EE, Jury J, Salapatek AM, Bowes T, Lam A, Thomas S, Ramnarain M, Nguyen V, & Mistry V (2000). Nitric oxide from enteric nerves acts by a different mechanism from myogenic nitric oxide in canine lower esophageal sphincter. *J Pharmacol Exp Ther* **294**, 270-279.

Farré R, Aulí M, Lecea B, Martínez E, & Clavé P. Pharmacological characterization of intrinsic mechanisms controlling tone and relaxation of porcine lower esophageal sphincter. J.Pharmacol.Exp.Ther. 2005.
Ref Type: In Press

Galligan JJ (2002). Ligand-gated ion channels in the enteric nervous system. *Neurogastroenterol Motil* **14**, 611-623.

Galligan JJ (1999). Nerve terminal nicotinic cholinergic receptors on excitatory motoneurons in the myenteric plexus of guinea pig intestine. *J Pharmacol Exp Ther* **291**, 92-98.

Galligan JJ, LePard KJ, Schneider DA, & Zhou X (2000). Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* **81**, 97-103.

Gilbert R, Rattan S, & Goyal RK (1984). Pharmacologic identification, activation and antagonism of two muscarine receptor subtypes in the lower esophageal sphincter. *J Pharmacol Exp Ther* **230**, 284-291.

Gonzalez AA, Farre R, & Clave P (2004). Different responsiveness of excitatory and inhibitory enteric motor neurons in the human esophagus to electrical field stimulation and to nicotine. *Am J Physiol Gastrointest Liver Physiol* **287**, G299-G306.

Konturek JW, Thor P, Lukaszyk A, Gabryelewicz A, Konturek SJ, & Domschke W (1997). Endogenous nitric oxide in the control of esophageal motility in humans. *J Physiol Pharmacol* **48**, 201-209.

Liebermann-Meffert D, Allgower M, Schmid P, & Blum AL (1979). Muscular equivalent of the lower esophageal sphincter. *Gastroenterology* **76**, 31-38.

Mattioli S, Pilotti V, Felice V, Di Simone MP, D'Ovidio F, & Gozzetti G (1993). Intraoperative study on the relationship between the lower esophageal sphincter pressure and the muscular components of the gastro-esophageal junction in achalasic patients. *Ann Surg* **218**, 635-639.

Mearin F, Mourelle M, Guarner F, Salas A, Riveros-Moreno V, Moncada S, & Malagelada JR (1993). Patients with achalasia lack nitric oxide synthase in the gastro-oesophageal junction. *Eur J Clin Invest* **23**, 724-728.

Muinuddin A, Chrones T, Preiksaitis HG, & Diamant NE. Distinct expression of M3 muscarinic receptors in feline lower esophageal sphincter smooth muscle. Neurogastroenterology and Motility 15[5], 591-681. 2003.
Ref Type: Abstract

Muinuddin A, Kang Y, Gaisano HY, & Diamant NE (2004a). Regional differences in L-type Ca2+ channel expression in feline lower esophageal sphincter. *Am J Physiol Gastrointest Liver Physiol* **287**, G772-G781.

Muinuddin A, Neshatian L, Gaisano HY, & Diamant NE (2004b). Calcium source diversity in feline lower esophageal sphincter circular and sling muscle. *Am J Physiol Gastrointest Liver Physiol* **286**, G271-G277.

Pasricha PJ, Ravich WJ, & Kalloo AN (1993). Effects of intrasphincteric botulinum toxin on the lower esophageal sphincter in piglets. *Gastroenterology* **105**, 1045-1049.

Paterson WG, Anderson MA, & Anand N (1992). Pharmacological characterization of lower esophageal sphincter relaxation induced by swallowing, vagal efferent nerve stimulation, and esophageal distention. *Can J Physiol Pharmacol* **70**, 1011-1015.

Perez Rubalcaba A. Real decreto sobre proteccion de los animales en el momento de su sacrificio o matanza. Boletin Oficial del Estado . 1995.
Ref Type: Journal (Full)

Preiksaitis HG & Diamant NE (1997). Regional differences in cholinergic activity of muscle fibers from the human gastroesophageal junction. *Am J Physiol* **272**, G1321-G1327.

Preiksaitis HG, Tremblay L, & Diamant NE (1994b). Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. *Dig Dis Sci* **39**, 770-775.

Preiksaitis HG, Tremblay L, & Diamant NE (1994a). Cholinergic responses in the cat lower esophageal sphincter show regional variation. *Gastroenterology* **106**, 381-388.

Richardson BJ & Welch RW (1981). Differential effect of atropine on rightward and leftward lower esophageal sphincter pressure. *Gastroenterology* **81**, 85-89.

Salapatek AM, Lam A, & Daniel EE (1998). Calcium source diversity in canine lower esophageal sphincter muscle. *J Pharmacol Exp Ther* **287**, 98-106.

Schulze K, Dodds WJ, Christensen J, & Wood JD (1977). Esophageal manometry in the opossum. *Am J Physiol* **233**, E152-E159.

Vicente Y, Da RC, Yu J, Hernandez-Peredo G, Martinez L, Perez-Mies B, & Tovar JA (2001). Architecture and function of the gastroesophageal barrier in the piglet. *Dig Dis Sci* **46**, 1899-1908.

Welch RW & Drake ST (1980). Normal lower esophageal sphincter pressure: a comparison of rapid vs. slow pull-through techniques. *Gastroenterology* **78**, 1446-1451.

Xue S, Valdez D, Collman PI, & Diamant NE (1996). Effects of nitric oxide synthase blockade on esophageal peristalsis and the lower esophageal sphincter in the cat. *Can J Physiol Pharmacol* **74**, 1249-1257.

Yamato S, Saha JK, & Goyal RK (1992). Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci* **50**, 1263-1272.

Yuan S & Brookes SJ (1999). Neuronal control of the gastric sling muscle of the guinea pig. *J Comp Neurol* **412**, 669-680.

Yuan S, Costa M, & Brookes SJ (1998). Neuronal pathways and transmission to the lower esophageal sphincter of the guinea Pig. *Gastroenterology* **115**, 661-671.

Capítol 4

Role of interstitial cells of Cajal (ICC) in neuromuscular transmission in the rat lower esophageal sphincter (LES)

4.1. Abstract

The distribution of ICC and neurotransmission were investigated in LES circular muscle strips from Sprague Dawley (SD), wild-type (+/+) and mutant (Ws/Ws) rats. Intramural c-kit positive cells were observed throughout both the circular and longitudinal layers from SD and +/+ rats. In contrast, these were absent in Ws/Ws. Strips from Ws/Ws rats showed an increased spontaneous motility. Those from SD and +/+ rats, electrical field stimulation (EFS) induced a relaxation which was L-NNA (1 mM) and apamin (1 μM) sensitive, followed by a contraction which was decreased by atropine (1 μM). In Ws/Ws rats, depolarisation with KCl (10 mM) increased the tone of the preparation and decreased the spontaneous motility. Similarly to +/+ rats, the relaxation was L-NNA and apamin-sensitive and the contraction was decreased by atropine. We conclude that in the rat LES, relaxation is mediated by nitric oxide and an apamin-sensitive mediator, and contraction by acetylcholine. Nerve-muscle interaction can be accomplished by a direct relationship between nerve endings and smooth muscle or alternatively, c-kit-negative interstitial cells could accomplish this function. The lack of c-kit-positive ICC increases the basal tension and the spontaneous motility.

Aquest capítol està en procés de revisió:
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4.2. Introduction

The lower oesophageal sphincter (LES) acts as a barrier at the gastroesophageal junction. Basal tone of LES is primarily myogenic in origin and is modulated by a combination of hormonal factors and neurogenic mechanisms involving enteric and extrinsic innervation. Inhibitory non-adrenergic non-cholinergic (NANC) enteric motor neurons are the final step of the neural pathway to LES, allowing swallowing-induced and transient LES relaxation that causes physiologic gastroesophageal reflux and belching (Chang *et al.*, 2003). It is well established that nitric oxide (NO) plays a primary role in LES relaxation in different species including humans (De Man *et al.*, 1991;Farre *et al.*, 2002;Gonzalez *et al.*, 2004;Kortezova *et al.*, 1996;Murray *et al.*, 1991). Other neurotransmitters have been proposed to share a role with NO in LES relaxation. These include an apamin-sensitive transmitter, possibly ATP (Imaeda & Cunnane, 2003;Yuan *et al.*, 1998) and vasoactive intestinal peptide (VIP) (Biancani *et al.*, 1984). However, other authors suggest that VIP has a negligible role in LES relaxation (Daniel *et al.*, 1983;Jury *et al.*, 1992). Acetylcholine is the main neurotransmitter in excitatory motor neurons (Gonzalez *et al.*, 2004;Tottrup *et al.*, 1991). However, non-cholinergic excitatory neurotransmitters, possibly substance P, have also been described in the LES (Krysiak & Preiksaitis, 2001). In the rat, vagal evoked responses involve the release of several neurotransmitters such as nitric oxide and a non-nitrergic inhibitory mediator whereas acetylcholine mediates excitation acting on muscarinic receptors (Kawahara *et al.*, 1997). However, the response “in vitro” using rat LES strips has not been characterized and the neurotransmitters involved in LES relaxation and contraction are unknown.

Interstitial cells of Cajal (ICC) are the pacemaker cells of the gut and might be involved in neurotransmission between nerve endings and smooth muscle (Daniel, 2001;Huizinga, 2001;Ward, 2000). Structural evidence supporting the role of ICC as an interface between nerve endings and smooth muscle was reported by Daniel in 1984 using the LES of the opossum (Daniel & Posey-Daniel, 1984). In this tissue varicosities are in close contact with ICC and ICC are coupled through gap junctions to smooth muscle. This observation supports a functional pathway involving nerve endings, ICC and smooth muscle.

Mutant rodents that lack specific populations of ICC are important biological tools to study a putative role of ICC in neurotransmission. The *c-kit* gene, located in the *white spotting (W)* locus, encodes Kit, a tyrosin kinase receptor. Stem cell factor (SCF)

is the natural ligand for the Kit receptor and the SCF-Kit pathway is essential for the differentiation and development of several cells such as melanocytes, mast cells and ICC. A mutation in the gene encoding the Stem Cell Factor is present in Sl/Sld mice, whereas a mutation in the gene encoding the c-kit receptor was detected in W/Wv mice and Ws/Ws rats. In the LES of W/Wv mice intramuscular ICC (ICC-IM) are absent and a reduction in nitrergic neurotransmission was found (Ward *et al.*, 1998) showing that ICC-IM might mediate inhibitory inputs from nerve endings to smooth muscle. Controversially, swallowing and vagal stimulation evoked a normal LES relaxation in Wv/Wv mice, suggesting that ICC-IM are not crucial cells mediating neurotransmission (Sivarao *et al.*, 2001). These discrepancies show that the involvement of ICC-IM in neurotransmission need further investigation with other mutant animals that might lack ICC (Sanders *et al.*, 2002).

Accordingly, the aim of this study was to characterize the neurotransmitters involved in the rat LES relaxation and contraction, and to study the response to EFS in the LES of Ws/Ws rats, which might lack ICC-IM, as an alternative model to the W/Wv mice previously studied.

4.3. Material and methods

4.3.1. Tissue preparation

Male Sprague-Dawley (SD), wild-type (+/+) and mutant (Ws/Ws) rats, (weighing 300–350 g) were used in the present study. Animals were fasted overnight (18 h) but allowed ad libitum access to water. Rats were killed by decapitation and bled. The entire stomach, including portions of the oesophagus and duodenum, was removed and placed in Krebs solution. The stomach and oesophagus were opened by an incision along the lesser curvature, continuing through the LES and the right side of the oesophagus. A second incision was made along the greater curvature from middle corpus to the apex of the fundus, revealing the junction between the oesophagus and the stomach. The LES was identified at the end of the oesophagus as the transition from striated to smooth muscle fibres in the longitudinal muscle as it had been previously reported (Kawahara et al., 1997). The experimental procedure of this work was previously approved by the Ethics Committee of the Universitat Autònoma de Barcelona.

4.3.2. C-kit immunohistochemistry

Immunohistochemical studies were performed on independent investigations. Three rats of each type were killed. The proximal stomach and lower oesophagus were isolated and fixed in paraformaldehyde (2%) in phosphate-buffered saline (PBS) (pH 7.3). The tissue was dehydrated, embedded in paraffin and oriented so that longitudinal sections could be obtained. Sections were cut at 4-μm thickness. Antigen retrieval was performed by microwaving at 95º C for 30 min using TEG buffer (Tris Basic Base and 1 M EGTA pH 9.0). Endogenous peroxidase activity was blocked by incubation in 1% hydrogen peroxide in methanol for 25 min. Sections were then preincubated in 20% goat non-immune serum in TBS for 2 hours before being incubated with a policlonal antibody against c-kit protein (Dakocytomation, Denmark) (DAKO rabbit anti-human 1:100 in 20% goat non-immune serum in TBS) at 4°C overnight. The antirabbit Envision visualization system (*DAKO ENVISION TM + System HRP Rabbit,* Dakocytomation, Denmark) was applied for 30 minutes and sections were developed in 195 mL imidazole buffer containing 5mL diaminobenzidine tetrahydrochoride (DAB) and 100μl H_2O_2. Paraffin sections were counterstained with haematoxylin stain. Control tissues were prepared in a similar manner, omitting c-kit antibody from the incubation solution.

4.3.3. Muscle bath studies

Full thickness preparations including the mucosa and both the circular and longitudinal muscle layers were obtained from the left side of LES. 1mm-wide and 3mm length strips were dissected parallel to the circular muscle fibres and placed organ baths containing 15 ml Krebs' solution, constantly bubbled with 5% CO_2 in O_2. Changes in tension of the strips were measured using isometric force transducers and recorded on a PC (Pentium III, processor speed 1500 MHz, 128 MB RAM) using the data acquisition software Acqknowledge 3.7.2 (Biopac Systems, Inc.) at a sampling frequency of 25Hz. Two strips from each animal were simultaneously studied. Strips were hung loosely, with no tension, in the organ bath for 20-30 minutes before the beginning of the study. Each strip was gently stretched up to 4.5-5 g and a stable tension was achieved after an equilibration period of 1hour. Spontaneous mechanical activity (area under curve -AUC- per minute) was measured in SD, wild-type +/+ and Ws/Ws LES strips.

4.3.4. Experimental procedure to study neuro-muscular transmission

Electrical field stimulation (EFS) was applied by means of two platinum wire electrodes (10 mm apart) located parallel to LES strips. The electrodes were connected to an electrical stimulator (Model S88, Grass Instruments Co) and a power booster (Stimu-Splitter II, Med-Lab Instruments, Loveland, CO, USA) in order to obtain identical and undistorted signals. Transmural EFS (pulses of 0.4 ms and 26V, train duration 5s at a increasing frequency from 0.3 to 20 Hz) were applied to LES strips (Gonzalez et al., 2004). To study the inhibitory and excitatory neurotransmission, the response was evaluated in the presence of the sequential addition of the nitric oxide syntase inhibitor L-NNA (1 mM), the muscarinic antagonist atropine (1 μM) and the small conductance Ca++-activated K+ channel blocker apamin (1 μM). In some Ws/Ws LES strips, KCl (10 mM) was used to contract the preparation and to evaluate the effect of these drugs in EFS-induced response.

4.3.5. Solutions and drugs

The composition of the Krebs solution was (in mM) 138.5 Na+, 4.6 K+, 2.5 Ca2+, 1.2 Mg+, 125 Cl-, 21.9 HCO3- ,1.2 H2PO4-, 1.2 SO4- and 11.5 glucose. KCl, apamin and L-NNA were obtained from Sigma-Aldrich Co (Madrid, Spain). And atropine sulphate was obtained from MERCK (Darmstadt, Germany). All drugs were dissolved in distilled water.

4.3.6. Data analysis

Tension was expressed in grams, and the spontaneous mechanical activity was evaluated by the area under curve per minute (AUCxmin-1). Relaxation was expressed as the percentage of LES tension at the end of the equilibration period. Contraction was expressed in grams. The number of experiments was represented by n (number of strips) and N (number of specimens). Data are expressed as mean ± SEM. Student's t-test was selected for comparisons, using the paired mode when appropriate. In order to characterize EFS responses, the comparison of frequency-response curves was performed using two-way ANOVA for repeated measures followed by a post-hoc test (Bonferroni). A p value <0.05 was considered statistically significant.

4.4. Results

4.4.1. C-kit immunohistochemistry

Specific c-Kit-positive ICC were distributed throughout the circular and longitudinal muscle layers (ICC-IM) in the LES of SD rats (N=3, Figure 1A and B) and +/+ rats (N=3, Figure 1C and D). Neither SD nor in +/+, c-Kit-positive ICC were found in the myenteric plexus region (ICC-MP), although ganglia appeared slightly stained (figure 1 A and C). In the absence of the primary antibody (c-kit), no immunoreactivity was found. In contrast to SD and +/+ rats, no c-kit immunlabelling was found in Ws/Ws rats (N=3) neither in the circular nor in longitudinal layers (Figure 1 E and F). These results show an absence of c-Kit- positive ICC in the LES of Ws/Ws animals.

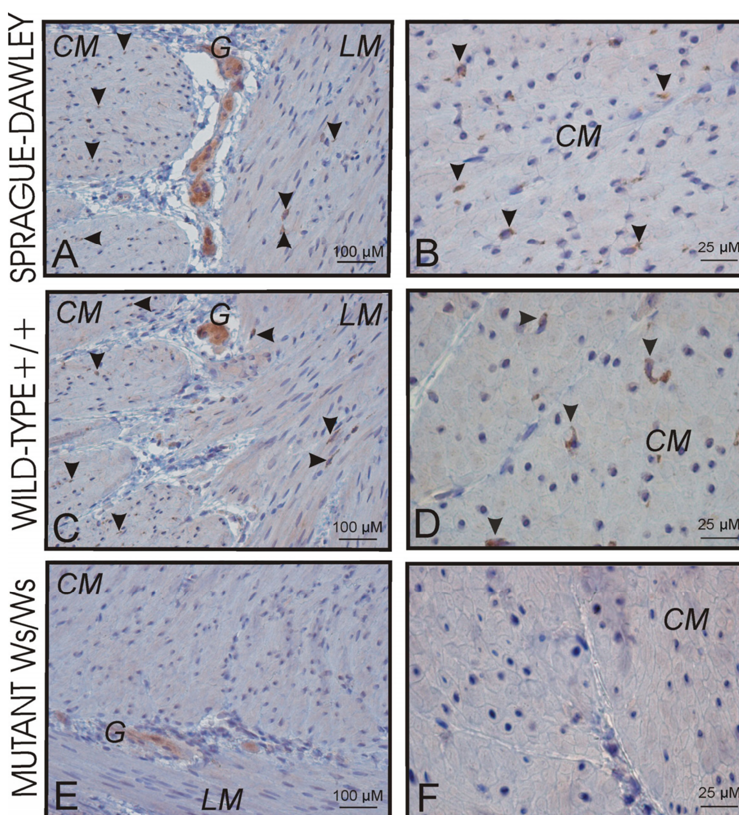

Figure 1. C-kit immunohistochemistry on the rat LES in SD, +/+ and Ws/Ws. Black arrows depict c-kit-positive ICC located in the circular (CM) and the longitudinal muscles (LM) of both SD (A) and wild-type (+/+) rats (C), these are absent in Ws/Ws rats (E). B, D and F show a magnification of the circular muscle. Ganglia (G)

4.4.2. Tension and spontaneous mechanical activity in SD, +/+ and Ws/Ws mutants

Initial stretch of LES strips did not differ between strains: 4.57±0.08g (SD, n=8, N=5), 4.88±0.41 g (+/+, n=9 N=5) and 5.20±0.35 g (Ws/Ws, n=12 N=6). After the initial stretch, tension decreased time-dependently reaching a stable tension (Figure 2 A and B). The final tension reached by the strips, expressed as the % of the initial value, was markedly higher in Ws/Ws strips (60%) than in +/+ and SD and (40 %) (ANOVA

p<0.0001, figure 2 A and B). After the equilibration period of 1 hour, Ws/Ws LES strips showed a higher stable tension (SD: 1.32±0.19 g; +/+: 1.51±0.16 g and Ws/Ws: 2.06±0.0.22 g, p<0.05) and an increased spontaneous motility (SD: 2.86±0.60 g/min ; +/+: 3.43±1.40 g/min and Ws/Ws: 23.78±6.82 g/min, p<0.002) (Figures 2 C and D).

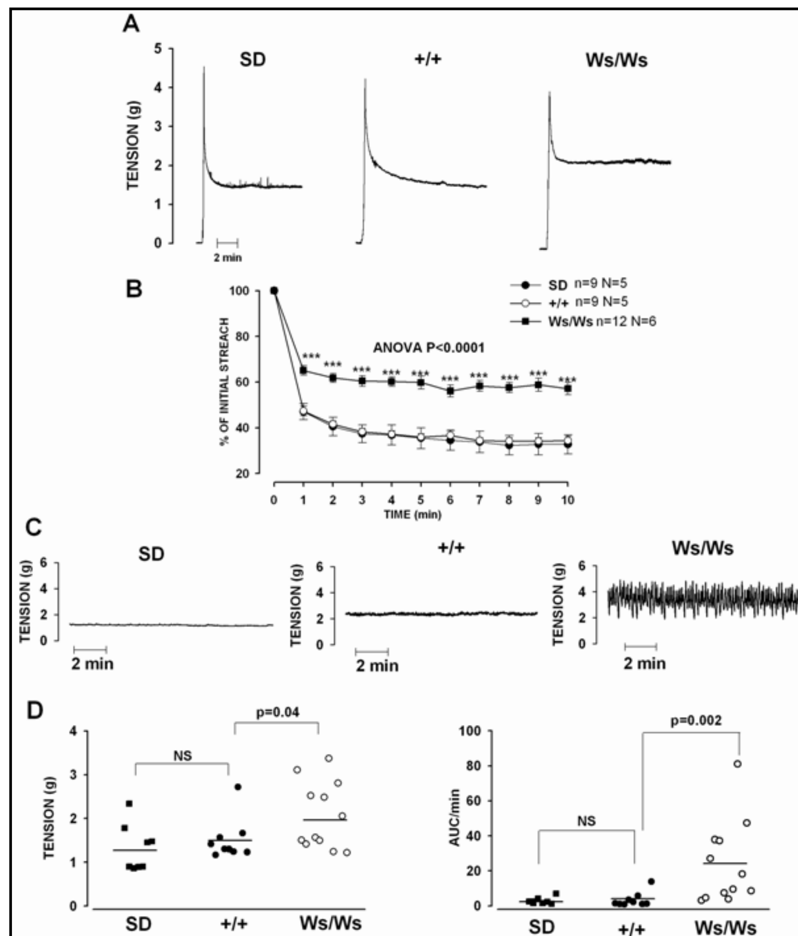

Figure 2. Tracings (A and C) and measurements of the tension after the stretching (B), tension after the equilibration period (D) and spontaneous mechanical activity developed by SD, +/+ and Ws/Ws LES strips

4.4.3. Neurotransmission in SD, +/+ and Ws/Ws rats

All SD LES strips responded to EFS with a sharp relaxation during the electrical stimulus ("on" relaxation) followed by a contraction at the end of the stimulus ("off" contraction) (n=8; N=5). The amplitude of both responses was frequency-dependent (Figure 3). Maximal relaxation (-46.16±6.50% of the tension) was reached at 3 Hz and the maximal contraction 1.29±0.37 g at 5 Hz. Regarding EFS-induced relaxation, L-NNA (1mM) significantly inhibited the response (ANOVA p<0.0001) with significant effects from 1 to 20 Hz. Subsequent addition of atropine caused an increase in EFS-

relaxation with significant effects from 10 to 20 Hz (ANOVA p<0.0001). Apamin (1µM) completely abolished this non-nitriergic relaxation (ANOVA p<0.0001). In relation to the “off” contraction, L-NNA increased the response (ANOVA p<0.001) with significant effects from 10 to 20 Hz. Subsequent addition of atropine further reduced the “off” contraction (ANOVA p<0.001) with significant effects from 3 to 20 Hz and finally, apamin reduced the “off” contraction (ANOVA p<0.001) with significant effects at 5-10 Hz (figure 3). All together, this results show that in SD rats, the inhibitory pathway involves a nitrergic and an apamin-sentive mediator whereas the excitatory pathway is mediated by acetylcholine and possibly another excitatory neurotransmitter.

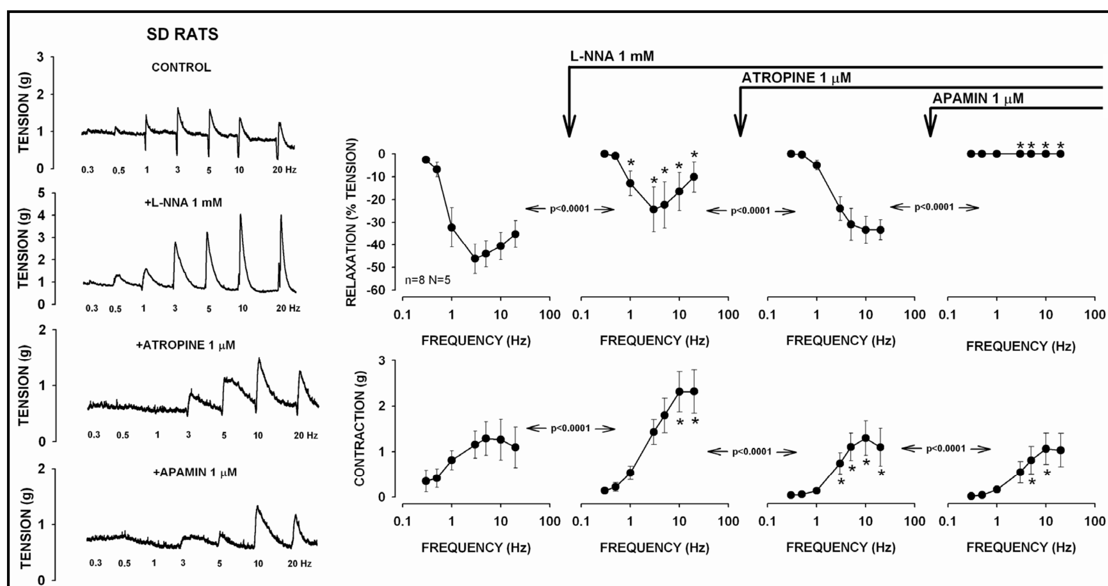

Figure 3. Effect of sequential addition of L-NNA, atropine and apamin in SD LES strips. Right and left panels show tracings and the frequency-related responses

All +/+ LES strips responded to EFS with a sharp relaxation during electrical stimulus (“on” relaxation) followed by a contraction at the end of the stimulus (“off” contraction) (n=9 N=5). The amplitude of both responses was frequency-dependent (Figure 4). Maximal relaxation (-44.17±5.38% of the tension) was reached at 5 Hz and the contraction at 20 Hz was 0.72±0.34 g. As for the EFS-induced relaxation, L-NNA (1mM) significantly inhibited the response (ANOVA p<0.0001) with significant effects from 1 to 20 Hz. Subsequent addition of atropine did not modify the EFS response and apamin abolished the non-nitrirgic relaxation (ANOVA p<0.0001). In relation to the “off” contraction, L-NNA increased the amplitude (ANOVA p<0.001) with significant effects from 5 to 20 Hz. The subsequent addition of atropine markedly reduced the “off”

contraction (ANOVA p<0.001) with significant effects from 5 to 20 Hz, and apamin did not modify amplitude of the non cholinergic contraction (figure 4). All together, this results show that in +/+ rats, the inhibitory pathway involves a nitrergic and an apamin-sentive component whereas the excitatory pathway is mainly mediated by acetylcholine.

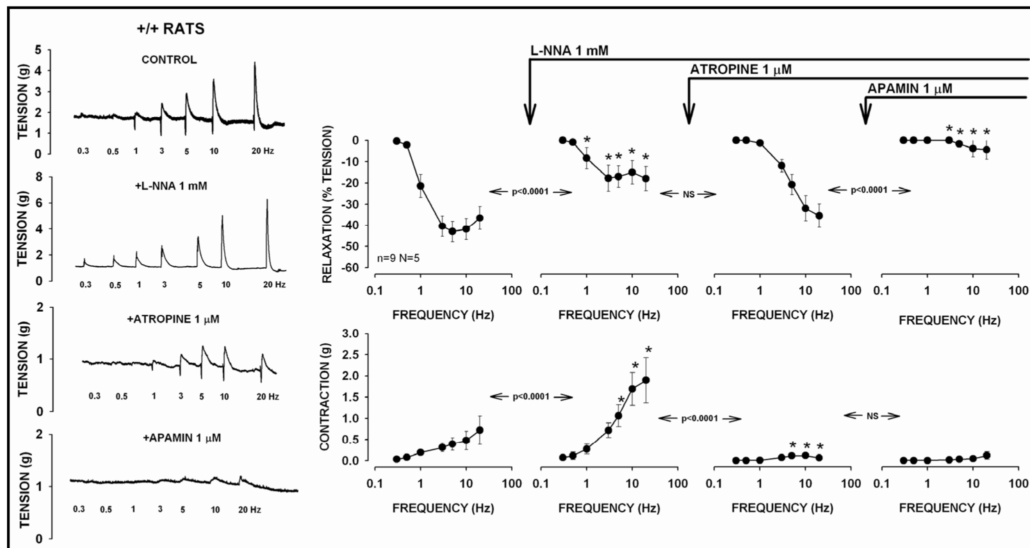

Figure 4. Effect of sequential addition of L-NNA, atropine and apamin in wild-type +/+ LES strips. Right and left panels show tracings and the frequency-related responses

Ws/Ws LES strips responded to EFS with two different patterns. In the first group of animals, 66% of the strips (n=8 out of 12 strips) responded with a sharp relaxation during electrical stimulus ("on" relaxation) followed by a contraction at the end of the stimulus ("off" contraction). This response was similar to those found in SD and +/+ LES strips. The amplitude of both responses was frequency-dependent. Maximal relaxation (-37.15±8.10% of the tension n=8 N=5) was reached at 3 Hz and the contraction at 20 Hz was 1.21±0.48 g (figure 5 group1).

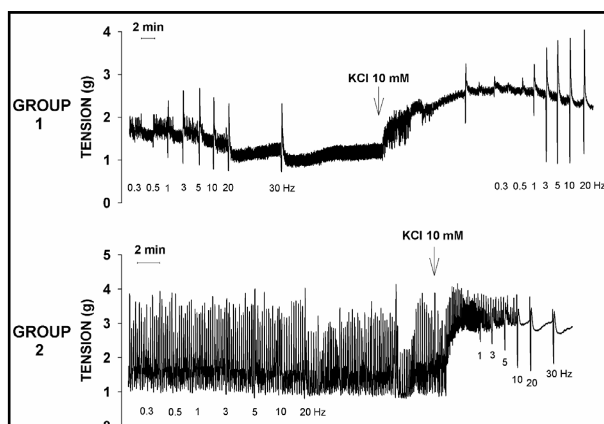

Figure 5. Tracings showing the two patterns of EFS-responses and the effect of KCl in Ws/Ws LES strips

The second group of animals (33%; n=4 out of 12 strips) responded with a frequency-dependent “off” contraction (figure 5 group 2).

The spontaneous activity of these two groups was significantly different ($p<0.01$). The strips that belonged to the second group of animals presented higher spontaneous motility than those functionally classified in the first group (group 1: 12.81±5.20 g/min and group 2: 45.72±12.00 g/min, $p<0.05$). When appropriate, to avoid a decrease in the basal tension and a spontaneous motility that might mask the muscle response, KCl 10 mM was added to the organ bath. Under these conditions all the muscles responded with an “on” relaxation followed by an “off” contraction, showing that the presence of a high motility pattern was masking the nerve-muscle interaction. Therefore under these conditions we wanted to pharmacologically characterize the mediators involved in both the excitatory and the inhibitory response. We evaluated the effect of sequential addition of L-NNA, atropine and apamin in Ws/Ws LES strips with (n=4 N=3) and without (n=2 N=2) the presence of KCl. All the strips responded to EFS with a sharp relaxation during electrical stimulus (“on” relaxation) followed by a contraction at the end of the stimulus (“off” contraction) (n=6 N=3). The amplitude of both responses was frequency-dependent (figure 6). Maximal relaxation (-48.51±9.40% of the tension) was reached at 10 Hz and the contraction at 20 Hz was 1.02±0.49 g. L-NNA inhibited the EFS-relaxation (ANOVA $p<0.0001$) with significant effects from 5 to 20 Hz. Subsequent addition of atropine did not modified EFS response. Apamin abolished the non-nitrergic relaxation (ANOVA $p<0.0001$). Regarding to the “off” contraction, L-NNA increased its amplitude (ANOVA $p<0.001$) with significant effects from 10 to 20 Hz. The subsequent addition of atropine markedly reduced the “off” contraction (ANOVA $p<0.001$) with significant effects at 20 Hz, and apamin did not modified the amplitude of the non-cholinergic contraction. Tracings of figure 6 show similar effects of the drugs in both experimental conditions (with and without KCl): L-NNA reduced the relaxation and increased the contraction, posterior addition of atropine reduced the contraction and, finally, the addition of apamin abolished the non-nitrergic relaxation. All together, these results show that also in Ws/Ws rats, the inhibitory pathway involves a nitrergic and an apamin-sentive component whereas the excitatory pathway is mainly mediated by acetylcholine.

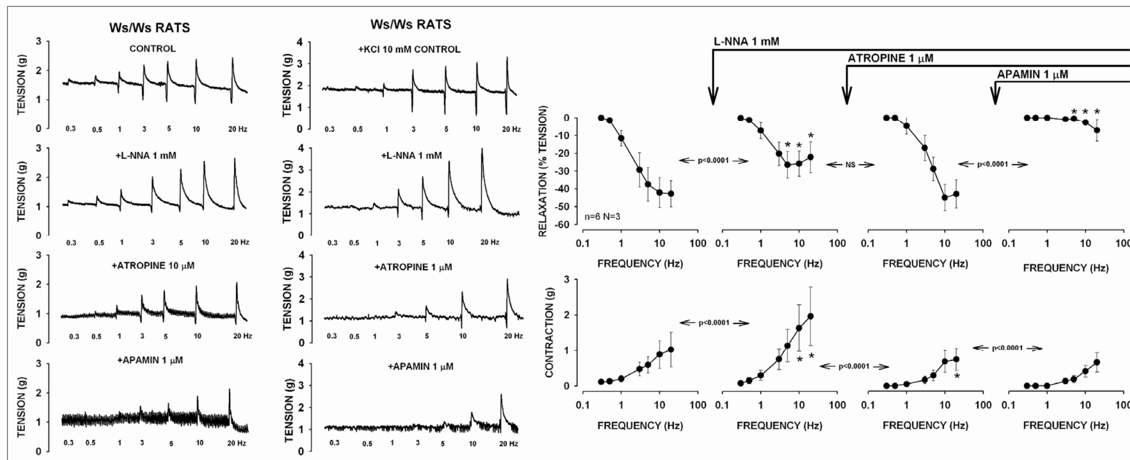

Figure 6. Effect of sequential addition of L-NNA, atropine and apamin in Ws/Ws LES strips under basal conditions and with the presence of KCl. Right panel shows tracings under basal conditions and with the presence of KCl, left panel shows graphic representation that includes strips of both experimental conditions

4.5. Discussion

The first aim of this work was to pharmacologically characterize the neurotransmitters that mediate contraction and relaxation at the neuromuscular junction in the LES of SD rats. In the present study we show that two major inhibitory neurotransmitters mediate this effect: nitric oxide and an apamin- sensitive component that might be ATP (Pluja et al., 1999;Yuan et al., 1998). Moreover, the excitatory component is cholinergic but a second excitatory neurotransmitter might be present. Moreover, these results are similar to those found in the LES of other species including humans (Gonzalez et al., 2004;Imaeda & Cunnane, 2003;Yuan et al., 1998). These results are similar to those found in vivo in rats after vagal stimulation, showing that in vitro EFS reproduce these effects (Kawahara et al., 1997). The immunohistochemistry against c-kit antigen show that c-kit-positive ICC are distributed throughout both muscle layers. Therefore, they were considered intramural ICC as it had been previously shown in the mice (Ward). These results show that ICC might be an interface between nerve endings and smooth muscle as it was structurally demonstrated in the LES of the opossum with electronmicroscopy (Daniel & Posey-Daniel, 1984) . Accordingly, the rat LES is a suitable model to study neuromuscular interactions because 1- In vivo effects are reproducible in vitro 2- the neurotransmitters involved in the relaxation and the

contraction are similar to those found in other species 3-ICC-IMs distribution follows the distribution of ICC in the LES of other animals.

Mutant animals that lack c-kit-positive ICC are important biological tools to investigate a putative role of ICC in LES neurotransmission. The results found in +/+ rats were similar to those found in SD rats: 1- smooth muscle inhibition is due to nitric oxide and an apamin-sensitive mediator, 2- excitation is mainly cholinergic and 3- c-kit-positive cells are distributed throughout both muscle layers. These results show that the different genetic background between strains do not modify the relationship between structure (ICC distribution) and function (neuromuscular transmission). In contrast, in Ws/Ws rats c-kit-positive cells are absent showing that the mutation impairs the development of c-kit-positive cells in the LES. This result is similar to the lack of c-kit-positive cells in sphincters of Wv/Wv mice (Ward *et al.*, 1998). However, the response to electrical field stimulation in Ws/Ws animals was partially similar to that found in control animals. In 66% of these strips, an "on" relaxation followed by an "off" contraction was recorded. This result shows that neurotransmission does not seem to be impaired despite the lack of c-kit-positive cells. In contrast, in 33% of the strips, the response was only excitatory suggesting a possible impairment of the inhibitory pathway. However, to check that nerve-mediated relaxation was effectively impaired, that is, not due to side effects, we used KCl to pre-contract the strips. Under these conditions, EFS caused a nitrergic and an apamin-sensitive relaxation and a cholinergic contraction. These results show that the lack of relaxation found in basal conditions is not probably due to the absence of ICC but might be related to the difference in excitability of smooth muscle cells (see below). According to these experiments, neural-mediated relaxation and contraction could be achieved despite the absence of c-kit-positive cells.

Consistent with these results, data from our laboratory show that the inhibitory junction potential in the colon of Ws/Ws rats is present and in this area of the gastrointestinal tract, c-kit-positive cells are extremely reduced (Alberti *et al.*, 2004). The major question is if ICC mediate the nerve-muscle interaction in the LES. Two hypotheses are possible: 1- c-kit-positive ICC-IM might not be essential for neurotransmission. This possibility should be considered because direct and indirect (via ICC) innervation has been ultrastructurally described in the rat stomach (Mitsui & Komuro, 2002) or 2- that this function might be accomplished by c-kit-negative interstitial cells that are in close association with nerve varicosities and form gap

junctions with smooth muscle cells (Ishikawa *et al.*, 1997). C-kit-negative interstitial cells have been characterized in the stomach of Ws/Ws rats and have been considered fibroblast-like cells (Ishikawa *et al.*, 1997;Wang *et al.*, 2003). These cells do not present the accumulation of abundant mitochondria found in normal ICC but, unlike fibroblasts, they have multiple gap junctions with smooth muscle cells. C-kit-negative interstitial cells can participate in neurotransmission because 1) cell to cell communication through the presence of gap junctions that couple these cells with smooth muscle cells has been described and 2) close associations between c-kit-negative interstitial cells have been reported. In consequence, a putative role in neurotransmission can be attributable to c-kit-negative interstitial cells. It is important to consider that some studies have reported that not all ICC identified with the electron microscopy are c-kit-positive. C-kit-negative ICC have been reported in the deep muscular plexus of the human small intestine. In this area, the cells have a weak or no immunoreactivity using several c-kit antibodies that stain ICC-AP in the same specimen, but have ultrastructural properties similar to ICC (Wang *et al.*, 2003).

Another important point to be considered is the difference in the time course to reach a stable tension and the difference in spontaneous motility found between groups of animals. Strips from animals with c-kit-positive ICC (SD and +/+ rats) reach a stable tension about 40% of the initial tension (4.5-5g) and tracings do not show spontaneous activity. Accordingly, strips from human or porcine LES do not have spontaneous motility (Farre *et al.*, 2002;Gonzalez *et al.*, 2004). In contrast, strips from Ws/Ws rats present a higher tension (60% of the initial tension) and present spontaneous motility. This is not an expected result because in vivo, the Wv/Wv mice LES is hypotensive compared to control animals (Sivarao *et al.*, 2001). We do not have a definitive explanation for this result but c-kit-positive ICC might be important elements that contribute to smooth muscle excitability. The lack of c-kit-positive ICC might depolarise smooth muscle cells and increase the probability of opening of calcium channels leading spontaneous contractions. In this sense, intestinal strips from Wv/Wv mice, which lack ICC at the Auerbach's plexus, display spontaneous rhythmic motility (Daniel *et al.*, 2004). In the other hand, it has been speculated that c-kit deficiency may affect the function and development of smooth muscle independently of the ICC-IM deficiency it entails (Sanders *et al.*, 2002); spontaneous motility could be explained, for example, by abnormalities in K^+ and/or Ca^{++} channels. The nature of the spontaneous

motility and abnormalities in smooth muscle of Ws/Ws mutant animals needs further investigation.

We conclude that nitric oxide, and an apamin-sensitive mediator, possibly ATP, are the main inhibitory neurotransmitters in the rat LES. Smooth muscle contraction is mainly mediated by acetylcholine. Ws/Ws animals lack c-kit- positive ICC but nerve-muscle interaction is preserved although in some cases strips need to be precontracted. Nerve-muscle interaction can be accomplished by a direct relationship between nerve endings and smooth muscle or alternatively, another subset of cells (c-kit-negative interstitial cells) could accomplish this function. The lack of c-kit-positive ICC modifies the basal tension and the spontaneous motility (both are increased in Ws/Ws rats). The mechanism responsible for such effects is unknown but it might be due to alteration in smooth muscle excitability.

References

Alberti E, Mikkelsen HB, Larsen JO, & Jimenez M (2004). Impaired electrical and mechanical patterns and distribution of interstitial cells of Cajal in the colon of Ws/Ws rats. *Gastroenterology* **126**, A221.

Biancani P, Walsh JH, & Behar J (1984). Vasoactive intestinal polypeptide. A neurotransmitter for lower esophageal sphincter relaxation. *J Clin Invest* **73**, 963-967.

Chang HY, Mashimo H, & Goyal RK (2003). Musings on the wanderer: what's new in our understanding of vago-vagal reflex? IV. Current concepts of vagal efferent projections to the gut. *Am J Physiol Gastrointest Liver Physiol* **284**, G357-G366.

Daniel EE (2001). Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. III. Interaction of interstitial cells of Cajal with neuromediators: an interim assessment. *Am J Physiol Gastrointest Liver Physiol* **281**, G1329-G1332.

Daniel EE, Boddy G, Bong A, & Cho W (2004). A new model of pacing in the mouse intestine. *Am J Physiol Gastrointest Liver Physiol* **286**, G253-G262.

Daniel EE, Helmy-Elkholy A, Jager LP, & Kannan MS (1983). Neither a purine nor VIP is the mediator of inhibitory nerves of opossum oesophageal smooth muscle. *J Physiol* **336**, 243-260.

Daniel EE & Posey-Daniel V (1984). Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal. *Am J Physiol* **246**, G305-G315.

De Man JG, Pelckmans PA, Boeckxstaens GE, Bult H, Oosterbosch L, Herman AG, & Van Maercke YM (1991). The role of nitric oxide in inhibitory non-adrenergic non-cholinergic neurotransmission in the canine lower oesophageal sphincter. *Br J Pharmacol* **103**, 1092-1096.

Farre R, Martinez E, Auli M, Jimenez M, Suñol X, & Clave P. Nitric oxide, adenosine triphosphate and vasoactive intestinal peptide interactions in lower oesophageal sphincter relaxation. Neurogastroenterol.Motil. 14[5], 588. 2002.
Ref Type: Abstract

Gonzalez AA, Farre R, & Clave P (2004). Different responsiveness of excitatory and inhibitory enteric motor neurons in the human esophagus to electrical field stimulation and to nicotine. *Am J Physiol Gastrointest Liver Physiol* **287**, G299-G306.

Huizinga JD (2001). Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. II. Gastric motility: lessons from mutant mice on slow waves and innervation. *Am J Physiol Gastrointest Liver Physiol* **281**, G1129-G1134.

Imaeda K & Cunnane TC (2003). Electrophysiological properties of inhibitory junction potential in murine lower oesophageal sphincter. *J Smooth Muscle Res* **39**, 119-133.

Ishikawa K, Komuro T, Hirota S, & Kitamura Y (1997). Ultrastructural identification of the c-kit-expressing interstitial cells in the rat stomach: a comparison of control and Ws/Ws mutant rats. *Cell Tissue Res* **289**, 137-143.

Jury J, Ahmedzadeh N, & Daniel EE (1992). A mediator derived from arginine mediates inhibitory junction potentials and relaxations in lower esophageal sphincter: an independent role for vasoactive intestinal peptide. *Can J Physiol Pharmacol* **70**, 1182-1189.

Kawahara H, Blackshaw LA, Lehmann A, & Dent J (1997). Responses of the rat lower oesophageal sphincter (LOS) to vagal efferent activation. *Neurogastroenterol Motil* **9**, 85-97.

Kortezova N, Mizhorkova Z, Milusheva E, Varga G, Vizi ES, & Papasova M (1996). Non-adrenergic non-cholinergic neuron stimulation in the cat lower esophageal sphincter. *Eur J Pharmacol* **304**, 109-115.

Krysiak PS & Preiksaitis HG (2001). Tachykinins contribute to nerve-mediated contractions in the human esophagus. *Gastroenterology* **120**, 39-48.

Mitsui R & Komuro T (2002). Direct and indirect innervation of smooth muscle cells of rat stomach, with special reference to the interstitial cells of Cajal. *Cell Tissue Res* **309**, 219-227.

Murray J, Du C, Ledlow A, Bates JN, & Conklin JL (1991). Nitric oxide: mediator of nonadrenergic noncholinergic responses of opossum esophageal muscle. *Am J Physiol* **261**, G401-G406.

Pluja L, Fernandez E, & Jimenez M (1999). Neural modulation of the cyclic electrical and mechanical activity in the rat colonic circular muscle: putative role of ATP and NO. *Br J Pharmacol* **126**, 883-892.

Sanders KM, Ward SM, & Daniel EE (2002). ICC in neurotransmission: hard to swallow a lack of involvement. *Gastroenterology* **122**, 1185-1186.

Sivarao DV, Mashimo HL, Thatte HS, & Goyal RK (2001). Lower esophageal sphincter is achalasic in nNOS(-/-) and hypotensive in W/W(v) mutant mice. *Gastroenterology* **121**, 34-42.

Tottrup A, Knudsen MA, & Gregersen H (1991). The role of the L-arginine-nitric oxide pathway in relaxation of the opossum lower oesophageal sphincter. *Br J Pharmacol* **104**, 113-116.

Wang XY, Paterson C, & Huizinga JD (2003). Cholinergic and nitrergic innervation of ICC-DMP and ICC-IM in the human small intestine. *Neurogastroenterol Motil* **15**, 531-543.

Ward SM (2000). Interstitial cells of Cajal in enteric neurotransmission. *Gut* **47 Suppl 4**, iv40-iv43.

Ward SM, Morris G, Reese L, Wang XY, & Sanders KM (1998). Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. *Gastroenterology* **115**, 314-329.

Yuan S, Costa M, & Brookes SJ (1998). Neuronal pathways and transmission to the lower esophageal sphincter of the guinea Pig. *Gastroenterology* **115**, 661-671.

Discussió general

La funció principal de l'esfínter esofàgic inferior (LES) és compartimentar dues zones del tracte gastrointestinal: l'esòfag i l'estómac. En condicions de repòs el LES està en contracció permanent (to muscular) i evita el pas del contingut gàstric cap a l'esòfag. En l'home, Liebermann-Meffert et al. (Liebermann-Meffert et al., 1979) van descriure que hi ha una asimetria muscular en el LES i van proposar que aquest està format per un capa muscular circular engruixida formant un anell parcial (en forma de U) o fibres de clasp, adjacents a la curvatura menor i per feixos musculars *sling-like* al costat de la curvatura major format per fibres gàstriques obliqües. En el porc la distribució anatòmica és idèntica a la de l'home. Ambdós tipus de fibres contribueixen al tancament del LES (to en repòs), i eviten així el pas del contingut àcid cap a l'esòfag. Per altra banda, durant la deglució i altres fenòmens fisiològics com per exemple les relaxacions transitòries del LES (TLESRs), es produeix l'activació d'una sèrie de mecanismes neurals que acaben amb la relaxació del LES per permetre, en el primer cas, el pas del bol de l'esòfag cap a l'estómac.

L'objectiu d'aquest treball ha estat investigar in vitro, usant la tècnica de bany d'òrgans, els diferents mecanismes implicats en la regulació de l'activitat motora de l'esfínter esofàgic inferior (LES). Per tant, hem realitzat estudis encaminats a:

a) estudiar el to muscular del LES i la seva influència neural

b) estudiar la neurotransmissió a nivell de la unió neuromuscular

c) avaluar les diferències entre les dues principals regions anatòmiques: clasp i sling

d) caracteritzar els neurotransmissors alliberats a nivell de la unió

e) comparar la resposta a l'estimulació de les motoneurones excitadores i inhibidores usant nicotina i estimulació elèctrica de camp (EFS)

f) estudiar el paper de les cèl·lules intersticials de Cajal (ICC) en el to i en la neurotransmissió inhibidora i excitadora

Per estudiar aquests diferents mecanismes hem utilitzat tres models:

-el porc, atesa la seva disponibilitat i similitud anatòmica amb l'home (Vicente et al., 2001),

-la rata, i en particular rates amb una mutació al gen Ws, el qual impedeix el desenvolupament d'algunes classes d'ICC,

-i, finalment, em pogut disposar de mostres de teixit humà, que és l'objectiu final de la recerca encaminada a entendre els mecanismes responsables del control de la motilitat en l'espècie humana.

Dintre de cadascuna de les discussions parcials s'ha abordat la problemàtica particular de cadascun dels aspectes tractats. Aquesta discussió general s'ha volgut plantejar de forma transversal de cara a donar una visió més integradora dels diferents mecanismes plantejats en els punts anteriors comparant, a més a més, les diferents espècies estudiades.

S'ha de tenir en compte que existeixen diferències importants entre les dues regions anatòmiques, clasp i sling, que formen el LES. Això s'ha caracteritzat en el porc per la facilitat d'obtenció del teixit. En canvi, en el cas de l'home, les preparacions estudiades han estat majoritàriament sling (encara que hem pogut disposar de dos espècimens de la zona clasp provinents de donants d'òrgans). Finalment els estudis en rata s'han fet amb la zona sling. A l'hora de discutir els resultats és difícil establir un ordre seqüencial perquè, per una banda, existeixen importants diferències funcionals entre zones anatòmiques i, per l'altra, s'han estudiat diverses espècies. Finalment, hem optat per la seqüència descrita anteriorment encara que molts dels resultats estan íntimament correlacionats els uns amb els altres. Per facilitar la lectura de cadascun dels apartats hem inclòs unes taules resum.

To muscular del LES i la seva influència neural

El to muscular del LES impedeix el pas del contingut gàstric a l'estómac. La pressió del LES varia en funció de l'espècie estudiada; així, en l'home, la pressió és d'uns 15 mmHg (Zaninotto et al., 1988), d'uns 60 mmHg en el gat (Zhang et al., 2005) i també d'uns 15 mmHg en els garrins (Vicente et al., 2001). La primera pregunta que cal fer-se és quina és l'equivalència entre el to *in vivo* (pressió) i el to in vitro que desenvolupen les preparacions estudiades. Així doncs, existeix in vitro:

a) un **to actiu** (*active resting tone*) que correspon a l'increment de tensió generat per la preparació després de la fase d'equilibri

b) un **to passiu** (*passive resting tone*) (Tottrup et al., 1990), que es deu a les propietats mecanoelàstiques de la preparació i que es pot evidenciar quan s'utilitzen agents relaxants

c) un **to total** que és la suma del to actiu més el to passiu i les seves propietats mecano-elàstiques. En aquest treball hem assumit que el to total equival al to generat *in vivo* pel LES, sense tenir en compte el component diafragmàtic que s'ha descrit que també contribueix a la barrera antireflux (Mittal et al., 1988).

1) Origen del to muscular

Per facilitar la comparació entre les diferents espècies, considerarem en aquest apartat les dades corresponents a les fibres *sling-like*. La comparació entre clasp i sling en el porc es farà en l'apartat corresponent. Atès el procés de dissecció, la majoria de les tires usades en l'estudi del LES humà provenen de la part esquerra de l'esfínter, és a dir, són fibres *sling-like*. El to actiu és similar en el porc i en l'home, 3,6±0,36 g i 3,85±0,44, respectivament. En canvi, en la rata, a diferència de l'home i del porc, les tires del LES no guanyen to actiu, tot i que relaxen a l'aplicació de l'EFS, fet que suggereix que tenen un to passiu molt important induït pel mateix estirament de la tira.

El to muscular del LES és d'origen miogènic i pot estar modulat per influències nervioses de tipus inhibidor i excitador.

Així doncs, la incubació del teixit amb un medi lliure de calci (to principalment miogènic) redueix el to actiu un 47,5±4,8% en l'home i 78,53±7,82% en el porc. Aquests resultats demostren que una part important del to muscular és d'origen miogènic i depèn de l'entrada de calci extracel·lular. Aquest resultat ha estat prèviament descrit en l'opòssum (Salapatek et al., 1998) i en l'home (Tottrup et al., 1990).

2) Influència neural

Existeix una influència neural sobre el to tant en l'home com en el porc, però existeixen diferències entre aquestes dues espècies. En el porc la incubació amb TTX no provoca cap modificació en la tensió de la preparació. Aquest resultat no es deu a l'absència d'influència neural perquè inhibint vies excitadores colinèrgiques amb atropina es produeix una disminució d'un 68% en el to actiu de la preparació i inhibint vies inhibidores nitrèrgiques es produeix un increment d'un 80%. Això suggereix, en el porc, que el to muscular del LES està influenciat tant per vies nervioses excitadores de tipus colinèrgic i inhibidores de tipus nitrèrgic.

En l'home la TTX provoca un augment del to actiu de magnitud similar a la produïda per l'L-NNA, en canvi l'atropina no provoca cap canvi ni tan sols en presència

de TTX i d'L-NNA. Això suggereix que en l'home el to muscular del LES està influenciat per vies nervioses principalment de tipus inhibidor.

Igual que en l'home i en el porc, en la rata el blocatge de la síntesi d'NO augmenta el to de la preparació. En canvi l'atropina no té cap efecte, com ocorre també en l'home. Aquests resultats suggereixen que en la rata, el to està modulat per motoneurones nitrèrgiques del plexe mientèric i que no existeix modulació important per part de les neurones colinèrgiques. En la taula 1 es compara el to actiu, el percentatge de dependència del calci extracel·lular i la presència o no d'influències neurals en la modulació del to entre les tres espècies estudiades.

Taula 1. Origen del to muscular i de la seva influència neural en l'home, el porc i la rata

	To actiu	**Dependència Ca^{++} extracel.**	**Vies implicades**	
espècies			Inhibidores nitrèrgiques	Excitadores colinèrgiques
Humà	3,6±0,36	47,5±4,8%	sí	no
Porc	3,85±0,44	78,53± 7,82%	sí	sí
Rata	0	NA	sí	no

NA no avaluat

Els resultats d'aquest estudi es poden correlacionar amb estudis *in vivo* amb gossos (Boulant et al., 1994) i opòssum (Yamato et al., 1992) i l'home (Konturek et al., 1997) en els quals s'han evidenciat que els inhibidors de la síntesi d'NO augmenten la pressió del LES. L'atropina redueix la pressió del LES *in vivo* en humans (Mittal et al., 1995), però l'administració d'un antagonista colinèrgic que no creua la barrera hematoencefàlica no produeix cap canvi en la pressió (Lidums et al., 1997). En el porc, l'administració d'atropina (Kiatchoosakun et al., 2002) i de BoTx (Pasricha et al., 1993) en el LES en disminueix la pressió un 50-60%. Aquests resultats, juntament amb les nostres troballes, suggereixen que *in vivo* els inhibidors de la síntesi d'NO augmenten la pressió de l'esfínter per un efecte local sobre les motoneurones inhibidores del plexe mientèric i, en canvi, l'efecte de l'atropina es deuria a un efecte a nivell central en l'home. Contràriament, en el porc s'evidencia una important modulació colinèrgica a nivell local.

Neurotransmissió a nivell de la unió neuromuscular

Per estudiar la neurotransmissió a nivell de la unió neuromuscular em fet servir dos tipus d'estímuls: l'estimulació per camp elèctric (EFS) i l'estimulació de les motoneurones mitjançant l'activació dels receptors nicotínics d'ACh (nAChRs). En aquest apartat es descriurà la caracterització de la resposta a aquests dos estímuls.

Estudis en preparacions del LES de diferents espècies com el conill porquí (Beveridge & Taylor, 1989), l'opòssum (Tottrup et al., 1991) i el gat (Fields et al., 1995) en els quals s'ha fet servir la tècnica de bany d'òrgans, mostren que la resposta a l'EFS consisteix en una relaxació durant l'estímul, anomenada relaxació en 'on', seguida d'una contracció un cop finalitzat l'estímul o contracció en 'off'. En les tres espècies estudiades –la rata, el porc i l'home– les tires d'esfínter esofàgic inferior responen també amb aquest patró característic. La resposta a l'EFS es bloqueja per TTX, i demostra que aquesta resposta és deguda a l'activació de motoneurones inhibidores i excitadores.

La freqüència en què es produeix la màxima resposta relaxadora és de 3 Hz en les tres espècies estudiades i la seva magnitud és molt semblant: en les fibres *sling-like* de la rata la resposta màxima va ser de -46,16±6,50%, en el porc de -58,02±2,22% i en l'home de -45,08±4,32% del to total. L'amplitud màxima de la contracció és, en la rata, d'1,29±0,37 g a 5 Hz, en el porc d'11,69±0,96 g a 10 Hz i en l'home d'1,60±0,25 g a 5 Hz. Així doncs, la resposta a l'EFS és igual quant a la morfologia de la resposta i força semblant quant a la seva magnitud i a la freqüència en què es produeix la resposta màxima. Així es demostra que, en les tres espècies, les motoneurones inhibidores s'estimulen a més baixes freqüències que no pas les excitadores.

En estudis *in vivo* en l'opòssum (Rattan & Goyal, 1975) i in vitro en el gat (Kortezova et al., 1994) i l'opòssum (Matsuda et al., 1997) s'ha vist que la nicotina indueix la relaxació del LES. En el nostre estudi, l'estimulació amb nicotina, indueix en les tires *sling-like* una resposta relaxadora de magnitud semblant tant en preparacions de porc com d'humà. Aquests resultats demostren que en el LES d'aquestes dues espècies, l'activació de nAChRs produeix l'activació de motoneurones inhibidores.

En estudis amb intestí de conill porquí en què s'ha usat la neurotoxina TTX (Schneider et al., 2000), s'ha descrit la presència de receptors nicotínics en les motoneurones colinèrgiques, tant a nivell del soma neural (somatodendrítics) com a nivell del terminal nerviós. L'activació dels receptors somatodendrítics indueix una

resposta que depèn d'un potencial d'acció neural que és degut a l'entrada de sodi i, per tant, TTX sensible) mentre que l'activació del receptors nicotínics a nivell del terminal provoca l'alliberació de neurotransmissors sense la necessitat de la gènesi d'un potencial d'acció (aquesta resposta és, doncs, TTX insensible). En el nostre estudi s'evidencia la presència de receptors somatodendrítics en les dues espècies (porc i humà). En el porc, la relaxació és deguda majoritàriament per l'activació d'aquest tipus de receptors, ja que la TTX transforma la relaxació induïda per nicotina en una contracció colinèrgica. Així doncs, semblaria que en el porc les vies inhibidores presenten receptors somatodendrítics i que les excitadores tindrien receptors nicotínics presinàptics. En canvi, en l'home la TTX disminueix la relaxació induïda per nicotina un 40%. Per tant, part de la resposta es deu a l'estimulació de receptors somatodendrítics, i una altra part a l'activació de receptors localitzats en el terminal nerviós. S'ha suggerit que, tot i no ser funcionals per l'Ach alliberada endògenament, aquests receptors nicotínics presinàptics poden ser dianes per al desenvolupament de nous agents procinètics (Galligan, 1999).

Diferencies entre les dues principals regions anatòmiques: clasp i sling

a) Control del to i influència neural

En aquest estudi i confirmant un estudi amb garrins (Vicente et al., 2001), es pot veure que en el porc, igual que en l'home, el LES és format per les fibres de clasp a la part dreta, formant una U, i les de *sling-like* a l'esquerra. Aquesta distribució és diferent en els altres models estudiats com el gat, l'opòssum i el gos, on les fibres de clasp formen un anell complet. Som, per tant, davant d'un model anatòmicament molt semblant a l'home.

Un dels objectius d'aquest estudi va ser explorar en preparacions de les dues regions del LES si, a part d'aquesta asimetria anatòmica, existia una asimetria funcional. Per avaluar aquesta hipòtesi, es va valorar el to miogènic, la influència neural sobre el to muscular i la resposta a l'estimulació elèctrica de camp i a la nicotina.

Els nostres resultats demostren que en el porc existeix una marcada asimetria pel que fa la modulació del to, tot i que el to actiu no difereix entre les fibres de clasp i les de *sling-like* i confirmen els resultats obtinguts en el gat (Preiksaitis et al., 1994a), existeixen importants diferències pel que fa al to total. El to total és superior en les tires

de sling (clasp: 4,72±0,67g vs. sling: 7,59±0,89 g). Tot i això, ambdós tipus de tires tenen la mateixa capacitat per relaxar quan se les exposa al donant d'NO nitroprusiat sòdic (SNP). Aquestes diferències en el to total es poden correlacionar amb estudis *in vivo* en el porc (Vicente *et al*., 2001), en el gat (Preiksaitis et al., 1994a) i en humans (Welch & Drake, 1980) en els quals la pressió generada per les fibres de sling és superior a les de clasp.

Pel que fa a l'origen d'aquest to, existeixen diferències entre ambdues regions anatòmiques. Tant en clasp com en sling, el to depèn del Ca^{++} extracel·lular però aquesta dependència és major en clasp, com s'observa també en estudis realitzats en el gat, on un medi lliure de Ca^{++} redueix més el to en clasp que en sling (Muinuddin et al., 2004).

Pel que fa a la influència neural sobre el to també vam observar importants diferències en funció de la regió anatòmica estudiada. La TTX no produeix canvis significatius en la tensió en cap de les dues regions estudiades. Això no vol dir que no hi hagi una influència neural sobre el to, perquè l'ODQ (inhibidor de la guanilat ciclasa) produeix un augment del to que només és significatiu en sling. Per altra banda, l'atropina provoca una disminució del to en ambdues preparacions, tot i que aquesta disminució és força superior en sling que en clasp. Això suggereix que el to muscular del LES està influenciat tant per vies nervioses inhibidores de tipus nitrèrgic (sling) com per excitadores de tipus colinèrgic (clasp i sling).

Així doncs, podem concloure que en el porc existeix una marcada asimetria en la dependència del Ca^{++} extracel·lular, força més important en clasp que en sling, i en la modulació del to per part de les motoneurones del plexe mientèric. En sling hi ha un important component nitrèrgic i colinèrgic, mentre que en clasp només existeix un component colinèrgic i de menor magnitud.

Taula 2. To actiu, to total, dependència del Ca^{++} extracel·lular i influència neural en les preparacions clasp i sling

	CLASP	**SLING**
To actiu	3,66±0,55 g	3,85±0,44 g
To total	4,72±0,67 g	7,59±0,89 g
Dependència Ca^{++} extracel.	70%	40%
Component nitrèrgic	NS	30%
Component colinèrgic	20%	40%

NS no significatiu

En la taula 2 es compara el to actiu, el to total, la dependència del Ca^{++} extracel·lular i la influència neural dels components nitrèrgic i colinèrgic entre clasp i sling.

b) Neurotransmissió a nivell de la unió neuromuscular i caracterització dels components implicats

En aquest estudi també es va avaluar l'existència d'una asimetria funcional a l'estimulació elèctrica de camp i a l'estimulació dels nAChRs. S'ha vist que les preparacions de clasp tenen una relaxació de major magnitud a tot el rang de freqüències estudiades, comparativament amb les preparacions de sling. En estudis preliminars amb teixit humà també vam veure aquesta diferència (resultats no mostrats), i així es van confirmar les troballes de Preiksaitis et al. (Preiksaitis & Diamant, 1997) en què va usar només una única freqüència d'estimulació. Igual que en la relaxació, en la contracció existeixen diferències en funció de la procedència de les tires, en el porc s'ha vist que les tires de clasp tenen una contracció en 'off' d'amplitud major que el sling a gairebé totes les freqüències testades. Aquests resultats es poden interpretar de dues maneres diferents, o bé 1) existeix una major sensibilitat o eficàcia per part de les fibres musculars als neurotransmissors inhibidors i excitadors, o bé 2) existeix una diferent innervació selectiva en aquestes dues regions. Per avaluar aquestes diferències vam aplicar un disseny experimental en fibres de clasp i en fibres de sling de forma aparellada, basat en avaluar 1) la caracterització farmacològica a la resposta a l'EFS, 2) la sensibilitat muscular a l'addició exògena de neurotransmissors inhibidors (addició d'ATP i del donador d'NO SNP) i excitadors (agonista muscarínic carbacol). Per estudiar la contribució dels diferents neurotransmissors en cadascuna de les regions anatòmiques es va avaluar la resposta atribuïble a cada neurotransmissor mitjançant la utilització d'antagonistes per cadascun d'ells. Així doncs, la resposta nitrèrgica es va calcular obtenint la diferència entre el control i el teixit incubat amb ODQ, la resposta colinèrgica amb posterior incubació amb atropina i la resposta purinèrgica amb posterior addició d'apamina. Els components nitrèrgic, colinèrgic i purinèrgic són presents en ambdues regions anatòmiques, però la contribució relativa de cadascun d'ells és molt diferent. A la regió clasp la contribució dels dos components inhibidors és major que a la regió sling; en canvi, la contribució del component colinèrgic és major a la regió sling. Aquesta diferent contribució es podria explicar per la diferent resposta que tenen les fibres de clasp i de sling a l'aplicació exògena de SNP, d'ATP i de carbacol. La mateixa dosi de SNP i d'ATP provoca relaxacions de magnitud superior en

clasp que en sling i la mateixa dosi de carbacol provoca contraccions de major magnitud en sling que en clasp, de gairebé el doble. Hem comparat les EC50 d'aquestes corbes concentració-resposta per avaluar si aquestes diferències es deuen a una diferent sensibilitat (si les EC50 són diferents) o bé a una diferent eficàcia (si les EC50 són iguals). Així trobem que existeix una diferent sensibilitat pel SNP i una diferent eficàcia per l'ATP i pel carbacol de les fibres musculars als neurotransmissors exògens. Aquests resultats suggereixen que la diferent contribució de cada neurotransmissor es pot deure a un mecanisme post unió. Resultats similars s'han descrit en el LES del gat (Preiksaitis et al., 1994a), on es mostra també una major eficàcia de les fibres de sling pel carbacol. De totes maneres no es pot descartar una contribució deguda a una diferent innervació, descrita prèviament en el LES del conill porquí. Estudis immunohistoquímics que van usar anticossos per marcar les motoneurones nitrèrgiques i les colinèrgiques demostren que existeix una major innervació nitrèrgica en la part dreta i una major innervació colinèrgica en la part esquerra del LES (Yuan et al., 1998;Yuan & Brookes, 1999). Aquests resultats necessiten ser confirmats en el porc.

La relaxació induïda per nicotina és de la mateixa magnitud en preparacions procedents d'una banda o de l'altra del LES, tot i que existeixen diferències en la localització dels receptors nicotínics a nivell neural entre les regions estudiades. En les mostres obtingudes de la zona clasp, els receptors de les motoneurones inhibidores es localitzen en ambdós nivells, a nivell presinàptic i a nivell somatodendrític. En canvi, en la zona sling els receptors es localitzen majoritàriament a nivell somatodendrític, ja que la TTX bloqueja totalment la relaxació induïda per nicotina i apareix una important contracció colinèrgica, fet que suggereix a la vegada la presència també de receptors nACh en les motoneurones excitadores a nivell del terminal nerviós.

L'addició de nicotina provoca una alliberació de diferents neurotransmissors en la zona clasp i el la sling. En les preparacions clasp existeix un component nitrèrgic i purinèrgic de tipus inhibidor molt important, mentre que en la zona sling només el primer hi és present. En ambdues zones existeix un component colinèrgic, però aquest és major en la zona sling. Així doncs, l'estimulació de motoneurones usant un estímul químic com la nicotina produeix l'alliberació de neurotransmissors inhibidors i excitadors, però la seva importància relativa és diferent en funció de la regió estudiada.

Estudis recents en el clasp del conill porquí (Yuan et al., 1998) mostren que l'estimulació vagal no provoca respostes excitadores mesurables ni amb el bloqueig de la via inhibidora amb L-NNA ni i amb apamina. Però el mateix grup, si troba una

resposta excitadora colinèrgica en la part sling, només un cop bloquejats els dos components inhibidors (Yuan & Brookes, 1999). Aquests resultats suggereixen que hi ha una diferent resposta de les motoneurones colinèrgiques a l'estimulació vagal en clasp i en sling. En el nostre estudi en el porc es troben resultats similars quan s'utilitza la nicotina per estimular les motoneurones del plexe, tot i que és molt més fàcil estimular les motoneurones colinèrgiques en preparacions procedents del sling.

Així doncs, les diferències entre clasp i sling es podrien resumir de la següent manera: 1) la tensió total és major en la zona sling que en la clasp, 2) la dependència del Ca^{++} extracel·lular en el manteniment del to és més gran en clasp, 3) la modulació neural del to muscular és diferent: en sling la modulació és nitrèrgica i colinèrgica mentre que en clasp només és colinèrgica, i aquesta és de menor importància relativa, 4) l'estimulació de motoneurones inhibidores i excitadores provoca respostes de major amplitud en la zona de clasp que la de sling, 5) la contribució dels neurotransmissors inhibidors és més gran en clasp que en sling i la dels neurotransmissors excitadors és més gran en sling, 6) la sensibilitat de l'administració exògena d'agonistes inhibidors és més gran en clasp mentre que la dels excitadors és més gran en sling, 7) l'estimulació de les motoneurones inhibidores alliberen NO i un neurotransmissor sensible a l'apamina (que és ATP com es veurà més endavant), en la zona clasp, mentre que principalment NO en la zona sling.

Taula 3. Contribució dels diferents components inhibidors i excitadors i resposta exògena dels neurotransmissors

	CLASP	**SLING**
Component nitrèrgic	+++	++
Component colinèrgic	++	+++
Component purinèrgic	+++	+
Efecte SNP	+++	++
Efecte carbacol	++	+++
Efecte ATP	+++	+

+ poc, ++ força, +++ molt

En la taula 3 es resumeixen les troballes realitzades en les preparacions procedents de clasp i de sling utilitzant un criteri subjectiu per donar una idea clara i ràpida de les diferències observades.

Tenim evidències que en l'home existeix també una forta asimetria pel que fa als components implicats en la relaxació. En dos espècimens procedents de donants d'òrgans es va avaluar la implicació del component nitrèrgic i del component colinèrgic en la resposta induïda per l'EFS i per la nicotina. En el sling de l'home, com es descriu en profunditat més endavant, existeix un component inhibidor nitrèrgic molt important i un segon component sensible a l'apamina i de magnitud molt inferior. També apareix un component colinèrgic força important. En canvi, en el clasp continua apareixent un component nitrèrgic, però apareix un segon component no nitrèrgic, igual d'important que el primer. El component colinèrgic és de magnitud similar a l'observat en el sling.

Aquesta asimetria observada en el porc i evidenciada també en l'home podria explicar-se pel diferent origen de les fibres de clasp i de sling. Les primeres s'originen per un engruiximent de la musculatura circular de l'esòfag i les segones són d'origen gàstric; les fibres de sling recorren els dos costats de la curvatura major fins arribar gairebé al pílor.

Caracterització dels neurotransmissors a nivell de la unió neuromuscular

a) En el porc

Atès que, com s'ha comentat anteriorment, en la zona clasp existeix un doble component inhibidor i que aquest és majoritàriament nitrèrgic en la zona sling, vam utilitzar la zona clasp per caracteritzar farmacològicament la neurotransmissió en el porc. Està ben establert que l'NO és el principal neurotransmissor inhibidor implicat en la relaxació del LES en diferents espècies incloent-hi l'home (de Man et al., 1991;Kortezova et al., 1996;Murray et al., 1991;Preiksaitis et al., 1994b). S'han proposat altres neurotransmissors que comparteixen un paper junt amb l'NO en la relaxació del LES. Aquest segon neurotransmissor s'ha descrit que pot ser el VIP (Biancani et al., 1984) o bé un neurotransmissor sensible a l'apamina, possiblement l'ATP (Yuan et al., 1998). Encara que altres autors suggereixen que el VIP no hi juga cap paper (Daniel et al., 1983;Jury et al., 1992).

Per avaluar els possibles neurotransmissors inhibidors implicats en la relaxació del LES del porc, es va avaluar l'efecte de l'aplicació exògena de SNP (donador d'NO), CORM-1 (donador de CO), d'ATP, de PACAP, de VIP i de CGRP. Tots els agonistes

usats excepte el CGRP van induir una relaxació, aquesta relaxació no es va veure modificada per la TTX, fet que suggereix un acció a nivell muscular.

A continuació vam testar diferents antagonistes, inhibidors i blocadors de canals per bloquejar específicament la resposta als diferents neurotransmissors aplicats exògenament. Així doncs, l'ODQ bloqueja específicament la relaxació induïda per SNP, l'apamina bloqueja el component ràpid de la relaxació induïda per ATP i PACAP i no la induïda pel SNP, l'α-quimotripsina (peptidasa) la relaxació induïda per VIP i per PACAP i per MRS2179 (antagonista $P2Y_1$) la relaxació induïda per ATP. Així doncs, aquests resultats suggereixen que l'NO administrat en forma de SNP actua exclusivament via sGC produint la síntesi de cGMP, i no activant canals SKCa com està descrit en la literatura (Serio et al., 2003b). En canvi, la relaxació ràpida induïda per l'ATP i el PACAP si que es produeix per activació de canals SKCa. L'L-NAME no modifica la relaxació induïda per VIP, PACAP ni per ATP, fet que demostra que cap d'aquests possibles neurotransmissors actuen via síntesi d'NO , com està descrit en la literatura (Jun et al., 2003;Xue et al., 2000;Zizzo et al., 2004).

Per avaluar els neurotransmissors alliberats per estimulació neural amb EFS i nicotina, vam realitzar un protocol experimental de bloqueig seqüencial amb diferents fàrmacs. Els resultats demostren que la relaxació és deguda a l'NO i a un component sensible a l'apamina. Usant ODQ o L-NAME s'obtenen resultats idèntics, fet que suggereix que és l'NO *per se* el responsable de la relaxació, ja que activa la sGC i que probablement l'NO no allibera cap neurotransmissor a nivell presinàptic com s'ha descrit en la literatura (Grider et al., 1992). A més, aquests resultats indiquen que l'NO alliberat endògenament per EFS actua exclusivament via sGC i no utilitza la via del canals SKCa (Serio et al., 2003b). El component apamin sensible està mediat per ATP o una purina relacionada i actua sobre receptors $P2Y_1$ com s'ha descrit prèviament en l'intestí del ratolí (de Man et al., 2003). Aquests resultats es posen de manifest per la important inhibició per part de l'MRS2179. Per altra banda, un efecte menor apareix en presència d'α-quimotripsina i d'NF279 (antagonista $P2X_{1,2,3}$). Aquest resultats demostren que el component sensible a l'apamina és degut a l'alliberació d'ATP o una purina relacionada i que el receptor implicat és un P2Y1 tal com els resultats obtinguts en el nostre laboratori demostren en el còlon humà (Aulí et al., 2005;Gallego et al., 2005) i en l'intestí de porc (Farré et al., 2005). En aquest últim treball es demostra que el potencial postunió inhibidor (IJP) està mitjançat per l'activació de receptors $P2Y_1$, i proporciona així una correlació electrofisiològica als resultats mecànics aportats en

aquest estudi. També pot haver-hi un petit paper pel PACAP, ja que la relaxació ràpida induïda per l'addició de PACAP exogen és bloquejada tant per apamina com per α-quimotripsina. Existeixen altres estudis on es demostra que la relaxació induïda per PACAP està mitjançada per canals SKCa, en què es suggereix la implicació d'aquest pèptid en el component sensible a l'apamina evidenciat per l'EFS (Zagorodnyuk et al., 1996;Serio et al., 2003a). La disminució de la relaxació produïda per l'NF279 suggereix la participació dels receptors P2X. S'ha descrit que aquests receptors estan implicats en la relaxació sensible a l'apamina en l'intestí del ratolí a nivell preunió i/o postunió (de Man et al., 2003). Estudis immunohistològics en el LES del porc demostren la presència de l'enzim de síntesi, hem oxigenasa tipus 2 (HO-2) en el LES del porc (Werkstrom et al., 1997). Tot i això, el nostre estudi suggereix que el CO no deu estar implicat en el component sensible a l'apamina, perquè l'inhibidor de l'hem oxigenasa, *tinprotoporfirin IX*, no té cap efecte sobre la relaxació no nitrèrgica.

Experiments amb EFS, evidencien que el component contràctil està mediat per un component nitrèrgic (bé sigui directe o bé rebot) i un component colinèrgic. En el nostre estudi s'observa que tant l'L-NAME com l'ODQ redueixen la contracció en 'off' induïda per EFS, la posterior addició d'atropina redueix aquesta contracció residual.

Aquests resultats confirmen les troballes descrites amb ratolins que no tenen els enzims nNOS i eNOS (*knock out*) (Kim et al., 1999) i en que s'han usat preparacions del LES in vitro, on s'observa que la nNOS és la responsable tant de la relaxació com de la contracció rebot, i també les troballes *in vivo* en el gat on es mostra que l'L-NNA redueix tant la relaxació com la contracció del LES induïda per la deglució (Sifrim & Lefebvre, 2001).

Aquests resultats suggereixen que l'estimulació de les motoneurones del plexe, tant amb EFS com per l'estimulació dels nAChRs, provoquen l'alliberament d'NO , que actua per la via del la sGC, més un segon neurotransmissor que és sensible a l'apamina. Aquest és majoritàriament ATP o una purina relacionada que actua sobre receptors $P2Y_1$ que provoquen l'obertura de canals SKCa. En menor magnitud, pot existir un paper per al PACAP i per als receptors $P2X_{1,2,3}$ a nivell presinàptic i/o postsinàptics. La contracció és deguda a un fenomen rebot mitjançat per l'NO més un component colinèrgic.

b) En l'home

La neurotransmissió en l'home s'ha caracteritzat majoritàriament en preparacions procedents de la regió de sling. Així, quan es comparin l'home amb el porc es compararan només els resultats obtinguts en aquesta regió.

Els nostres resultats confirmen que l'NO és el principal neurotransmissor en la relaxació induïda per EFS en el LES del home (Preiksaitis et al., 1994b) i suggereix que s'allibera un altre neurotransmissor inhibidor perquè apareix una relaxació sensible a TTX després del bloqueig nitrèrgic, de manera similar a com s'observa en l'opòssum (Uc et al., 1999). En aquest estudi es testen, sense èxit, antagonistes del VIP i del CGRP però no s'avalua la implicació d'un component sensible a l'apamina. En el nostre estudi es pot veure que, igual que en el porc, en l'home existeix un component nitrèrgic majoritari més un altre component de magnitud molt menor que és completament sensible a l'apamina en l'home i només ho és parcialment en el porc. Aquests resultats són semblants als trobats en el sling de l'opòssum (Yuan & Brookes, 1999), on també es descriu un component sensible a l'apamina a més d'un de nitrèrgic. Com s'ha vist anteriorment, aquest neurotransmissor sensible a l'apamina pot ser ATP o una purina relacionada que actua principalment sobre receptors $P2Y_1$. Aquest és el primer estudi on s'evidencia un component possiblement purinèrgic en el LES de l'home.

En el porc i en l'home, a més de bloquejar la relaxació, l'L-NNA, transforma la contracció en 'off' en una contracció en 'on' colinèrgica. Resultats idèntics han estat prèviament descrits també en l'home (Preiksaitis et al., 1994b). Aquest important component colinèrgic apareix en les dues espècies, però la seva magnitud és força superior en el porc. A 20 Hz, l'amplitud en l'home és d'uns 6 g i en el porc d'uns 20 g. Aquesta diferència es pot explicar per una major sensibilitat o eficàcia de l'ACh en el LES del porc o bé per una major innervació colinèrgica.

Igual que quan s'estimula amb EFS, quan s'estimula amb nicotina s'allibera majoritàriament NO més un segon neurotransmissor sensible a l'apamina que representa només un 10% de la relaxació total. Resultats similars s'observen en un altre estudi amb LES humà (Smid & Blackshaw, 2000), on es mostra que la relaxació induïda per l'estimulació dels receptors nACh amb DMPP també és principalment deguda a l'alliberament d'NO .

Així doncs, en el LES humà, l'NO és el principal però no l'únic neurotransmissor inhibidor alliberat, existeix també un paper menor per un neurotransmisor sensible a l'apamina.

c) En la rata

Els components implicats en la relaxació del LES de la rata són els mateixos que en porc i en l'home. En el sling de la rata, la relaxació està mitjançada per un component nitrèrgic i un altre component sensible a l'apamina, tot i que la magnitud d'aquest últim component és major que en les altres dues espècies estudiades. Igual que en l'home i el porc, la contracció es deu a un component colinèrgic, però existeix un segon component igual d'important que el colinèrgic i que no s'evidencia en les altres dues espècies. Estudis en què s'usa estimulació vagal en rata (Kawahara et al., 1997) demostren que la inhibició és deguda a NO i un altre component no adrenèrgic i que tampoc és degut a NO, i que podria ser, com es mostra en aquest estudi, un neurotransmissor sensible a l'apamina. En aquest mateix estudi també es descriu un important component colinèrgic contràctil. Igual que en l'home, el component sensible a l'apamina podria ser degut a l'alliberació d'ATP o una purina relacionada que actua sobre receptors $P2Y_1$, com es posa de manifest en el clasp del porc.

Resposta a l'estimulació de les motoneurones excitadores i inhibidores usant nicotina i estimulació elèctrica de camp (EFS)

De manera idèntica a com ocorre en el porc, en l'home la nicotina desemmascara una petita contracció un cop bloquejat el component inhibidor amb L-NNA i apamina. Aquesta contracció és deguda a l'estimulació de les neurones excitadores colinèrgiques, la seva amplitud és molt menor a la de la contracció induïda per EFS un cop bloquejat també el component inhibitori. Per altra banda, hem vist anteriorment que és molt fàcil estimular les motoneurones inhibidores amb nicotina. Així doncs, existeix una diferent resposta de les motoneurones colinèrgiques quan s'estimulen per EFS o quan s'estimulen per activació dels nAChRs. Aquests resultats es poden relacionar amb estudis realitzats en el sling del conill porquí, en què s'usa com a tècnica l'estimulació vagal, (Yuan & Brookes, 1999), i també es mostra que per evidenciar una resposta de les motoneurones colinèrgiques excitadores es necessita haver bloquejat el component inhibidor amb L-NNA i apamina. La transmissió sinàptica a nivell ganglionar cap a les motoneurones inhibidores i excitadores, està descrita clàssicament que és deguda a l'alliberació d'ACh que actua sobre nAChs; no obstant això, la neurotransmissió en el LES de l'home pot ser deguda a un component no nicotínic. Així doncs, estudis en el LES de l'opòssum demostren que receptors

muscarínic M_1 i serotoninèrgics (5-HT_3) també estan implicats en la transmissió dels *inputs* vagals cap a les motoneurones inhibidores (Gilbert et al., 1984;Paterson et al., 1992). Estudis recents (Galligan et al., 2000;Galligan, 2002) mostren que la majoria dels potencials postsinàptics excitadors ràpids (fEPSPs) en el plexe mientèric són deguts en una petita part per l'ACh que actua sobre receptors nACh i que existeix una forta contribució de l'ATP que actua sobre receptors purinèrgic P2X o de la serotonina que ho fa sobre receptors 5-HT_3.

D'aquests resultats podem concloure que les motoneurones inhibidores del LES de l'home i també del porc són fàcilment i eficientment estimulades tant per EFS com pels nAChRs. Per contra, encara que les motoneurones excitadores poden ser eficientment estimulades per EFS, la seva estimulació per nAChRs és molt difícil: requereix el bloqueig de les vies inhibidores i causa només una petita resposta. Aquest fet suggereix que les fibres vagals poden estimular fàcilment i eficientment les motoneurones inhibidores i que l'estimulació de les motoneurones excitadores requereixen neurotransmissors no nicotínics o altres circuits que necessiten ser investigats.

Paper de les ICC en el to i en la neurotransmissió inhibidora i excitadora

Les cèl·lules intersticials de Cajal són les cèl·lules marcapassos del tracte digestiu i poden estar implicades en la neurotransmissió inhibidora i excitadora entre el terminal nerviós i la musculatura llisa (Daniel, 2001;Huizinga, 2001;Ward, 2000). Estudis de microscòpia electrònica en el LES del opòssum (Daniel & Posey-Daniel, 1984) mostren que les varicositats estan en contacte estret amb les ICC, les quals estan acoblades per mitjà d'unions gap al muscle llis. Aquestes observacions suporten l'existència d'una via funcional en la neurotransmissió que implica el terminal nerviós, les ICC i la musculatura llisa.

Per estudiar el paper de les ICC en la neurotransmissió s'han emprat rosegadors mutants que no tenen poblacions especifiques d'ICC. El gen c-kit està localitzat en el *locus white spotting* (W) i codifica el receptor c-kit, un receptor associat a tirosina cinasa. El lligand natural per aquest receptor és el *stem cell factor* (SCF), la unió d'aquest lligand amb el seu receptor és essencial per la diferenciació i desenvolupament d'alguns tipus

cel·lulars, així com els melanòcits, els mastòcits i les ICC. Aquesta mutació en el gen que codifica el receptor c-kit s'ha detectat en ratolins W/Wv i en rates Ws/Ws.

En un estudi en què s'han usat preparacions de LES in vitro de ratolins W/Wv es conclou que les ICC juguen un paper important en la relació nitrèrgica del LES (Ward et al., 1998); en canvi, en un altre estudi *in vivo* es conclou que aquestes cèl·lules no són importants (Sivarao et al., 2001). Vista aquesta controvèrsia, l'objectiu d'aquest estudi va ser estudiar in vitro en tires de LES els neurotransmissors implicats en la resposta a EFS, usant el model de la rata (rates mutants Ws/Ws i les seves germanes no mutants +/+). Aquest és el primer treball que avalua el paper de les ICC en la neurotransmissió al LES usant com a model animal aquest tipus de rates, la gran majoria dels estudis per estudiar el paper d'aquestes cèl·lules en la neurotransmissió han estat fets amb ratolins W/Wv. Estudis anteriors en el nostre laboratori demostraven que en el còlon, aquestes rates mutants no tenien IM-ICC, que són les que poden estar implicades en la neurotransmissió (Alberti et al., 2004). Primer vam confirmar per tècniques immunohistoquímiques l'absència de cèl·lules c-kit positives intramusculars en el LES de les rates Ws/Ws, la presència d'aquestes en les seves germanes no mutants +/+ i en les Sprague-Dawley.

Després vam veure que, igual que en les rates Sprague-Dawley, en les rates +/+ la relaxació induïda per EFS és deguda a l'NO i a un segon component sensible a l'apamina. Per altra banda, la contracció és deguda a l'alliberament principalment d'ACh més un segon neurotransmissor no colinèrgic. En preparacions de rates mutants Ws/Ws, la relaxació també té els mateixos dos components de magnituds idèntiques i la contracció es deu també en part a l'alliberació d'ACh. Per evidenciar aquests components, algunes preparacions (3/6) es van haver de precontraure amb KCl per raó de l'enorme motilitat que tenien i per raó també que, per causes desconegudes, es va veure que en les tires de rates Ws/Ws el to passiu era menys estable i que era molt fàcil quedar-se sense a la meitat del protocol utilitzat. Aquests resultats demostren que el LES de les rates amb absència de cèl·lules c-kit positives Ws/Ws presenten en la relaxació un component nitrèrgic i un component sensible a l'apamina i en la contracció un component colinèrgic, de magnituds similars a les que presenten les seves germanes no mutants. Aquests resultats suggereixen que les ICC c-kit positives no són essencials ni en el component nitrèrgic ni en el sensible a l'apamina, ni tampoc en el component colinèrgic de la contracció. Existeixen, a més, altres treballs on es posa en dubte la

implicació de les ICC en la neurotransmissió nitrèrgica. En el IAS del ratolí en estudis *in vivo* i in vitro, es descriu que, tot i que aquests ratolins tenen l'esfínter hipotens, la innervació nitrèrgica no es veu afectada en ratolins que no tenen ICC (de Lorijn F. et al., 2005;Terauchi et al., 2005). Aquests autors suggereixen que les ICC que expressen c-kit no són essencials en el reflex rectoanal inhibidor (RAIR). Estudis mecànics i electrofisiològics en el còlon d'aquestes mateixes rates, han descrit que el component nitrèrgic hi és disminuït però no hi és absent (Alberti et al., 2004). Pel que fa al component sensible a l'apamina, s'ha descrit que les ICC no intervenen en aquest tipus de neurotransmissió (Alberti et al., 2004). Estudis en el còlon de la rata, on l'amplitud de l'IJP és sensible a l'apamina, es demostra que aquesta no es veu alterada en les rates mutants Ws/Ws. En el nostre estudi el component no nitrèrgic de la relaxació és completament sensible a apamina tant en les rates +/+ com en les Ws/Ws, fet que suggereix que aquest component no està alterat en aquest tipus de mutació i corrobora aquestes troballes.

Pel que fa a la contracció, s'ha descrit en el fundus gàstric del ratolí que les IM-ICC juguen un paper molt important en els imputs colinèrgics que reben del sistema nerviós entèric (Ward et al., 2000). En aquests estudis s'observa que l'EJP està molt disminuït en ratolins W/Wv. En canvi, estudis electrofisiològics recents (Zhang & Paterson, 2005) demostren que en el LES dels mateixos ratolins, l'EJP és present en ratolins mutants W/Wv i que aquest és sensible a l'atropina. Aquests resultats suggereixen que, a diferència de l'estómac, el component excitador colinèrgic no està afectat en el LES d'aquests ratolins. Aquest resultat concorda amb les nostres troballes, on s'evidencia també un important component colinèrgic en el LES de rates amb absència de cèl·lules c-kit positives.

Creiem que a la pregunta de si les ICC juguen un paper en la interacció nervi-múscul en el LES, poden ser possibles dues hipòtesis: en la primera, les ICC-IM c-kit positives poden no ser essencials en la neurotransmissió. Aquesta possibilitat pot ser considerada perquè en estudis de microscòpia electrònica s'ha descrit una innervació directa i indirecta (via ICC) en l'estómac de la rata (Mitsui & Komuro, 2002). En la segona hipòtesi, aquesta funció la poden dur a terme cèl·lules intersticials c-kit negatives que estan en contacte estret amb la varicositat terminal, i a més formen unions gap amb les cèl·lules musculars llises (Ishikawa et al., 1997). Les cèl·lules intersticials c-kit negatives han estat caracteritzades en l'estómac de les rates Ws/Ws i s'han considerat cèl·lules *fibroblast-like* (Ishikawa et al., 1997;Wang et al., 2003). La

presència i la implicació de cèl·lules c-kit negatives en la neurotransmissió del LES de les rates Ws/Ws necessita més estudis.

D'aquest estudi podem concloure que l'òxid nítric i un component sensible a l'apamina, possiblement l'ATP, són els principals neurotransmissors inhibidors en el LES de la rata, i que la contracció està mitjançada per ACh. Les rates mutants Ws/Ws no presenten ICC c-kit positives però la relació neurona-múscul està preservada. Aquesta relació entre el terminal nerviós i la cèl·lula muscular llisa pot produir-se directament. Alternativament, un altre tipus de cèl·lules poden dur a terme aquesta funció (cèl·lules intersticials c-kit negatives).

Referències

Alberti E, Mikkelsen HB, Larsen JO, & Jimenez M (2004). Impaired electrical and mechanical patterns and distribution of interstitial cells of Cajal in the colon of Ws/Ws rats. *Gastroenterology* **126**, A221.

Aulí M, Farré R, Hernandez P, Gallego D, Martinez E, Marti-Rague J, Suñol X, Jimenez M, & Clave P. Nitrergic and purinergic co-neurotransmission in human sigmoid colon. Neurogastroenterology and Motility 17[Supplement 2], 27. 2005.
Ref Type: Abstract

Beveridge AA & Taylor GS (1989). Evidence for a lower oesophageal sphincter in the guinea-pig. *Comp Biochem Physiol C* **93**, 293-301.

Biancani P, Walsh JH, & Behar J (1984). Vasoactive intestinal polypeptide. A neurotransmitter for lower esophageal sphincter relaxation. *J Clin Invest* **73**, 963-967.

Boulant J, Fioramonti J, Dapoigny M, Bommelaer G, & Bueno L (1994). Cholecystokinin and nitric oxide in transient lower esophageal sphincter relaxation to gastric distention in dogs. *Gastroenterology* **107**, 1059-1066.

Daniel EE (2001). Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. III. Interaction of interstitial cells of Cajal with neuromediators: an interim assessment. *Am J Physiol Gastrointest Liver Physiol* **281**, G1329-G1332.

Daniel EE, Helmy-Elkholy A, Jager LP, & Kannan MS (1983). Neither a purine nor VIP is the mediator of inhibitory nerves of opossum oesophageal smooth muscle. *J Physiol* **336**, 243-260.

Daniel EE & Posey-Daniel V (1984). Neuromuscular structures in opossum esophagus: role of interstitial cells of Cajal. *Am J Physiol* **246**, G305-G315.

de Lorijn F., de Jonge WJ, Wedel T, Vanderwinden JM, Benninga MA, & Boeckxstaens GE (2005). Interstitial cells of Cajal are involved in the afferent limb of the rectoanal inhibitory reflex. *Gut* **54**, 1107-1113.

de Man JG, de Winter BY, Seerden TC, De Schepper HU, Herman AG, & Pelckmans PA (2003). Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. *Br J Pharmacol* **140**, 1108-1116.

de Man JG, Pelckmans PA, Boeckxstaens GE, Bult H, Oosterbosch L, Herman AG, & Van Maercke YM (1991). The role of nitric oxide in inhibitory non-adrenergic non-cholinergic neurotransmission in the canine lower oesophageal sphincter. *Br J Pharmacol* **103**, 1092-1096.

Farré R, Gallego D, Domenech A, Clave P, & Jiménez M. Purinergic inhibitory neuromuscular transmission is mediated trough $P2Y_1$ receptor in the porcine ileum. Neurogastroenterology and Motility 17[Supplement 2], 1-85. 2005.
Ref Type: Abstract

Fields JZ, Jacyno M, Wasyliw R, Winship D, & Keshavarzian A (1995). Ethanol inhibits contractility of esophageal smooth muscle strips. *Alcohol Clin Exp Res* **19**, 1403-1413.

Gallego D, Hernandez P, Marti-Rague J, Clave P, & Jimenez M. P2YI receptor mediates inhibitory purinergic neuromuscular transmission in the human sigmoid colon. Gastroenterology 128[4], A13. 2005.
Ref Type: Abstract

Galligan JJ (2002). Ligand-gated ion channels in the enteric nervous system. *Neurogastroenterol Motil* **14**, 611-623.

Galligan JJ (1999). Nerve terminal nicotinic cholinergic receptors on excitatory motoneurons in the myenteric plexus of guinea pig intestine. *J Pharmacol Exp Ther* **291**, 92-98.

Galligan JJ, LePard KJ, Schneider DA, & Zhou X (2000). Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. *J Auton Nerv Syst* **81**, 97-103.

Gilbert R, Rattan S, & Goyal RK (1984). Pharmacologic identification, activation and antagonism of two muscarine receptor subtypes in the lower esophageal sphincter. *J Pharmacol Exp Ther* **230**, 284-291.

Grider JR, Murthy KS, Jin JG, & Makhlouf GM (1992). Stimulation of nitric oxide from muscle cells by VIP: prejunctional enhancement of VIP release. *Am J Physiol* **262**, G774-G778.

Huizinga JD (2001). Physiology and pathophysiology of the interstitial cell of Cajal: from bench to bedside. II. Gastric motility: lessons from mutant mice on slow waves and innervation. *Am J Physiol Gastrointest Liver Physiol* **281**, G1129-G1134.

Ishikawa K, Komuro T, Hirota S, & Kitamura Y (1997). Ultrastructural identification of the c-kit-expressing interstitial cells in the rat stomach: a comparison of control and Ws/Ws mutant rats. *Cell Tissue Res* **289**, 137-143.

Jun CH, Lee TS, & Sohn UD (2003). NO/cyclic GMP pathway mediates the relaxation of feline lower oesophageal sphincter. *Auton Autacoid Pharmacol* **23**, 159-166.

Jury J, Ahmedzadeh N, & Daniel EE (1992). A mediator derived from arginine mediates inhibitory junction potentials and relaxations in lower esophageal sphincter: an independent role for vasoactive intestinal peptide. *Can J Physiol Pharmacol* **70**, 1182-1189.

Kawahara H, Blackshaw LA, Lehmann A, & Dent J (1997). Responses of the rat lower oesophageal sphincter (LOS) to vagal efferent activation. *Neurogastroenterol Motil* **9**, 85-97.

Kiatchoosakun P, Dreshaj IA, bu-Shaweesh JM, Haxhiu MA, & Martin RJ (2002). Effects of hypoxia on respiratory neural output and lower esophageal sphincter pressure in piglets. *Pediatr Res* **52**, 50-55.

Kim CD, Goyal RK, & Mashimo H (1999). Neuronal NOS provides nitrergic inhibitory neurotransmitter in mouse lower esophageal sphincter. *Am J Physiol* **277**, G280-G284.

Konturek JW, Thor P, Lukaszyk A, Gabryelewicz A, Konturek SJ, & Domschke W (1997). Endogenous nitric oxide in the control of esophageal motility in humans. *J Physiol Pharmacol* **48**, 201-209.

Kortezova N, Mizhorkova Z, Milusheva E, Varga G, Vizi ES, & Papasova M (1996). Non-adrenergic non-cholinergic neuron stimulation in the cat lower esophageal sphincter. *Eur J Pharmacol* **304**, 109-115.

Kortezova N, Velkova V, Mizhorkova Z, Bredy-Dobreva G, Vizi ES, & Papasova M (1994). Participation of nitric oxide in the nicotine-induced relaxation of the cat lower esophageal sphincter. *J Auton Nerv Syst* **50**, 73-78.

Lidums I, Bermingham H, & Holloway RH (1997). Effect of hyoscine butylbromide on gastroesophageal reflux and lower esophageal sphincter function in healthy volunteers. *Gastroenterology* **112**, A776.

Liebermann-Meffert D, Allgower M, Schmid P, & Blum AL (1979). Muscular equivalent of the lower esophageal sphincter. *Gastroenterology* **76**, 31-38.

Matsuda NM, Oliveira RB, & Ballejo G (1997). Characterization of nerve-induced relaxation of gastrointestinal sphincteric smooth muscle in a South American opossum (Didelphis albiventris). *Braz J Med Biol Res* **30**, 793-799.

Mitsui R & Komuro T (2002). Direct and indirect innervation of smooth muscle cells of rat stomach, with special reference to the interstitial cells of Cajal. *Cell Tissue Res* **309**, 219-227.

Mittal RK, Holloway R, & Dent J (1995). Effect of atropine on the frequency of reflux and transient lower esophageal sphincter relaxation in normal subjects. *Gastroenterology* **109**, 1547-1554.

Mittal RK, Rochester DF, & McCallum RW (1988). Electrical and mechanical activity in the human lower esophageal sphincter during diaphragmatic contraction. *J Clin Invest* **81**, 1182-1189.

Muinuddin A, Neshatian L, Gaisano HY, & Diamant NE (2004). Calcium source diversity in feline lower esophageal sphincter circular and sling muscle. *Am J Physiol Gastrointest Liver Physiol* **286**, G271-G277.

Murray J, Du C, Ledlow A, Bates JN, & Conklin JL (1991). Nitric oxide: mediator of nonadrenergic noncholinergic responses of opossum esophageal muscle. *Am J Physiol* **261**, G401-G406.

Pasricha PJ, Ravich WJ, & Kalloo AN (1993). Effects of intrasphincteric botulinum toxin on the lower esophageal sphincter in piglets. *Gastroenterology* **105**, 1045-1049.

Paterson WG, Anderson MA, & Anand N (1992). Pharmacological characterization of lower esophageal sphincter relaxation induced by swallowing, vagal efferent nerve stimulation, and esophageal distention. *Can J Physiol Pharmacol* **70**, 1011-1015.

Preiksaitis HG & Diamant NE (1997). Regional differences in cholinergic activity of muscle fibers from the human gastroesophageal junction. *Am J Physiol* **272**, G1321-G1327.

Preiksaitis HG, Tremblay L, & Diamant NE (1994a). Cholinergic responses in the cat lower esophageal sphincter show regional variation. *Gastroenterology* **106**, 381-388.

Preiksaitis HG, Tremblay L, & Diamant NE (1994b). Nitric oxide mediates inhibitory nerve effects in human esophagus and lower esophageal sphincter. *Dig Dis Sci* **39**, 770-775.

Rattan S & Goyal RK (1975). Effect of nicotine on the lower esophageal sphincter. Studies on the mechanism of action. *Gastroenterology* **69**, 154-159.

Salapatek AM, Lam A, & Daniel EE (1998). Calcium source diversity in canine lower esophageal sphincter muscle. *J Pharmacol Exp Ther* **287**, 98-106.

Schneider DA, Perrone M, & Galligan JJ (2000). Nicotinic acetylcholine receptors at sites of neurotransmitter release to the guinea pig intestinal circular muscle. *J Pharmacol Exp Ther* **294**, 363-369.

Serio R, Alessandro M, Zizzo MG, Tamburello MP, & Mule F (2003a). Neurotransmitters involved in the fast inhibitory junction potentials in mouse distal colon. *Eur J Pharmacol* **460**, 183-190.

Serio R, Zizzo MG, & Mule F (2003b). Nitric oxide induces muscular relaxation via cyclic GMP-dependent and -independent mechanisms in the longitudinal muscle of the mouse duodenum. *Nitric Oxide* **8**, 48-52.

Sifrim D & Lefebvre R (2001). Role of nitric oxide during swallow-induced esophageal shortening in cats. *Dig Dis Sci* **46**, 822-830.

Sivarao DV, Mashimo HL, Thatte HS, & Goyal RK (2001). Lower esophageal sphincter is achalasic in nNOS(-/-) and hypotensive in W/W(v) mutant mice. *Gastroenterology* **121**, 34-42.

Smid SD & Blackshaw LA (2000). Neuromuscular function of the human lower oesophageal sphincter in reflux disease and Barrett's oesophagus. *Gut* **46**, 756-761.

Terauchi A, Kobayashi D, & Mashimo H (2005). Distinct Roles of Nitric Oxide Synthases and Interstitial Cells of Cajal in Rectoanal Relaxation. *Am J Physiol Gastrointest Liver Physiol*.

Tottrup A, Forman A, Uldbjerg N, Funch-Jensen P, & Andersson KE (1990). Mechanical properties of isolated human esophageal smooth muscle. *Am J Physiol* **258**, G338-G343.

Tottrup A, Svane D, & Forman A (1991). Nitric oxide mediating NANC inhibition in opossum lower esophageal sphincter. *Am J Physiol* **260**, G385-G389.

Uc A, Oh ST, Murray JA, Clark E, & Conklin JL (1999). Biphasic relaxation of the opossum lower esophageal sphincter: roles of NO., VIP, and CGRP. *Am J Physiol* **277**, G548-G554.

Vicente Y, Da RC, Yu J, Hernandez-Peredo G, Martinez L, Perez-Mies B, & Tovar JA (2001). Architecture and function of the gastroesophageal barrier in the piglet. *Dig Dis Sci* **46**, 1899-1908.

Wang XY, Paterson C, & Huizinga JD (2003). Cholinergic and nitrergic innervation of ICC-DMP and ICC-IM in the human small intestine. *Neurogastroenterol Motil* **15**, 531-543.

Ward SM (2000). Interstitial cells of Cajal in enteric neurotransmission. *Gut* **47 Suppl 4**, iv40-iv43.

Ward SM, Beckett EA, Wang X, Baker F, Khoyi M, & Sanders KM (2000). Interstitial cells of Cajal mediate cholinergic neurotransmission from enteric motor neurons. *J Neurosci* **20**, 1393-1403.

Ward SM, Morris G, Reese L, Wang XY, & Sanders KM (1998). Interstitial cells of Cajal mediate enteric inhibitory neurotransmission in the lower esophageal and pyloric sphincters. *Gastroenterology* **115**, 314-329.

Welch RW & Drake ST (1980). Normal lower esophageal sphincter pressure: a comparison of rapid vs. slow pull-through techniques. *Gastroenterology* **78**, 1446-1451.

Werkstrom V, Ny L, Persson K, & Andersson KE (1997). Carbon monoxide-induced relaxation and distribution of haem oxygenase isoenzymes in the pig urethra and lower oesophagogastric junction. *Br J Pharmacol* **120**, 312-318.

Xue L, Farrugia G, & Szurszewski JH (2000). Effect of exogenous ATP on canine jejunal smooth muscle. *Am J Physiol Gastrointest Liver Physiol* **278**, G725-G733.

Yamato S, Saha JK, & Goyal RK (1992). Role of nitric oxide in lower esophageal sphincter relaxation to swallowing. *Life Sci* **50**, 1263-1272.

Yuan S & Brookes SJ (1999). Neuronal control of the gastric sling muscle of the guinea pig. *J Comp Neurol* **412**, 669-680.

Yuan S, Costa M, & Brookes SJ (1998). Neuronal pathways and transmission to the lower esophageal sphincter of the guinea Pig. *Gastroenterology* **115**, 661-671.

Zagorodnyuk V, Santicioli P, Maggi CA, & Giachetti A (1996). The possible role of ATP and PACAP as mediators of apaminsensitive NANC inhibitory junction potentials in circular muscle of guinea-pig colon. *Br J Pharmacol* **119**, 779-786.

Zaninotto G, DeMeester TR, Schwizer W, Johansson KE, & Cheng SC (1988). The lower esophageal sphincter in health and disease. *Am J Surg* **155**, 104-111.

Zhang X, Geboes K, Depoortere I, Tack J, Janssens J, & Sifrim D (2005). Effect of repeated cycles of acute esophagitis and healing on esophageal peristalsis, tone, and length. *Am J Physiol Gastrointest Liver Physiol* **288**, G1339-G1346.

Zhang Y & Paterson WG (2005). Selective impairment of nitrergic neurotransmission in lower esophageal sphincter (LES) circular smooth muscle (CSM) of interstitial cell of cajal (ICC)-deficient W/W-V mice. *Gastroenterology* **128**, A629.

Zizzo MG, Mule F, & Serio R (2004). Interplay between PACAP and NO in mouse ileum. *Neuropharmacology* **46**, 449-455.

Conclusions

1- Les preparacions in vitro d'esfínter esofàgic inferior (LES) de les tres espècies estudiades (l'home, el porc i la rata) desenvolupen espontàniament un to d'origen miogènic. Aquest to muscular depèn de l'entrada de calci extracel·lular i està modulat de manera diferent, depenent de l'espècie, per l'activitat tònica de motoneurones entèriques nitrèrgiques i colinèrgiques.

2. L'activació de les motoneurones inhibitòries amb estimulació elèctrica de camp (EFS) provoca una relaxació durant l'estímul. Les motoneurones inhibitòries poden ser també fàcilment activades mitjançant l'estimulació dels receptors nicotínics d'acetilcolina (nAchRs), fet que també indueix una relaxació. Les motoneurones excitadores són fàcilment excitables amb EFS, en canvi la seva estimulació per l'activació dels nAChRs és molt difícil i causa una resposta de poca amplitud. Aquest fet suggereix la participació de mecanismes no nicotínics en la neurotransmissió vagal i/o interneuronal cap a les motoneurones excitadores en el LES humà i del porc.

3- En les tres espècies, la relaxació del LES com a conseqüència de l'activació de les motoneurones inhibitòries depèn de l'alliberació d'òxid nítric (NO) i d'un component sensible a l'apamina. La contracció en resposta a l'estimació neural depèn de l'alliberació d'acetilcolina. La contribució de cada un dels components es diferent en cada espècie.

4- L'NO produeix la relaxació actuant sobre la guanilat ciclasa muscular, mentre que el component sensible a l'apamina es degut a l'alliberació d'ATP actuant principalment sobre receptors $P2Y_1$ però també sobre receptors P2X pre- i/o postsinàptics, existeix una petita part deguda a l'acció del PACAP. Aquests neurotransmissors actuen en paral·lel i sense interaccions important entre ells. Ni l'ATP ni el PACAP estan associats a la síntesis d'NO. El VIP i el monòxid de carboni (CO) no estan implicats en la transmissió neuromuscular en el LES del porc.

5-Existeix una marcada asimetria funcional pel que fa al control del to i als components implicats en la relaxació i en la contracció del LES. Les fibres de la dreta (clasp) tenen una major dependència del calci extracel·lular en el manteniment del to, en canvi les fibres de l'esquerra (sling) depenen més de les influencies neurals nitrèrgiques i colinèrgiques. La major relaxació de les fibres de clasp envers les d'sling és deguda a una major contribució del component inhibitori nitrergic i purinèrgic i a una menor contribució del component excitador colinèrgic.

6- Les cèl·lules intersticial de Cajal (ICC) c-kit positives no tenen un paper limitant ni en la relaxació ni en la contracció del LES. Aquest fet fa pensar que els terminals nerviosos poden innervar directament la cèl·lula muscular llisa i/o existir ICC c-kit negatives involucrades en la neurotransmissió.

7- El coneixement dels mecanismes i neurotransmissors que intervenen en el manteniment del to, la relaxació i la contracció del LES té una gran importància per entendre la fisiopatologia de malalties que afecten al LES com poden ser la malaltia per reflux gastroesofàgic (GERD), els trastorns motors esofàgics i l'acalàsia.