PHARMACOLOGIC CHARACTERIZATION OF INTRINSIC MECHANISMS CONTROLLING TONE AND RELAXATION OF PORCINE LOWER ESOPHAGEAL SPHINCTER [a]

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Running title: Inhibitory co-neurotransmitters in porcine LES.

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Nonstandard Abbreviations: AChRs: acetylcholine receptors; ADPβS: adenosine 5'-O-2-thiodiphosphate; cGMP: 3'-5' cyclic guanosine monophosphate; CGRP: calcitonin gene-related peptide; CORM-1: tricarbonyl dichlororuthenum dimer; EFS: electrical field stimulation; GEJ: gastro-esophageal junction; LES: lower esophageal sphincter; L-NAME: L-N^G-nitroarginine methyl ester; MRS 2179: 2'-Deoxy-N^6-methyladenosine 3',5'-bisphosphate tetraammonium salt; NANC, non-adrenergic, non-cholinergic; NF 279: 8,8¢-[Carbonylbis(imino-4,1-phenylenecarbonylimino-4,1-phenylenecarbonylimino)]bis-1,3,5-naphthalenetrisulfonic acid hexasodium salt; SnPP-IX: tin protoporphyrin IX; ODQ: 1H-[1,2,4]oxadiazole-[4,3-α]quinoxalin-1-one; PACAP: pituitary adenylate cyclase-activating peptide; VIP: Vasoactive intestinal peptide.

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ABSTRACT

Aims: The neurotransmitters mediating relaxation of lower esophageal sphincter (LES) were studied using circular LES strips from adult pigs in organ baths**. Results**: LES relaxation by sodium nitroprusside (1nM-3μM), VIP and PACAP (1nM-1μM), ATP (10μM-30mM), and tricarbonyldichlororuthenum dimer (CORM-1 1μM-1mM) was unaffected by tetrodotoxin (1μM) or L-NAME (100μM). Calcitonin gene-related peptide (CGRP 1nM-1μM) did not affect LES tone. ATP-relaxation was blocked by 1μM apamin and the $P2Y_1$ antagonist N^6-methyl 2'-deoxyadenosine 3',5'-bisphosphate (MRS 2179 10μM). Apamin inhibited PACAP-relaxation. VIP- and PACAP-relaxation was blocked by α-chymotrypsin 10U/ml. L-NAME (-62.52±13.13%) and 1H-[1,2,4]oxadiazole-[4,3-α]quinoxalin-1-one (ODQ 10μM, -67.67±6.80%) similarly inhibited electrical LES relaxation, and apamin blocked non-nitrergic relaxation. Nicotine relaxation (100μM) was inhibited by L-NAME (-60.37±10.8%) and ODQ (-41.90±7.89%); and apamin also blocked non-nitrergic relaxation. Non-nitrergic and apamin-sensitive LES relaxation by electrical stimulation or nicotine was strongly inhibited by MRS 2179, slightly inhibited by α-chymotrypsin and the $P2X_{1,2,3}$ receptor antagonist NF 279 (10μM), and unaffected by tin protoporphyrin IX (100μM). **Conclusion:** Porcine LES relaxation following stimulation of intrinsic inhibitory motor neurons is mediated by two main neuromuscular pathways: a) nitric oxide through guanylate cyclase signaling and apamin-insensitive mechanisms, and b) by non-nitrergic apamin-sensitive neurotransmission mainly mediated by ATP, ADP or a related purine acting on $P2Y_1$ receptors and a minor contribution of purinergic $P2X_{1,2,3}$ receptors and PACAP. 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 carbon monoxide in porcine LES relaxation.

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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 involve local enteric motor neurons and extrinsic nerves and are not yet fully understood. Non-adrenergic, non-cholinergic (NANC) enteric motor neurons 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), nitric oxide, 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 enteric motor neurons 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 motor neurons (Imaeda and Cunnane, 2003; Ny et al., 1995b; Ny et al., 1997; Uc et al., 1997; Yuan et al., 1998). However, evidence suggests that nitric oxide 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 nitric oxide-mediated release of other neurotransmitters (Grider et al., 1992), or pre- and/or postjunctional

modulation of nitric oxide synthesis by other neurotransmitters (Ergun and 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 motor neurons and little data is available on the effects of these inhibitory neurotransmitters following other more specific stimuli for the inhibitory neurons. In a recent *in vitro* study on human LES, we found that inhibitory enteric motor neurons are efficiently stimulated both by EFS and nicotinic acetylcholine receptors (AChRs) located in somatodendritic regions and nerve terminals, whereas esophageal excitatory motor neurons are also efficiently stimulated by electrical field stimulation (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 and Timmermans, 2004; Pasricha et al., 1993). The size, histology (smooth muscle cells) and neurochemical code of porcine LES motor neurons 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 controlling tone and relaxation of porcine lower esophageal sphincter (LES) following stimulation of inhibitory enteric motor neurons by EFS and through nicotinic AChRs.

METHODS

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. 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 and 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.

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

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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.

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, 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 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), the selective heme oxygenase inhibitor tin protoporphyrin IX (SnPP-IX), and the $P2Y_1$ agonist adenosine 5'-O-2-thiodiphosphate (ADPβS) 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).

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 nitric oxide-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 nitric oxide-mediated release of any other putative neurotransmitters.

a) The effect of neurotransmitters on LES tone and the selection of specific antagonists. Concentration-related curves of the effects of sodium nitroprusside, 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 1nM-3 μM for sodium nitroprusside; 1 nM-1 μM for VIP, PACAP, and CGRP; 10 μM-30 mM for ATP, and 1μM-1mM for CORM-1. Reported concentrations are final bath concentrations. Responses to 10 μM-1 mM ADPβS were also assessed to further characterize the purinergic receptors involved in LES relaxation (De Man et al., 1991; Yoshioka and Nakata, 2004). Submaximal LES responses to sodium nitroprusside, ATP, VIP, PACAP, and CORM-1 and ADPβS were studied in the presence of the neurotoxin tetrodotoxin (1 μM) and the following substances: ODQ (10 μM), an specific inhibitor of the soluble guanylyl cyclase that has been widely used as a pharmacological tool to characterize the NO-mediated component of EFS-induced LES relaxation (Shahin et al., 2000); apamin (1 μM), blocker of small conductance Ca2+-activated K^+ (SK) channels; MRS 2179 (10 μM), competitive antagonist for $P2Y_1$ receptors (Boyer et al., 1998; De Man et al., 2003) and NF279 (10 μM) for

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$P2X_{1,2,3}$ receptors (Damer et al., 1998; De Man et al 2003); the peptidase α-chymotrypsin (10 U/mL) that cleaves VIP and PACAP at the level of tyrosine residues (Mule and Serio, 2003), and the VIP antagonist, VIP 6-28 (100 nM); and also following the nitric oxide-synthase inhibitor L-NAME (100 μM) to explore the possible involvement of nitric oxide 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).

b) 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 tetrodotoxin 1 μM and to a Ca^{2+}-free medium for 30 min. The influence of tonic activity of inhibitory motor neurons 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 10μM for 30 min; or α-chymotrypsin 10 U/mL for 10 min. The influence of cholinergic motor neurons upon LES tone was assessed by atropine 1 μM for 30 min.

c) Stimulation of enteric motor neurons by EFS, the effects of the neurotransmitters released, and characterization of nitric oxide-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 tetrodotoxin 1 μM and hexamethonium 100 μM. First, two experiments were conducted during direct stimulation of inhibitory motor neurons 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 nitric oxide, allowing the effect of any neurotransmitter coupled in series with nitric oxide, or released by nitric oxide. 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.

d) Stimulation of enteric motor neurons with nicotine, identification of the neurotransmitters released and characterization of nitric oxide-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 enteric motor neurons 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 tetrodotoxin 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 motor neurons 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 nitric oxide allowing the effect of any neurotransmitter coupled in series with nitric oxide, or released by

nitric oxide. 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 motor neurons 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).

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 (Gonzalez et al., 2004). 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.

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RESULTS

LES strips developed an active resting tone of 4.90±0.30g (n=34). Sodium nitroprusside, VIP, PACAP, ATP, ADPβS, and CORM-1 relaxed LES strips; in contrast CGRP had no effect at any dose tested (Figure 1 and 2). The nitric oxide-donor sodium nitroprusside, the carbon monoxide donor CORM-1 and VIP (Figure 1) induced a monophasic LES relaxation. PACAP and ADPβS 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 sodium nitroprusside, CORM-1 and VIP was slower than initial fast relaxation induced by PACAP and ATP (Table 1). The nitric oxide donor, VIP, PACAP, CORM-1, ATP, and ADPβS 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 nitric oxide 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.

The doses of neurotransmitters causing submaximal LES relaxation selected for studies with antagonists were sodium nitroprusside (1μM), VIP (100 nM), CORM-1 (500 μM), ATP (1mM), ADPβS (10 μM) and PACAP (100 nM), the effects of antagonists having been evaluated in individual experiments using 3-7 specimens. Inhibition of nitric oxide synthesis by L-NAME did not significantly modify any of the responses induced by sodium nitroprusside (+0.50±4.02%, ns), VIP (-8.56±2.91%, ns), ATP (fast relaxation +0.30±4.86%, fast contraction -13.24±4.47%, slow relaxation +4.01±11.27%, ns), or PACAP (fast relaxation -11.64±10.92%, slow relaxation -13.40± 5.28%, ns). Nitric oxide-induced relaxation by sodium nitroprusside 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 tetrodotoxin (-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%, $p<0.05$). Tetrodotoxin did not significantly affect the relaxation induced by sodium nitroprusside (–3.04 ±2.17%, ns), VIP (-5.62±5.49%, ns), ATP (fast relaxation +27.31± 9.21% and slow relaxation +21.30± 9.57%, ns), ADPβS 10 μM (fast relaxation +6.84±9.81%; slow relaxation +15.32±14.45%, ns), and PACAP (fast relaxation -20.26±16.89%, ns and slow relaxation -30.57± 8.93%, ns).

b) 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 tetrodotoxin 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 motor neurons as well as by continuous influence of inhibitory motor neurons.

c) Inhibitory neurotransmitters 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 tetrodotoxin at all frequencies tested (-100%, n=5, $p<0.001$), and unaffected by hexamethonium (+6.78±14.65% at 3 Hz, n=3, ns) showing the origin of these responses in the activation of enteric motor neurons. 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 nitric oxide 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 nitric oxide. 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 enteric motor neurons 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 neurotransmitters released by nitric oxide. Apamin also caused similar and comparable effects in both experiments.

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). Results from experiments E-1 suggest that EFS relaxation is mainly mediated by nitric oxide 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 carbon monoxide during EFS-relaxation.

d) 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 tetrodotoxin 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). 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 enteric motor neurons 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 motor

neurons 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 motor neurons through nicotinic AChRs is mainly caused by nitric oxide 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 carbon monoxide in LES relaxation following stimulation of inhibitory motor neurons through nicotinic AChRs.

DISCUSSION

Our study shows that porcine LES tone is mainly myogenic and is modulated by tonic input from excitatory and inhibitory enteric motor neurons. Relaxation of porcine LES following stimulation of inhibitory motor neurons is caused to a similar extent by two pathways: a) a nitrergic one mediated by nitric oxide through guanylate cyclase signaling and apamin-insensitive mechanisms, and b) a nonintrergic pathway coupled to apamin-sensitive small conductance Ca^{2+}-activated K^+ channels mainly mediated by ATP, ADP or a related purine acting through $P2Y_1$ receptors and a minor contribution of purinergic $P2X_{1,2,3}$ receptors and PACAP. 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, and although carbon monoxide and VIP caused a direct LES relaxation, we did not find any major role for them in neuroeffector LES relaxation.

Porcine, like human, LES is formed by the sling muscle on the angle of Hiss and the clasp component in lesser curvature (Preiksaitis and Diamant, 1997). We only included clasp strips that developed active tension and relaxed during stimulation of inhibitory motor neurons, ensuring that we had selected LES strips with intact innervation. We tested the effect of a nitric oxide-donor, VIP, ATP, PACAP, a carbon monoxide-donor and CGRP because these substances colocalize in esophageal inhibitory motor neurons (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 nitric oxide-donor, VIP, ATP, ADPβS, the carbon monoxide-donor, and PACAP relaxed LES strips through a tetrodotoxin-insensitive mechanism suggesting a direct effect on smooth muscle cells. Nitric oxide, ATP, and PACAP induced a complete LES relaxation, and CORM-1 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). Relaxation induced by sodium nitroprusside was specifically blocked by ODQ in our study showing a direct nitric oxide effect through guanylate cyclase pathways (Shahin et al., 2000). VIP relaxation was unaffected by L-NAME or by ODQ arguing against the theory of a "serial cascade" involving nitric oxide production by VIP (Ergun and Ogulener, 2001; Grider et al., 1992). Apamin inhibited ATP and PACAP but not nitric oxide, CORM-1 or VIP relaxation; and α-chymotrypsin antagonized both VIP and fast-PACAP relaxation without affecting the nitric oxide 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. Neither L-NAME or α-chymotrypsin affected ATP-induced relaxation, also arguing against an "in series" relation between nitric oxide or VIP and ATP in our study (Xue et al., 2000).

We assessed the physiological relevance of nitric oxide, ATP, PACAP, carbon monoxide and VIP by testing the effect of their antagonists on LES resting tone and during stimulation of inhibitory motor neurons by EFS or through nicotinic AChRs. 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 motor neurons; and L-NAME, ODQ, and apamin enhanced LES tone also showing a tonic influence of inhibitory motor neurons. Nitric oxide 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 the action of nitric oxide from nerves, but not that from muscle (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 motor neurons in porcine LES that could affect treatments for achalasia 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 studies on human LES (Gonzalez et al., 2004). 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 nitric oxide inhibition in both series of experiments. The apamin-sensitive component has been indirectly attributed to ATP in previous studies (Imaeda et al., 1998). We found that the apamin-sensitive component of EFS-relaxation 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 $P2X_{1,2,3}$ 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 $P2X_{1,2,3}$ receptors on inhibitory neurotransmission were found in the mouse jejunum (De Man et al., 2003) and ATP-induced relaxation of rat pylorus has

been attributed to muscular P2X purinoceptors (Ishiguchi et al., 2000). 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 carbon monoxide. In addition, the similarity of the responses in experiments with ODQ (where nitric oxide is synthesized and released and could induce the release of any other neurotransmitter) and L-NAME (where nitric oxide is not synthesized) argues against release of VIP or any other neurotransmitter by nitric oxide (Grider et al., 1992) and also against nitric oxide 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 enteric motor neurons with nicotine. Nicotinic AChRs are located in somatodendritic regions of enteric motor neurons and participate in the transmission of vagal inputs (Chang et al., 2003; Galligan, 1999). We also found nicotinic AChRs at pre-junctional sites of inhibitory motor neurons in human LES (Gonzalez et al., 2004). In the present study, nicotine-induced LES relaxations were partly antagonized by tetrodotoxin, further suggesting the presence of nAChRs in nerve terminals of the inhibitory neurons of porcine LES. Previous studies on cats (Kortezova et al., 1994) and our studies on human LES also found a residual relaxation induced by nicotine following nitric oxide blockade. Nitric oxide synthesis inhibitors reduced nicotine-relaxation 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, the nicotinic agonist DMPP induced both VIP and nitric oxide release, VIP release being further facilitated by nitric oxide production in the guinea-pig ileum (Grider et al., 1992). In our present study, the relaxation resistant to nitric oxide blockade was fully antagonized by apamin and a weak contraction induced by stimulation of cholinergic neurons 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 $P2Y_1$ receptors, a second minor component antagonized by NF279 through $P2X_{1,2,3}$ receptors, and a minor third component antagonized by α-chymotrypsin and also probably mediated by PACAP (Smid and 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 motor neurons 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 nitric oxide blockers— and partly caused by the probable participation of a 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 pathways induced only a slight contraction in LES, suggesting that nicotinic AChRs stimulate excitatory motor neurons much less efficiently than inhibitory ones; as we found in human LES (Gonzalez et al., 2004). These results further suggest that whereas vagal fibers or interneurons could easily and efficiently stimulate inhibitory motor neurons, full stimulation of intrinsic excitatory enteric motor neurons requires non-nicotinic neurotransmitters (Galligan et al., 2000) or other circuits that need further investigation.

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FIGURE LEGENDS

Figure 1. Representative tracings showing the morphology of LES relaxation induced by each proposed neurotransmitter. The nitric oxide donor sodium nitroprusside, VIP, and the carbon monoxide donor CORM-1 induced a monophasic relaxation. PACAP and ADPβS 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 (sodium nitroprusside, CORM-1, VIP, CGRP). Right panel: Neurotransmitters with polyphasic responses (PACAP, ADPβS, ATP). Effects are expressed in terms of percentage changes of active LES tone (n=3 at each point).

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

Figure 4. Characterization of neurotransmitters released during stimulation of LES enteric motor neurons 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 nitric oxide 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.

Figure 5. Experiment E-2. Blockade of nitric oxide 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.

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Figure 6. Characterization of neurotransmitters 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 $P2X_{1,2,3}$ 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.

Figure 7. Characterization of neurotransmitters released during stimulation of LES enteric motor neurons 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 nitric oxide 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 nitric oxide 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.

Figure 8. Characterization of neurotransmitters 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.

Figure 9. Schematic representation of two main parallel pathways of inhibitory neurotransmission in porcine LES: a) nitric oxide through guanylate cyclase signaling and apamin-insensitive mechanisms, and b) nonintrergic apamin-sensitive neurotransmission mainly mediated by ATP, ADP or a related purine acting on $P2Y_1$ receptors but also with a minor contribution of purinergic $P2X_{1,2,3}$ receptors and PACAP. Our study shows no role for CGRP, and, although carbon monoxide 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.

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Table 1. EC_{50}, maximal effect (E Max), and time to achieve E Max of putative neurotransmitters on porcine LES. Results are expressed in percentage decrease (-) or increase (+) of active resting tone. FR= fast relaxation, FC= fast contraction, SR= slow relaxation. * $p < 0.05$ denotes fast responses.

Agonist	EC_{50}	E Max	Time to E Max (S)
SNP	61.2 nM	-92.1±6.3%	83.51±6.34
VIP	52.3 nM	-47.1±12.2%	161.01±12.84
CORM-1	120.2 μM	-30.94±6.7%	103.08±12.28
ATP (FR)	133.6 μM	-96.0±12%	9.73±0.50*
ATP (SR)	606.7 μM	-86.2±19%	148.03±25.18
ATP (FC)	27.9 μM	+20.7±11.7%	11.65±1.27*
PACAP (FR)	81.1 nM	-98.0±4.7%	24.91±3.68*
PACAP (SR)	20.1 nM	-112.9±11%	119.26±16.19
CGRP	-	0%	-

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Figure 1

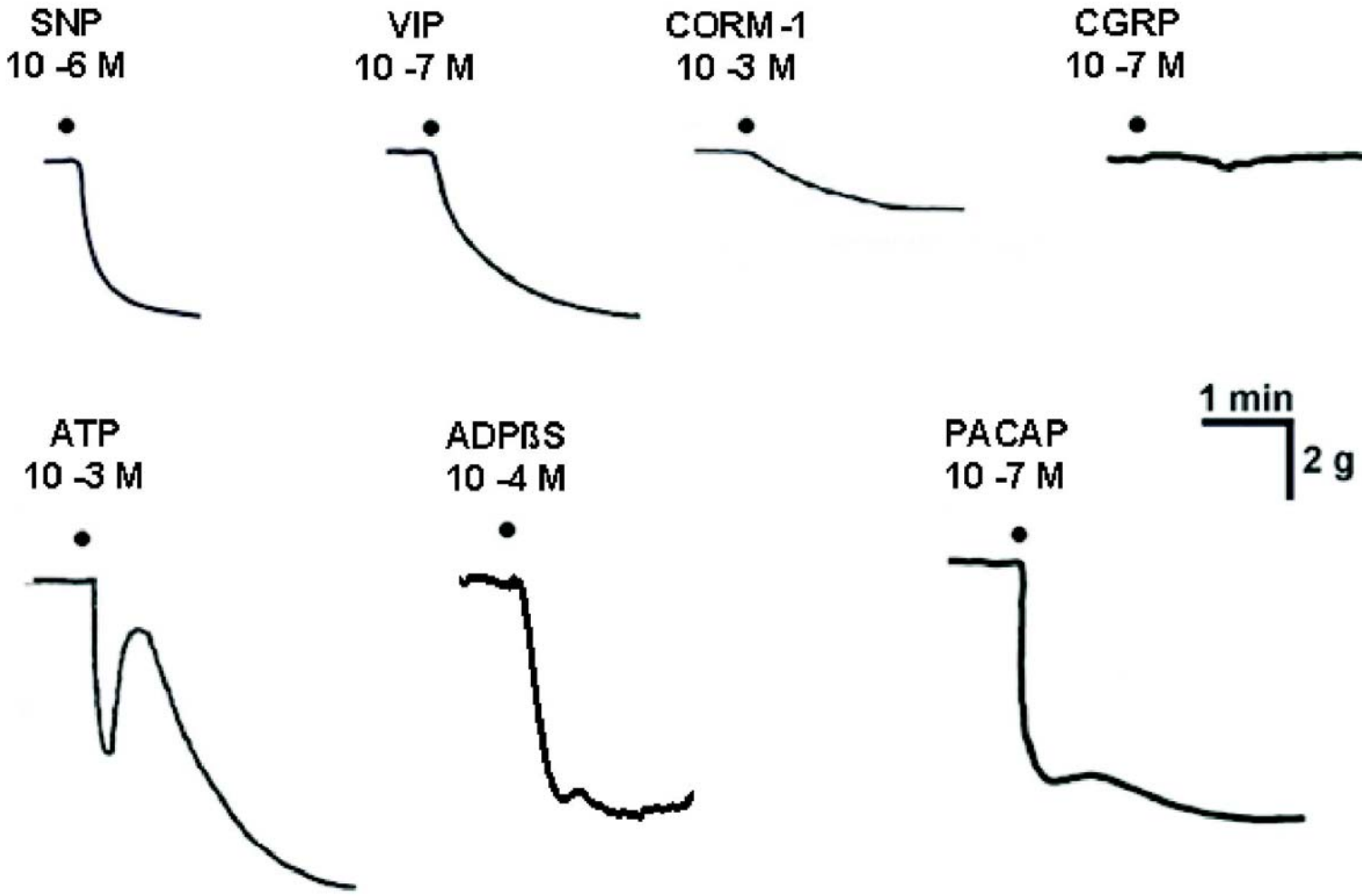

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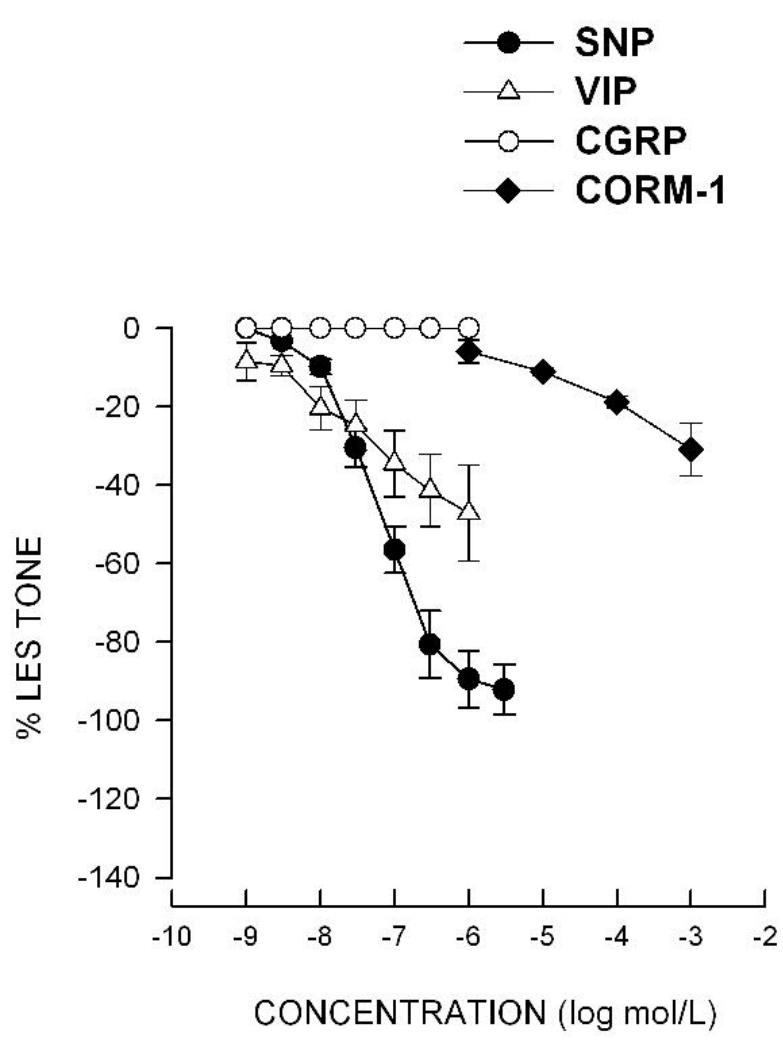
Figure 2



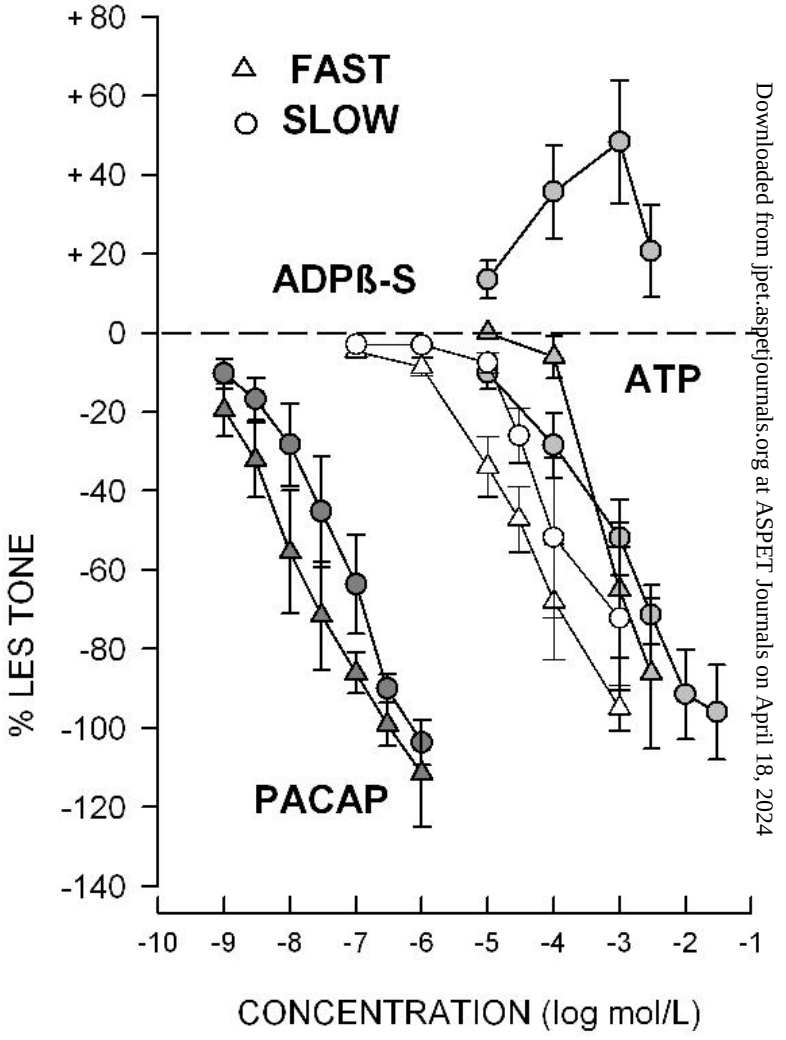


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Figure 3

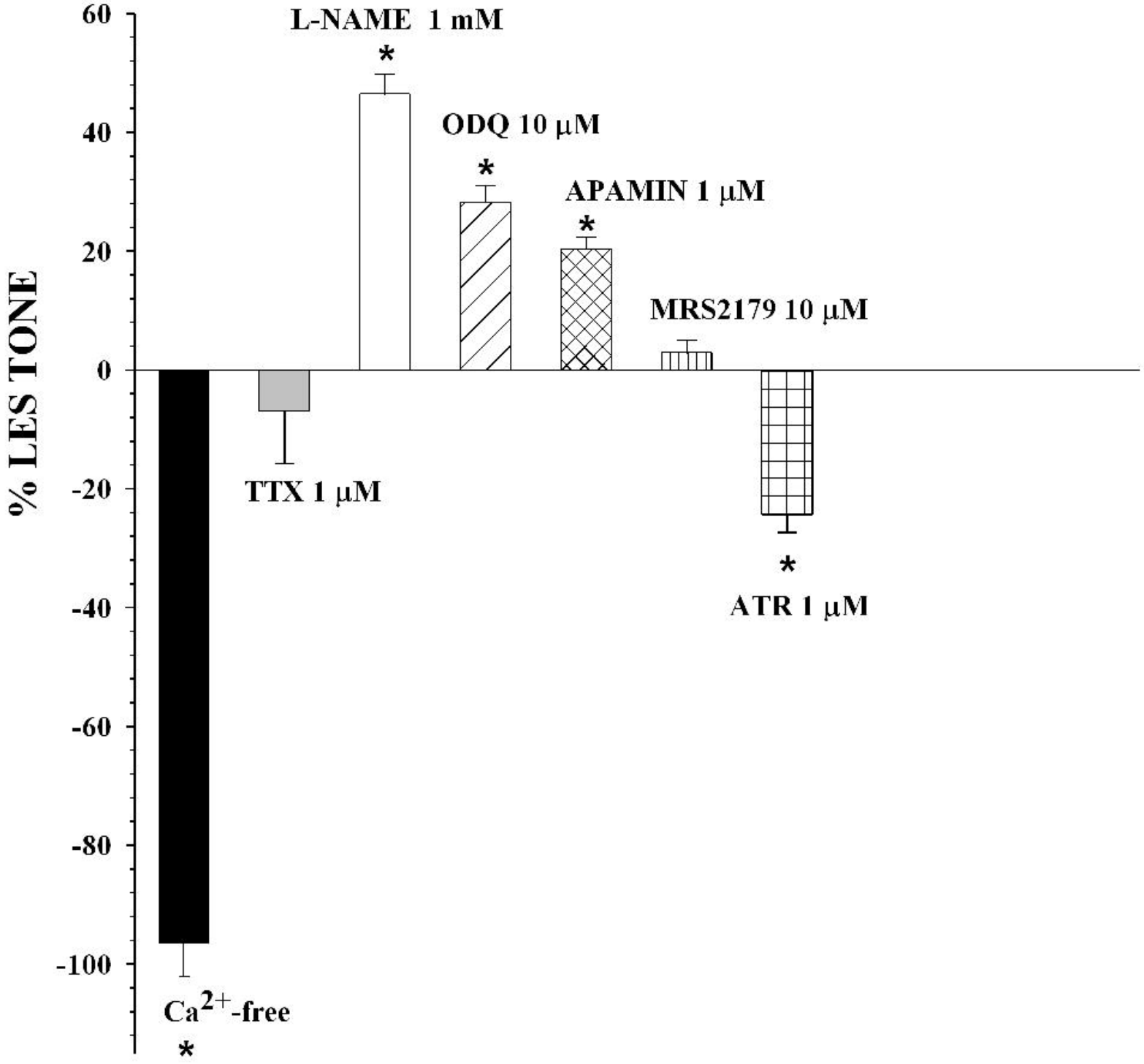

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Figure 4

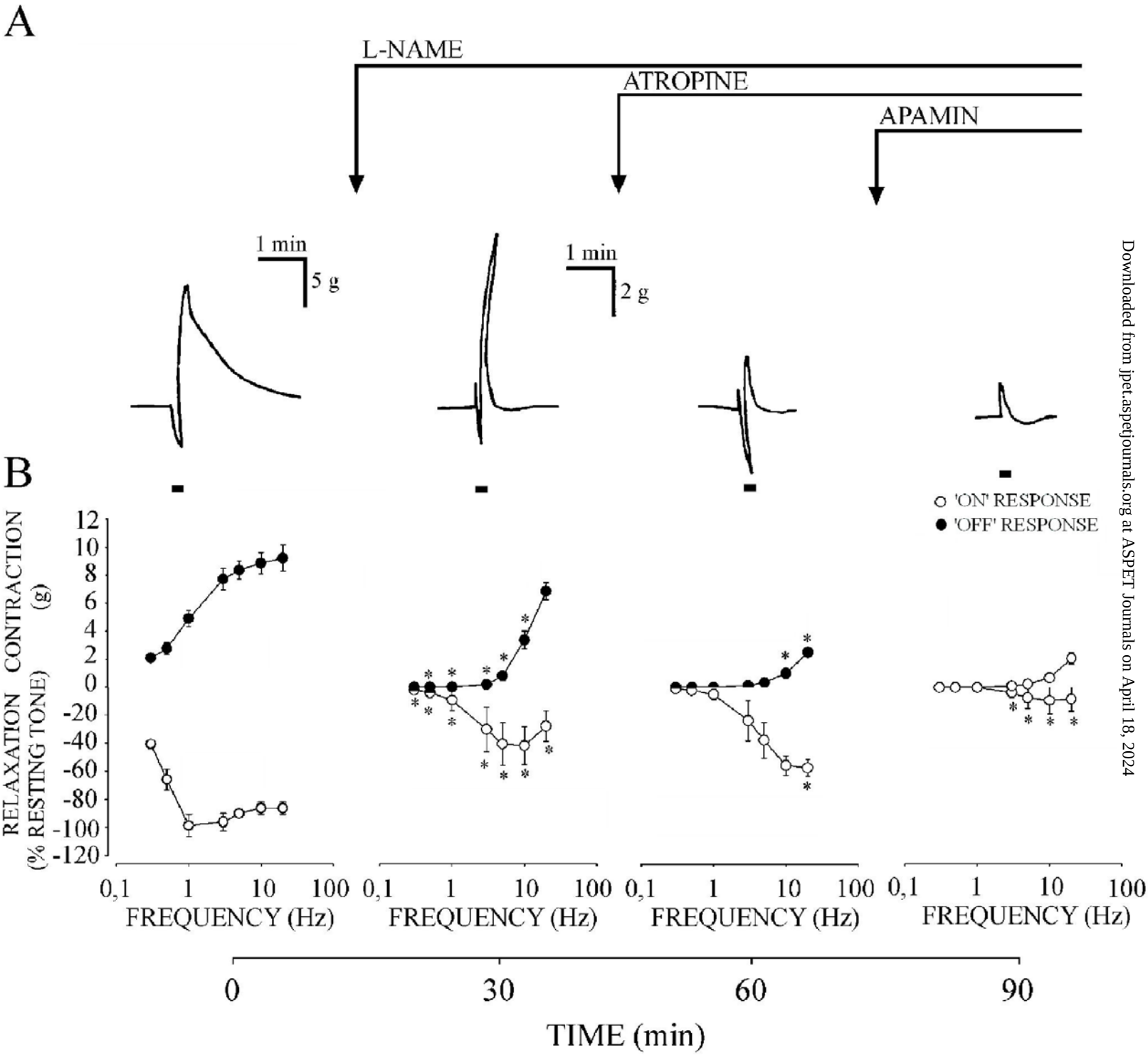

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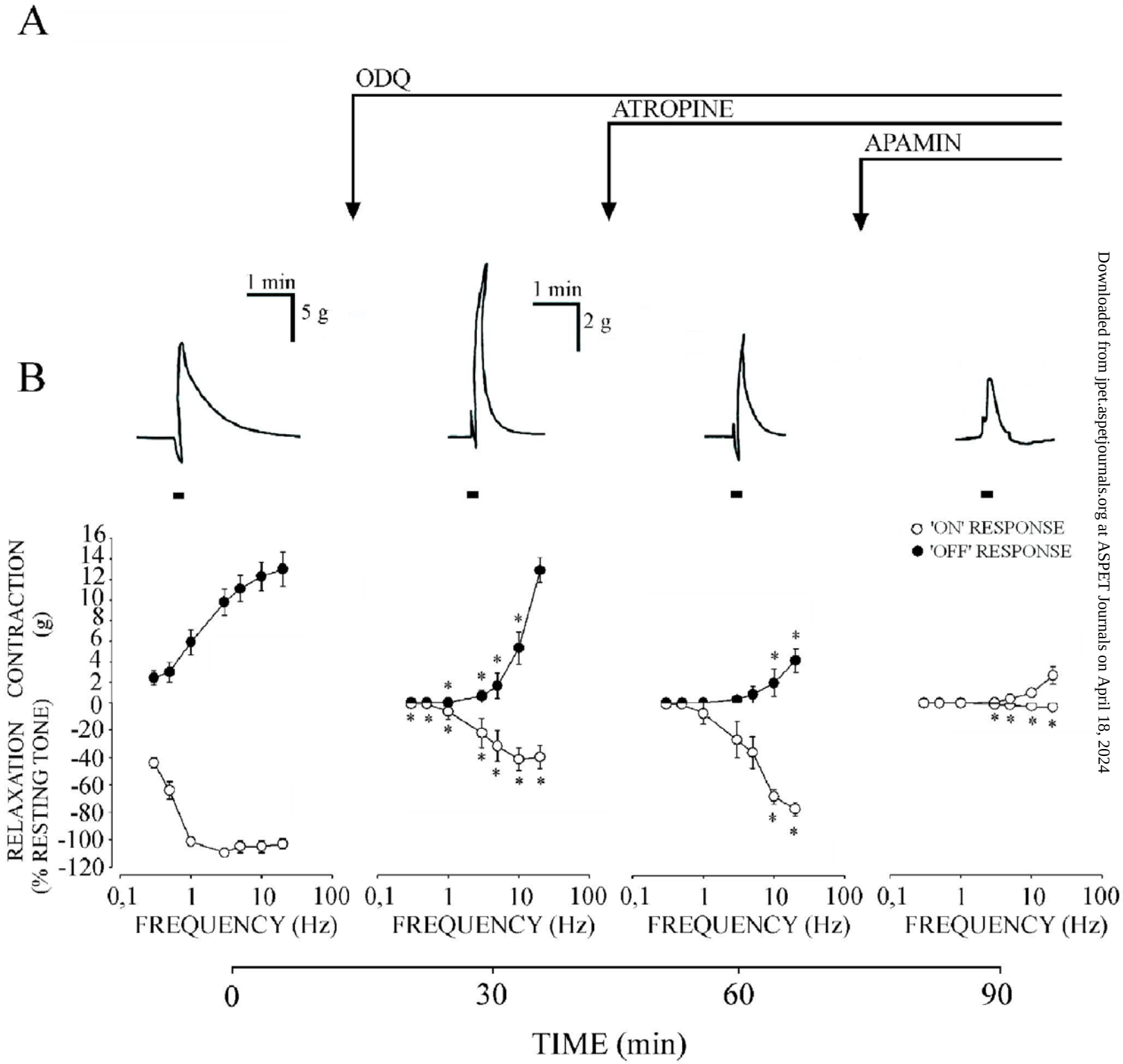
Figure 5



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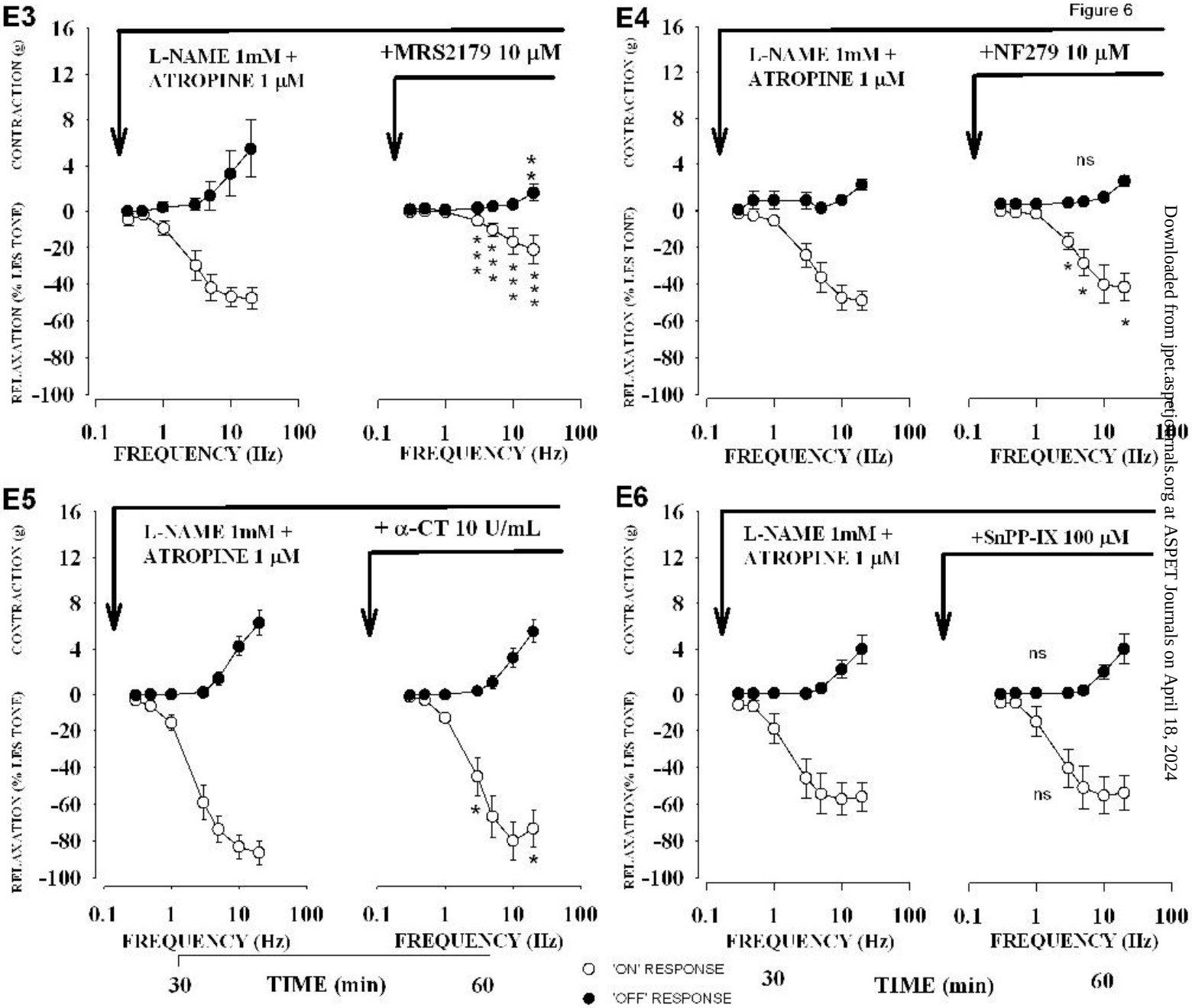

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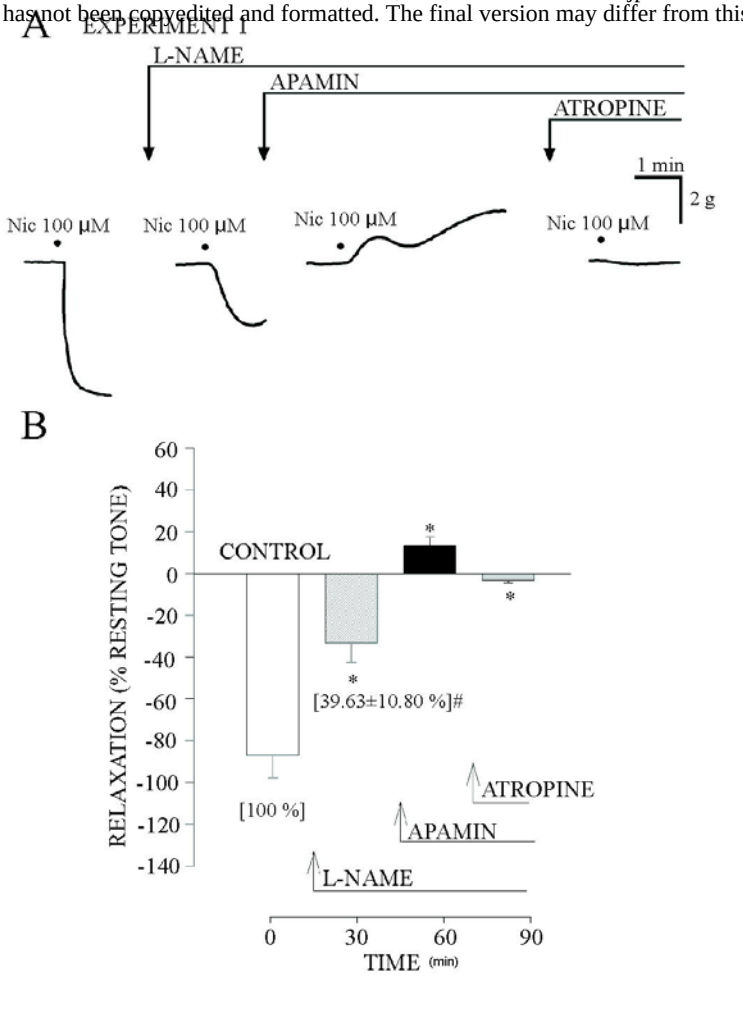

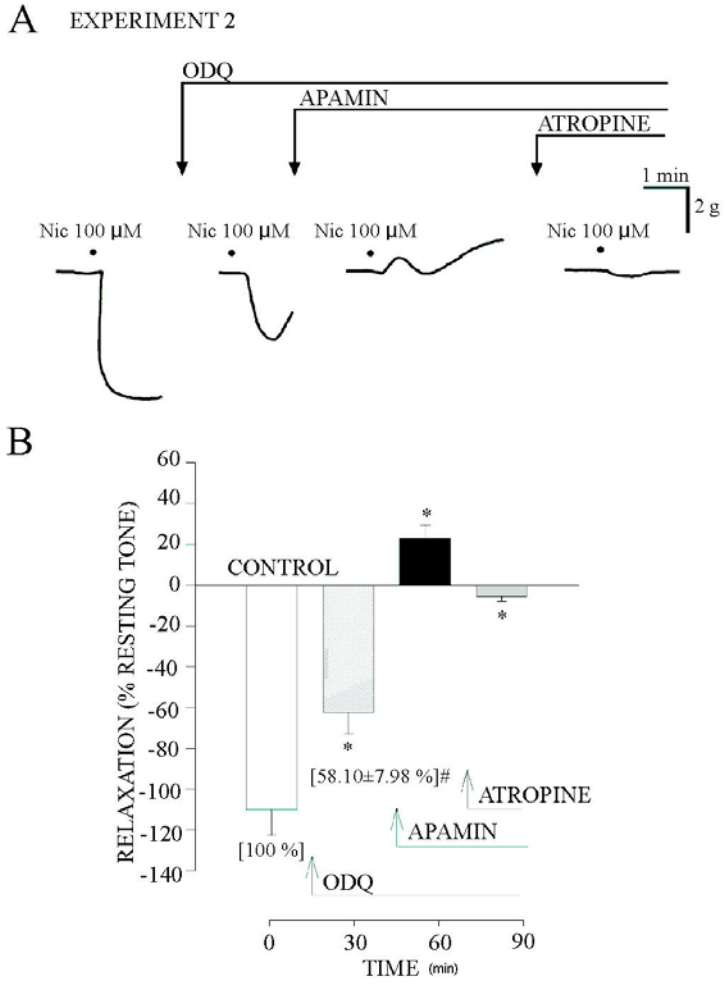

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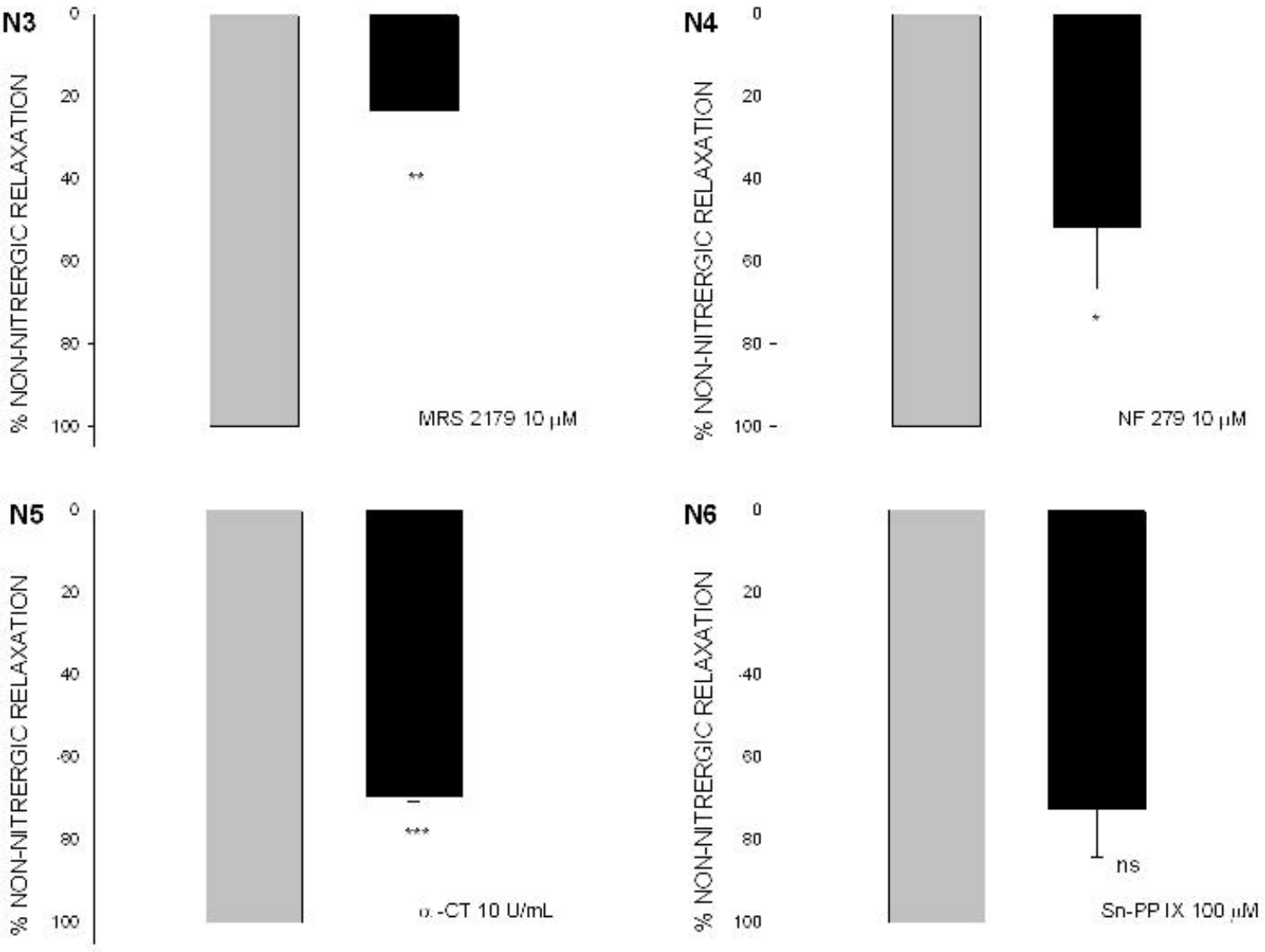
Figure 8



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