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Role of NK_1 and NK_2 receptors in mouse gastric mechanical activity

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1 The aim of the present study was to examine the role of NK_1 and NK_2 receptors in the control of mechanical activity of mouse stomach. In this view, the motor effects induced by NK_1 and NK_2 receptor agonists and antagonists were analyzed, measuring motility as intraluminal pressure changes in mouse-isolated stomach preparations. In parallel, immunohistochemical studies were performed to identify the location of NK_1 and NK_2 receptors on myenteric neurons and smooth muscle cells.

2 Substance P (SP) induced biphasic effects: a contraction followed by relaxation; neurokinin A (NKA) and [β-Ala^8]-NKA(4–10), selective agonist of NK_2 receptors, evoked concentration-dependent contractions, whereas [Sar^9, Met(O_2)^{11}]-SP, selective agonist of NK_1 receptors, induced concentration-dependent relaxation.

3 SR48968, NK_2 receptor antagonist, did not modify the spontaneous activity and reduced the contractile effects induced by tachykinins without affecting the relaxation. SR140333, NK_1 receptor antagonist, did not modify the spontaneous activity and antagonized the relaxant response to tachykinins, failing to affect the contractile effects.

4 The relaxation to SP or to [Sar^9, Met(O_2)^{11}]-SP was abolished by tetrodotoxin (TTX) and significantly reduced by N_ω-nitro-L-arginine methyl ester (L-NAME).

5 NK_2-immunoreactivity (NK_2-IR) was seen at the level of the smooth muscle cells of both circular and longitudinal muscle layers. NK_1-immunoreactive (NK_1-IR) neurons were seen in the myenteric ganglia and NK_1/nNOS double labeling revealed that some neurons were both NK_1-IR and nNOS-IR.

6 These results suggest that, in mouse stomach, NK_1 receptors, causing relaxant responses, are present on nitrergic inhibitory myenteric neurons, whereas NK_2 receptors, mediating contractile responses, are present at muscular level.

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Abbreviations: [β-Ala^8]-NKA(4–10), [β-Ala^8]-Neurokinin A (4–10); CCh, carbachol; ISO, isoproterenol; L-NAME, N_ω-nitro-L-arginine methyl ester; NANC, nonadrenergic noncholinergic; NKA, neurokinin A; NKB, neurokinin B; NK_1-IR, NK_1-immunoreactivity; NK_2-IR, NK_2-immunoreactivity; nNOS-IR, neuronal nitric oxide synthase-immunoreactivity; SP, substance P; [Sar^9, Met(O_2)^{11}]-SP, [Sar^9, Met(O_2)^{11}]-substance P; NO, nitric oxide; NOS, nitric oxide synthase; cNOS, constitutive nitric oxide synthase; nNOS, neuronal nitric oxide synthase; PBS, phosphate buffered saline; TTX, tetrodotoxin

Introduction

Tachykinins constitute a family of peptides, including substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), which are present in a large number of neurons of the enteric nervous system and seem to play a key role in the regulation of motility, secretion and blood flow in the intestine (Holzer & Holzer-Petsche, 1997a, b). Regarding motor activity there is considerable evidence of the involvement of tachykinins in the control of intestinal peristalsis (Barthò *et al*., 1982; Holzer *et al*., 1998; Toulouse *et al*., 2001) and in the excitatory component of the nonadrenergic noncholinergic (NANC) transmission (Maggi *et al*., 1994; Holzer & Holzer-Petsche, 1997a; Serio *et al*., 1998). Tachykinins are found in intrinsic and extrinsic primary afferent neurons as well as in excitatory motor neurons supplying the circular and longitudinal muscle layers, and in interneurons (Holzer & Holzer-Petsche, 1997a). Thus, they control both neuroneuronal and neuromuscular transmission within the enteric nervous system.

Tachykinins exert their effects through the interaction with three types of receptors, termed NK_1, NK_2 and NK_3, preferring SP, NKA and NKB, respectively (Holzer-Petsche, 1995). The type of tachykinin receptors as well as their localization on muscle cells and/or neurons determines the response of smooth muscle of the gut. In fact, although stimulation of motility is the prevailing response to SP, tachykinins can also inhibit intestinal motor activity *via* a neural site of action (Holzer & Holzer-Petsche, 1997a; Bian *et al*., 2000). For example, tachykinins can relax the muscle of

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the guinea-pig gut through the activation of both NK_1 (Johnson *et al*., 1998; Lecci *et al*., 1999) and NK_3 receptors (Jin *et al*., 1993; Maggi *et al*., 1993b). Neurochemical and pharmacological evidence has indicated that NK_1 receptors are located on the cell bodies of enteric neurons (Jin *et al*., 1993; Holzer-Petsche, 1995; Portbury *et al*., 1996a; Vannucchi & Faussone-Pellegrini, 2000), but they can be also located on the musculature (Schmidt & Holst, 2000). NK_2 receptors are prominent on the smooth muscle cells but have also been seen in some nerve terminals (Grady *et al*., 1996, Portbury *et al*., 1996b, Vannucchi *et al*., 2000; Vannucchi & Faussone-Pellegrini, 2000). NK_3 receptors appear to be located mainly on neurons and affect the motility through the release of other mediators (Maggi *et al*., 1994; Johnson *et al*., 1998; Vannucchi & Faussone-Pellegrini, 2000). However, as it appears by literature data, substantial differences exist between species in the regional and tissue distribution of the neurokinin receptors.

In the stomach, tachykinins stimulate motility in all its parts, even if they appear to inhibit it in certain situations and the precise role of tachykinins in the various motor programs in the stomach is not clear (Schmidt & Holst, 2000). For example, the stomach maintains a basal tonic contraction (gastric tone) which is neurally modulated (Mulè & Serio, 2002), but the tachykinins seem not to be involved in the modulation of gastric tone (Tonini *et al*., 2001; Crema *et al*., 2002).

To date, there are only a few studies on the characterization and classification of the tachykinin receptors in the mouse (Fontaine & Lebrun, 1989; Allogho *et al*., 1997; Saban *et al*., 1999; Zizzo *et al*., 2005) although this animal is most frequently used for knocking out the expression of messenger molecules as well as their receptors in studies directed to determine the physiological role of both entities. Information on the gastrointestinal tachykinin system in the normal mouse is an essential prerequisite for such an approach. Functional studies performed on the mouse small intestine have suggested that NK_1 is the primary tachykinin receptor involved in the excitatory NANC transmission in ileum (Saban *et al*., 1999), whereas both NK_1 and NK_2 receptors mediate NANC-evoked contractions in duodenum (Zizzo *et al*., 2005). The distribution of tachykinin receptors has been analyzed just for mouse ileum (Vannucchi & Faussone-Pellegrini, 2000) whereas information is lacking for the stomach, although it is expected to find them as a large number of SP-immunoreactive fibers and neurons have been found in the myenteric plexus and circular muscle layer of mouse stomach (Ekblad *et al*., 1985).

The objectives of the present study were to characterize, in mouse stomach, the responses induced by exogenous tachykinins and to verify if basal release of endogenous tachykinins is involved in the control of gastric tone. So, measuring motility as pressure changes from isolated whole-organ, we analyzed the motor effects induced by NK_1 and NK_2 receptor agonists and antagonists. In parallel, immunohistochemical studies were performed to examine the location of NK_1 and NK_2 on myenteric neurons and/or muscle cells.

Methods

The experiments were authorized by Ministero della Sanità (Rome, Italy). Adult mice (C57BL/10SnJ) were killed by cervical dislocation. The abdomen was immediately opened, the esophagus was tied proximal to lower esophageal sphincter, and the entire stomach was excised. Preparations were mounted in a custom-designed horizontal organ bath, which was continuously perfused with oxygenated (95% O_2 and 5% CO_2) and heated (37°C) Krebs solution with the following composition (mM): NaCl 119; KCl 4.5; $MgSO_4$ 2.5; $NaHCO_3$ 25; KH_2PO_4 1.2; $CaCl_2$ 2.5; glucose 11.1. The pyloric end was cannulated and connected to a standard pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA, U.S.A.). The mechanical activity was recorded on ink-writer polygraph (Grass model 7D). Preparations were allowed to equilibrate for about 60 min before starting the experiment.

Experimental protocol

In a first series of experiments, non-cumulative concentration–response curves for SP, NKA, [Sar^9, $Met(O_2)^{11}$]-SP, NK_1 receptor agonist, and [β-Ala^8]-NKA(4−10), NK_2 receptor agonist, were established before and after 40 min pretreatment with SR140333 (0.1 μM), NK_1 receptor antagonist, or SR48968 (0.1 μM), NK_2 receptor antagonist. The agonists were added to the bath for 4 min. Concentrations were progressively increased and tested at 40-min intervals to avoid tachyphylaxis. Then, the responses to NK_1 and NK_2 receptor agonists were tested in the presence of tetrodotoxin (TTX) (1 μM), a voltage-dependent Na^+-channel blocker, and the relaxations induced by SP or by [Sar^9, $Met(O_2)^{11}$]-SP were tested in the presence of L-NAME (300 μM), inhibitor of nitric oxide synthase (NOS). The inhibitors were added to the perfusing solution at least 30 min before testing the tachykinins.

In the experiments designed to evaluate the relative contribution of NK_1 and NK_2 receptors in the maintenance of gastric tone, SR140333 (0.01–1 μM), NK_1 receptor antagonist, or SR48968 (0.1–1 μM), NK_2 receptor antagonist, were added to the Krebs solution in consecutively increasing concentrations, with intervals of at least 30 min, and the effects on spontaneous mechanical activity were examined.

Data analysis and statistical tests

The contractile response of gastric preparations was defined as the change in the resting tone (the mean level of the pressure oscillations). Contractile and relaxant responses to agonists were expressed as a percentage of the response produced by carbachol (CCh, 1 μM) or isoproterenol (ISO, 1 μM), respectively. The concentration (EC_{50}) with 95% confidence intervals (Cls) producing half-maximum response was calculated using Prism 4.0, GraphPad (San Diego, CA, U.S.A.). All data are expressed as mean values $\pm$ s.e.m. The letter n indicates the number of experiments and it is equivalent to the number of experimental animals. Statistical analysis was performed by means of paired Student's t-test. A probability value of less than 0.05 was regarded as significant.

Drugs

The following drugs were used and stock solutions were prepared using distilled water or as indicated below. The working solutions were prepared freshly on the day of the experiments by diluting the stock solutions in Krebs. Tetrodotoxin (TTX), N_ω-nitro-L-arginine methyl ester

(L-NAME), SP and NKA were purchased from Sigma Chemical Corp. (St Louis, MO, U.S.A.). ((S)-*N*-methyl-*N*[4-(4-acetyl-amino-4-phenylpiperi-dino)-2-(3,4-dichloro-phenyl)-butyl]benzamide (SR48968), (S)-1-[2-[3-(3,4-dichlorphenyl)-1 (3-isopropoxy-phenylacetyl)piperidin-3yl]ethyl]-4-phenyl-1 azaniabicyclo[2.2.2] octane chloride (SR140333), gifts from Sanofi Recherche (Montpellier Cédex, France), were dissolved in dimethyl sulphoxide (0.1% final concentration). [Sar^9, $Met(O_2)^{11}$]-SP, dissolved in diluted acetic acid, and [β-Ala^8]-NKA(4−10), dissolved in diluted ammonia, were from Calbiochem-Novabiochem AG (Laufelfingen, Switzerland). Control experiments using the different solvents alone showed that none had effects on the tissue responses studied.

Immunohistochemistry

Full thickness pieces of stomach were cleaned of digestive material with saline and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.4, for 4–6 h at 4°C and then placed in 30% sucrose in PBS, at 4°C. These specimens were embedded in OCT compound (Miles, Elkhart, IN, U.S.A.) and frozen at −80°C. Sections, 14 μm thick, were cut with a cryostat and collected on polylysine (0.1% in distilled water)-coated slides.

After washing in PBS containing 3% normal goat serum and 0.5% Triton X-100, one series of sections was incubated with NK_1 (a generous gift of Dr P. Vigna) and another series with NK_2 (#94179) (a generous gift of Dr N.W. Bunnett) receptor polyclonal antibodies These antibodies were raised in rabbit against synthetic fragments corresponding to the intracellular C-terminal portion of the rat receptors and extensively characterized by radioimmunoassay, immunohistochemistry, Western blotting and preadsorption tests (Vigna *et al*., 1994; Grady *et al*., 1996). A third series of sections was incubated with a nNOS monoclonal antibody (N31020) raised in mouse against a human nNOS protein (residues 1095–1289) (Transduction Laboratories, Lexington, KY, U.S.A.) that recognizes a nNOS protein with a molecular weight of 155 kDa. The primary antisera NK_1 and NK_2 were used at dilution 1 : 2500 and 1 : 500, respectively, and the nNOS antiserum at dilution 1 : 1 : 500, for 24–48 h at 4°C. For double labeling, a fourth series of sections was simultaneously incubated with the monoclonal nNOS antiserum and the polyclonal NK_1 antiserum. At the end of incubation, all the sections were rinsed three times in 10 min washes in PBS. After the final wash, the polyclonal primary antisera were revealed by using fluorescein (FITC)-AffiniPure F(ab′)$_2$ fragment goat anti-rabbit IgG (H + L; Jackson Immuno-Research, West Grove, PA, U.S.A.) secondary antibody, diluted 1 : 100, for 2 h at room temperature. nNOS monoclonal antibody was revealed by incubating the sections in the presence of TEXAS RED® anti-mouse IgG, AffiniPure (H + L; Vector Laboratories, Burlingame, CA, U.S.A.) secondary antibody, diluted 1 : 200, for 2 h at room temperature. The sections were then mounted in an aqueous medium (Gel Mount, Biomeda Corp., Foster City, CA, U.S.A.) and the immunoreaction products were observed under an epifluorescence Zeiss Axioskop microscope and photographed.

Negative controls were performed by omitting the primary antibodies or substituting them with a non-immune rabbit or mouse serum in order to check the specificity of the immunostaining. To avoid aspecific binding by the monoclonal anti-nNOS antibody to mouse tissues, the sections were pretreated with unlabeled goat anti-mouse whole IgG molecules (Sigma, St Louis, MO, U.S.A.), diluted 1 : 50 in PBS and applied to the sections for 15 min, followed by rinsing in PBS. To evaluate the specificity of the staining, a preadsorption test was also done with constitutive NOS (cNOS) rat neuronal recombinant (Calbiochem, San Diego, CA, U.S.A.) used at two different cNOS concentrations. A volume of 8 or 18 μl of cNOS (5.5 mg ml^{-1}) were added to 2 μl of nNOS undiluted antibody; the solution was incubated for 30 min at 37°C and 30 min at room temperature, cooled at 4°C and centrifuged in a refrigerated microfuge. Supernatant was diluted as appropriate and used immediately for control staining (see above). The best results were obtained using the larger volume of the recombinant cNOS solution.

Results

Pharmacology

As previously described (Mulè & Serio, 2002) gastric preparations showed small spontaneous phasic contractions, the amplitude of which ranged from 0.5 to 1 cm H_2O with a frequency of about 5 c.p.m.

Substance P (0.01–1 μM) induced biphasic effects: the preparation initially contracted on exposure to the agonist and then showed a late relaxation (Figure 1). Both responses were dependent on the concentrations (Figure 2). In particular, the maximal contractile response was obtained at a concentration of 0.3 μM (about 40% of the response to 1 μM CCh) yielding an EC_{50} value of 29 nM (Cls 1–814 nM). The contractile component was significantly antagonized by SR48968 (0.1 μM), selective NK_2 receptor antagonist, but it was not modified appreciably by SR140333 (0.1 μM), NK_1 receptor antagonist (EC_{50} value 810 nM Cls 106–6267 nM after SR48968 and 35 nM Cls 0.4–2812 nM after 140333) (Figure 2). The maximal relaxant response was obtained at a concentration of 0.3 μM (about 30% of the relaxation to 1 μM isoproterenol) with an EC_{50} value of 28 nM (Cls 0.9–790 nM).

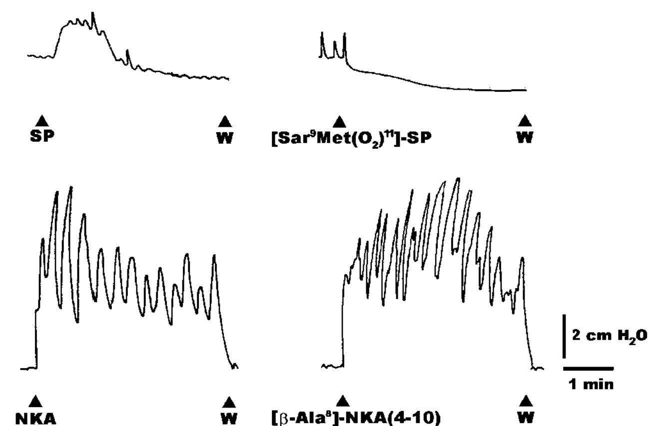

Figure 1 Typical tracings showing the effects of the natural tachykinins, SP (0.3 μM) and NKA (0.3 μM), and of NK_1 and NK_2 receptor selective agonists, [Sar^9, $Met(O_2)^{11}$]-SP (1 μM) and [β-Ala^8]-NKA(4−10) (0.3 μM), respectively, in mouse gastric preparations. Arrows show the application of the agonists. W = wash out.

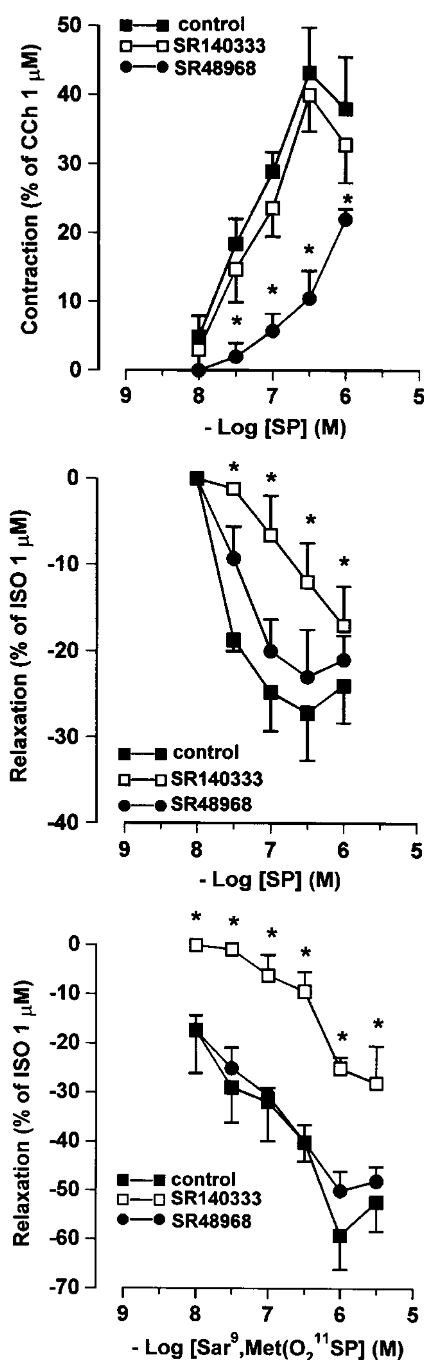

Figure 2 Concentration–response curves for the effects induced by SP ($n=6$) or by [Sar[9], Met(O$_2$)[11]]-SP ($n=5$), selective agonist of NK$_1$ receptors, in mouse gastric preparations in control conditions and after pretreatment with SR140333 (0.1 μM) or SR48968 (0.1 μM), NK$_1$ receptor antagonist and NK$_2$ receptor antagonist, respectively. Contractile responses are expressed as percent of the contraction induced by carbachol (CCh, 1 μM) and relaxant responses are expressed as percent of the relaxation induced by isoproterenol (ISO, 1 μM). All values are means$\pm$s.e.m. *$P<0.05$ compared to the control.

The relaxation was greatly antagonized by the NK$_1$ receptor antagonist, SR140333 (0.1 μM), but it was not affected by the NK$_2$ receptor antagonist, SR48968 (0.1 μM) (EC$_{50}$ 220 nM Cls 10–1650 nM after SR140333 and 33 nM Cls 3–910 nM after SR48968) (Figure 2).

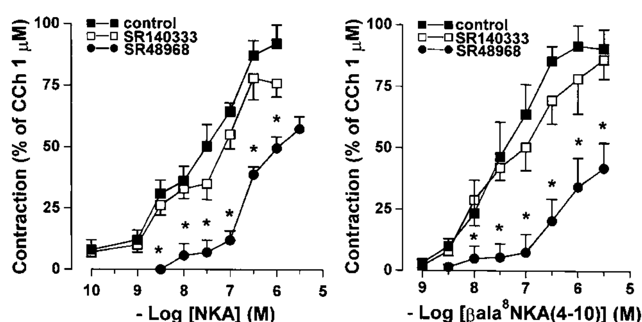

Figure 3 Concentration–response curves for the contractile effects induced by NKA ($n=4$) or by [β-Ala[8]]-NKA(4–10) ($n=5$), selective agonist of NK$_2$ receptors, in mouse gastric preparations in control conditions and after pretreatment with SR48968 (0.1 μM) or SR140333 (0.1 μM), NK$_2$ receptor antagonist and NK$_1$ receptor antagonist, respectively. Contractile responses are expressed as percent of the contraction induced by carbachol (CCh, 1 μM). All values are means$\pm$s.e.m. *$P<0.05$ compared to the control.

NKA (0.1 nM–1 μM) induced concentration-dependent contractile effects, characterized by a fast increase in the tone with superimposed phasic contractions (Figures 1 and 3). NKA was more effective than SP with a maximal response at 0.3 μM of about 90% of contraction to 1 μM CCh. The NKA-induced response was significantly antagonized by the NK$_2$ receptor antagonist, SR48968 (0.1 μM), but it was unaffected by the NK$_1$ receptor antagonist, SR140333 (0.1 μM) (EC$_{50}$ 80 nM Cls 39–160 nM in the control; 290 nM Cls 59–1476 nM after SR48968; and 77 nM Cls 9–637 nM after SR140333) (Figure 3).

To further assess the specificity of the observed effects, we tested the selective NK$_1$ receptor agonist, [Sar[9], Met(O$_2$)[11]]-SP, and the selective NK$_2$ receptor agonist, [β-Ala[8]]-NKA(4–10).

[Sar[9], Met(O$_2$)[11]]-SP (10 nM–3 μM) exclusively induced a relaxation, which developed slowly and enhanced with the increase in the concentration (Figures 1 and 2). The maximal response was obtained at 1 μM (about 60% of the relaxation to 1 μM isoproterenol). The [Sar[9], Met(O$_2$)[11]]-SP-induced response was significantly antagonized by the NK$_1$ receptor antagonist, SR140333 (0.1 μM), but it was not affected by the NK$_2$ receptor antagonist, SR48968 (0.1 μM) (EC$_{50}$ 170 nM Cls 12–2382 nM in the control; 550 nM Cls 120–2520 nM after SR140333; and 120 nM Cls 33–434 nM after SR48968) (Figure 2).

[β-Ala[8]]-NKA(4–10) (1 nM–3 μM), NK$_2$ receptor agonist, induced a contractile response, that was maintained for the entire application time. It was characterized by a concentration-dependent increase in the basal tone, with superimposed phasic contractions (Figures 1 and 3). The maximal response was obtained at a concentration of 0.1 μM (90% of the contraction to 1 μM CCh). The response was antagonized by SR48968 (0.1 μM), NK$_2$ receptor antagonist but it was unaffected by the NK$_1$ receptor antagonist, SR140333 (0.1 μM) (EC$_{50}$ 44 nM Cls 19–101 nM in the control, 360 nM Cls 166–803 nM after SR48968, and 55 nM Cls 29–102 nM after SR140333) (Figure 3).

In order to distinguish the action of the tachykinins on neurons and smooth muscle cells, the effects of TTX were tested on the response evoked by the NK$_1$ or NK$_2$ receptor agonists. TTX (1 μM) abolished the relaxation to a submaximal concentration of SP (0.1 μM) or of [Sar[9], Met(O$_2$)[11]]-SP (0.3 μM) suggesting that the relaxation is mediated by an

inhibitory neurotransmitter (Figure 4). It failed to affect the contraction evoked by a submaximal concentration of SP (0.1 μM) (31.5$\pm$6.5% in the control and 34$\pm$5.2% after TTX; $n=4$, $P>0.05$) or of [β-Ala^8]-NKA(4–10) (0.3 μM) (85.6$\pm$5.8% in the control and 101$\pm$4% after TTX; $n=4$, $P>0.05$).

Lastly, the relaxations induced by SP (0.1 μM) or by [Sar^9, Met(O_2)^{11}]-SP (0.3 μM) were significantly reduced in the presence of L-NAME (300 μM), inhibitor of the NOS (Figure 4).

Perfusion with the selective tachykinin NK_1 receptor antagonist SR140333 (0.1–1 μM) or with the selective tachykinin NK_2 receptor antagonist SR48968 (0.1–1 μM) produced neither modifications in the amplitude of the pressure waves nor in the basal tone (Figure 5).

Immunohistochemistry

NK_2-immunoreactivity (NK_2-IR) was seen at the level of the smooth muscle cells of both circular and longitudinal muscle layers (Figure 6a) and labeling was slightly more intense in the longitudinal than in the circular smooth muscle cells. Nerve terminals NK_2-IR were not seen in the stomach.

NK_1-immunoreactive (NK_1-IR) neurons were seen in the myenteric ganglia (Figure 6b). Labeling was intense and mainly distributed along the pericaryal contour.

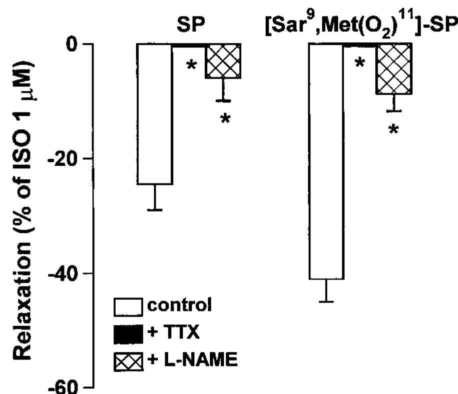

Figure 4 Effects of TTX (1 μM, $n=4$) or of L-NAME (300 μM, $n=5$) on the relaxation evoked by SP (0.1 μM) or by [Sar^9, Met(O_2)^{11}]-SP (0.3 μM), selective agonist for NK_1 receptors. Relaxant responses are expressed as percent of the relaxation induced by isoproterenol (ISO, 1 μM). All values are means$\pm$s.e.m. $^*P<0.05$ compared to the respective control conditions.

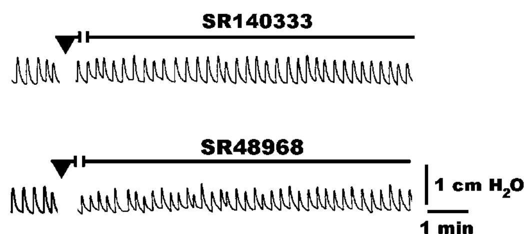

Figure 5 Typical tracings showing the spontaneous mechanical activity of mouse gastric preparations before and after SR140333 (0.1 μM) and SR48968 (0.1 μM), selective antagonists for NK_1 and NK_2 receptors, respectively.

nNOS-immunoreactive (nNOS-IR) neurons were also present in the myenteric ganglia (Figure 6b). In these cells, the labeling was intense and distributed within pericarya and nerve processes. NK_1/nNOS double labeling revealed that some neurons were both NK_1-IR and nNOS-IR (Figure 6b).

Discussion

The present results indicate that in mouse stomach NK_1 receptor activation evokes a neurogenic inhibitory relaxation that involves a nitrergic pathway. The NK_2 receptor activation induces a non-neural, likely myogenic, contractile response. Data from immunohistochemistry support these conclusions as NK_1 receptors were located on myenteric neurons, which may co-express nNOS, and NK_2 receptors were present on the smooth muscle cells. However, NK_1 and NK_2 receptors do not appear to be involved in the control of gastric tone.

The involvement of tachykinin NK_1 and NK_2 receptors in the modulation of gastric contractions has been shown in different species (Zagorodnyuk & Maggi, 1997; Schmidt & Holst, 2000). However, substantial differences exist between species in the tissue distribution of the neurokinin receptors (Burcher *et al*., 1986; Sternini *et al*., 1995; Holzer & Holzer-Petsche, 1997a). Few studies have examined function and mechanism of action of the tachykinin receptors in the regulation of the gastrointestinal motility in the mouse (Allogho *et al*., 1997; Saban *et al*., 1999; Zizzo *et al*., 2005). Their location in the muscle coat of mouse gut is reported just for the ileum (Vannucchi & Faussone-Pellegrini, 2000).

This study shows that in mouse stomach selective tachykinin analogs and natural tachykinins interact with distinct NK_1 receptors located on myenteric nitrergic neurons and NK_2 receptors located on muscle cells. In our experiments, NKA and [β-Ala^8]-NKA(4–10) caused fast contraction with superimposed rhythmic oscillations, whereas SP was less effective

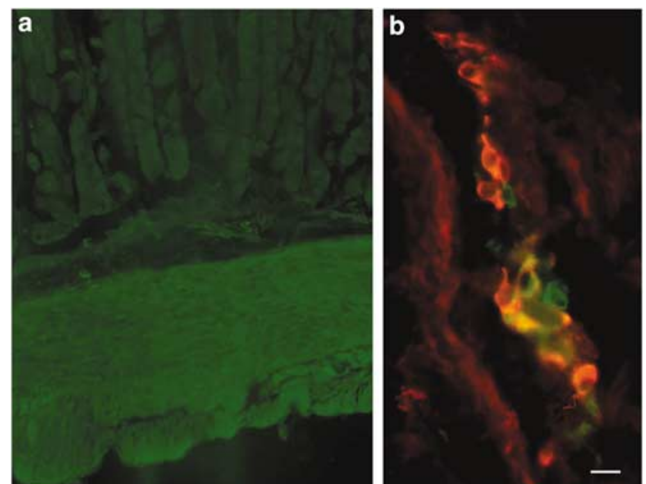

Figure 6 Mouse stomach. (a) Immunolabeling with the NK_2 antibody. Immunoreactivity (in green) is present on the smooth muscle cells of both circular and longitudinal muscle layers. From upper to lower side: mucosa, submucosa, muscle coat. (b) Double labeling with NK_1 and nNOS antibodies. In a myenteric ganglion, some neurons are NK_1-IR (in green), some are nNOS-IR (in red) and some others are NK_1/nNOS-IR (in orange). Calibration bars: a = 25 μm; b = 40 μm.

and [Sar^9, Met(O$_2$)^{11}]-SP had no contractile effects, indicating that NK$_2$ receptors are the mainly responsible for the contractile response. The contractile effects induced by NKA or [β-Ala^8]-NKA(4−10) were antagonized by SR48968, but not by SR140333 suggesting that NK$_2$ receptors exclusively mediate contraction. In support of the hypothesis that NK$_2$ receptors are the primary receptors for stimulation of mechanical activity in mouse gastric preparations there is the observation that the weaker contraction induced by SP was antagonized by SR48968, selective antagonist of NK$_2$ receptors, but not by SR140333, selective antagonist of NK$_1$ receptors, suggesting that SP-induced contractile effects were mediated by NK$_2$ receptors. Our finding in mouse gastric preparation in which NK$_2$ receptors are the main contributors to contraction of the smooth muscle is in agreement with data obtained in isolated rat perfused stomach (Holzer *et al*., 1997). In addition, the failure of TTX to inhibit the contractile responses induced by SP or [β-Ala^8]-NKA(4−10) indicates that the evoked contractions may be due to direct smooth muscle stimulation, however, not involving a neuronal component. This possibility is supported by immunohistochemistry that did not reveal NK$_2$-IR nerve terminals in the mouse stomach.

Interestingly, we observed also relaxation responses induced by SP which were mimicked by [Sar^9, Met(O$_2$)^{11}]-SP, selective NK$_1$ receptor agonist, suggesting that they were mediated by the NK$_1$ receptors. This is confirmed by the observation that the relaxant responses were antagonized by SR140333, selective antagonist of NK$_1$ receptors, but not by SR48968. On the other hand, the finding that each antagonist displaced only the concentration–response curve of the respective agonist to the right demonstrates the specificity of action of the drugs used. SR140333 and SR48968 also induced a reduction of the maximal response to the respective agonists, suggesting that they do not exert a purely competitive antagonism. The non-competitive nature of these antagonists has been shown in some *in vitro* functional experiments (Edmonds-Alt *et al*., 1993; Maggi *et al*., 1993a), including mouse gastric strips (Allogho *et al*., 1997).

It was previously shown that in guinea-pig small intestine SP first stimulates and then inhibits propulsive motility (Holzer *et al*., 1995). Indeed, the analysis of the time course of the effects of SP indicates that the late inhibitory effects are masked or overwhelmed by the concomitant activation of excitatory mechanisms. Our data differs from previously published results that showed exclusively contraction in mouse gastric strips by NK$_1$ agonists (Allogho *et al*., 1997). This apparent discrepancy may be attributable to the differece in the methodology. We recorded the pressure changes from the whole stomach; consequently, the response of gastric smooth muscle to tachykinins is the net result of direct and neurally mediated effects from the different part of the organ. The relaxation induced by SP or by selective NK$_1$ agonist, [Sar^9, Met(O$_2$)^{11}]-SP was abolished by TTX suggesting that it is entirely mediated by enteric neurons. In addition, it was strongly inhibited by L-NAME indicating that it is mainly due to the release of nitric oxide (NO) from neural source. On the other hand, the experiments with NK$_1$/nNOS double labeling revealed that some neurons were both NK$_1$-IR and nNOS-IR supporting the hypothesis that NK$_1$ receptors are present on nitrergic neurons. Other functional studies have indicated that NK$_1$ receptor inhibitory effects on the motility of the small intestine can be mediated by NO production (Holzer, 1997; Lecci *et al*., 1999; Bian *et al*., 2000) and NO is involved in the guinea-pig gastric relaxation induced by SP (Jin *et al*., 1993). Moreover, colocalization of the NK$_1$-IR and NOS-IR or NADPH-diaphorase labeling has been showed just for guinea-pig small and large intestine (Portbury *et al*., 1996a; Lecci *et al*., 1999; Bian *et al*., 2000). In our preparation, part of the NK$_1$ receptor-evoked inhibitory effects were L-NAME-resistant, suggesting that likely another transmitter, different from NO, is involved in the relaxation following NK$_1$ receptor activation. This is not surprising since multiple nonadrenergic noncholinergic inhibitory transmitters are known to mediate NANC relaxation in mouse stomach (Mulè & Serio, 2003) and, for instance, ATP, in addition to NO, has been involved in the motor response to NK$_1$ agonists in guinea-pig small intestine (Shahbazian & Holzer, 2000).

Although this study shows the functional presence of NK$_1$ and NK$_2$ receptors in gastric tissue, none of the selective antagonists had any effect on basal gastric tone at the concentration showed to block the respective receptors. Therefore, these findings suggest that tachykinins are not involved in maintaining of gastric tone in mouse, at least in normal conditions. In other preparations (dog, rat), no change in gastric tone was observed after blockade of NK$_1$ or NK$_2$ receptors (Tonini *et al*., 2001; Crema *et al*., 2002); however, we cannot rule out the possibility that NK receptor antagonists affect gastric compliance in patients with defective gastric accomodation.

In conclusion, our studies demonstrate that in the mouse stomach there are NK$_1$ receptors on nitrergic, inhibitory myenteric neurons, which in turn would induce muscular relaxation, whereas excitatory NK$_2$ receptors are present only at the muscular level.

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