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Original Article

Contractile effect of tachykinins on rabbit small intestine

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Aim: To study the role of the tachykinin receptors in spontaneous contractions of longitudinal and circular smooth muscle from rabbit small intestine and to determine the mechanism of action of Substance P (SP).
Methods: Rabbit duodenum, jejunum and ileum segments were prepared. The spontaneous contractions of longitudinal and circular smooth muscle were recorded using a computer via an isometric force transducer. The specific agonists and antagonists of tachykinin receptors were added into the organ bath.
Results: The agonists of tachykinin NK1 receptor (SP and [Sar9] SP), NK2 receptor (NKA and (β-Ala8)-NKA), and NK3 receptor (NKB and Senktide) all induced contractions in the small intestine. The contractions were diminished by NK1 receptor antagonist L-733,060, NK2 receptor antagonist GR-94800, and NK3 receptor antagonist SB 218795. Contractions caused by SP were also reduced by atropine, verapamil, PKC inhibitor staurosporine, and PLC inhibitor U73122.
Conclusion: Ttachykinin NK1, NK2, and NK3 receptors mediate the contractions of the smooth muscle in rabbit intestine. Furthermore, SP acts directly on smooth muscle cells through the tachykinin NK1 receptor.

Keywords: tachykinin receptors; substance P; small intestine; rabbits; staurosporine; U73122; L-733,060; GR-94800; SB 218795

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Introduction

Tachykinins (TKs) are a family of neuropeptides distributed throughout the mammalian central and peripheral nervous systems. TKs act as neurotransmitters on neurons and cells (such as smooth muscle, secretory epithelium, and glands) in the gastrointestinal tract of mammals[1–3]. They are important excitatory neurotransmitters in the enteric nervous system, are involved in the coordination of gastrointestinal motility, and are powerful spasmogens in almost every region of the mammalian intestine[1–6]. Substance P (SP), neurokinin A (NKA) and neurokinin B (NKB) are tachykinins derived from two preprotachykinin (PPT) genes[1, 7–10]. Substance P and neurokinin A (NKA) are present in neurons of the enteric nervous system, where they appear to coexist with acetylcholine (ACh)[11]. SP, NKA, and NKB contract nearly all parts of the gastrointestinal tract, acting on different types of receptors[4, 12].

Receptors of TKs have been implicated in normal, defensive, and pathological gastrointestinal (GI) functions[13–16]. The three types of tachykinin receptors, which have been identified based on their genomic and molecular structure, are currently termed NK1, NK2, and NK3 tachykinin receptors[1]. They are heterogeneously distributed within each species. The tachykinin NK1 receptor is widely expressed in the nervous system at both the central and the peripheral level, and it is present in neurons, muscle, and different types of immune cells[2, 3]. The tachykinin NK2 receptor is detected primarily in the periphery nerves, and its expression in the central nervous system (CNS) appears to be restricted to specific brain nuclei[3, 17]. Tachykinin NK1, NK2, and/or their receptors are expressed by neurons, interstitial cells of Cajal, intestinal muscle, epithelium, vasculature and the immune system in a cell-specific, region-specific, and species-specific manner[1, 15, 18, 19]. In contrast, the tachykinin NK3 receptor is primarily expressed in the CNS and has been detected only in certain peripheral tissues, such as human and rat uterus, rat mesenteric vein, and certain enteric neurons from the gut of various species[15, 20, 21].

TKs influence gastrointestinal motor activity not only through their direct effect on the muscle but also through their action on other motility-regulating systems[8]. The aims of this work were to study *in vitro* the role of tachykinin receptors on

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spontaneous contractions of longitudinal and circular smooth muscle from rabbit small intestine using specific agonists and antagonists of each tachykinin receptor as well as to determine the mechanism of action of SP.

Materials and methods

Male New Zealand rabbits weighing 2–2.5 kg were maintained at a constant temperature (22 °C) with standard rabbit fodder and free access to water. The equipment used and the handling and sacrifice of animals complied with European Council legislation 86/609/EEC concerning experimental animal protection. The experimental protocols were approved by the Ethical Committee of the University of Zaragoza (Spain).

Solutions and substances

The Krebs solution contained the following (in mmol/L): NaCl 120, KCl 4.7, $CaCl_2$ 2.4, $MgSO_4$ 1.2, $NaHCO_3$ 24.5, KH_2PO_4 1, and glucose 5.6 at 37 °C to achieve pH 7.4. Some experiments were conducted with a Ca^{2+}-free Krebs solution from which $CaCl_2$ was omitted and to which EGTA 0.5 mmol/L was added.

Acetylcholine (ACh), atropine, guanethidine, verapamil, hexamethonium, N^{ω}-nitro-*L*-arginine (*L*-NNA), ethylene glycol-*bis* (β-aminoethylether)-*N*,*N*′-tetraacetic acid (EGTA), ryanodine, and Substance P (SP) were purchased from Sigma (Madrid, Spain). Neurokinin A (NKA), neurokinin B (NKB), [Sar9] SP, (β-Ala8)-neurokinin A [(β-Ala8)-NKA], [succinyl-Asp6, Me-Phe8]-SP (senktide), and GR-94800 were obtained from American Peptide (Sunnyvale, CA, USA). Tetrodotoxin (TTX), staurosporine, U 73122, L-733060, and SB 218795 were acquired from Tocris (Bristol, UK). Thapsigargin was kindly donated by Alomone Labs (Jerusalem, Israel). All chemicals were of analytical grade. TTX and staurosporine were dissolved in acidic buffer (pH 4.8) and ethanol, respectively. Thapsigargin, ryanodine, and U 73122 were prepared in dimethyl sulphoxide (DMSO). The remaining drugs were dissolved in Milli-Q water. All solutions were stored at -20 °C, and fresh dilutions were made daily.

Preparation of smooth muscle segments

After 24 h of fasting, animals were humanely euthanized by means of a blow to the head. Pieces of rabbit duodenum, jejunum, and ileum were removed, washed, freed from mesenteric attachment, and cut into smaller segments. Whole thickness segments (10 mm long) were suspended in the direction of longitudinal and circular smooth muscle fibers in a thermostatically controlled (37 °C) organ bath (10 mL capacity) containing Krebs solution and were continuously gassed with 95% O_2 and 5% CO_2.

Each segment was connected to an isometric force transducer (Pioden UF1, Graham Bell House, Canterbury, UK) and passively stretched to an initial tension of 20 mN. The signal output of the mechanical activity was amplified, recorded on a computer for later analysis using Mac Lab System/8e computer program (AD Instruments Inc, Milford, MA, USA), and digitized at two samples per second per channel. Prior to testing, segments were allowed to equilibrate in Krebs solution for 60 min.

Experimental protocols

Each experimental protocol was systematically performed on two or three segments of duodenum, jejunum, and ileum taken from the same rabbit and repeated in three or four different animals. Segments that showed no spontaneous activity were discarded; thus, each preparation served as its own control.

Noncumulative concentration-response curves of SP (agonist of NK1, NK2, and NK3 receptors) were established by adding SP (1 nmol/L to 10 μmol/L) to the bath for 3 min.

To identify the tachykinin receptor subtypes, we tested several specific agonists of tachykinin receptors in the bath for 3 min: [Sar9] SP (100 nmol/L, agonist of NK1 receptor), NKA and (β-Ala8)-NKA (100 nmol/L, agonists of NK2 receptor), and NKB and Senktide (100 nmol/L, agonists of NK3 receptor). Furthermore, we assayed L-733060 (1 μmol/L), GR-94800 (100 nmol/L), and SB 218795 (1 μmol/L), antagonists of NK1, NK2, and NK3 receptors, respectively, on SP-invoked contractions. In addition, L-733060, GR-94800, and SB 218795 were assayed on [Sar9] SP-, (β-Ala8)-NKA-, and Senktide-invoked contractions, respectively, in longitudinal and circular muscle of small intestine. The antagonists or inhibitors used in this work were added to the bath 15 min before the respective agonist was added.

To examine neuronal transmission, the segments were incubated with tetrodotoxin (1 μmol/L) or hexamethonium (100 μmol/L) for 15 min before adding [Sar9] SP, (β-Ala8)-NKA, or Senktide.

To investigate cholinergic transmission, segments were incubated with atropine (1 μmol/L); the adrenergic transmission was assessed by incubating segments with atropine (1 μmol/L)+guanethidine (1 μmol/L); neuronal transmission was studied by adding atropine (1 μmol/L)+tetrodotoxin (1 μmol/L), and NO release was studied by incubating the samples with atropine (1 μmol/L)+*L*-NNA (100 μmol/L).

To study the effect of Ca^{2+} on the SP-invoked contractions of longitudinal and circular smooth muscle in the small intestine, segments were exposed to Ca^{2+}-free Krebs solutions containing 0.5 mmol/L EGTA; verapamil (100 nmol/L), a voltage-dependent Ca^{2+}-channel inhibitor; thapsigargin (100 nmol/L), an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases; or ryanodine (100 nmol/L), an inhibitor of Ca^{2+} release from the sarcoplasmic reticulum. Furthermore, effects of staurosporine (100 nmol/L), a protein kinase C (PKC) inhibitor, and U 73122 (100 nmol/L), a phospholipase C (PLC) inhibitor, on SP-invoked contractions were tested.

Data analysis

All intestinal segments included in the analyses showed spontaneous contractions. The tachykinin receptor agonists' motor responses (MR) were measured in terms of integrated mechanical activity (IMA) per second, expressed as mN/s, and normalized per square millimeter of cross-sectional area

(CSA), as we have previously described[22]. Results were expressed as a percentage of the control values of the various agonists (100%).

Median effective concentration (EC_{50}, the concentration of SP required to produce 50% of the effect) and 95% confidence limits were calculated using a linear least-squares regression.

Values are expressed as means±SEM. Comparisons between means were made using one-way analysis of variance (ANOVA), and *P*-values were verified using the Scheffé *F* test. Differences in *P*-values of <0.05 were considered statistically significant.

Results

Effects of tachykinin receptor agonists on spontaneous motility

Muscle of rabbit duodenum, jejunum and ileum exhibited cyclic, phasic and rhythmic spontaneous contractions *in vitro*[23]. To study the role of the tachykinin receptors in the spontaneous motility of rabbit small intestine, we tested specific agonists of these receptors. SP (1 nmol/L to 10 μmol/L), an NK1, NK2, and NK3 receptor agonist, induced tonic contractions in longitudinal and circular smooth muscle of rabbit duodenum, jejunum, and ileum. These SP-induced contractions were concentration-dependent (Table 1 and Figure 1). The EC_{50} calculated from the noncumulative concentration-response curves in longitudinal and circular smooth muscle, were 40 nmol/L and 160 nmol/L in the duodenum, 120 nmol/L and 200 nmol/L in the jejunum, and 80 nmol/L and 200 nmol/L in the ileum, respectively.

[Sar9] SP (100 nmol/L, NK1 receptor agonist), NKA and (β-Ala-8)-NKA (100 nmol/L, NK2 receptor agonists), and NKB and Senktide (100 nmol/L, NK3 receptor agonists) induced contractions in three segments of the longitudinal and circular muscle of the intestine (Figure 2). We compared the contractile responses of the different agonists with the response to SP (Table 2). [Sar9] SP-evoked contractions were similar to those evoked by SP in both types of smooth muscle of the three segments of small intestine. (β-Ala8)-NKA, NKB, and Senktide invoked weaker contractions than SP in both types of smooth muscle. The order of potency of agonists tested was [Sar9] SP>SP>NKA>NKB>(β-Ala8)-NKA=Senktide (Table 2).

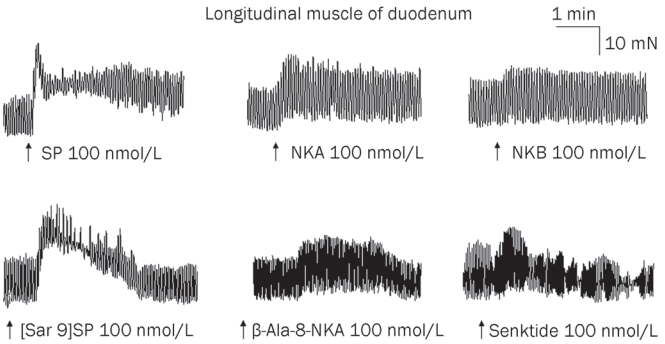

Figure 2. Effect of SP (100 nmol/L), NKA (100 nmol/L), NKB (100 nmol/L), [Sar9] SP (100 nmol/L), (β-Ala-8)-NKA (100 nmol/L), and Senktide (100 nmol/L) on spontaneous contractions in longitudinal smooth muscle of rabbit duodenum. Arrowheads indicate the addition of agents.

Effects of tachykinin receptor antagonists

We also tested the effect of specific antagonists of TK receptors

Table 1. Effects of different doses of substance P (SP). Average values of the motor response ($mN \cdot s^{-1} \cdot mm^{-2}$) to SP of the longitudinal and circular muscle of the duodenum, jejunum and ileum of rabbits. In brackets it expresses in number of segments.

	Duodenum		Jejunum		Ileum	
	L	C	L	C	L	C
SP 1 nmol/L	0.0±0.0 (8)	0.0±0.0 (13)	0.0±0.0 (10)	0.0±0.0 (11)	0.1±0.0 (9)	0.1±0.0 (11)
SP 10 nmol/L	0.0±0.0 (9)	0.0±0.0 (13)	0.0±0.0 (10)	0.0±0.0 (11)	0.0±0.0 (9)	0.0±0.0 (11)
SP 100 nmol/L	0.2±0.0 (9)	0.0±0.0 (13)	0.3±0.1 (10)	0.1±0.0 (11)	0.4±0.1 (9)	0.1±0.0 (11)
SP 1 μmol/L	0.1±0.0 (9)	0.0±0.0 (13)	0.2±0.1 (10)	0.1±0.0 (11)	0.4±0.1 (9)	0.1±0.0 (11)
SP 10 μmol/L	0.1±0.0 (8)	0.1±0.0 (12)	0.5±0.0 (8)	0.2±0.0 (10)	0.5±0.1 (8)	0.1±0.0 (10)

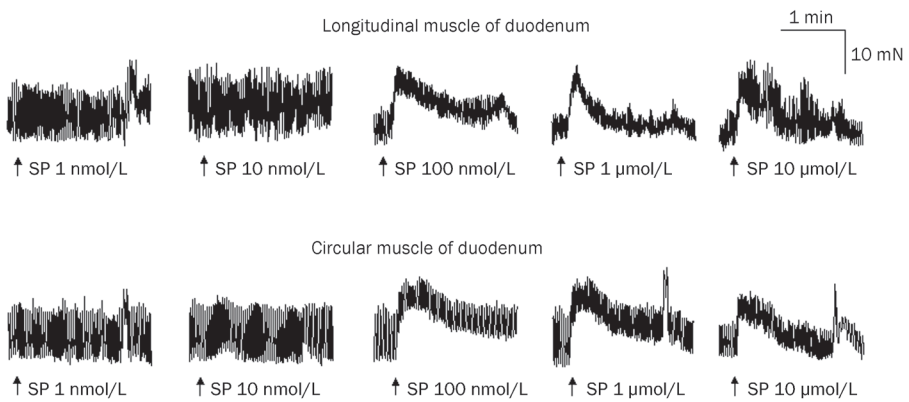

Figure 1. Concentration-dependent effects of SP (1 nmol/L–10 μmol/L) on spontaneous contractions in longitudinal and circular smooth muscle of rabbit duodenum. Arrowheads indicate the addition of agents.

Table 2. Comparison of the effects of [Sar9] SP (100 nmol/L), NKA (100 nmol/L), (β-Ala-8) NKA (100 nmol/L), NKB (100 nmol/L), and Senktide (100 nmol/L) with respect to SP (100 nmol/L, 100%), on contractions of longitudinal (L) and circular (C) smooth muscle of rabbit duodenum, jejunum, and ileum. Values of integrated mechanical activity (% of SP) are the mean±SEM. Numbers in brackets indicate number of segments. $^{c}P<0.01$

	Duodenum		Jejunum		Ileum	
	L	C	L	C	L	C
SP	100 (11)	100 (8)	100 (8)	100 (12)	100 (11)	100 (10)
[Sar9] SP	103.1±15.2 (8)	128.3±28.4 (8)	134.5±29.6 (8)	131.1±21.3(8)	116.2±19.2 (12)	144.1±20.7 (8)
NKA	87.9±4.1 (10)	89.5±8.5 (6)	90.9±2.1 (9)	96.0±2.3 (5)	98.2±5.0 (9)	97.2±3.9 (8)
(β-Ala-8)NKA	15.8±2.3 (8)^{c}	22.4±6.4 (8)^{c}	39.5±7.4 (8)^{c}	17.2±6.4 (8)^{c}	32.8±5.2 (10)^{c}	24.7±8.4 (9)^{c}
NKB	69.1±4.5 (10)^{c}	79.4±12.5 (6)	72.5±4.1 (9)^{c}	91.2±5.1 (5)	73.7±4.8 (14)^{c}	80.2±6.4 (5)
Senktide	26.1±3.4 (10)^{c}	36.3±17.8 (5)	8.9±2.2 (8)^{c}	8.3±2.6 (5)^{c}	6.5±1.0 (9)^{c}	6.9±0.4 (5)^{c}

on the SP-, [Sar9] SP-, (β-Ala8)-NKA-, and Senktide-induced contractions. L-733060 (1 μmol/L), GR-94800 (100 nmol/L), and SB 218795 (1 μmol/L), antagonists of NK1, NK2, and NK3, respectively, reduced contractions caused by SP (100 nmol/L) (Table 3). L-733060 (1 μmol/L) reduced contractions caused by [Sar9] SP (100 nmol/L) in all intestinal segments except for the longitudinal muscle of the ileum (Figure 3A, 3D). GR-94800 (100 nmol/L) reduced contractions caused by (β-Ala8)-NKA (100 nmol/L) in all intestinal segments except for the circular muscle of the duodenum (Figure 3B, 3E). SB 218795 (1 μmol/L) slightly reduced the Senktide-induced contractions (100 nmol/L), although this reduction was only statistically significant for the circular muscle of the ileum (Figure 3C, 3F).

Effects of tetrodotoxin (TTX) and hexamethonium on contractions induced by TK receptor agonists

We examined whether TK receptor agonists act directly on the muscle or indirectly at the nerve level with the use of TTX (1 μmol/L), a blocker of Na^{+} channels in neurons, and hexamethonium (100 μmol/L), a blocker of nicotinic receptors. TTX and hexamethonium reduced the contractions induced by [Sar9] SP (100 nmol/L) in all segments except for the longitudinal muscle of the jejunum (Figure 3A, 3D). TTX and hexamethonium decreased the contractions induced by (β-Ala8)-NKA (100 nmol/L) in the circular muscle of all three intestinal segments (Figure 3E). Hexamethonium, but not TTX, decreased these contractions in the longitudinal muscle (Figure 3B). TTX reduced the contractions induced by Senktide (100 nmol/L) in all intestinal segments except for the jejunum and the longitudinal muscle of the ileum (Figure 3C, 3F). Hexamethonium reduced these contractions in all intestinal segments except for the circular muscle of the jejunum (Figure 3C, 3F).

Effect of atropine, guanethidine, TTX, and *L*-NNA on the effects of SP

To examine the mechanism involved in SP responses, we investigated whether SP acted directly on the muscle or indirectly at the nerve level. Pretreatment of the intestinal segments for 15 min with atropine (1 μmol/L) decreased the SP-induced contractions in longitudinal and circular muscle of the small intestine (Figure 4A, 4B). No additive effects were observed with respect to contractions to SP reduced by atropine (1 μmol/L) when the three intestinal segments were incubated with atropine (1 μmol/L) plus guanethidine (1 μmol/L), atropine (1 μmol/L) plus TTX (1 μmol/L), or atropine (1 μmol/L) plus *L*-NNA (100 μmol/L) (Figures 4A, 4B).

Intracellular mechanisms for the action of SP

SP-induced contractions (100 nmol/L, 3 min) were reduced in Ca^{2+}-free Krebs solution containing 0.5 mmol/L EGTA or in the presence of verapamil (100 nmol/L, 15 min), a voltage-dependent Ca^{2+}-channel inhibitor, in longitudinal and circular muscle of the duodenum, jejunum, and ileum (Figures 5A, 5B). However, incubation of intestinal segments for 15 min

Table 3. Comparison of the effects of different tachykinin receptor antagonists with respect to Substance P. Effect of L-733060 (1 μmol/L), GR-94800 (100 nmol/L), and SB 218795 (1 μmol/L) on SP (100 nmol/L) contractions on longitudinal (L) and circular (C) smooth muscle of rabbit duodenum, jejunum, and ileum. Values of integrated mechanical activity (% of SP) are the mean±SEM. Numbers in brackets indicate number of segments. $^{b}P<0.05$; $^{c}P<0.01$.

	Duodenum		Jejunum		Ileum	
	L	C	L	C	L	C
SP	100 (11)	100 (8)	100 (8)	100 (12)	100 (11)	100 (10)
L-733060+SP	73.7±3.2 (9)^{c}	77.7±2.3 (10)^{c}	70.1±3.4 (8)^{c}	77.6±6.3 (9)^{c}	74.5±7.7 (7)^{c}	96.5±1.1 (7)
GR-94800+SP	74.6±5.5 (6)^{c}	75.9±5.2 (7)^{c}	92.5±3.6 (5)^{b}	86.7±6.2 (7)	85.2±1.4 (5)^{c}	70.9±6.3 (6)^{c}
SB 218795+SP	70.9±6.1 (8)^{c}	79.7±9.1 (8)^{c}	61.5±11.4 (9)^{c}	81.9±1.5 (9)^{c}	71.7±12.5 (8)^{b}	70.7±14.8 (8)

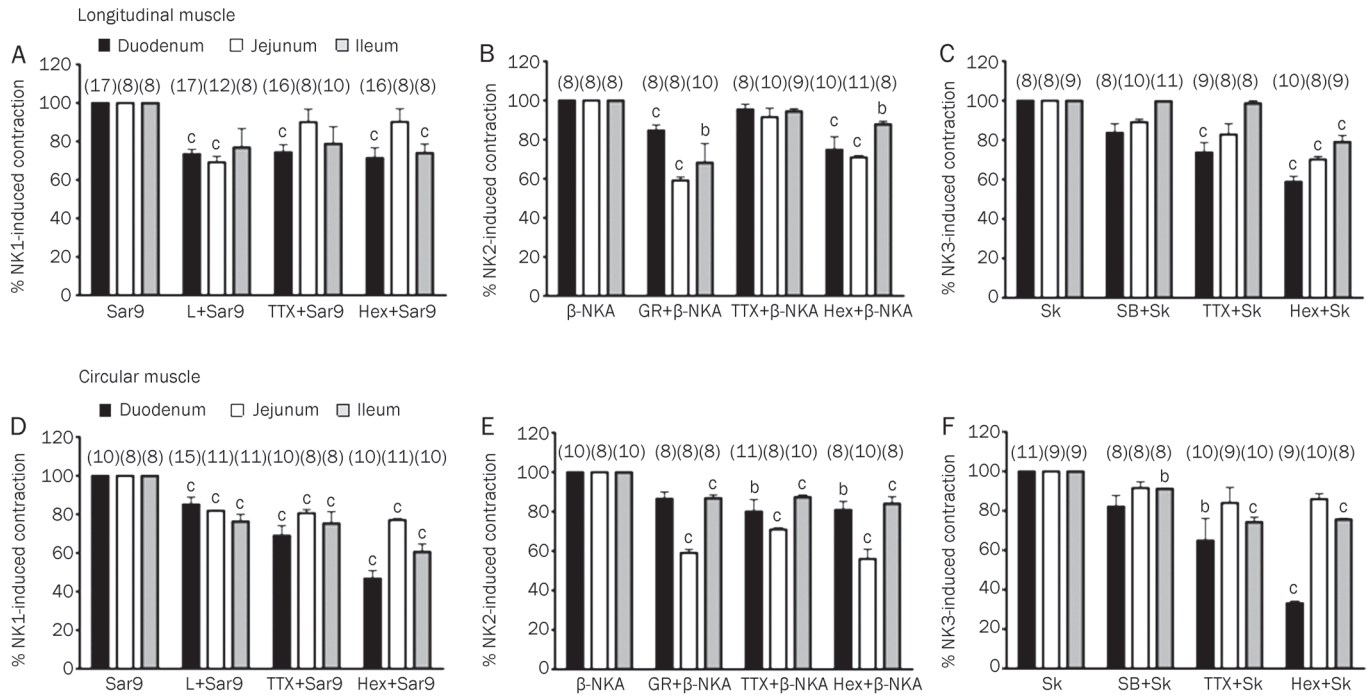

Figure 3. (A and D) Effects of L-733060 (L, 10^{-6} mol/L), tetrodotoxin (TTX, 10^{-6} mol/L), and hexamethonium (Hex, 10^{-4} mol/L) on contractions caused by the agonist of tachykinin NK1, [Sar9] SP (Sar9, 100 nmol/L), in longitudinal and circular smooth muscle from rabbit duodenum, jejunum, and ileum. (B and E) Effects of GR-94800 (GR, 100 nmol/L), TTX, and Hex on contractions caused by the agonist of tachykinin NK2, (β-Ala 8)-NKA (β-NKA, 100 nmol/L), in longitudinal and circular smooth muscle from rabbit duodenum, jejunum, and ileum. (C and F) Effects of SB 218795 (SB, 100 nmol/L), TTX, and Hex on contractions caused by the agonist of tachykinin NK3, Senktide (Sk, 100 nmol/L), in longitudinal and circular smooth muscle from rabbit duodenum, jejunum, and ileum. Columns indicate the mean values of integrated mechanical activity (% of TK agonist), and vertical bars indicate SEM. $^{b}P<0.05$, $^{c}P<0.01$. Numbers in brackets indicate the number of segments.

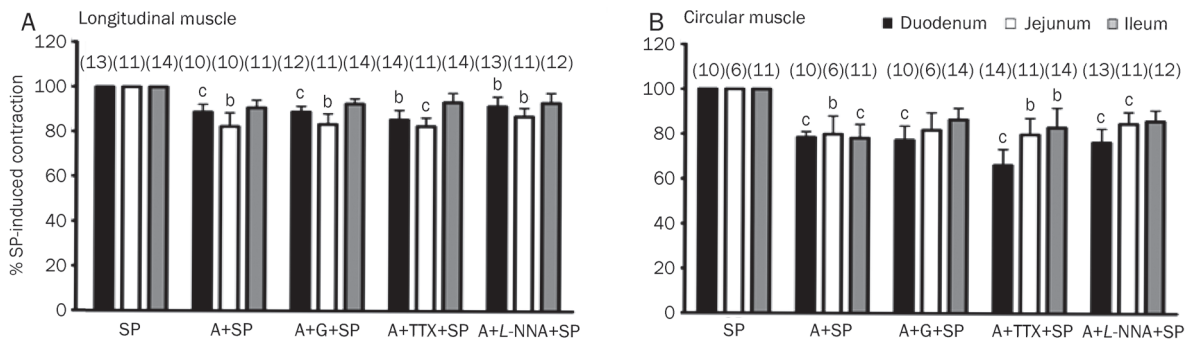

Figure 4. Effect of atropine (A, 1 μmol/L), atropine plus guanethidine (A+G, 1 μmol/L), atropine plus TTX (A+TTX, 1 μmol/L), and atropine (1 μmol/L) plus *L*-NNA (100 μmol/L) (A+*L*-NNA) on contractions caused by SP (100 nmol/L) in longitudinal (A) and circular (B) smooth muscle of rabbit duodenum, jejunum, and ileum. Columns indicate the mean values of integrated mechanical activity (% of SP), and vertical bars indicate SEM. $^{e}P<0.05$, $^{c}P<0.01$. Numbers in brackets indicate the number of segments.

with thapsigargin (100 nmol/L), an inhibitor of sarco-endoplasmic reticulum Ca^{2+}-ATPases, or ryanodine (100 nmol/L), an inhibitor of Ca^{2+} release from sarcoplasmic reticulum, did not change the contractile response to SP (Figures 5A, 5B); however, SP-induced contractions were reduced in the presence of staurosporine (1 μmol/L, 15 min), a PKC inhibitor, and U 73122 (100 nmol/L, 15 min), a PLC inhibitor, in longitudinal and circular muscle of the small intestine (Figures 5A, 5B).

Discussion

In the present study, SP and [Sar9] SP, agonists of the NK1 receptor, invoked contractions in the longitudinal and circular smooth muscle of rabbit small intestine; the contractions were higher in the presence of [Sar9] SP. L-733060, a potent NK1 antagonist, significantly reduced the SP- and [Sar9] SP-induced contractions. This suggests the existence of NK1 receptors that modulate these contractions in the rabbit small

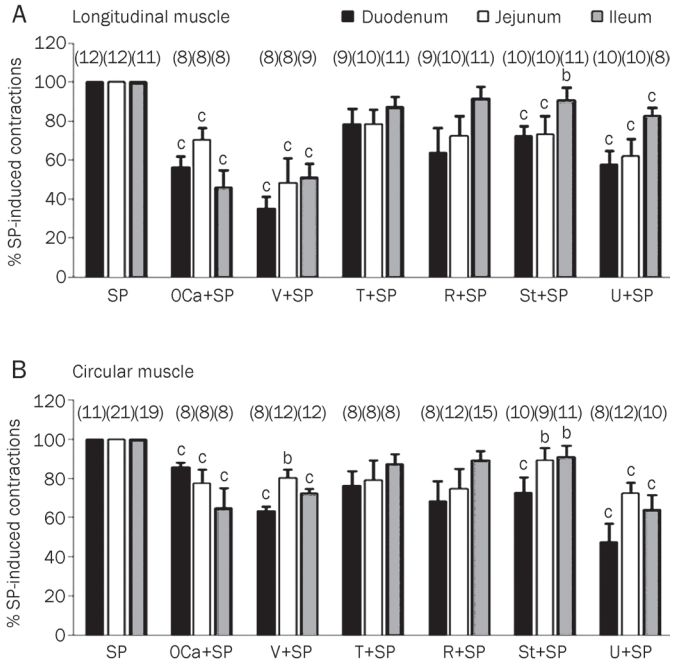

Figure 5. Effect of Ca^{2+}-free solutions containing 0.5 mmol/L EGTA (0Ca), verapamil (V, 100 nmol/L), thapsigargin (T, 100 nmol/L), ryanodine (R, 100 nmol/L), staurosporine (St, 100 nmol/L), and U 73122 (U, 100 nmol/L) on contractions caused by SP (100 nmol/L) in longitudinal (A) and circular (B) smooth muscle of rabbit duodenum, jejunum, and ileum. Columns indicate the mean values of integrated mechanical activity (% of SP), and vertical bars indicate SEM. $^{b}P<0.05$, $^{c}P<0.01$. Numbers in brackets indicate the number of segments.

intestine. Our results agree with other authors' findings that tachykinin NK1 receptors are implicated in intestinal peristalsis[24] and in excitatory nonadrenergic and noncholinergic (NANC) transmission in the mouse ileum[25].

As demonstrated in our experimental model, NKA and (β-Ala8)-NKA, as well as NKB and Senktide, agonists of tachykinin NK2 and NK3 receptors, respectively, invoked contractions in the longitudinal and circular smooth muscle of rabbit small intestine, but these contractions were weaker than those that were SP-invoked. At the same concentrations, NKA and NKB caused stronger contractions than (β-Ala8)-NKA and Senktide. Moreover, GR-94800 and SB 218795, potent and selective NK2 and NK3 antagonists, respectively, diminished SP-invoked contractions, and GR-94800 reduced (β-Ala8)-NKA-invoked contractions. SB 218795 partly reduced contractions caused by Senktide in all of the intestinal segments, although not significantly. In rabbit, peristalsis regulation in the isolated distal colon is most likely mediated by the activation of postjunctional excitatory tachykinin NK1 receptors[24]. NK1 receptors are also implicated in the descending relaxant reflex responses and in ascending contraction[26]. NK1 and NK2 receptors are activated in the contractile responses induced by SP and NKA in canine ileum circular muscle[27] and mediate nonadrenergic, noncholinergic excitatory neurotransmission in hamster ileum[28]. In muscle cells of rat intestine, the coexistence of NK1, NK2, and NK3 tachykinin receptors has been described[29].

In our study, the potency of the agonists tested was ranked as follows: [Sar9] SP>SP>NKA>NKB>(β-Ala8)-NKA=Senktide. This finding is in accordance with other studies for the three subtypes of TK receptors in which the rank order of potency for NK1 receptors was SP=hHK-1≥NKA>NKB, while it is NKA>NKB>SP>hHK-1 (Human hemokinin 1) for the NK2 receptor and NKB>NKA>hHK-1 (Human hemokinin 1)>SP for the NK3 receptor[5, 30–32].

We examined whether the TK receptor agonists act directly on the muscle or indirectly at the nerve level using TTX, a blocker of Na^+ channels in neurons, and hexamethonium, a blocker of nicotinic receptors. TTX and hexamethonium reduced the contractions induced by [Sar9] SP, (β-Ala8)-NKA, and Senktide in longitudinal and circular smooth muscle, suggesting that preganglionar neural pathways are involved. However, the fact that only a small part of the TK agonist response was blocked by TTX or hexamethonium suggests that the main contractility response is due to TK receptors located on smooth muscle cells. Indeed, TTX and hexamethonium do not alter the contractions caused by various TK receptor agonists in the Suncus murinus ileum[6, 33]. Tachykinin NK1, NK2, and/or their receptors have been reported to be expressed by neurons, interstitial cells of Cajal, intestinal muscle, epithelium, vasculature, and the immune system in a cell-specific, region-specific and species-specific manner[1, 3, 18, 19]. In contrast, the tachykinin NK3 receptor is primarily expressed in the central nervous system and has been detected only in certain peripheral tissues, such as the human and rat uterus, the rat mesenteric vein, and certain enteric neurons from the gut of various species[3, 20].

In this study, we investigated the mechanism of action of SP on smooth muscle in rabbit small intestine. SP induced concentration-dependent contractions in longitudinal and circular smooth muscle of the duodenum, jejunum, and ileum. The EC_{50}s in circular muscle were slightly higher than those in longitudinal muscle of the small intestine. These results are in accordance with the contractions caused by SP described in isotonic recordings in longitudinal muscle of rabbit ileum[34].

Our results showed that the contractions induced by SP were reduced in Ca^{2+}-free solutions and in the presence of verapamil, whereas they were not modified in the presence of thapsigargin or ryanodine. These results show that extracellular Ca^{2+} is more important in SP-induced contractions than intracellular Ca^{2+} and that extracellular Ca^{2+} enters the cell through voltage-dependent Ca^{2+} channels. Staurosporine, a PKC inhibitor, and U 73122, a PLC inhibitor, diminished SP-invoked contractions in small intestine longitudinal and circular muscle, suggesting a role for these intracellular messengers. Verapamil reduces the effect of SP on rabbit ileum[34]. Ca^{2+} antagonists such as verapamil, nifedipine, and diltiazem diminish spontaneous activity in sheep duodenum[35]. In murine colonic myocites, SP at low concentrations hyperpolarizes the muscle cells and, at higher concentrations, increases basal cytoplasmic Ca^{2+} concentration by increasing Ca^{2+} influx

through L-type Ca^{2+} channels. Furthermore, nifedipine and GF 109203, a PKC inhibitor, blocked SP-induced effects[36]. NK1 antagonists competitively inhibit the activation of phospholipase C by [Pro9] SP in cultured cortical astrocytes[37]. In previous studies, the amplitude of spontaneous contractions of intestine was diminished by Ca^{2+}-free solutions, verapamil and nifedipine and was increased by thapsigargin and cyclopiazonic acid; however, extracellular and intracellular Ca^{2+} mediate ACh- and KCl-induced contractions[23], and K^+ channels mediate spontaneous contractions in rabbit intestine[38].

In this study, atropine (1 μmol/L) decreased SP-induced contractions in longitudinal and circular muscle of the duodenum, jejunum, and ileum, whereas atropine plus guanethidine, atropine plus TTX (1 μmol/L), or atropine plus *L*-NNA did not invoke additional effects when compared with atropine alone. These results suggest that in SP-invoked contractions, a cholinergic neural pathway is involved through the activation of muscarinic receptors. In contrast, our results do not favor a role of adrenergic and nitrergic pathways because guanethidine and *L*-NNA do not alter SP responses; however, atropine (0.35 μmol/L) or TTX (0.31 μmol/L) added to the bath 2 min before the addition of SP has no impact on SP-induced effects in rabbit ileum[34]. This author tested the short-time effects of atropine on the amplitude of contractions at a lower concentration. Atropine (1 μmol/L) inhibits the velocity of propulsion of rabbit colon, which is mediated by NK2 and reduces the effect of TK receptor agonists in Sancus murinus ileum[33, 39]. Our results are partially consistent with those of other reports where the contractions induced by NK1 agonists were reduced by atropine and augmented by *L*-NNA[25, 40]. These authors propose that excitatory nonadrenergic and non-cholinergic transmission in the circular muscle layer is mediated by tachykinins that principally act on NK1 receptors on cholinergic nerves and smooth muscle cells.

In conclusion, our study demonstrates that tachykinin NK1, NK2, and NK3 receptors invoke contractions in the smooth muscle of rabbit intestine. Furthermore, extracellular Ca^{2+}, PKC, phospholipase C, and cholinergic neurons mediate the contractions caused by SP. We suggest that the SP acts directly on smooth muscle cells through the tachykinin NK1 receptor.

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Author contribution

María Divina MURILLO designed the study and wrote the paper. Marta Sofía VALERO and Diego Santos FAGUNDES performed research and analyzed data. Laura GRASA wrote the paper. Miguel Angel PLAZA and María Pilar ARRUEBO contributed to the Discussion section of the paper.

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