Tachykinin Receptor Expression and Function in Human Esophageal Smooth Muscle

Jason R. Kovac, Tom Chrones, Harold G. Preiksaitis, and Stephen M. Sims

Departments of Physiology and Pharmacology (J.R.K., T.C., H.G.P., S.M.S.) and Medicine (H.G.P.), Schulich School of Medicine & Dentistry, University of Western Ontario, London, Ontario, Canada

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ABSTRACT

Tachykinins are present in enteric nerves of the gastrointestinal tract and cause contraction of esophageal smooth muscle; however, the mechanisms involved are not understood. Our aim was to characterize tachykinin signaling in human esophageal smooth muscle. We investigated functional effects of tachykinins on human esophageal smooth muscle using tension recordings and isolated cells, receptor expression with reverse transcription (RT)-polymerase chain reaction (PCR) and immunoblotting, intracellular Ca²⁺ responses using fluorescent indicator dyes, and membrane currents with patch-clamp electrophysiology. The mammalian tachykinins [substance P and neurokinin (NK) A and NKB] elicited concentration-dependent contractions of human esophageal smooth muscle. These responses were not affected by muscarinic receptor or neuronal blockade indicating a direct effect on smooth muscle cells (SMCs). Immunofluorescence and RT-PCR identified tachykinin receptors (NK1, NK2, and NK3) on SMCs. Contraction was mediated through a combination of Ca²⁺ release from intracellular stores and influx through L-type Ca²⁺ channels. NK2 receptor blockade inhibited the largest proportion of tachykinin-evoked responses. NKA evoked a nonselective cation current (IＮＳＣ) with properties similar to that elicited by muscarinic stimulation. The following paradigm is suggested: tachykinin receptor binding to SMCs releases Ca²⁺ from stores along with activation of IＮＳＣ, which in turn results in membrane depolarization, L-type Ca²⁺ channel opening, rise of Ca²⁺ concentration, and contraction. These studies reveal new aspects of tachykinin signaling in human esophageal SMCs. Excitatory tachykinin pathways may represent targets for pharmacological intervention in disorders of esophageal dysmotility.

Tachykinins, present in the neurons of myenteric and submucosal plexi, affect both circular and longitudinal esophageal muscles (Holzer and Holzer-Petsche, 1997a; Furness et al., 2004). The mammalian tachykinin family includes substance P (SP), neurokinin (NK) A, and NKB (Holzer and Holzer-Petsche, 2001). All three peptides share a common carboxyl-terminal motif (Phe-X-Gly-Leu-Met-NH₂) and are derived from precursor preprotachykinins via proteolytic processing. Tachykinins interact with three receptor subtypes (NK1, NK2, and NK3) that exhibit preferential affinities for SP, NKA, and NKB, respectively (Holzer and Holzer-Petsche, 1997a).

NKA is usually colocalized in enteric neurons with SP and acetylcholine (ACh) (Holzer and Holzer-Petsche, 1997a; Furness et al., 2004). In the smooth muscle portion of the human esophageal body (EB), tachykinin-positive neurons are abundant (Wattchow et al., 1987; Singaram et al., 1991). Indeed, a substantial portion of the nerve-mediated EB contraction is not blocked by the muscarinic receptor antagonist atropine (Krysiak and Preiksaitis, 2001). This component is largely mediated by tachykinins, with blockade by NK2 receptor antagonists (Krysiak and Preiksaitis, 2001). These findings are consistent with a role for tachykinins in general, and NKA in particular, in the regulation of esophageal motility. Both cholinergic and nonadrenergic, noncholinergic neuronal pathways contribute to gastrointestinal peristalsis (Furness et al., 2004). Nerves in the gastrointestinal tract are closely apposed to interstitial cells of Cajal (ICC), which are...
now recognized to play a role in the excitation of smooth muscles (Daniel, 2001; Ward and Sanders, 2001). Although ICCs of the murine small intestine express NK1 receptors (Iino et al., 2004; Jun et al., 2004), and NK1 receptor deficient mice exhibit altered nerve-mediated ileal motility, prominent NK2-mediated components remain (Saban et al., 1999). In situ studies using pharmacologic manipulation also support key roles for NK2 receptors in intestinal peristalsis (Tomini et al., 2001).

Muscle contraction studies of human EB support a role for tachykinins in regulating nonadrenergic, noncholinergic-mediated excitation (Huber et al., 1993a; Krysiak and Preiksaitis, 2001); however, little is known about the effects of tachykinins on individual esophageal smooth muscle cells (SMCs). The presence of NK1 (Portbury et al., 1996) and NK3 (Holzer and Holzer-Petsche, 1997b) receptors on enteric neurons in tissue strips, the ability of SP-positive neurons to corelease tachykinins and ACh, and the capability of tachykinins to bind multiple receptor subtypes (Domoto et al., 1993; Furness et al., 2004) all indicate the need for further examination of tachykinin signaling in esophageal muscle.

Tachykinin signaling differs among smooth muscles. In guinea pig ileum as well as rabbit and human colon, excitation involves influx of extracellular Ca\(^{2+}\) through nifedipine-sensitive, L-type Ca\(^{2+}\) channels (Maggi et al., 1997; O’Riordan et al., 2001). In contrast, contractions of human sigmoid colon are less dependent on Ca\(^{2+}\) influx but are abolished by blockade of the sarcoplasmic reticulum Ca\(^{2+}\) ATPase by thapsigargin (Cao et al., 2000). In canine colonic SMCs, tachykinins activate a nonselective cation current (I\(_{\text{NSC}}\)) (Lee et al., 1995). Given the importance of esophageal peristalsis in health and disease, it is important to understand the signaling mechanisms involved in tachykinin-induced contraction.

In the present study, our aim was to characterize tachykinin signaling in human esophageal smooth muscle. We identified three tachykinin receptors (NK1, NK2, and NK3) and found that SP, NKA, and NKB predominantly acted through NK2 receptors located on SMCs. The mechanism of excitation involved a combination of release of Ca\(^{2+}\) from intracellular stores, influx through L-type Ca\(^{2+}\) channels, and activation of nonselective cation current, aspects of tachykinin signaling not previously reported for esophageal muscle.

**Materials and Methods**

**Tissue Retrieval, Isolation of Cells, and SMC Culture.** Tissue collection was carried out in accordance with the guidelines of the University of Western Ontario Research Ethics Board for the Review of Health Sciences Research Involving Human Subjects. Tissues were obtained from patients undergoing esophageal resection as described previously (Preiksaitis and Diamant, 1997; Sims et al., 1997; Kovac et al., 2005). Samples were removed from disease-free regions of the distal third, placed in ice-cold, oxygenated Krebs bicarbonate solution (see below), and transported to the laboratory. The circular muscle (CM) or longitudinal muscle (LM) layers were carefully dissected based on their morphology and orientation, cleaned of nerves, blood vessels, fat, and fascia. Portions of muscle were frozen on dry ice (-70°C) for RNA extraction or further dissected for acute studies. Freshly dispersed SMCs were studied within 6 h or maintained in primary culture as described previously (Sims et al., 1997; Wang et al., 2000; Kovac et al., 2005). In total, muscle was studied from 55 specimens.

**Tissue Bath Studies.** Muscle strips were mounted in tissue baths containing 10 ml of Krebs bicarbonate solution continuously bubbled with 5% CO\(_2\)-95% O\(_2\) at 37°C as described previously (Sims et al., 1997; Wade et al., 1999; Kovac et al., 2005).

**Reverse Transcription-Polymerase Chain Reaction.** Total RNA was extracted from EB smooth muscle by phenol-chloroform extraction using frozen samples or cultured cells grown to near confluence (10–15 days). Using a first strand cDNA synthesis kit (Invitrogen, Burlington, ON, Canada), 2 \(\mu\)g of total RNA was reverse-transcribed for 60 min at 42°C with Oligo(dT)\(_{12-18}\). Polymerase chain reaction (PCR) was performed in 50 \(\mu\)l of PCR buffer containing 3 mM MgCl\(_2\), 200 \(\mu\)M dNTPs, 0.1 nM primer, 0.3 \(\mu\)M of TaqDNA polymerase (Fermentas Life Sciences, Burlington, ON, Canada), and 5–7 \(\mu\)l of cDNA reaction mixture. PCR was carried out in an Eppendorf Mastercycler Gradient (Eppendorf, Westbury, NY) for 35 cycles with cycling parameters of 0.5 min at 94°C, 1 min at 58°C, 1.5 min at 72°C, and a final 10-min extension at 72°C. The PCR primers used to amplify cDNA are listed in Table 1. Primers for \(\beta\)-actin were used to confirm fidelity of the PCR reaction and to detect genomic DNA contamination.

**Immunofluorescence.** Cells were fixed in ice-cold 95% ethanol (15–30 min), blocked with 1% goat serum in PBS (60 min), and incubated with \(\beta\)-antibody (from Dr. Krause, Neurogen Inc., Branford, CT) at 4°C overnight. 1° antibodies were diluted in PBS, pH 7.4, containing 1% goat serum. The antibodies were used at the following dilutions: NK1 (1:500), NK2 (1:500), and NK3 (1:250). After washing with PBS (2 × 10 min), cells were incubated (60 min at 22°C) with fluorescein isothiocyanate-linked goat, anti-rabbit secondary \(\beta\)-antibody (1:50; Jackson Laboratories, West Grove, PA) to detect NK1 receptors, and with Cy-3-linked donkey, anti-mouse \(\beta\)-antibody (1: 200; Jackson Labs) to detect NK2 and NK3 receptors. Following washes with PBS (2 × 10 min), TO-PRO-I or TO-PRO-3 dimeric cyanine dye (5 \(\mu\)M, 60 min at 22°C; Molecular Probes, Eugene, OR) was used to stain nuclei. Samples were again washed with PBS (2 × 10 min), and coverslips were mounted on slides with FluoroGuard Antifade (Bio-Rad, Hercules, CA). Western blot analysis of cell homogenates confirmed the specificity of the antibodies. Single bands were apparent at predicted molecular weight values, and absent when primary antibodies were preabsorbed with the peptide immuno-
nogens (data not shown). For immunofluorescence controls, parallel preparations were processed without primary antibody.

**Measurement of Ca**²⁺ **Concentration.** Cells were loaded by incubation with fura-2-acetoxymethyl ester (0.2 μM) or fluo-4-acetoxyethyl ester (5 μM) at room temperature (21–24°C) for 40 min, as described previously (Kovac et al., 2005). Cells loaded with fluo-4 were illuminated with 488 nm of light from an argon ion laser and emissions detected at 510 to 560 nm using a Photometrics Cascade camera (Photometrics, Tucson, AZ) controlled by PTI ImageMaster software (Photon Technology Int. Inc., Birmingham, NJ).

**Electrophysiological Recordings.** Dispersed cells were allowed to settle and adhere to the bottom of a perfusion chamber mounted on the stage of a Nikon inverted microscope (Nikon, Tokyo, Japan) and perfused with Na⁺-HEPES bathing solution, as described previously (Kovac et al., 2005). Dispersed cells were studied at room temperature (21–24°C).

**Solutions.** The Krebs’ solution consisted of 116 mM NaCl, 5 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 2.2 mM NaH₂PO₄, 25 mM NaHCO₃, and 10 mM d-glucose, equilibrated with 5% CO₂-95% O₂, pH 7.4. The bathing solution for fluorescence studies and electrophysiological recordings contained 130 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 20 mM HEPES, and 10 mM d-glucose (adjusted to pH 7.4 with NaOH). Ca²⁺-free solutions contained 0.5 mM EGTA and omitted CaCl₂. In patch recordings, KCl electrode solution contained 140 mM KCl, 20 mM HEPES, 1 mM MgCl₂, and 0.1 mM EGTA (adjusted to pH 7.2 with KOH). CsCl electrode solution contained 130 mM CsCl, 20 mM HEPES, 1 mM MgCl₂, 10 mM TEACl, 0.4 mM CaCl₂, and 1 mM EGTA (adjusted to pH 7.2 with CsOH). Cs-glutamate electrode solution contained 40 mM CaCl₂, 100 mM glutamate, 20 mM HEPES, 1 mM MgCl₂, 0.4 mM CaCl₂, and 0.01 mM EGTA (adjusted to pH 7.2 with CsOH).

**Chemicals.** Chemicals were from Sigma (St. Louis, MO), BDH Inc. (Toronto, ON, Canada), or Calbiochem (San Diego, CA) unless otherwise stated. SR48968, SR140333, and SR142801 were from Tocris Bioscience (Bristol, UK) and prepared as described previously (Holzer et al., 1997a). Carbachol (CCh, 1 μM) and the muscarinic receptor agonist atropine (1 μM) were from Tocris Bioscience.

**Statistics.** Values are the means ± S.E.M. with sample sizes (n) indicating the number of cells or muscle strips studied. All traces are representative of at least three experiments on muscle or cells from two or more esophageal specimens. For Ca²⁺ fluorescence and patch-clamp experiments, only one recording was obtained per cell. Comparisons were made using the Student’s paired t test, analysis of variance, and Tukey’s post test as indicated. p < 0.05 was considered significant.

**Results**

**Tachykinins Contract Human EB Smooth Muscle Strips.** We first established the functional relevance of tachykinins by monitoring contraction of smooth muscle strips to exogenously applied tachykinins. NKA, NKB, and SP caused release of ACh from enteric neurons via NK3 and NK1 receptors (Portbury et al., 1996; Holzer and Holzer-Petsche, 1997b). To investigate whether tachykinins had a direct action on SMCs, we blocked nerve transmission with tetrodotoxin (TTX, 1 μM) and muscarinic receptors with atropine (1 μM). Carbachol-evoked contractions were largely unaffected by TTX, whereas atropine abolished the response (Fig. 2A). NKA-evoked contractions were also insensitive to TTX, but atropine caused a small but significant decrease in the NKA-evoked contraction (Fig. 2B), with summary of the results and statistical analysis in Fig. 2C. Traces and mean values in Fig. 2 illustrate responses of circular muscle, and similar results were observed in independent experiments on longitudinal muscle. These findings are consistent with tachykinins acting directly on SMC receptors, prompting us to further investigate the presence of tachykinin receptors on these cells.

**Human EB SMCs Express Tachykinin Receptors.** We examined tachykinin receptor expression using reverse transcription (RT)-PCR. Primers were designed to amplify sequences for the tachykinin receptors based on human sequences (Table 1). Transcripts were identified in both CM and LM of fresh EB (Fig. 3A, n = 10). The PCR products were of the expected sizes (see Table 1; Fig. 3), and the identity of the PCR product was confirmed by sequencing. The amplified β-actin sequence was selected to span a 206-bp intron so the finding of a single band at 314 bp verified the absence of genomic DNA (Table 1; Fig. 3). Control experiments were also performed both with reverse transcriptase omitted (control lanes, Fig. 3) and water only (data not shown). To confirm that the positive responses were due to smooth muscle, we carried out further experiments using mRNA isolated from EB SMC maintained in culture, previously established to be pure SMCs (Wang et al., 2000). A similar pattern of
tachykinin receptor mRNA expression was confirmed in these cells (Fig. 3B, n = 6).

Using immunofluorescence, we next examined the expression of tachykinin receptors in EB SMCs (n = 6 for each NK1, NK2, and NK3). Positive immunostaining of the tachykinin receptors was seen in all cells (Fig. 4). Immunostaining was diffuse throughout the cells, rather than localized or restricted to the plasma membrane. No immunolabeling was observed when 1st antibodies were omitted, and cells were incubated with 2nd antibody only (Fig. 4). Similar findings were obtained for SMCs isolated from longitudinal and circular muscle layers.

**Direct Effect of NKA on Human Esophageal SMCs.** With confirmation of tachykinin receptors on individual esophageal SMCs, we proceeded to study the effects of tachykinins on freshly isolated and cultured human EB SMCs. Freshly isolated cells ranged in length from 70 to 200 μm and appeared spindle shaped with a phase bright periphery. In response to tachykinin stimulation, SMCs rapidly and reversibly contacted to ~60% of resting state. Subsequent ACh stimulation evoked similar reversible contractions (Fig. 5A). Maximal shortening occurred in all cases within 20 s of agonist application.

We next sought to establish the signaling mechanisms underlying tachykinin-evoked contractions. Ca^{2+}-sensitive dyes were used to determine whether changes of intracellular free Ca^{2+} concentration ([Ca^{2+}]_i) occurred in response to tachykinins. Focal application of NKA onto freshly isolated SMCs resulted in contraction accompanied by an increase of [Ca^{2+}]_i (Fig. 5B, left). ACh resulted in similar responses (Fig. 5B, right), consistent with previous studies (Sims et al., 1997). As such, we went on to study the mechanisms of these responses.

**NKA-Evoked Contractions Involve Release of Ca^{2+} from Stores and Influx.** It has been previously shown that cholinergic excitation induces both Ca^{2+} influx and release from stores (Sims et al., 1997); however, the Ca^{2+} sources involved in tachykinin-evoked contractions are unknown. NKA caused a rapid, transient rise of [Ca^{2+}]_i in cells from primary culture (Fig. 6). When Ca^{2+} was eliminated from the bathing solution (with addition of 0.5 mM EGTA), repeated applications of NKA resulted in diminishing responses (Fig. 6A). Recovery occurred following readdition of extracellular...
confirming that the decline in the amplitude of the responses was not due to cellular run-down or desensitization (Fig. 6A). The timing of the response varied from cell to cell, so representative traces are presented. Similar responses were recorded in four additional cells from multiple esophageal specimens.

To evaluate directly the contribution of intracellular stores, we blocked the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase with cyclopiazonic acid (CPA; 10 \(\mu\)M). CPA induced a slow, gradual rise in \([Ca^{2+}]_i\), and an initial application of NKA elicited a rapid and slightly prolonged \([Ca^{2+}]_i\), increase. However, subsequent application of NKA had no effect, indicating depletion of stores. This reduction was reversible upon washout of CPA and reinduction of extracellular \(Ca^{2+}\), indicating intact receptor signaling (Fig. 6B, \(n = 5\)).

To examine the contribution of \(Ca^{2+}\) entry to the observed transients, L-type \(Ca^{2+}\) channels were blocked with nifedipine (10 \(\mu\)M, Fig. 6C), a dose shown previously to be effective in abolishing L-type \(Ca^{2+}\) current in EB SMCs (Kovac et al., 2005). A significant decrease in NKA-evoked rise of \([Ca^{2+}]_i\), was observed (Fig. 6D; control rise of 248 \(\pm\) 66 \(n\)M versus rise in the presence of nifedipine of 156 \(\pm\) 26 \(n\)M, \(p < 0.01\), \(n = 8\)). These data support a role for both \(Ca^{2+}\) entry through L-type \(Ca^{2+}\) channels and \(Ca^{2+}\) release from intracellular stores in tachykinin signaling.

**Tachykinin Responses Are Mediated by the NK2 Receptor.** We next examined which receptor subtype(s) contributed to signaling in esophageal muscle. Tachykinin receptor subtypes NK1, NK2, and NK3 are activated preferentially by SP, NKA, and NKB, respectively (Holzer and Holzer-Petsche, 1997a). Given that NKA evoked the largest EB muscle strip contractions (Fig. 1A), we asked which receptors might mediate the actions of NKA. Although a 3-min perfusion of the NK1 antagonist SR140333 (2 \(nM\), \(n = 7\); Fig. 7A) and the NK3 antagonist SR142801 (2 \(nM\), \(n = 7\); Fig. 7B) caused only slight inhibition of NKA-evoked \([Ca^{2+}]_i\) transients, the NK2 antagonist SR48968 (2 \(nM\), \(n = 7\); Fig. 7C) effectively abolished NKA responses. Recovery was apparent following a 10-min washout. This concentration of tachykinin antagonists is effective at blocking contractions of guinea pig esophageal muscularis mucosa and ileum, rat duodenum, and rabbit colonic muscle (Croci et al., 1995; Kerr et al., 2000; Onori et al., 2000). Thus, our data are consistent with the literature and the selective action of tachykinin antagonists at this concentration. We next pretreated cells with the NK2 blocker SR48968 and found that both NKB (1 \(\mu\)M, \(n = 10\), Supplemental Fig. 1A)- and SP (1 \(\mu\)M, \(n = 6\), Supplemental Fig. 1B)-evoked \([Ca^{2+}]_i\), transients were significantly inhibited.

We verified the results obtained above in cultured cells using freshly dissociated EB SMCs. Removal of extracellular \(Ca^{2+}\) (\(n = 5\), L-type \(Ca^{2+}\) channel blocker with nifedipine (\(n = 11\)), as well as NK1 (\(n = 10\)), NK2 (\(n = 10\)) and NK3 (\(n = 8\)) receptor blockade experiments all yielded results that were essentially the same as those obtained in cultured cells (Supplemental Fig. 2). The observation that NK1 and NK3 antagonists reduced responses to NKA could reflect promiscuity in receptor blockade because we saw no differences in expression of NK1 or NK3 in cultured cells versus freshly isolated cells using PCR and immunolocalization. As a con-
control, tachykinin antagonists had no effect on carbachol-evoked transients. Therefore, in cases where the recovery time following washout of tachykinin receptor blockers in fresh cells was prolonged, we applied ACh to confirm cellular viability (Supplemental Fig. 2C).

**Agonists Activate Nonselective Cation Current.** The ability of nifedipine to inhibit [Ca$^{2+}$]$_i$ elevations led us to hypothesize that tachykinins could elicit excitatory inward, depolarizing currents. When esophageal cells were held under voltage clamp at −60 mV, NKA induced a transient inward current (Fig. 8A). Responses were reproducible and recovered following washout in both fresh and cultured cells. Recordings of whole-cell currents using voltage ramp commands (from −100 to 50 mV) were used to evaluate the voltage dependence and reversal potential of the NKA-evoked current (Fig. 8A). With cesium (Cs) in the recording electrode solution to block outward K$^+$ currents (Wade et al., 1999), the evoked current was linear and reversed direction at −5 ± 2 mV (n = 4, Fig. 8A). Since this reversal potential was close to the equilibrium potential for Cl$^-$, NKA may have activated either a nonselective INSC and/or Cl$^-$ current. When Cl$^-$ was replaced with glutamate (shifting the Cl$^-$ equilibrium potential from 0 to −30 mV), the substitution had no effect on the reversal potential (−6 ± 4 mV, n = 4), indicating negligible contribution of anions.

ACh activated a similar inward current (Fig. 8B) that was linear and reversed at 0 ± 3 mV with a CsCl electrode solution (Fig. 8C; n = 16), at −1 ± 4 mV with a KCl electrode solution (n = 7), and at −2 ± 2 mV with a Cs-glutamate electrode solution (n = 10). These findings are consistent with the activation of an INSC in response to tachykinins and muscarinic agonist.

**Discussion**

The mechanisms of tachykinin signaling in human esophageal excitation were examined using a combination of intact muscle strips to evaluate contraction, RT-PCR and immunofluorescence to study receptor expression, as well as isolated SMCs to evaluate Ca$^{2+}$ responses and to characterize ionic currents. We show the existence of multiple tachykinin receptors and demonstrate, for the first time, that tachykinins cause excitation through a combination of mechanisms: Ca$^{2+}$ influx, Ca$^{2+}$ release from stores, and activation of INSC.

Tachykinin-containing enteric nerves innervate large portions of the gastrointestinal tract and, with projections to both longitudinal and circular muscle layers, are thought to play important roles in regulating peristalsis (Holzer and Holzer-Petsche, 1997a). Tachykinin-containing neurons have been described in human esophagus (Wattchow et al., 1987; Singaram et al., 1991), where they have been suggested to play a role in the physiological regulation of esophageal func-

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**Fig. 7.** NK2 receptor-mediated rise of [Ca$^{2+}$]$_i$ in cultured human EB SMCs. Subtype-selective tachykinin receptor antagonists were applied for 3 min before NKA. A and B, NK1 antagonist SR140333 (2 nM) and the NK3 antagonist SR142801 (2 nM) profoundly blocked the NKA-evoked response. In all experiments, responses recovered following a 10-min washout of the antagonists. D, summary of the data obtained in A to C, suggesting that NKA acted through all three tachykinin receptors with NK2-mediating responses to a greater degree. Values expressed as a percentage of control responses; *, p < 0.05 compared with control responses; †, p < 0.01 compared with samples treated with antagonists.

**Fig. 8.** Tachykinin and cholinergic agonists activate INSC in human EB SMCs. A, representative trace from a cultured SMC held at −60 mV with Cs$^+$ present to block outward currents. NKA (1 μM), applied for the duration of the bar, activated inward current. Voltage ramp commands were applied periodically to evaluate current-voltage relationships. Subtraction of control current from the NKA-evoked current revealed the activated current (ΔI, right) that reversed close to 0 mV, consistent with activation of a INSC. B and C, representative trace from a freshly dissociated SMC. ACh (10 μM) elicited similar whole-cell inward currents that reversed close to 0 mV. For both tachykinin and cholinergic agonists, similar results were obtained in cultured and fresh cells using KCl, CaCl$_2$, and Cs-glutamate electrode solutions, confirming the presence of an agonist-evoked INSC in human EB SMCs.
tion. In addition to direct effects on smooth muscle cells, enteric nerves are suggested to mediate excitation through actions on ICCs. Indeed, c-Kit-positive cells (a marker for ICC) have been identified in the LM and CM layer of human esophagus (Torihashi et al., 1999). In other gastrointestinal tissues, ICCs express tachykinin receptors and receive excitatory inputs from enteric neurons (Daniel, 2001; Ward and Sanders, 2001). Although our data do reveal that tachykinins can act directly on the SMCs, they do not exclude the additional involvement of ICCs in regulating esophageal peristalsis in vivo.

To establish the functional effects of exogenous tachykinins on esophageal contraction, our initial experiments employed whole-tissue strips. With neuronal transmission blocked using TTX and muscarinic receptors antagonized with atropine (Preiksaitis et al., 2000), tachykinins evoked contraction of EB smooth muscle. Previous studies employing muscle strips have shown tachykinins to contract several gastrointestinal smooth muscles, including those from opossum and human esophagus (Crist et al., 1986; Huber et al., 1993a; Krysiak and Preiksaitis, 2001). However, the potential presence of heterogeneous cells within these preparations (Porthbury et al., 1996; Holzer and Holzer-Petsche, 1997b) motivated us to confirm our muscle strip findings in cultured and freshly isolated SMCs. In our studies, atropine caused a small but significant inhibition of the NKA-activated contraction, suggesting a minor cholinergic component to the tachykinin contraction, similar to that reported for human colonic muscle (Liu et al., 2002).

In early studies, tachykinin receptors were identified using a combination of contraction and receptor binding studies (Holzer and Holzer-Petsche, 1997a). However, given that each tachykinin is capable of acting as an agonist at all receptor types, the results are difficult to interpret. The advent of nonpeptide antagonists (Snider et al., 1991) allowed a more detailed examination of binding characteristics. Despite this, early compounds such as the NK1 antagonist CP-96,345 used by Huber et al. (1993a,b) were later found to have confounding effects, including a strong affinity to block the L-type Ca\(^{2+}\) channel (Guard et al., 1993). As such, in the present study, we have extended the pioneering observations by Huber et al. (Huber et al., 1993a,b) by using a newer generation of selective receptor antagonists (Holzer and Holzer-Petsche, 1997a). Furthermore, by employing RT-PCR and immunofluorescence, we have been able to confirm that tachykinin receptors are present on human esophageal SMCs.

Indeed, when freshly dissociated cells were stimulated with tachykinins, all agonists evoked substantial SMC contractions. Few studies have examined the transduction mechanisms underlying tachykinin-evoked smooth muscle contraction and to our knowledge, none of these have investigated human esophageal muscle. Previous studies suggested that Ca\(^{2+}\) influx through L-type Ca\(^{2+}\) channels was critical for tachykinin-evoked contraction in guinea pig, rabbit, and human colonic smooth muscles (Maggi et al., 1997; O’Riordan et al., 2001). In contrast, a major role for Ca\(^{2+}\) stores has been suggested in human sigmoid colon muscle by Cao et al. (2000). The authors noted tachykinin-evoked contractions and [Ca\(^{2+}\)]\(_i\) elevations were abolished by sarcoplasmic reticulum Ca\(^{2+}\) ATPase blockade with thapsigargin, but contractions were unaffected by Ca\(^{2+}\) removal (Cao et al., 2000). In the present study, we showed that tachykinin signaling in human EB SMC involves a combination of Ca\(^{2+}\) influx and release from intracellular stores.

We have identified, for the first time, the presence of a I\(_{NSC}\) current in human esophageal smooth muscle. The I\(_{NSC}\) evoked by acetylcholine in human EB SMCs is similar to that identified in several gastrointestinal tissues including guinea pig (Inoue and Isenberg, 1990) and rabbit (Benham et al., 1985) small intestine, canine colonic (Lee et al., 1993) as well as canine gastric corpus (Sims, 1992) smooth muscles. Tachykinins are reported to evoke I\(_{NSC}\) in canine colonic smooth muscles (Lee et al., 1995), although the magnitude is somewhat smaller than we found in human esophagus. It has not been established whether the I\(_{NSC}\) in esophageal SMCs allows entry of Ca\(^{2+}\). Nevertheless, this inward current, evoked by both cholinergic and nonadrenergic, noncholinergic agonists, would cause membrane depolarization. The resultant activation of voltage-dependent L-type Ca\(^{2+}\) channels, previously characterized in these cells (Kovae et al., 2005), would lead to Ca\(^{2+}\) influx that, along with the Ca\(^{2+}\) released from stores, would initiate contraction.

In summary, the present study reveals that tachykinins bind and activate human esophageal SMCs directly to initiate contraction. Excitation involves a combination of Ca\(^{2+}\) release from intracellular stores, influx through L-type Ca\(^{2+}\) channels, and I\(_{NSC}\) activation, features of physiological regulation not previously reported. It has been suggested that tachykinins play an important role in the pathologic regulation of smooth muscle contraction. Indeed, under inflammatory conditions, tachykinin-secreting immune cells including macrophages, lymphocytes, and mast cells are recruited to the gastrointestinal tract (O’Connor et al., 2004). Since prevention of mast cell degranulation reduces injury-induced esophageal contraction (Paterson, 1998), our studies may have relevance to therapeutic intervention in the treatment of esophageal motility disorders.

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Address correspondence to: Dr. Stephen M. Sims, Department of Physiology and Pharmacology, Schulich School of Medicine & Dentistry, University of Western Ontario, London, ON, Canada N6A 5C1. E-mail: stephen.sims@schulich.uwo.ca.