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Tachykinin receptor expression and function in human esophageal smooth muscle

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

Department of Physiology and Pharmacology (JRK, TC, HGP and SMS)

Department of Medicine (HGP)

Schulich School of Medicine & Dentistry, The University of Western Ontario, London, Ontario, Canada N6A 5C1

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Corresponding Author: Dr. Stephen M. Sims

Department of Physiology and Pharmacology

Schulich School of Medicine & Dentistry

University of Western Ontario

London, Ontario, Canada, N6A 5C1

Tel: (519) 661-3768

Fax: (519) 850-2459

Email: stephen.sims@schulich.uwo.ca

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Non-standard abbreviations: ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; CCh, carbachol; CM, circular muscle; EB, esophageal body; LM, Longitudinal muscle; NANC, Non-adrenergic non-cholinergic; NKA, Neurokinin A; NKB, Neurokinin B; SMC, smooth muscle cell; SP, Substance P; TTX, tetrodotoxin; I_{NSC}, nonselective cation current; ICC, interstitial cells of Cajal; CPA, cyclopiazonic acid

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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 signalling 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 RT-PCR and immunoblotting, intracellular Ca^{2+} responses using fluorescent indicator dyes, and membrane currents with patch clamp electrophysiology. The mammalian tachykinins (substance P, SP; neurokinin A, NKA; neurokinin B, 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 Ca^{2+} release from intracellular stores and influx through L-type Ca^{2+} channels. NK2 receptor blockade inhibited the largest proportion of tachykinin-evoked responses. NKA evoked a nonselective cation current (I_{NSC}) with properties similar to that elicited by muscarinic stimulation. The following paradigm is suggested: tachykinin receptor binding to SMCs releases Ca^{2+} from stores along with activation of I_{NSC}, which in turn results in membrane depolarization, L-type Ca^{2+} channel opening, rise of Ca^{2+} concentration and contraction. These studies reveal new aspects of tachykinin signalling in human esophageal SMCs. Excitatory tachykinin pathways may represent targets for pharmacological intervention in disorders of esophageal dysmotility.

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INTRODUCTION

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 A (NKA) and neurokinin B (NKB) (Holzer and Holzer-Petsche, 2001). All three peptides share a common carboxy-terminal motif (Phe-X-Gly-Leu-Met-NH_2) 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 co-localized 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 (NANC) 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). While 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 (Tonini et al., 2001).

Muscle contraction studies of human EB support a role for tachykinins in regulating NANC-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 co-release tachykinins and ACh, and the capability of tachykinins to bind multiple receptor subtypes (Domoto et al., 1983; Furness et al., 2004) all indicate the need for further examination of tachykinin signalling in esophageal muscle.

Tachykinin signalling 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_{NSC}) (Lee et al., 1995). Given the importance of esophageal peristalsis in health and disease, it is important to understand the signalling mechanisms involved in tachykinin-induced contraction.

In the present study, our aim was to characterize tachykinin signalling 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 signalling not previously reported for esophageal muscle.

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 *Solutions*) 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 hours or maintained in primary culture as previously described (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 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 (RT-PCR). 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 μg of total RNA were reverse-transcribed for 60 min. at 42ºC with Oligo$(dT)_{12\text{-}18}$. PCR was performed in 50 μl of PCR buffer containing 3 mM $MgCl_2$, 200 μM dNTPs, 0.1 nM of primer, 0.3 μl of *Taq* DNA polymerase (Fermentas Life Sciences, Burlington, ON, Canada) and 5-7 μl 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 94ºC, 1 min. at 58ºC, 1.5 min. at 72ºC, and a final 10 min. extension at 72ºC. The PCR primers used to amplify cDNA are listed in Table 1. PCR primers for β-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 1º antibody (from Dr. Krause at Neurogen Inc., Branfort, CT) at 4ºC overnight. 1º 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 (2x10 min.), cells were incubated (60 min. at 22ºC) with FITC-linked goat, anti-rabbit secondary (2º) antibody (1:50, Jackson Labs, West Grove, PA) to detect NK1 receptors, and with Cy-3-linked donkey, anti-mouse 2º antibody (1:200, Jackson Labs, West Grove, PA) to detect NK2 and NK3 receptors. Following washes with PBS (2x10 min.), TO-PRO-1 or TO-PRO-3 dimeric cyanine dye (5 μM, 60 min. at 22ºC; Molecular Probes, Eugene, OR) was used to stain nuclei. Samples were again washed with PBS (2x10 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 weights, and absent when primary antibodies were pre-absorbed with the peptide immunogens (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 (fura-2 AM, 0.2 μM) or fluo-4 AM (5 μM) at room temperature (21-24°C) for 40 minutes, as described previously (Kovac et al., 2005). Cells loaded with fluo-4 were illuminated with 488 nm light from an argon ion laser and emissions detected at 510-560 nm using a Photometrics Cascade camera controlled by PTI ImageMaster software.

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 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 (in mM): 116 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 2.2 NaH_2PO_4, 25 $NaHCO_3$ and 10 D-glucose, equilibrated with 5% CO_2-95% O_2 (pH 7.4). The bathing solution for fluorescence studies and electrophysiological recordings contained (in mM): 130 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, and 10 D-glucose (adjusted to pH 7.4 with NaOH). Ca^{2+}-free solutions contained ethylene glycol-bis (ß-aminoethyl ether)-*N*,*N*,*N'*,*N'*–tetraacetic acid (EGTA, 0.5 mM) and omitted $CaCl_2$. In patch recordings, KCl electrode solution contained (in mM): 140 KCl, 20 HEPES, 1 $MgCl_2$ and 0.1 EGTA (adjusted to pH 7.2 with KOH). CsCl electrode solution contained (in mM): 130 CsCl, 20 HEPES, 1 $MgCl_2$, 10 TEACl, 0.4 $CaCl_2$, and 1 EGTA (adjusted to pH 7.2 with CsOH). CsGlutamate electrode solution contained (in mM): 40 CsCl, 100 Glutamate, 20 HEPES, 1 $MgCl_2$, 0.4 $CaCl_2$, and 0.01 EGTA (adjusted to pH 7.2 with CsOH).

Chemicals. Chemicals were from Sigma (St. Louis, MO), BDH Inc. (Toronto, ON) or Calbiochem (San Diego, CA) unless otherwise stated. SR48968 ((S)-N-methyl-N[4-(4-acetyl-amino-4-phenylpiperi-dino)-2-(3,4-dichloro-phenyl)butyl]benzamide, SR140333 (S)-1-[2-[3-(3,4-dichlorphenyl)-1 (3-isopropoxy-phenylacetyl)piperidin-3yl]ethyl]-4-phenyl-1 azaniabicyclo [2.2.2] octane chloride, and SR142801 ((R)-(N)-[1-[3-[1-benzoyl-3-(3,4-dichlorophenyl)piperidin-3-yl]propyl]-4-phenylpiperidin-4-yl]-N-methylacetamide were from Sanofi Recherche (Montpellier, France). Drugs were prepared in distilled water or dimethyl sulfoxide (DMSO) and diluted into bathing solution. Control experiments in which vehicle alone was applied to tissues or cells revealed no effects.

Statistics. Values are the means ± SEM with sample sizes (n) indicating the number of cells or muscle strips studied. All traces are representative of at least 3 experiments on muscle or cells from two or more esophageal specimens. For Ca^{2+} fluorescence and patch clamp experiments only one recording was obtained per cell. Comparisons were made using the Student's paired *t*-test, ANOVA and Tukey's post-test as indicated. A value of $p<0.05$ 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 reproducible increases in EB tension (Fig. 1A). NKA-evoked contractions were larger than those stimulated by NKB or SP (Fig. 1A) in agreement with our previous study of human esophagus (Krysiak and Preiksaitis, 2001). Concentration-dependent contractions induced by NKA were consistently greater in CM than in LM (Fig. 1B, *left panel*). Depolarization with high extracellular K^+ (Fig. 1B, *right panel*) evoked similar contractions amongst EB CM and LM. Accordingly, we focused on the functional regulation of CM for this report.

Tachykinin-positive neurons co-express choline acetyltransferase and excitation releases both tachykinins and ACh from nerve endings (Domoto et al., 1983; Holzer et al., 1998; Furness et al., 2004). Furthermore, tachykinins may cause release of ACh from enteric neurons via NK3 and NK1 receptors (Holzer and Holzer-Petsche, 1997b); (Portbury et al., 1996). 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.

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Human EB SMCs expresses tachykinin receptors.

We examined tachykinin receptor expression using 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 1º antibodies were omitted and cells were incubated with 2º 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 seconds of agonist application.

We next sought to establish the signalling mechanisms underlying tachykinin-evoked contractions. Ca^{2+}-sensitive dyes were used to determine if 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 panel*). ACh resulted in similar responses (Fig. 5B, *right panel*), 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 re-addition of extracellular Ca^{2+} 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 4 additional cells from multiple esophageal specimens.

To directly evaluate the contribution of intracellular stores, we blocked the sarcoplasmic reticulum Ca^{2+}-ATPase with cyclopiazonic acid (CPA, 10 μM). 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 re-introduction of extracellular

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Ca^{2+}, indicating intact receptor signalling (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 μM, 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±46 nM vs. rise in the presence of nifedipine of 156±26 nM, $p<0.05$, 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 signalling.

Tachykinin responses are mediated by the NK2 receptor.

We next examined which receptor subtype(s) contributed to signalling 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. Whereas a three minute 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 minute 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 is 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 μM, n=10, Supplemental Fig. 1A) and SP (1 μM, 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 blockade 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, as we saw no differences in expression of NK1 or NK3 in cultured cells vs. freshly isolated cells using PCR and immunolocalization. As a 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 cation current (I_{NSC}) 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 CsGlutamate electrode solution (n=10). These findings are consistent with the activation of an I_{NSC} in response to tachykinins and muscarinic agonist.

DISCUSSION

The mechanisms of tachykinin signalling 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 I_{NSC}.

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 function. 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). While 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 (Portbury 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-evoked 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 non-peptide antagonists (Snider et al., 1991) allowed a more detailed examination of binding characteristics. In spite of this, early compounds such as the NK1 antagonist CP-96,345 used by Huber and colleagues (Huber et al., 1993a; Huber et al., 1993b) 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 and colleagues (Huber et al., 1993a; Huber et al., 1993b) 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 and coworkers (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 signalling 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 non-adrenergic, non-cholinergic agonists, would cause membrane depolarization. The resultant activation of voltage dependent L-type Ca^{2+} channels, previously characterized in these cells (Kovac 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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Footnotes

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LEGENDS FOR FIGURES

Figure 1: Tachykinins evoke concentration-dependent contraction of human esophageal body (EB) smooth muscle. *A.* Exogenous application of the mammalian tachykinins neurokinin A (NKA, 1 μM), neurokinin B (NKB, 1 μM) and substance P (SP, 1 μM) as well as depolarization with a high extracellular K^+ solution (60 mM) and carbachol (CCh, 1 μM) contract human EB. *B:* NKA (*left panel*) evoked minimal contractions in longitudinal muscle (LM; ■, solid line; n=9-15) and large, concentration-dependent contractions in circular muscle (CM; ▫, dashed line, n=11-17). High extracellular K^+ (*right panel*; CM and LM n=17-19) produced similar concentration-dependent contractions in CM and LM. The quantification of contraction in panel *B* are standardized for wet tissue weight, and other responses are presented as raw traces.

Figure 2: Neurokinin A exerts a direct effect on esophageal smooth muscle. *A:* CCh (1 μM, applied at arrow) evoked large, sustained contractions in EB CM. Following washout, tissues were perfused with tetrodotoxin (TTX, 1 μM) to block nerve transmission (left panel), or atropine (1 μM, right panel) to block muscarinic receptors. As expected, CCh responses were abolished in the presence of atropine. *B:* NKA (1 μM), applied at the arrows, evoked contractions that were largely unaffected by TTX. Atropine caused a small but significant inhibition of the NKA-evoked contraction, as assessed by two-way analysis of variance followed by Tukey's post test (Fig. 2C, * $p<0.05$).

Figure 3: Tachykinin receptor mRNA in human esophageal smooth muscle. PCR products of the expected sizes were obtained in both fresh tissue (*A*) and cultured cells (*B*) as follows: NK1, 596 bp; NK2, 557 bp; and NK3, 542 bp (see primers in Table 1). Product identity was confirmed by sequencing of the amplicons. Products were identified in LM and CM layers. β-

actin spanned a 206-bp intron so finding a single band of 314 bp verified the absence of genomic DNA contamination. Controls (CON) contained the same RNA samples without reverse transcriptase. Ladder (Lad) contained standard molecular weight markers.

Figure 4: Immunofluorescent labelling of tachykinin receptors in cultured human EB SMCs. Subtype-specific antibodies were used to label NK1 (green, *top left*), NK2 (red, *top middle*), and NK3 receptors (red, *top right*). Receptor expression was observed as diffuse labelling in all cells (only CM shown). For controls, the 1º antibody was omitted and no labelling was observed (control, *bottom panels*). Nuclei were stained red with TO-PRO-3 (*left* panels) and green with TO-PRO-1 (*middle* and *right* panels). Calibration bar applies to all panels.

Figure 5: Tachykinins cause reversible contraction and increased $[Ca^{2+}]_i$ in human esophageal SMCs. *A:* A series of video frames illustrating a typical cell before stimulation (far *left*) and then 10 seconds following application of NKA (1 μM). The cell contracted to ~60% of its original length with recovery to ~85% of initial length after a 10 minute washout. Acetylcholine (ACh, 10 μM) elicited a similar contraction that was also reversible following washout (far *right*). Agonists were applied by pressure ejection from an application pipette (seen at lower left in the NKA panel). *B:* In conjunction with both tachykinin (*left panel*) and cholinergic (*right panel*) stimulation, a rapid and transient increase in $[Ca^{2+}]_i$ is observed (Δf, % change of baseline fluorescence), consistent with the role for Ca^{2+} in initiating contraction.

Figure 6: NKA-mediated excitation involves release of Ca^{2+} from stores and influx. *A:* NKA (1 μM, applied for 10 s at arrowheads) caused a transient rise of $[Ca^{2+}]_i$ in cultured EB SMCs. Responses were diminished in a Ca^{2+}-free solution (0.5 mM EGTA) consistent with the depletion

of intracellular stores. Breaks in the trace represent 5 min. intervals for washout and recovery. Following a 10 minute reperfusion with a Ca^{2+}-containing solution, NKA-evoked Ca^{2+} transients recovered (at *right*). *B:* Blockade of the sarcoplasmic reticulum Ca^{2+}-ATPase with cyclopiazonic acid (CPA, 10 μM, applied for duration indicated by solid bar), resulted in a gradual rise of $[Ca^{2+}]_i$. The first response to NKA in the presence of CPA was prolonged whereas the second stimulation had no effect, indicating depletion of stores. Store depletion was reversible on washout of CPA and reintroduction of bath Ca^{2+}. *C:* The Ca^{2+} channel blocker nifedipine (10 μM, 90 s) decreased NKA-induced rise of $[Ca^{2+}]_i$. *D:* Summary of the experiments in *C* with mean values ± SEM (* $p < 0.01$), indicating a role for Ca^{2+} influx in NKA-evoked responses.

Figure 7: NK2 receptor-mediated rise of $[Ca^{2+}]_i$ in cultured human EB SMCs. Subtype selective tachykinin receptor antagonists were applied for 3 minutes before NKA. *A, B:* The NK1 antagonist SR140333 (2 nM) and the NK3 antagonist SR142801 (2 nM) had significant, but modest effects on the NKA-evoked $[Ca^{2+}]_i$ transients. *C:* In contrast, the NK2 antagonist SR48968 (2 nM) profoundly blocked the NKA-evoked response. In all experiments, responses recovered following a 10 minute washout of the antagonists. *D:* Summary of the data obtained in *A – C* suggesting that NKA acted through all three tachykinin receptors with NK2 mediating responses to a greater degree. Values expressed as a % of control $[Ca^{2+}]_i$ transients (‡, $p < 0.05$ compared with control responses; *, $p < 0.01$ compared with samples treated with antagonists).

Figure 8: Tachykinin and cholinergic agonists activate non-selective cation current (I_{NSC}) 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, at *right*) that reversed close to 0 mV, consistent with activation of a I_{NSC}. *B, 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, CsCl and CsGlutamate electrode solutions - confirming the presence of an agonist-evoked I_{NSC} in human EB SMCs.

TABLE 1

Primer sequences used in RT-PCR to identify neurokinin receptor subtypes in human esophageal muscle.

Receptor Type	Accession Number	Primer Pair Sequence: Sense/Antisense	Location	Predicted Size (bp)
NK1	M76675	5' -GACTGTGCTGATCTACTTCC- 3' 5' -AGCTCTCTGTCATGGTCTTG- 3'	600-1195	596
NK2	NM_001057	5' -TTATTGCTGGCATCTGGCTG- 3' 5' -GAGCTTATCTTCCTTGGTGG- 3'	452-1008	557
NK3	NM_001059	5' -TTGCGGTGGACAGGTATATG- 3' 5' -CAGACAGCAGTAGATGATGG- 3'	678-1220	542
β-actin	X00351	5' - CACTCTTCCAGCCTTCCTTC - 3' 5' - CTCGTCATACTCCTGCTTGC - 3'	820-1133	314*

* β-actin PCR primer pair was selected to span a 206-bp intron.

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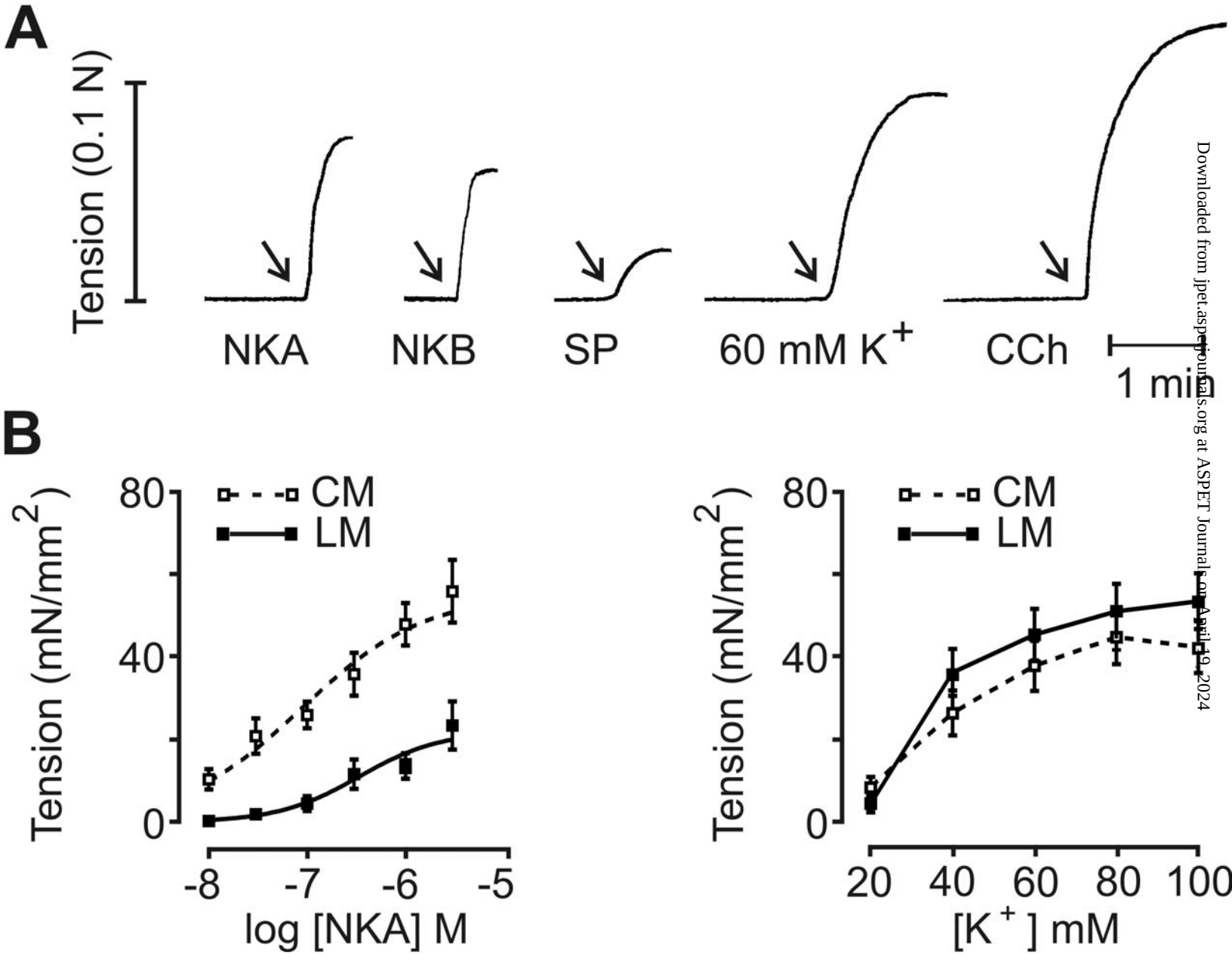

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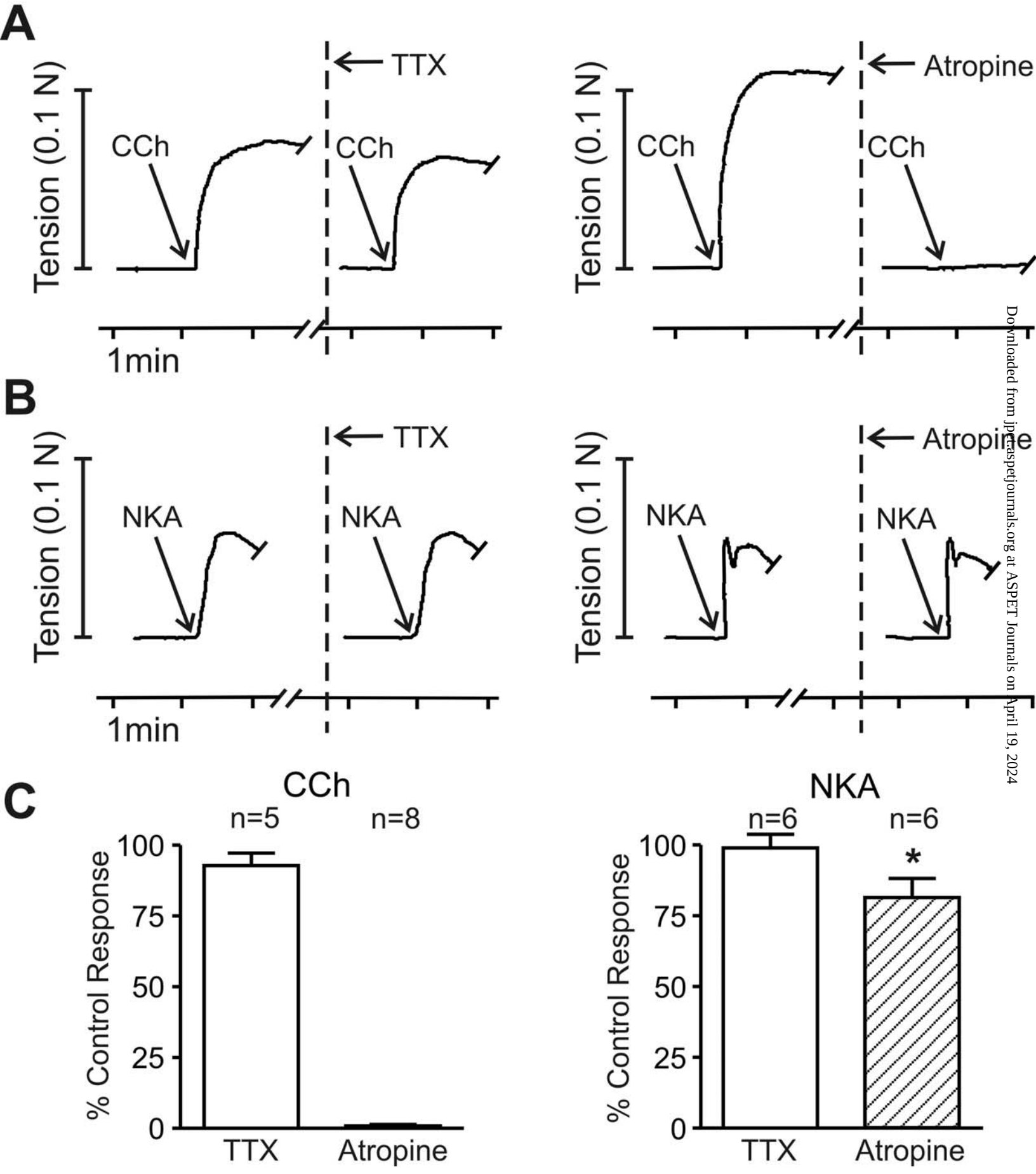

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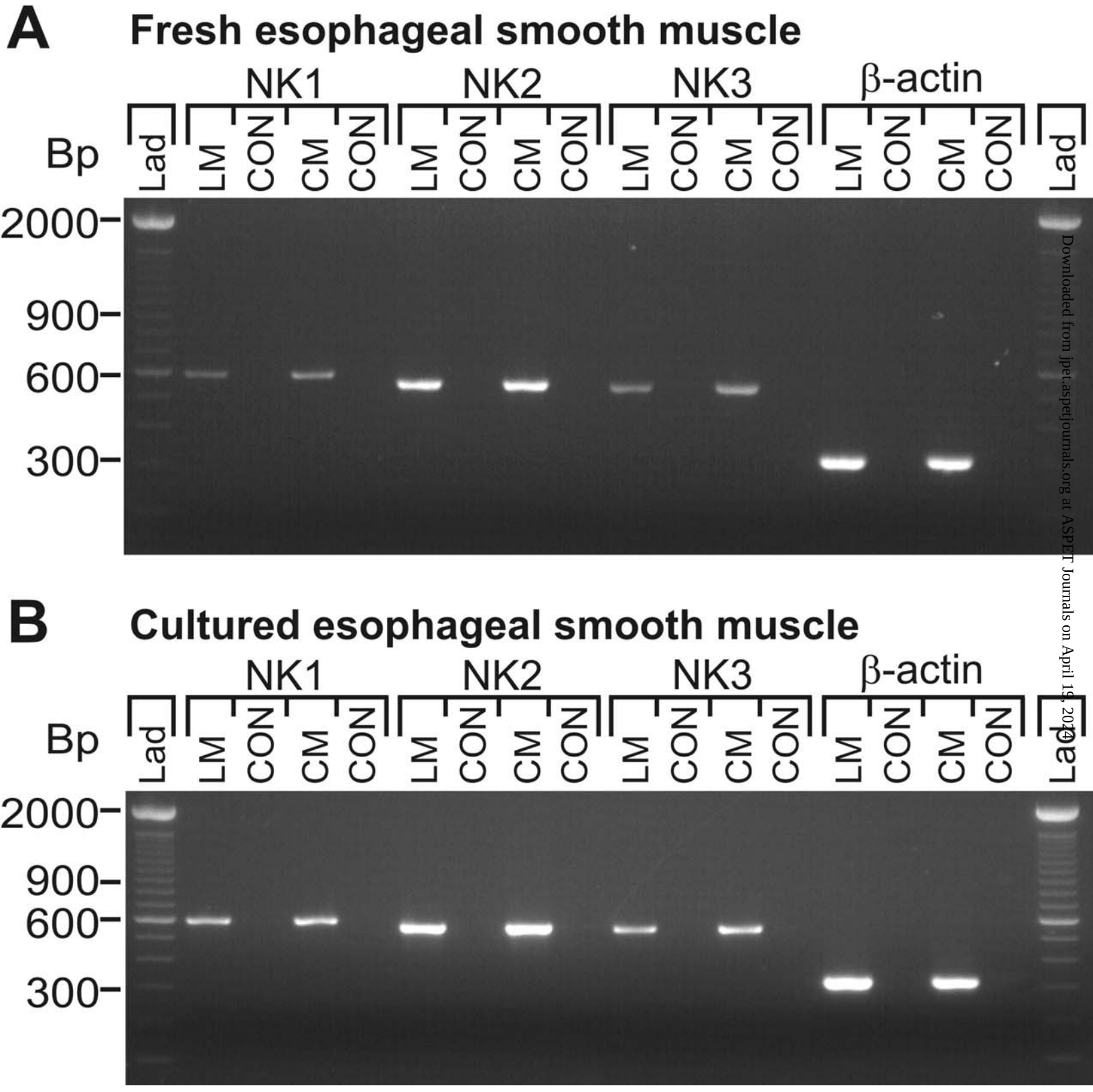

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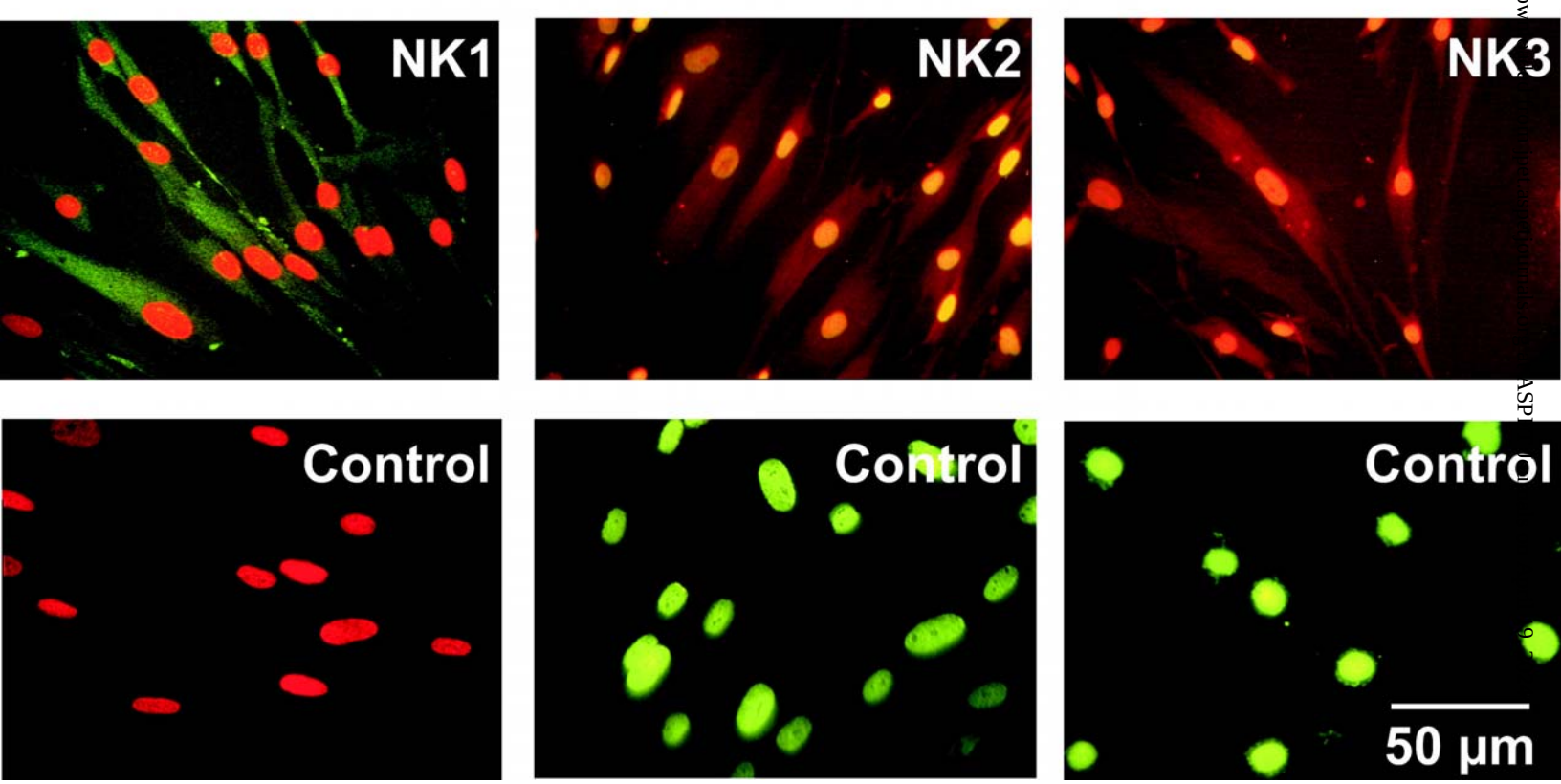

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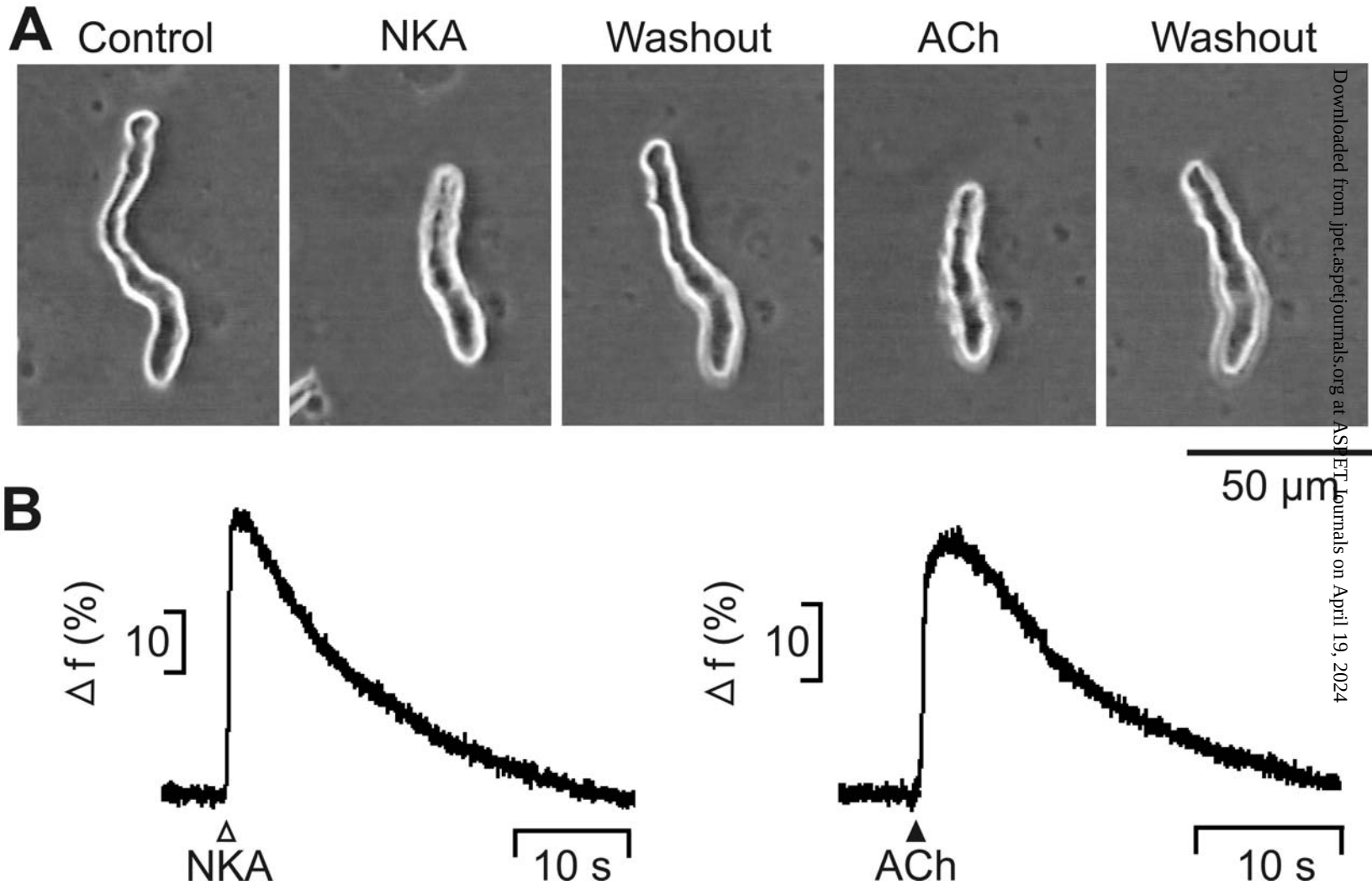

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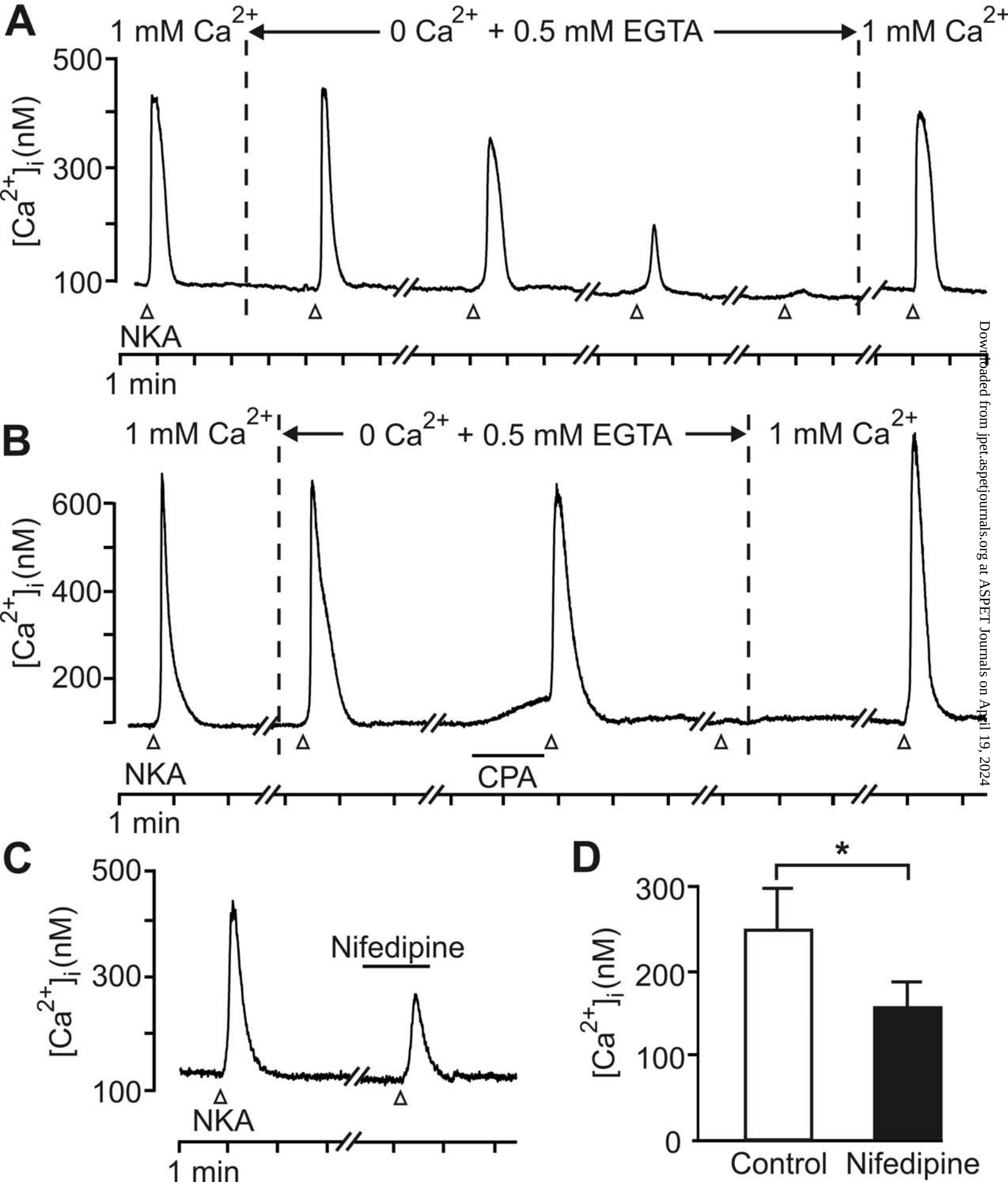

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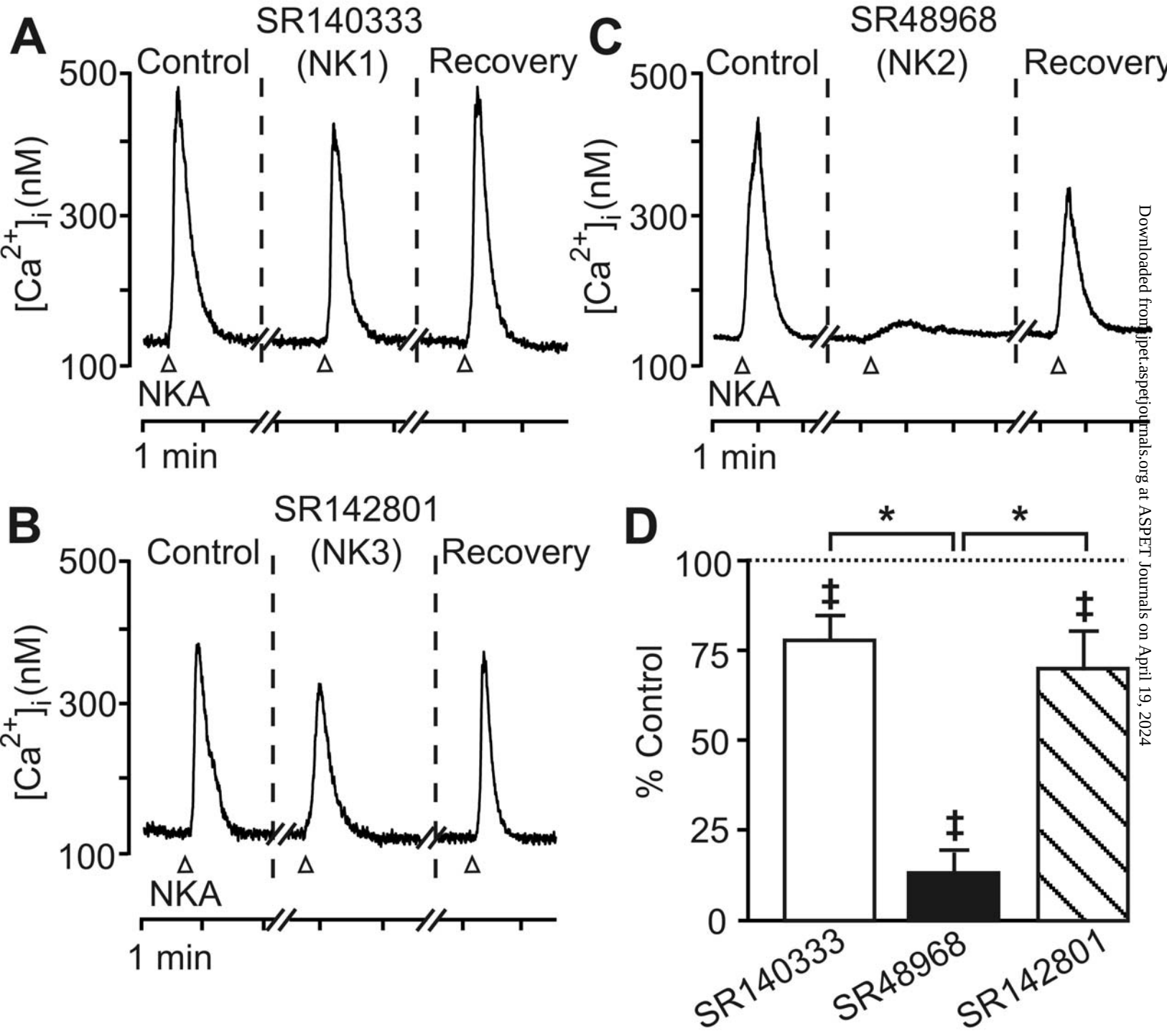

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