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SNAP-25 inhibits L-type Ca^{2+} channels in feline esophagus smooth muscle cells☆

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Abstract

We recently reported that non-secretory gastrointestinal smooth muscle cells also possessed SNARE proteins, of which SNAP-25 regulated Ca^{2+}-activated (K_{Ca}) and delayed rectifier K^+ channels (K_V). Voltage-gated, long lasting (L-type) calcium channels (L_{Ca}) play an important role in excitation–contraction coupling of smooth muscle. Here, we show that SNAP-25 could also directly inhibit the L-type Ca^{2+} channels in feline esophageal smooth muscle cells at the SNARE complex binding synprint site. SNARE proteins could therefore regulate additional cell actions other than membrane fusion and secretion, in particular, coordinated muscle membrane excitability and contraction, through their actions on membrane Ca^{2+} and K^+ channels.

Keywords: SNARE proteins; SNAP-25; L-type calcium channel; Smooth muscle; Esophagus; Feline; Peristalsis

SNAREs (soluble *N*-ethylmaleimide sensitive factor attachment protein receptors) were originally found in neurons and endocrine cells to mediate vesicular transport, particularly exocytosis [1–3]. New isoforms of SNARE proteins have been identified in non-neuronal cells to regulate exocytosis [4–9]. The plasma membrane SNARE proteins, SNAP-25, and syntaxin 1A, have also been found to bind and physically interact with and functionally modify a number of membrane ion channels in these secretory cells, such as N- and L-type Ca^{2+} channels [10–12], CFTR (cystic fibrosis transmembrane conductance regulator) Cl^- channel [13], and delayed rectifier K^+ channels [14,15]. Recently, we found numerous neuronal and non-neuronal isoforms of these SNARE proteins to be present in the non-secretory smooth muscle cells of the feline esophagus [16], of which SNAP-25 regulated the large-conductance Ca^{2+}-activated K^+ channels [17] and delayed rectifier K^+ channels [17]. These SNARE proteins therefore could regulate cell functions other than secretion, and in particular muscle contractility such as esophageal peristalsis. In this work we further demonstrate this potential by demonstrating the interaction of SNAP-25 with the Ca^{2+} channels of the feline esophageal smooth muscle cell [18].

Materials and methods

Materials. The Lc peptide is a gift from Professor Daphne Atlas (The Hebrew University of Jerusalem, Jerusalem, Israel). Antibodies to the subunits of the L-type calcium channels were obtained from Alomone Labs (Jerusalem, Israel). Bay K 8644, nifedipine, and all enzymes and other chemical reagents were obtained from Sigma (St. Louis, MO) unless stated otherwise. Both Bay K 8644 and nifedipine were prepared in DMSO with a final DMSO concentration in solution of 0.1%.

Tissue preparation and cell dissociation. Smooth muscle cells were isolated from circular smooth muscle of feline esophagus following a

☆ *Abbreviations:* SNAP-25, synaptosome-associated protein of 25 kDa; GST, glutathione *S*-transferase, L_{Ca}, L-type calcium channel; SNARE, soluble *N*-ethylmaleimide sensitive factor attachment protein receptors.

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previously described protocol [17] with minor modification. Briefly, adult cats of either sex were euthanized by intravenous injection of overdose sodium pentobarbital following a protocol approved by the University Health Network Animal Care Committee. The esophagus was quickly excised, placed in Krebs solution bubbled with 5% CO_2–95% O_2 at room temperature, and maintained at pH 7.4 ± 0.05. After the mucosa and most of the submucosa were stripped off, circular muscle from the smooth muscle portion of the esophageal body was dissected out and cut into squares of $\sim 2\,mm^2$. These squares were placed in a test tube with 1 ml dissociation solution composed of (in mM): 110.0 NaCl, 5.0 KCl, 0.16 $CaCl_2$, 2.0 $MgCl_2$, 10.0 Hepes, 10.0 $NaHCO_3$, 0.5 KH_2PO_4, 0.5 NaH_2PO_4, 0.49 EDTA, and 10.0 glucose (pH 7.0). Papain (2 mg/ml) as well as collagenase blends F (1.3 mg/ml), 1,4-dithio-L-threitol (154 μg/ml), and bovine serum albumin (1 mg/ml) were added to the test tube that was then incubated in a water bath at 35 °C for 45 min. After that, tissues were rinsed with enzyme-free dissociation solution 3 times and gently agitated with a glass pipette. Spindle-shaped single smooth muscle cells were used for patch clamp study within the following 5 h after dissociation.

Whole-cell patch clamp. Isolated muscle cells suspended in dissociation solution were placed in a 1-ml glass-bottom dish mounted on the stage of an inverted microscope and allowed to adhere to the bottom for 30 min. The chamber was perfused with the external solution composed of (mM): 20 $BaCl_2$, 90 NaCl, 5 CsCl, 1 $MgCl_2$, 20 TEA–Cl, and 10 Hepes (pH 7.4). Pipette resistance was about 2–3 MΩ after being filled with the pipette solution containing the following components (mM): 105 Cs–aspartate, 1 $MgCl_2$, 5 EGTA, 4 Mg–ATP, and 20 Hepes (pH 7.2). Recordings were performed by using Axopatch 1D and 200B amplifiers (Axon Instruments, Union City, CA). Whole-cell voltage-clamp protocols were generated by pClamp8 software (Axon Instruments, Union City, CA). Experiments were only carried out on cells which had stable access resistance less than 20 MΩ. All signals were filtered at 1 kHz by an on-board eight-pole Bessel filter before digitization with a Digiata 1200 analog-to-digital converter (Axon Instruments, Union City, CA). Cell capacitance was determined by integration of the capacity transient. All experiments were performed at room temperature of 22–24 °C.

Immunoblotting. Membrane protein samples were prepared from the circular muscle layer. Muscle tissues stored at −80 °C were minced into small pieces and homogenized on ice in a glass tissue grinder containing 600 μl buffer solution with the following components: 250 mmol/L sucrose, 50 mmol/L MOPS, 0.1 mmol/L phenylmethylsulfonyl fluoride, 5 mg/ml leupeptin, 5 g/ml antipapain, and 5 mg/ml aprotinin A (pH 7.4 adjusted with NaOH). After sonication, large tissue debris and nuclear fragments were removed by low centrifuge spins (1000*g* for 10 min) at 4 °C and the pellet of membrane protein was obtained after a subsequent centrifugation at 100,000*g* at 4 °C for 30 min. Ten microgram proteins of this sample were electrophoretically size-separated on a 15% SDS–PAGE and transferred to PVDF membranes (Millipore, Bedford, MA), and the α1C protein was identified with a rabbit anti-$\alpha 1C_{818-835}$ (1:200) antibody, which was raised against residues 818–835 of the intracellular loop between II and III domains of α1C-subunit of the L-type Ca^{2+} channel (Alomone Labs, Jerusalem, Israel).

Statistics. Data are presented as means ± standard error of the mean (SEM). A Student's *t* test was used to compare data between two groups, whereas analysis of variance (ANOVA) was used among three or more groups. A value of $P < 0.05$ was considered to have significant difference.

Results

Freshly isolated esophageal circular smooth muscle cells were spindle-shaped and appeared phase bright under phase contrast microscopy. Contractions were observed when these cells were exposed to acetylcholine (10–100 μM) in calcium containing external solution, indicating that the cells are viable and have retained contractile function after enzymatic digestion.

Identification of L_{Ca} in the esophagus circular smooth muscle cells

To study the depolarization-activated inward current in isolation, K^+ currents were blocked with Cs^+ and TEA in the recording electrode, while Ba^{2+} was added to the bath solution to enhance the L_{Ca} conductance (see Materials and methods). When cells were held at −50 mV and stepped to various test potentials, inward current first became apparent at approximately −30 mV, reached maximal value at +10 mV, and reversed at about +50 mV (Fig. 1B). The inward current showed a transient component, peaking then declining. Fig. 1A shows inward currents in response to depolarization at +10 mV from a holding potential of −50 mV. Stimulation of the cells with the dihydropyridine Ca^{2+} channel agonist Bay K 8644 caused an increase in inward current, with a scaling up of both the transient and sustained components of the current (Fig. 1A). The peak inward current was augmented over the entire voltage range and caused a shift in the *I–V* relationship of 10 mV to the left in the hyperpolarization direction (Fig. 1B). Bay K 8644 also decelerated the deactivation of the tail current (Fig. 1A). The dihydropyridine Ca^{2+} channel blocker nifedipine reduced both transient and sustained components of the inward current, with only a small current remaining (Figs. 1A and B). Enhancement with Bay K 8644 and suppression of the Ca^{2+} current with nifedipine, together with the high voltage activation, are consistent with the inward current being carried primarily by L-type Ca^{2+} channels [19,20].

We next confirmed the expression of L_{Ca} channels in the esophageal circular smooth muscle by immunoblot analysis against positive controls, including insulinoma HIT-T15 cells and rat brain lysates. Fig. 1C shows that doublet immunoreactive bands were detected at 200 and 240 kDa. These doublet bands correspond to the predicted sizes of the short and full length forms of the α1C-subunit protein of the L_{Ca} channel, respectively [21], which were also observed with the control rat brain and insulinoma HIT-T15 cells [12].

SNAP-25 inhibits calcium current in esophageal smooth muscle

Whole-cell L_{Ca} currents were evoked by step depolarizing voltage pulses from a holding potential of −50 to +10 mV. After establishing the whole-cell configuration, maximal inward current was regularly achieved within 180 s. The dialysis of recombinant glutathione *S*-transferase (GST)–SNAP-25 fusion protein (10^{-8} M),

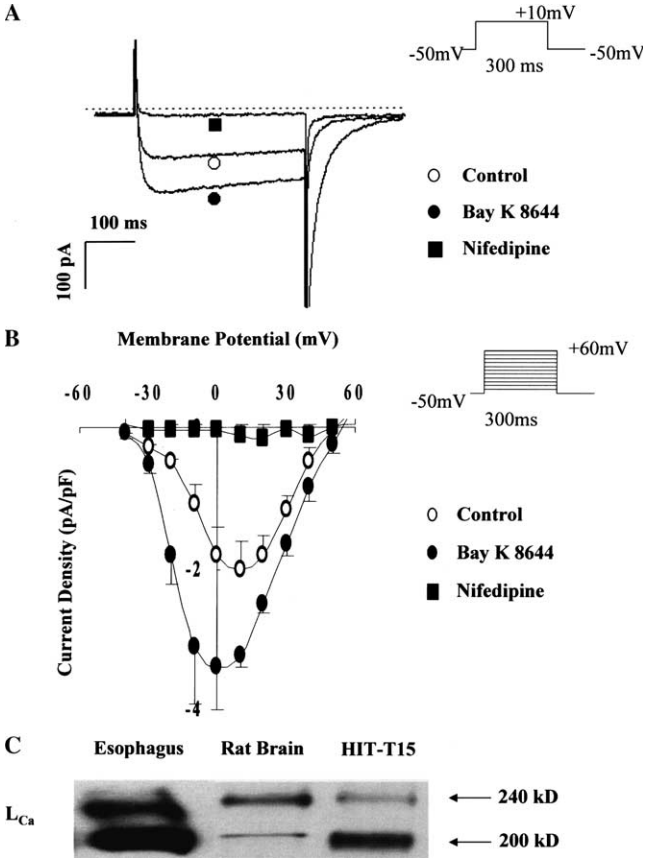

Fig. 1. Identification of L_{Ca} currents in esophageal circular smooth muscle cells. (A) Inward currents were elicited by depolarization to +10 mV for 300 ms from a holding potential of −50 mV. (B) Current–voltage (*I–V*) relationships obtained from smooth muscle cells by plotting peak inward currents against test pulse voltages. A family of inward current traces were evoked by cell depolarization to test pulse voltages from −40 to +60 mV for 300 ms in 10 mV increments with an inter-pulse interval of 15 s. The holding potential was −50 mV. The dotted line indicates the zero current level. Addition of the specific L_{Ca} agonist Bay K 8644 (1 μM) to the recording chamber caused significant increase in inward current amplitude; addition of L_{Ca} antagonist nifedipine (1 μM) significantly inhibited inward current (A and B). (C) Expression of the α1C-subunit in circular smooth muscle membrane fractions from the esophageal body. Each lane was loaded with 10 μg protein. Lystates of insulinoma HIT-T15 and rat brain were used as the positive controls. Arrows pointing to the long and short forms of α1C-subunit were also expressed in both the positive control tissues. This is a representative of two separate experiments.

generated as we have previously described [12,14] into the muscle cells via patch pipette for 10 min, caused a dramatic decrease in inward current ($44.5 \pm 4.0\%$, $n = 8$) occurring at +10 mV (Fig. 2). SNAP-25 did not shift the *I–V* curve of the inward current (data not shown). Cells dialyzed with 10^{-9} M GST ($n = 7$) as controls caused slight ($15.6 \pm 2.7\%$) but significant inhibition of L_{Ca} currents by 780 s (10 min). This inhibition was not significantly different than when using normal Cs–Asp (GST-free) pipette solution. Lc peptide ($Lc_{753-893}$) is a recombinant protein corresponding to the cytoplasmic II–III loop of L_{Ca} [11,12]. Remarkably, the gradual reduction in whole-cell L_{Ca} currents induced by the intracellular application of GST–SNAP-25 was completely blocked by the co-application of 10^{-6} M $Lc_{753-893}$ (Fig. 2), resulting only in a slight inhibition ($18.1 \pm 2.4\%$, $n = 5$) in L_{Ca} that is similar to the GST protein control values.

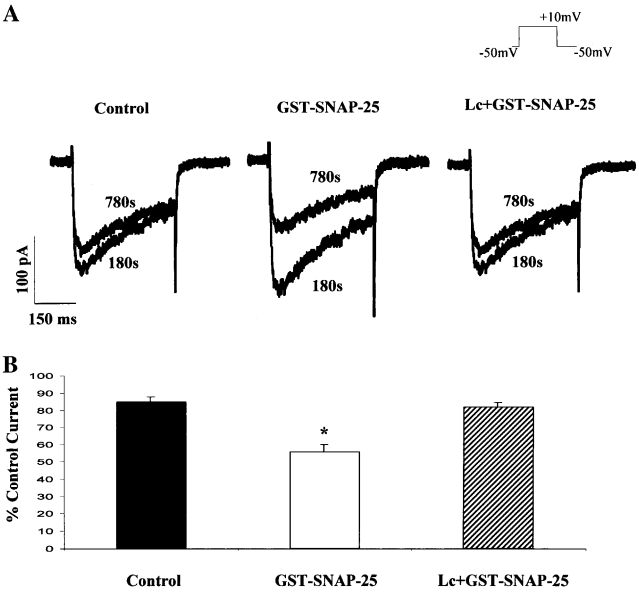

Fig. 2. Effect of GST–SNAP-25 on voltage-gated L-type calcium channel currents in feline smooth muscle cells. Cells dialyzed with 10^{-9} M GST alone as control caused slight inhibition of L_{Ca} currents by 780 s (10 min). Dialysis of 10^{-8} M GST–SNAP-25 via patch pipette caused a significant reduction of L_{Ca} current with recording time. Cells dialyzed with 10^{-6} M $Lc_{753-893}$ together with 10^{-7} M GST–SNAP-25 display only slight run-down in L_{Ca} similar to control. Dotted lines are the zero current level. $^*P < 0.05$ was considered statistically significant.

Discussion

A complex pattern of Ca^{2+} mobilization regulates the motility of the circular smooth muscle cells in the esophagus. These pathways for calcium mobilization include the release of Ca^{2+} from intracellular stores and Ca^{2+} influx through receptor-operated and voltage-gated Ca^{2+} channels [22,23]. In response to different stimuli, these various pathways are recruited to raise intracellular-free Ca^{2+} concentrations ($[Ca]_i$) which subsequently mediate smooth muscle contraction. Voltage-dependent Ca^{2+} channels have been identified in virtually all smooth muscles and play a key role in initiating contraction [24]. In the esophagus, inward calcium currents have been studied using patch clamp techniques in rabbit esophageal muscularis mucosae [25] and esophageal circular smooth muscle from the opossum [26]. In the cat, though inward calcium currents have been demonstrated [27], functional and molecular data demonstrating calcium channel sub-type have been lacking. In this study we combined electrophysiological

and biochemical assays to demonstrate the presence of L-type calcium channels of the C-type in the circular smooth muscle of the feline esophagus (Fig. 1).

Our previous histochemical studies showed that endogenous SNAP-25 is ideally situated in the plasma membrane where L_{Ca} and other ion channels reside [16]. In this study, we demonstrate for the first time that SNAP-25 can modulate Lc sub-type Ca^{2+} channel activity in non-secretory muscle cells. More specifically, SNAP-25 interacts with the voltage-gated L_{Ca} to limit Ca^{2+} entry, and hence has the potential to play an important role in smooth muscle contractility. For instance, L_{Ca} contributes to the action potential in most gastrointestinal smooth muscle including the esophagus [26] and also plays a role in refilling intracellular calcium stores. In the opossum esophagus, a modest inhibition of L_{Ca} of only 20% was found to be enough to impair spike generation [26]. In our studies, dialysis of a small amount of SNAP-25 into a cell through the patch pipette inhibited L_{Ca} by approximately 47%. This inhibition is similar to the inhibition of the large-conductance Ca^{2+}-activated K^+ channels [17] and delayed rectifier K^+ channels [17] by SNAP-25 when assessed in the same manner. The inhibition of K^+ channels was shown to be physiological as indicated by an increase in the channel currents when the SNAP-25 was cleaved by Botulinum neurotoxin A. It is likely therefore that SNAP-25 also plays a physiological role in modulating action potential amplitude and duration through its action on L_{Ca}, and hence in regulating smooth muscle contraction.

We show that this inhibitory effect of SNAP-25 was related to its direct interaction with the $Lc_{753–893}$ cytoplasmic domain corresponding to the II and III intracellular loop of the α1C-subunit of the L_{Ca}. The L_{Ca} peptide corresponding to this domain, when dialyzed with GST–SNAP-25, resulted in complete blockade of SNAP-25 inhibition of L_{Ca}, even when the SNAP-25 concentration was 10 times greater than that of SNAP-25 used alone. Indeed, this L_{Ca} domain, conserved in neuronal Ca^{2+} channels, is the precise binding domain to SNAP-25 [28]. These findings are also consistent with findings in pancreatic β cells where intracellular application of the Lc peptide together with SNAP-25 resulted in blockade of SNAP-25 inhibition of L_{Ca} [12]. In the latter report, we also found that the SNAP-25 protein possesses positive and negative regulatory domains in the regulation of islet β cell Ca^{2+} channels. This raises the possibility that specific domains within SNAP-25 may as well have distinct positive and negative regulatory actions on the L_{Ca} of esophageal smooth muscle. Syntaxin-1 is the cognate SNARE partner of SNAP-25 which modulates many of the same target proteins as SNAP-25, and is also present in the plasma membrane of esophageal circular smooth muscle cells [16]. Syntaxin-1A is able to complex with SNAP-25 to bind and regulate neuronal Ca^{2+} channels [11,28] and the delayed rectifier K^+ channel K_V 1.1 [14,15,29]. As noted above, in esophageal smooth muscle cells, both K_{Ca} and K_V currents are inhibited by SNAP-25 [17]. As K_{Ca} has been shown to limit excitation in esophageal tissue [30], and K_V currents contribute to resting membrane potential [31,32], SNAP-25 regulation of these K^+ channels holds the potential to modulate membrane excitability at several points. Whereas both SNAP-25 and syntaxin-1A have independent inhibitory effects on these Ca^{2+} and K_V channels in other tissues, their combination showed a distinct positive regulatory effect on the Ca^{2+} channels [11]. That is, it seems that distinct domains within each SNARE protein and additional conformations conferred when they are in complex would have profound and distinct regulatory actions on both Ca^{2+} and K_V channels. This provides extreme versatility in mediating the plasticity of membrane excitability. However, further studies are needed to elucidate the functional domains of each of these SNARE proteins and their complex interactions in regulating the membrane ion channels. In this context, the effects of SNAP-25 on L_{Ca} and K_V currents in esophageal smooth muscle cells raise interesting new possibilities for therapeutic targets to treat a number of esophageal motility disorders.

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