Regional differences in cholinergic regulation of potassium current in feline esophageal circular smooth muscle

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Muinuddin, Ahmad, Khurram Naqvi, Laura Sheu, Herbert Y. Gaisano, and Nicholas E. Diamant. Regional differences in cholinergic regulation of potassium current in feline esophageal circular smooth muscle. Am J Physiol Gastrointest Liver Physiol 288: G1233–G1240, 2005. First published February 3, 2005; doi:10.1152/ajpgi.00310.2004.—Potassium channels are important contributors to membrane excitability in smooth muscles. There are regional differences in resting membrane potential and K⁺-channel density along the length of the feline circular smooth muscle esophagus. The aim of this study was to assess responses of K⁺-channel currents to cholinergic (ACh) stimulation along the length of the feline circular smooth muscle esophageal body. Perforated patch-clamp technique assessed K⁺-channel responses to ACh stimulation in isolated smooth muscle cells from the circular muscle layer of the esophageal body at 2 (distal)- and 4-cm (proximal) sites above the lower esophageal sphincter. Western immunoblots assessed ion channel and receptor expression. ACh stimulation produced a transient increase in outward current followed by inhibition of spontaneous transient outward currents. These ACh-induced currents were abolished by blockers of large-conductance Ca²⁺-dependent K⁺ channels (BKCa), Distal cells demonstrated a greater peak current density in outward current than cells from the proximal region and a longer-lasting outward current increase. These responses were abolished by atropine and the specific M3 receptor antagonist 4-DAMP but not the M1 receptor antagonist pirenzepine or the M2 receptor antagonist methoctramine. BKCa expression along the smooth muscle esophagus was similar, but M1 receptor expression was greater in the distal region. Therefore, ACh can differentially activate a potassium channel (BKCa) current along the smooth muscle esophagus. This activation probably occurs through release of intracellular calcium via an M3 pathway and has the potential to modulate the timing and amplitude of peristaltic contraction along the esophagus.

esophagus; acetylcholine; potassium channel

Regional differences in neural influence have been described along the circular smooth muscle esophageal body (EB), including the excitatory (cholinergic) influence most prominent proximally (5, 8–10, 12, 30), and the inhibitory (nitroglycerine-nitric oxide) influence most active distally (2, 11, 40, 41). Hence, peristaltic contractions are considered to be the result of the local muscle responding passively to neural signals. However, when adequately stimulated in the face of neural blockade (with tetrodotoxin), single or repetitive contractions can traverse the smooth muscle (SM) esophagus at velocities similar to primary peristalsis (13, 14, 26). Therefore, in the esophagus, as in other regions of the gastrointestinal tract, myogenic properties are present that could contribute to the regulation of muscle contraction patterns. Indeed, regional differences in muscle properties have also been described and hold the potential to contribute to these gradients in excitatory and inhibitory neural influences. In support of this hypothesis, it has previously been found that 1) the resting membrane potential of the circular muscle is progressively more depolarized toward the distal esophagus (28); 2) circular muscle cells from the proximal region of the esophagus possess a higher density of large-conductance Ca²⁺-activated K⁺ channel (BKCa) current and delayed rectifier K⁺-channel (KDR) current (28); 3) there is a greater L-type calcium-channel current density in the proximal smooth muscle esophagus (18); and 4) proximal esophageal muscle develops significantly higher tension with stretch and in response to cholinergic stimulation even in the face of neural blockade (18, 19).

Potassium channels are important contributors to membrane excitability in smooth muscle cells. In feline, opossum, and human esophageal smooth muscles, the outward currents consist primarily of K⁺ currents including a transient outward current (KTo), KDR, and BKCa (1, 15, 28, 36). Blockade of the BKCa has been shown to increase the amplitude of ACh-induced contraction in human esophageal circular SM (15). Hence, activation of BKCa could serve to limit the duration of excitation in esophageal muscles (15).

In light of the differential cholinergic influence along the SM-EB and the central role of BKCa in contributing to membrane excitability, the aim of this study was to assess responses of potassium channel currents to cholinergic stimulation in the proximal and distal regions of the feline esophageal SM-EB through 1) electrophysiological assessment of the currents on stimulation and 2) assessment of the BKCa and M3 cholinergic receptor expression in these regions. A portion of this study has been published elsewhere in abstract form (20).

METHODS

Tissue preparation and cell dissociation. The experimental protocol was approved by the University Health Network Animal Care Committee following the guidelines of the Canadian Council on Animal Care. SM cells were isolated from circular SM of feline esophagus following a previously described protocol (28) with minor modifications. Briefly, adult cats of either sex were euthanized by intravenous injection of overdose pentobarbital sodium. The esophagus was quickly excised and placed in Krebs solution. After the mucosa and most of the submucosa were stripped off, circular muscle was dissected out and cut into squares of ~2 mm². These squares were placed in a test tube with 1 ml dissociation solution composed of

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nuclear fragments were removed by low-centrifuge spins (1000 g/ml leupeptin, 5 g/ml antipapain, and 5 mg/ml aprotinin A (pH 7.0), containing 600 mM sucrose, 50 mM MOPS, 0.1 mM phenylmethylsulfonyl fluoride, and 5.5 glucose (pH 7.0). Papain (2 mg/ml) as well as collagenase blends F and G (1:200), which is a polyclonal antibody (Research & Diagnostic Antibodies, Benicia, CA) or BKCa α1-subunit antibody (1:200), which is a polyclonal antibody (Alomone Labs, Jerusalem, Israel). Detection was made possible by enhanced chemiluminescence (Amersham, Arlington Heights, IL), and the specific bands were quantified by densitometric scanning of the blots using Image Pro 5 software (Media Cybernetics, Silver Spring, MD).

Immunoblotting. Membrane protein samples were prepared from the circum muscle layer of distal and proximal sites as previously described (25, 39). 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 mM sucrose, 50 mM MOPS, 0.1 mM 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 muscle 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. The protein concentration was determined by the Bio-Rad method using bovine serum albumin as a standard. Equivalent amounts of total protein (25 µg) from five tissue samples were electrophoretically size-separated on a 12% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Subsequently, the membranes were incubated for 2 h at room temperature with a rabbit anti-M₃(1:9,000), which is a polyclonal antibody (Research & Diagnostic Antibodies, Benicia, CA) or BKCa α₁-subunit antibody (1:200), which is a polyclonal antibody (Alomone Labs, Jerusalem, Israel). Detection was made possible by enhanced chemiluminescence (Amersham, Arlington Heights, IL), and the specific bands were quantified by densitometric scanning of the blots using Image Pro 5 software (Media Cybernetics, Silver Spring, MD).

Application of agents by picospritzer. In the patch-clamp recording chamber two methods were employed to test the effect of different agents. Bulk addition of the agent to the recording chamber allowing time for the bath to equilibrate to the final concentration and application of ACh by pico-spritzer (3 lb/in²) directly onto the muscle cell through a pipette positioned 50 µm away from the cell (16, 32) to study the transient induced membrane ion channel events. Studies with only external solution in the pipette solution confirmed no significant artifacts or contribution of mechanical spritzing force on membrane currents.

Drugs. Acetylcholine, atropine, 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP), methoctramine, pirenzipine, and all other chemical reagents were obtained from Sigma (St. Louis, MO). Iberiotoxin and dendrotoxin were purchased from Alomone Labs and prepared in ddH₂O. Acetylcholine was prepared fresh as a 10 mM stock in ddH₂O and diluted in external solution.

Statistical analysis. Data are presented as means ± SE. A Student’s t-test was used to compare data between two groups (using Instat Graph Pad software Version 3). P < 0.05 was considered to reflect significant difference. In all of these studies, cells are representative of three or more feline esophageal specimens.

RESULTS

Freshly isolated esophageal circular SM cells were spindle-shaped and appeared phase bright under phase-contrast microscopy. Application of ACh (100 µM, 1 s) by picospritzer micropipette placed 50 µm away from the center of the cell caused reversible contraction in these cells (n = 20; Fig. 1) indicating that the cells were viable and had retained contractile function after enzymatic digestion.

Effect of ACh on membrane currents and membrane potential. In voltage-clamp mode, with the holding potential (HP) set more positive than −50 mV, spontaneous transient outward currents (STOCs) were observed in muscle cells from both proximal and distal regions (Fig. 2A) as previously described (32). The amplitude of these STOC events increased as the HP was set to more depolarized potentials (n = 6). In current-clamp mode, spontaneous hyperpolarizations were routinely observed (Fig. 2B) under the same control conditions. Resting membrane potential of cells from the distal circular SM region were more depolarized than proximal cells (−49.5 ± 2.5 vs. −41.7 ± 1.2, n = 10 each region, P < 0.05). In voltage clamp, at HP = −30 mV, when ACh (100 µM) was applied for 1 s by picospritzer, the cell responded to ACh with a transient increase in outward currents followed by inhibition of STOCs (n = 14; Fig. 3A). STOCs gradually returned after several minutes of perfusion in external solution. This response was abolished in the presence of the general muscarinic receptor antagonist atropine (1 µM; n = 10) as well as the specific M₃ receptor antagonist 4-DAMP (0.1 µM; n = 5). Neither the

![Control](https://via.placeholder.com/150) ![+ ACh](https://via.placeholder.com/150)

Fig. 1. Effect of ACh on length of relaxed single circular smooth muscle cells. Cells studied were spindle shaped and appeared phase bright under phase-contrast microscopy. Application of ACh (100 µM, 1 s) by picospritzer micropipette placed 50 µM away from the center of the cell caused contraction in all cells tested.
specific M1 antagonist pirenzipine (0.1 μM; n = 5) nor the M2 receptor antagonist methoctramine (0.1–1.0 μM; n = 5) had any significant inhibitory effect. In current clamp, application of ACh caused initial hyperpolarization of the cell followed by gradual and variable depolarization of between 5 and 15 mV (n = 10; Fig. 3B). The membrane potential usually returned to resting levels within 5 min.

Identification of ACh-induced potassium currents. In voltage clamp at HP = −30 mV, outward baseline current and STOCs were present in both proximal and distal cells (Fig. 4A). ACh

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**Fig. 2.** Spontaneous transient outward currents (STOCs) recorded from circular esophageal muscle cells (2 cm above lower esophageal sphincter) in voltage clamp (A) at holding potential (HP) = −50 mV. STOCs lasted 50–150 ms, and the shape varied considerably. B: in current clamp, spontaneous transient hyperpolarizations occur frequently.

**Fig. 3.** Typical response of membrane currents and membrane potential to cholinergic stimulation. A: in voltage clamp mode at HP = −30 mV, the cell responded to ACh (100 μM, 1 s) with increase in outward K⁺ currents followed by inhibition of STOCs. STOCs gradually returned after several minutes of perfusion. B: in current-clamp mode, spontaneous hyperpolarizations were routinely observed. Application of ACh caused initial hyperpolarization of the cell followed by gradual depolarization and suppression of spontaneous hyperpolarizations.

**Fig. 4.** ACh activates large-conductance Ca²⁺-dependent K⁺ channels (BKCa). A: in voltage clamp at HP = −30 mV outward baseline current and STOCs were present. ACh transiently activated the outward K⁺ current and this activation was followed by inhibition of STOCs. B: in the presence of the selective BKCa antagonist iberiotoxin (200 nM; n = 5), baseline outward current and STOCs were inhibited; stimulation with ACh did not result in activation of outward current.
transiently activated the outward potassium current, and this activation was followed by inhibition of STOCs. As shown in the inset, the current present was noisy at higher potentials. In the presence of the selective BKCa antagonist iberiotoxin (IbTX; 100 nM), baseline outward current and STOCs were inhibited (Fig. 4B). Stimulation with ACh did not result in activation of outward current in the presence of IbTX (Fig. 4B).

Regional differences in ACh-induced BKCa currents. Outward BKCa currents were activated in response to cholinergic stimulation at lower (−30 mV) and high (0 mV) holding potentials (Fig. 6A). The current density of ACh-induced BKCa currents was greater in the distal esophagus at HP more positive than −30 mV (Fig. 6, A and B). At HP = −30 mV, the current density in distal vs. proximal cells was 9.8 ± 2.5 vs. 4.35 ± 0.9 pA/pF (Fig. 6B, n = 5, P < 0.05). Latency to onset of the increase in outward current in response to ACh stimulation was similar in cells from both regions (Fig. 7B). Similarly, the latency to reach peak outward current was not significantly different in cells from both regions in response to ACh (Fig. 7B). However, the distal esophagus exhibited a longer-lasting effect of the ACh-induced outward current increase (time to decay to half of the peak amplitude 1,687 ± 320 vs. 839 ± 194 ms, n = 5, P < 0.05; Fig. 7).

BKCa α-subunit expression in EB. BKCa channel expression along the EB was examined to help explain the regional differences in ACh-induced BKCa current density. Western blot analyses revealed the α-subunit of BKCa to be at ~125 kDa (Fig. 8A), and this location is consistent with the size reported for the α-subunit of BKCa in other gastrointestinal SM preparations (17). When normalized to α-actin, there were no significant differences in BKCa α-subunit expression between the proximal and distal regions of the circular SM esophagus (n = 4, P > 0.05; Fig. 8B).

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Fig. 6. Regional differences in ACh-induced BKCa current density. A: outward currents are activated in response to cholinergic stimulation at low (−30 mV) and high (0 mV) holding potentials. B: at HP = −30 mV, the current density as determined by peak amplitude of response normalized to cell capacitance was greater in distal cells than cells from the proximal region.

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Fig. 5. Inhibition of delayed rectifier K channel (KDR) does not inhibit ACh-induced outward K+ current. A: in control cells at HP = −30 mV, ACh transiently activated the outward K+ currents. B: in the presence of 4-AP (5 mM; n = 5), there is no inhibition of ACh-induced outward currents, confirming that the outward current activated by ACh is BKCa.
Muscarinic receptor expression in EB. Muscarinic receptor expression along the EB was also examined to help explain the regional differences in ACh-induced BKCa current activity. Western blot analyses revealed the M3 receptor to be at ~113 kDa (Fig. 9A) and was confirmed by incubation with peptide. This is consistent with the size reported for M3 in other muscle preparations (38). When normalized to α-actin, M3 receptor was expressed more abundantly in the distal SM than in the proximal esophageal tissues (n = 5, P < 0.05; Fig. 9B). With the antibody used, Western blot analyses were unable to specifically detect M2 receptor in 15 tissue preparations tested. Hence, we were unable to assess regional variation in the expression of M2 receptors in feline esophageal tissues.

DISCUSSION

These studies demonstrate regional differences in the activation of BKCa along the SM-EB by ACh. We show that 1) ACh activates BKCa via an M3 receptor-mediated pathway leading to membrane hyperpolarization, 2) there is a greater current density of ACh-induced BKCa current in the distal than in the proximal esophagus, 3) ACh-induced BKCa current takes longer to return to baseline in distal cells, and 4) there is a greater muscarinic M3 receptor expression in circular muscle from the distal region compared with that in the proximal region, whereas BKCa expression is similar in both regions.

BKCa channels are present in almost all cells and play an important role in regulating membrane excitability. BKCa is considered to play a role in repolarization of the membrane of SM following action potentials (7, 37) and more recently to cause relaxation of arteriolar SM (22). In the circular SM of the LES, continuous activation of BKCa by nitric oxide (NO) is believed to modulate LES muscle cell membrane potential and excitability (29). Localized elevation of Ca2+ activates BKCa leading to hyperpolarization of cells, reduction of Ca2+ entry by L-type calcium channels, and lowering of resting muscle tone. NO has also been shown to activate BKCa in many gastrointestinal tissues including circular SM from the esophagus (21). Hence, BKCa channels can participate in both regulation of membrane potential and have an inhibitory effect on contraction during excitation of SM.
In this study the effect of cholinergic stimulation on \(K^+\) currents was examined in circular SM cells from the feline esophagus. Previous studies (28) in our laboratory have characterized the outward \(K^+\) currents in these SM cells to be comprised of primarily BKCa and KDR. Both low-dose tetraethylammonium and IbTX inhibited cholinergic activation of the outward currents, whereas 4-AP had no significant effect, hence demonstrating that ACh was acting primarily on BKCa at the holding potentials employed in this study. Our present results are therefore consistent with earlier studies in demonstrating activation of BKCa by ACh in feline and human esophageal circular SM (15, 32).

We also demonstrate that this cholinergic activation of BKCa occurs via an M3-signaling pathway, because both atropine and the specific M3 receptor antagonist 4-DAMP inhibited BKCa activation by ACh, whereas inhibitors of M1 and M2 receptors did not. In circular SMs from the feline, M3 receptors are believed to be coupled to phospholipase C, stimulating the production of inositol 1,4,5-trisphosphate (IP3), which triggers the release of calcium from intracellular stores (3, 4, 24, 25, 33, 34). Hence, the increase in BKCa induced by ACh probably occurs through release of intracellular \(Ca^{2+}\) from stores via an M3 pathway (31).

We initially hypothesized that ACh would cause a greater increase in BKCa in the proximal esophagus as a greater BKCa current density has been previously reported in this region (28). On the contrary, the main findings in the present work demonstrate that ACh activates BKCa to a greater degree in distal than proximal sites as demonstrated by current density and time to decay of outward current. In this study, we also show that M3 receptor expression is greater in the distal circular SM esophagus compared with the more proximal region. Hence, a greater BKCa response in the distal region may be due to greater M3-IP3 pathway activation (6, 31) resulting in greater release of \(Ca^{2+}\) from intracellular \(Ca^{2+}\) stores rather than being dependent only on the BKCa current density inherent to each region (28). However, the present study cannot rule out the possibility of differences in either calcium store capacity or uptake between proximal and distal segments that may also impact on BKCa activation.

As BKCa has been shown to limit excitability in human esophageal tissues (15), greater BKCa activation in the distal region by ACh may serve to have a greater limiting effect on excitation in the distal tissue. This effect could potentially operate in two ways. First, this activation could increase latency before onset of contraction (2). The BKCa activation and membrane hyperpolarization occurs almost immediately and is followed by membrane depolarization. This series of events, membrane hyperpolarization followed by depolarization and contraction, has been reported to occur with swallow-induced peristalsis (23, 27, 35). The hyperpolarization and increasing delay to onset of contraction distally are usually attributed to the release of the main inhibitory neurotransmitter NO (2, 40, 41) and, as noted above, NO can activate BKCa in circular SM from the esophagus (21). As the main excitatory neurotransmitter in the esophagus (5), it is interesting that ACh

Fig. 9. Expression of M3 muscarinic receptor in circular smooth muscle from EB by immunoblotting. A: M3 was present at molecular mass of 113 kDa. As shown on the right, the dominant band was suppressed by adsorption of the antibody with the immunogen peptide. B: densitometric analysis of Western blot normalized to \(\alpha\)-actin expression demonstrated that M3 was expressed more abundantly in the distal smooth muscle than the proximal smooth muscle esophageal tissues. (\(n = 5, P < 0.05\)).
activation of BK<sub>Ca</sub> could also potentially serve to increase delay, the delay most marked distally. Together with the release of NO, both neurotransmitters appear programmed to act to limit initial excitation with increasing influence distally. That is, the cholinergic influence has the potential to contribute to the distal movement of the peristaltic contraction through its potential to progressively increase the delay to onset of the contraction. Further experiments in the intact tissue and animal are required to establish this complementary activity. Second, the increased BK<sub>Ca</sub> activation distally could serve to also reduce contraction amplitude and duration (40). Presumably, the action of the BK<sub>Ca</sub> to hyperpolarize the membrane could act to reduce Ca<sup>2+</sup> influx through the voltage-gated L-type Ca<sup>2+</sup> channel and to provide a further mechanism for the decrease in contraction amplitude seen distally in the feline EB (18, 19, 40).

In summary, this study reports regional differences in cholinergic regulation of BK<sub>Ca</sub> by electrophysiological techniques. In addition, we show differential expression of muscarinic receptors in the circular SM portion of the EB. These differences in BK<sub>Ca</sub> activation by ACh and muscarinic receptor expression may allow for differential muscular responses to innervation and varied muscular contribution to esophageal contractility.

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