Role of cADPR in sodium nitroprusside-induced opossum esophageal longitudinal smooth muscle contraction


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Campbell RK, Wells RW, Miller DV, Paterson WG. Role of cADPR in sodium nitroprusside-induced opossum esophageal longitudinal smooth muscle contraction. Am J Physiol Gastrointest Liver Physiol 292: G1543–G1548, 2007. First published February 15, 2007; doi:10.1152/ajpgi.00111.2006.—Nitric oxide (NO) relaxes most smooth muscle, including the circular smooth muscle (CSM) of the esophagus, whereas in the adjacent longitudinal smooth muscle (LSM), it causes contraction. The second messenger pathways responsible for this NO-induced LSM contraction are unclear, given that these opposing effects of NO are both cGMP dependent. In intestinal LSM, but not CSM, cADP ribose (cADPR)-dependent pathways participate in Ca\(^{2+}\) mobilization and muscle contraction; whether similar differences exist in the esophagus is unknown. The purpose of this study was to determine whether cADPR plays a role in the NO-mediated contraction of opossum esophageal LSM. Standard isometric tension recordings were performed using both LSM and CSM strips from opossum distal esophagus that were hung in 10-ml tissue baths perfused with oxygenated Krebs solution. cADPR produced concentration-dependent contraction of LSM strips with an EC\(_{50}\) of 1 nM and peak contraction of 57% from initial resting length. NO-induced LSM contraction was abolished by the cADPR antagonist 8-bromo-cADPR and 8-amino-cADPR, as well as ryanodine receptor antagonists ryanodine and tetracaine, significantly inhibited the SNP-induced contraction. In conclusion, in the opossum esophagus, 1) cADPR induces contraction of LSM, but not CSM, and 2) NO-induced contraction of LSM appears to involve a cADPR-dependent pathway.

Most research on esophageal physiology and pathophysiology has focused on the lower esophageal sphincter (LES) and circular smooth muscle (CSM) of the esophageal body. This is not surprising given the importance of the LES in preventing acid reflux and the critical role of the CSM in peristaltic contractions. In contrast, relatively little attention has been directed toward the external longitudinal smooth muscle (LSM) layer of the esophagus. Studies performed in recent years have suggested that LSM dysfunction may contribute to impaired bolus transit (20) and esophageal chest pain (1). Furthermore, intraluminal acid can induce sustained contraction of LSM and esophageal shortening of both the opossum (18, 19, 26) and human esophagus (6), which might contribute to the etiology of hiatus hernia. Once formed, the hernia will then contribute to ongoing episodes of gastroesophageal reflux (2, 15, 16). To understand the role of esophageal LSM in the pathophysiology of certain esophageal disorders, a better understanding of the normal function of this muscle layer is required.

Little is known about the specific second messenger pathways responsible for eliciting esophageal LSM contraction. It has been reported that intestinal LSM and CSM utilize markedly different Ca\(^{2+}\) mobilizing pathways for the initial phase of agonist-induced contractions (10, 11, 14, 17). In both muscle layers, most agonists activate G protein-coupled receptors. In CSM, this leads to the formation of phospholipase C (PLC), which catalyzes the cleavage of phosphoinositide (PI) to eventually form inositol 1,4,5-trisphosphate (IP\(_3\)). IP\(_3\) is capable of mobilizing Ca\(^{2+}\) from the sarcoplasmic reticulum (SR), which leads to the activation of contractile proteins. Ca\(^{2+}\) mobilization in LSM involves a distinct PI-independent pathway. Receptors for contractile agonists in intestinal LSM cells are coupled via a pertussis toxin-sensitive G protein that leads to the activation of PLA\(_2\) and formation of arachidonic acid. It is believed that arachidonic acid activates chloride (Cl\(^{-}\)) channels, leading to depolarization of the cell via efflux of Cl\(^{-}\) (14). This activates voltage-sensitive Ca\(^{2+}\) channels, allowing Ca\(^{2+}\) influx as well as the stimulation of Ca\(^{2+}\) release from the SR via ryanodine receptors (Ca\(^{2+}\)-induced Ca\(^{2+}\) release). It has been proposed that the Ca\(^{2+}\)-mobilizing molecule cADPR ribose (cADPR), which had been demonstrated to function in intestinal LSM but not CSM (10, 11), may be responsible for Ca\(^{2+}\)-induced Ca\(^{2+}\) release from SR stores following agonist binding.

An important and puzzling difference between esophageal LSM and CSM is that nitric oxide (NO) contracts LSM and relaxes CSM, yet these opposing effects are both cGMP dependent (22, 30). Previous work from our laboratory (30) has shown that the esophageal LSM contraction induced by NO donors is antagonized by 1H-[1,2,4]oxidiazolo[4,3-α]quin-oxalin-1-one, L-type Ca\(^{2+}\) channel blockers, or Cl\(^{-}\) channel blockers, which is consistent with the mechanism of agonist-induced contraction proposed for intestinal LSM (14). It remains unclear how cGMP triggers an excitatory pathway in esophageal LSM, given that cGMP activation is usually associated with eliciting relaxation in smooth muscle. Protein kinase G (PKG) has been thought to mediate the relaxant effect through a variety of mechanisms that lead to a decrease in intracellular Ca\(^{2+}\) (14). Although it remains unknown why cGMP activation by NO in esophageal LSM does not lead to...
similar PKG-mediated events, it has been noted that PKG leads to activation of cADPR and Ca\(^{2+}\) release in sea urchin eggs (8, 28). Given this background, it is possible that in esophageal LSM, PKG activates cADPR (which we hypothesize is not active in CSM), which then induces Ca\(^{2+}\) mobilization and cell contraction.

The aims of this study were to elucidate whether 1) similar to intestinal LSM, contraction of esophageal LSM involves the cADPR Ca\(^{2+}\)-mobilizing pathway; and 2) cADPR plays a role in the NO-induced contraction of esophageal LSM.

**METHODS AND MATERIALS**

**Animal Preparation**

The protocol was approved by the Animal Care Committee of Queen’s University, in accordance with the regulations of the Canadian Council on Animal Care. Adult opossums (*Didelphis virginiana*) of either sex, weighing between 2 and 7.3 kg (North Eastern Wildlife, South Plymouth, NY), were fasted for 12 h before experimentation but were allowed free access to water at all times. Anesthesia was induced at the beginning of each experimental day via a tail vein injection of 40 mg/kg body wt of Somnotol. A pediatric cuffed endotracheal tube was placed and inflated to prevent aspiration, and the animal was ventilated using a Harvard Rodent Ventilator (model 63; Ealing Scientific, St. Laurent, PQ, Canada) at bodyweight-appropriate rate and tidal volume according to the manufacturer’s instructions. The thoracic cavity was then exposed via midline sternotomy, and the distal esophagus (8–10 cm) along with cuff of proximal stomach was excised after its resting in vivo length was measured. The animal was then killed by an intracardiac injection overdose of Somnotol.

**Muscle Strip Experiments**

**Tissue preparation.** The esophagus was pinned out in a silicon-coated-bottom dissecting dish at its measured in vivo length and immersed in oxygenated (95% O\(_2\)-5% CO\(_2\)) Krebs solution maintained at 35°C and pH 7.4. The connective tissue surrounding the esophagus was removed using fine dissecting scissors. It was then opened longitudinally and pinned out with the mucosa side up. The mucosa and submucosa were removed by sharp dissection to expose the underlying smooth muscle layers. Paired circular and longitudinal muscle strips (10 × 5 mm) were obtained by cutting along the transverse and long axis of the esophagus, respectively, from 3–5 cm above the LES (26).

**Circular and longitudinal muscle mechanical recordings.** Paired circular and longitudinal muscle strips were hung in double-chambered organ baths containing 10 ml of Krebs solution (35°C) and gassed with 95% O\(_2\)-5% CO\(_2\). Silk ligatures secured one end of the strip to a hook within the bath and the other end to a force transducer (Myograph F-2000; Narco Bio-Systems) used to record isometric tension. Outputs from each of the transducers were displayed on an IBM-compatible personal computer using Windaq/200 data acquisition software (DataQ Instruments). With the use of a micromanipulator, the force transducers were carefully adjusted to stretch the strips to ~150% of initial length. This was followed by an equilibration period of 1 h, with the Krebs solution being replaced every 15 min. Muscle strip viability was determined by assessing the contractile response to a 5-min application of Krebs solution that had been modified to contain 60 mM KCl. Subsequent washings with regular Krebs solution occurred at 5-min intervals for 30 min to ensure complete washout of the 60 mM KCl solution and to allow the tissue to achieve a stable baseline tension. Strips that displayed no or minimal response (less than a 20% increase from baseline tension) or ripped upon application of 60 mM KCl were omitted from the study.

**Experimental protocol.** Cumulative concentration-response curves for CSM and LSM to cADPR were obtained at bath concentrations from 1 × 10\(^{-12}\) to 1 × 10\(^{-6}\) M. A 15-min interval was allotted in between applications of the next concentration, and concentrations were added in a sequential fashion from lowest to highest. Responses to cADPR were depicted as percentages of the maximal response produced by 60 mM KCl. The NO donor sodium nitroprusside (SNP; 300 μM) was incubated with CSM and LSM strips for 15 min (30) to establish a control response to this agent. At this point, the tissues were washed with Krebs solution at 5-min intervals for 20 min. The cADPR antagonists 8-bromo-cADPR (8-Br-cADPR; 100 μM) and 8-amino-cADPR (4 μM) and the ryanodine receptor antagonists ryanodine (20 μM) and tetracaine (100 μM) were then added to LSM bath preparations and allowed to incubate for 1 h. These concentrations had previously been shown to provide optimal inhibition of cADPR (10, 24) and the ryanodine receptor (12), respectively, in similar smooth muscle preparations. After incubation, another application of 300 μM SNP was added. Control experiments were also performed in which responses to SNP before and after addition of the respective vehicles (distilled water for 8-Br-cADPR, 8-amino-cADPR, and tetracaine; dimethyl sulfoxide for ryanodine). Because there was no significant difference between the effects of the two different vehicles on the SNP response, these control experiments were pooled. Initial and postantagonist applications of SNP were expressed as percentages of the maximal response produced by 60 mM KCl. In a similar fashion, the effect of 8-Br-cADPR on carbachol (1 μM)-induced contractions was determined to exclude nonspecific actions of this antagonist.

**Single Cell Experiments**

**LSM and CSM cell isolation.** A modified version of a previously described method (3, 4, 23) was used to isolate LSM and CSM cells. Briefly, the esophagus was removed, and a segment 3–5 cm proximal to the LES was excised, placed in Krebs solution, and pinned out. With sharp dissection, the mucosa and submucosa were removed, and the CSM was separated from the LSM layer. Once isolated, the smooth muscle layer was cut into 1 × 0.5-cm pieces and placed into enzymatic solution containing papain (0.5 mg/ml), bovine serum albumin (1 mg/ml), 1,4-dithio-D,L-threitol (1 μM), and collagenase type F (1 mg/ml for CSM, 2.5 mg/ml for LSM) dissolved in HEPES physiological salt solution-digestion formula (in mM: 125 NaCl, 10 glucose, 10 Na-HEPES, 1 MgCl\(_2\), 4 KCl, 1 CaCl\(_2\), 0.25 EDTA, and 10 taurine, at pH 7.4) for 2 h at 37°C. The beakers were then superfused with 100% O\(_2\) at room temperature (22°C) for 20 min and subsequently incubated at 31°C for 10 min. The solution was poured over a nylon filter (200-μm pore diameter) and rinsed with Dulbecco’s modified Eagle’s medium (DMEM) to remove excess enzyme solution. The tissue was resuspended in 5 ml of DMEM and gently agitated until the cells dispersed. Finally, this solution was filtered through nylon mesh (500-μm pore diameter) to remove undigested tissue and was stored at 5°C until experimentation.

**Single cell experimental protocol.** An aliquot of isolated CSM or LSM cells was placed on a 35-mm glass-bottom dish and viewed on an inverted phase-contrast microscope (Olympus IMT-2) connected to a closed-circuit video camera. The cells were constantly perfused with Krebs solution at a rate of 2 ml/min. As a control for cell contractility, ACh (1 μM in Krebs solution) was applied to individual cells by pressure application via a puffer pipette (500-ms application at 10 lb./fin.\(^2\) through a 1 MΩ resistance tip), which was micromanipulated to within 200 μm of the cell. Subsequent responses of a new aliquot of cells to cADPR (1 nM) were captured using the Image Pro-5 analysis program (Media Cybernetics, Carlsbad, CA) at a rate of 10 Hz. Videos of the responses were saved, coded, and analyzed by an observer who was unaware of the drug application used. Length measurements of cells were obtained by manually

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tracing through the midline of the cell using the Image Pro-5 image-analysis program.

Data Analysis

Esophageal LSM and CSM contractile responses induced by cADPR and SNP were expressed as percentages of the response obtained from application of 60 mM KCl. The data are expressed as means ± SE, with n referring to the number of animals. All data comparisons were statistically analyzed using either ANOVA or the paired Student’s t-test where appropriate. A P value of <0.05 was considered significant.

Solutions and Drugs

Sodium pentobarbital (Somnotol) was obtained from Bimeda-MTC Pharmaceuticals (Cambridge, ON, Canada). The Krebs solution contained (in mM) 118 NaCl, 25 NaHCO3, 11 glucose, 4.75 KCl, 2.5 CaCl2, 1.2 MgSO4, and 1 NaH2PO4. The high-KCl Krebs solution contained (in mM) 63.75 NaCl, 60 KCl, 25 NaHCO3, 11 glucose, 2.5 CaCl2, 1.2 MgSO4, and 1 NaH2PO4. cADPR, 8-Br-cADPR, SNP, tetracaine, and all enzymatic solution chemicals were obtained from Sigma (Mississauga, ON, Canada), whereas 8-amino-cADPR and DMEM were obtained from Invitrogen (Burlington, ON, Canada), and ryanodine was from Molecular Probes (Eugene, OR).

RESULTS

Effect of cADPR on Opossum Esophageal LSM and CSM

cADPR produced concentration-dependent contraction of LSM muscle strips with an EC50 of ~1 nM (n = 7) and a peak contraction of 57 ± 18% of the KCl response (P < 0.05 for all cADPR concentrations between LSM and CSM). cADPR had no significant effect on CSM strips at concentrations up to 10^-6 M (Fig. 1). Experiments were conducted on isolated CSM and LSM cells to determine whether responses similar to those elicited by cADPR in muscle strips could be observed. In isolated LSM cells, ACh (1 μM) resulted in a mean contraction of 24 ± 2% (decrease in cell length; n = 4 animals at 4–7 cells per animal) with a maximal contraction of 46% (Fig. 2A). The effect of the cADPR EC50 on isolated LSM cells was significantly less than that of ACh but nevertheless caused a mean contraction of 19 ± 2% with a maximal contraction of 37% (n = 4 at 5–14 cells per animal; P < 0.05) (Fig. 2A). Representative images of isolated LSM cells responding to ACh and 1 nM cADPR, respectively, are depicted in Fig. 2B. In contrast, 1 μM ACh elicited a mean contraction of 18 ± 6% (n = 4 animals at 7–8 cells per animal) in isolated CSM cells (Fig. 3A) with a maximal observed contraction of 54%. The EC50 for cADPR (1 nM) caused a negligible (3 ± 1%) contraction of isolated CSM cells (n = 4 animals at 8–16 cells per animal), which was significantly less than the responses elicited by ACh in CSM cells and cADPR in LSM cells (Fig. 3A). However, of the 45 CSM cells exposed to cADPR, 6 exhibited contraction with the maximal peak contraction observed at 21%; these 6 cells were all from the same animal and likely represent contamination of the preparation by CSM cells. Representative images of isolated CSM cells responding to ACh and 1 nM cADPR are depicted in Fig. 3B.

Effect cADPR and Ryanodine Receptor Antagonists on SNP-Induced LSM Contraction

SNP (300 μM) induced contraction of LSM strips that averaged 66.7 ± 4.8% of the 60 mM KCl-induced contrac-
Student’s t in LSM strips than in CSM strips (n length, whereas cation of 1 60 mM KCl (n tissue strips, expressed as a percentage of the maximal response produced by and 8-Br-cADPR (100 4 animals). SNP elicited contraction was significantly greater (A) cADPR (1 nM) elicited minimal contraction of CSM cells (CSMC), and contraction was significantly less than contraction elicited by 1 μM ACh (n = 4 at 8–16 cells per animal and n = 4 at 7–8 cells per animal for cADPR and ACh experiments, respectively). *P < 0.05 by a paired Student’s t-test. B: typical images of isolated CSM cells; a and c represent images of cells at resting length, whereas b and d represent images of contraction elicited after application of 1 μM ACh and 1 nM cADPR, respectively.

Fig. 3: cADPR caused contraction of LSM but not CSM. Furthermore, selective antagonists of cADPR bound avidly to permeabilized LSM cells but not to CSM cells. This is consistent with the present observations in esophageal smooth muscle and suggests that CSM may lack the accessory protein required for interaction of cADPR with the ryanodine receptor. In muscle strip studies, we saw no significant increase in basal tension of any of our CSM preparations when cADPR was added at concentrations as high as 10 μM. Similar findings were observed in isolated LSM and CSM cells, where cADPR consistently caused phasic shorten-

Fig. 4: Effect of sodium nitroprusside (SNP; 300 μM) on CSM and LSM tissue strips, expressed as a percentage of the maximal response produced by 60 mM KCl (n = 4 animals). SNP elicited contraction was significantly greater in LSM strips than in CSM strips (n = 4 animals). *P < 0.05 by a paired Student’s t-test.

Fig. 5. Effect 8-amino-cADPR (4 μM; n = 5), 8-bromo-cADPR (100 μM; n = 7), ryanodine (30 μM; n = 5), and tetracaine (100 μM; n = 5) on LSM contraction induced by SNP (300 μM). The magnitude of contraction is expressed as a percentage of the 60 mM KCl-induced contraction. All antagonists significantly inhibited the SNP-induced contraction, whereas application of vehicle (n = 10) had no significant effect. Filled bars represent control responses, whereas open bars represent the responses following application of antagonist or vehicle. *P < 0.01 by a paired Student’s t-test.

DISCUSSION

The present study sought to confirm whether agonist-induced contraction of esophageal LSM involves the cADPR Ca2+-mobilizing molecule, the same second messenger pathway that has been shown to mediate intestinal LSM contraction (10, 11). Consistent with the studies in intestinal smooth muscle, cADPR elicited concentration-dependent contractions in LSM preparations, whereas it had no effect in CSM preparations. In isolated cells, cADPR produced comparable results obtained from muscle strips. Furthermore, selective antagonists of cADPR significantly inhibited the SNP-induced contraction in LSM muscle strips, suggesting that NO-mediated contraction in esophageal LSM is partly due to cADPR.

Previous studies on the mammalian intestine have demonstrated that the Ca2+ mobilization pathways mediating contraction in LSM and CSM are different (10, 14). Agonist-induced contraction of the CSM depends on the activation of PLC leading to the formation of IP3, which releases Ca2+ from SR stores. In contrast, agonist-induced contraction of the LSM is mediated in part through activation of cADPR, which releases Ca2+ from ryanodine-sensitive SR stores. Studies using single smooth muscle cells from rabbit small intestine revealed that cADPR caused contraction of LSM but not CSM. Furthermore, cADPR bound avidly to permeabilized LSM cells but not to CSM cells. This is consistent with the present observations in esophageal smooth muscle and suggests that CSM may lack the accessory protein required for interaction of cADPR with the ryanodine receptor. In muscle strip studies, we saw no significant increase in basal tension of any of our CSM preparations when cADPR was added at concentrations as high as 10−6 M. Similar findings were observed in isolated LSM and CSM cells, where cADPR consistently caused phasic shorten-
ing of LSM cells. In the vast majority of isolated CSM cells, application of cADPR had no detectable effect on cell length; however, in a small number of cells (6 of 45) cADPR did induce phasic shortening. However, these six cells all came from the same preparation, and it is likely that they represent inadvertent contamination from the LSM layer. Although it has been reported that permeabilization is required for exogenous cADPR entry into cells, this study has verified that unpermeabilized cells do in fact respond to extracellular cADPR. This is in keeping with a recent study on the effects of cADPR in tracheal smooth muscle (7).

Previous work by us (30) and others (22) has demonstrated that NO causes contraction of opossum esophageal LSM, whereas in the adjacent CSM, NO causes inhibition (5). This is an intriguing difference given that they are both cGMP dependent. How cGMP triggers this excitatory pathway in LSM, given that cGMP activation is usually associated with relaxation of smooth muscle, remains unresolved. The relaxant effect is thought to be mediated through generation of PKG, which through a variety of mechanisms leads to a decrease in intracellular Ca2+. It is unclear why cGMP activation by NO in LSM would not also cause such PKG-mediated events. The results of the current study provide some insight into this apparent paradox.

SNP, a putative NO donor, was initially applied to CSM and LSM tissue strips to confirm previous studies demonstrating opposing effects. As reported previously, SNP had little effect on CSM strips, whereas it produced significant contraction of LSM strips. Antagonists of cADPR were then added 1 h before a second application of SNP, and it was found that the SNP-induced contraction was significantly inhibited. The experiments in the second phase of the study used different antagonists to infer selectivity of the agents used had been demonstrated in other smooth muscle preparations (10, 24). Nevertheless, it was recognized that nonselective effects may occur, and thus we attempted to confirm selectivity by demonstrating that the antagonist did not affect the carbachol-induced contraction, which is known to occur via an IP3-dependent pathway. As expected, 8-Br-cADPR had no effect on the carbachol-induced contraction. On the other hand, the SNP-induced contraction was also antagonized by two different blockers of the ryanodine receptor, which is consistent with previous work showing that the cADPR works via this SR receptor to mediate Ca2+-induced Ca2+ release (8). In several non-smooth muscle tissues, it has been reported that NO causes Ca2+ release from ryanodine-dependent stores (9, 21, 27), and, in sea urchin eggs, this has been shown to involve cADPR (28). Like the SNP-induced esophageal LSM contraction (30), this involves a cGMP-dependent pathway, presumably via G-kinase activation of ADPR cyclase (8, 28). On the other hand, in vascular (29) and airway smooth muscle (25), NO has been shown to inhibit cADPR cyclase without affecting cADPR hydrolyase. This likely occurs through S-nitrosylation of sulphydryl groups in the enzyme, independently of cGMP (25). The net effect is a decrease in intracellular Ca2+ and muscle relaxation. We believe that if such a mechanism is operant in LSM, it is likely overshadowed by cGMP-dependent activation of cADPR and/or other mechanisms leading to Ca2+ influx.

Although the incomplete inhibitory effect caused by the selective cADPR or ryanodine receptor antagonists on SNP-induced contraction may be due to suboptimal antagonist concentrations, another distinct possibility is that the contraction involves other parallel pathways. As noted above, cADPR is believed to mediate Ca2+-induced Ca2+ release that is first triggered by influx of extracellular Ca2+. Our previous studies (30) found that the NO-induced esophageal LSM contraction is also antagonized by blockade of either Cl− channels or L-type Ca2+ channels, suggesting that membrane depolarization with subsequent influx of extracellular Ca2+ via voltage-sensitive channels is also involved. Why such an SNP-induced mechanism occurs in LSM but not CSM warrants further study.

In conclusion, the present study is the first to report that cADPR induces contraction in esophageal LSM but not CSM. These results support the hypothesis that, similarly to intestinal smooth muscle, contraction of esophageal LSM involves a different second messenger system than CSM. Furthermore, known antagonists of cADPR inhibited the SNP-induced contraction in LSM tissue, suggesting that at least a component of the SNP-induced contraction of LSM involves a cADPR-dependent pathway.

REFERENCES


