Role of cyclic ADP-ribose in the regulation of $[\text{Ca}^{2+}]_i$ in porcine tracheal smooth muscle

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Praakash, Y. S., Mathur S. Kannan, Timothy F. Walseth, and Gary C. Sieck. Role of cyclic ADP-ribose in the regulation of $[\text{Ca}^{2+}]_i$ in porcine tracheal smooth muscle. Am. J. Physiol. 274 (Cell Physiol. 43): C1653–C1660, 1998.—The purpose of the present study was to determine whether cyclic ADP-ribose (cADPR) acts as a second messenger for $\text{Ca}^{2+}$ release through ryanodine receptor (RyR) channels in tracheal smooth muscle (TSM). Freshly dissociated porcine TSM cells were permeabilized with $\beta$-escin, and real-time confocal microscopy was used to examine changes in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$). cADPR (10 nM–10 µM) induced a dose-dependent increase in $[\text{Ca}^{2+}]_i$, which was blocked by the cADPR receptor antagonist 8-amino-cADPR (20 µM) and by the RyR blockers ruthenium red (10 µM) and ryanodine (10 µM), but not by the inositol 1,4,5-trisphosphate receptor blocker heparin (0.5 mg/ml). During steady-state ($[\text{Ca}^{2+}]_i$); oscillations induced by acetylcholine (ACh), addition of 100 nM and 1 µM cADPR increased oscillation frequency and decreased peak-to-trough amplitude. ACh-induced $[\text{Ca}^{2+}]_i$ oscillations were blocked by 8-amino-cADPR; however, 8-amino-cADPR did not block the $[\text{Ca}^{2+}]_i$ response to a subsequent exposure to caffeine. These results indicate that cADPR acts as a second messenger for $\text{Ca}^{2+}$ release through RyR channels in TSM cells and may be necessary for initiating ACh-induced $[\text{Ca}^{2+}]_i$ oscillations.

ryanodine receptor; second messenger; confocal microscopy; sarcoplasmic reticulum; $\beta$-escin; intracellular calcium concentration

Several studies in a variety of tissues have shown that cyclic ADP-ribose (cADPR), a metabolite of $\beta$-NAD, can induce sarcoplasmic reticulum (SR) $\text{Ca}^{2+}$ release through ryanodine receptor (RyR) channels. However, cADPR does not appear to directly activate RyR channels. Instead, the effect of cADPR on $\text{Ca}^{2+}$ release is apparently mediated by high-affinity cADPR binding sites in the SR membrane. Accordingly, it has been demonstrated that SR $\text{Ca}^{2+}$ release is inhibited by 8-amino-cADPR, a selective cADPR receptor antagonist. The concentration dependence of the $\text{Ca}^{2+}$ response to cADPR appears to vary across tissues. For example, in sea urchin eggs, where the $\text{Ca}^{2+}$ response to cADPR has been best characterized, the $\text{Ca}^{2+}$ response is saturated at nanomolar concentrations. In contrast, in skeletal and cardiac muscles, micromolar concentrations of cADPR are required to elicit maximal $\text{Ca}^{2+}$ responses.

The effect of cADPR on SR $\text{Ca}^{2+}$ release in smooth muscle cells has been recently studied. In SR vesicles from intestinal smooth muscle, Kummerle and Makhlouf (9) demonstrated that cADPR induces $\text{Ca}^{2+}$ release. Kummerle and Makhlouf also demonstrated the existence of such high-affinity cADPR binding sites in intestinal smooth muscle. In permeabilized coronary artery smooth muscle cells, we demonstrated that cADPR induces SR $\text{Ca}^{2+}$ release even in the presence of heparin. However, in coronary artery smooth muscle, ryanodine blockade of RyR channels did not completely inhibit cADPR-induced $\text{Ca}^{2+}$ release. Furthermore, depletion of the caffeine-sensitive SR $\text{Ca}^{2+}$ stores did not prevent the $\text{Ca}^{2+}$ response to cADPR. Thus we concluded that cADPR-induced $\text{Ca}^{2+}$ release in coronary artery smooth muscle is not mediated solely by RyR channels.

Recently, we and others have shown that activation of muscarinic receptors in porcine tracheal smooth muscle (TSM) cells by acetylcholine (ACh) results in oscillations in intracellular $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_i$). These ACh-induced $[\text{Ca}^{2+}]_i$ oscillations in TSM cells persist even when SR $\text{Ca}^{2+}$ release through inositol 1,4,5-trisphosphate (IP$_3$) receptors is inhibited by heparin. Furthermore, ACh-induced $[\text{Ca}^{2+}]_i$ oscillations in TSM cells are inhibited by ruthenium red, which blocks RyR channels. Therefore, we concluded that ACh-induced $[\text{Ca}^{2+}]_i$ oscillations in TSM cells arise from repetitive release of SR $\text{Ca}^{2+}$ through RyR channels. However, the second messenger involved in triggering $\text{Ca}^{2+}$ release through RyR channels in TSM cells remains to be determined.

The purpose of the present study was to examine whether cADPR induces SR $\text{Ca}^{2+}$ release in porcine TSM cells. The role of RyR channels in the $[\text{Ca}^{2+}]_i$ response to cADPR was investigated. Furthermore, the modulation of ACh-induced $[\text{Ca}^{2+}]_i$ oscillations in TSM cells by cADPR was assessed.

METHODS

Cell preparation. Porcine tracheas were obtained from a local abattoir. Single TSM cells were isolated using techniques described previously. Briefly, the tissue was minced in Hanks’ balanced salt solution (HBSS) containing 10 mM glucose and 10 mM HEPES (pH 7.4). The tissue was then incubated first in 20 U/ml papain and 2,000 U/ml DNase and subsequently in 1 mg/ml type IV collagenase (Worthington Biochemical, Freehold, NJ). TSM cells were released by trituration, centrifuged, and suspended in minimum essential medium containing 10% FCS. Confocal ($[\text{Ca}^{2+}]_i$) imaging. Dissociated TSM cells were plated on glass coverslips coated with rat tail collagen and incubated for 1–2 h at 37°C in 5% CO$_2$. The cells were used for experiments at 2–16 h after plating. Exclusion of trypan blue was used to determine cell viability (>90%). Some cell samples were reacted with anti-smooth muscle myosin antibody (Sigma Chemical, St. Louis, MO) to determine the relative proportions of myocytes and fibroblasts (50:1 ratio).
Coverslips with attached TSM cells were incubated in 5 µM fluo 3-AM (Molecular Probes, Eugene, OR) at 37°C for 30 min and then placed on an open slide chamber (Warner Instruments, Hamden, CT) mounted on a Nikon Diaphot inverted microscope. The chamber was perfused with HBSS at 2–3 ml/min at room temperature.

Detailed techniques for real-time confocal imaging of [Ca²⁺]i in TSM cells have been recently described. Briefly, fluo 3-loaded cells were visualized using an Odyssey XL real-time confocal system (Noran Instruments, Middleton, WI) attached to the Nikon microscope and equipped with an Ar-Kr laser. Although the confocal system was capable of acquiring images at 480 frames/s, in previous studies we determined that a rate of 30 frames/s was sufficient to determine the dynamic [Ca²⁺]i response of TSM cells without frequency aliasing (the appearance of higher-frequency signals being of lower frequency because of inadequate data sampling). An Olympus ×40, 1.3 numerical aperture, oil-immersion objective lens was used for imaging, with image size set to 640 × 480 pixels (0.06 µm²/pixel). Optical section thickness was set to 1 µm. With regions of interest of 5 × 5 pixels (1.5 µm²), [Ca²⁺]i measurements were obtained from volumes of 1.5 µm³. On the basis of previous calibrations of [Ca²⁺]i in TSM cells, a fixed combination of laser intensity (20% of maximum) and photomultiplier gain (1,700 from a maximum of 4,096) was set a priori to ensure that pixel intensities within regions of interest ranged from 25 to 255 gray levels. In intact TSM cells the gray level data were converted to nanomolar Ca²⁺ on the basis of a previously described calibration procedure. However, in β-escin-permeabilized TSM cells the amount of fluo 3 leakage from each cell could not be determined; thus accurate calibration of [Ca²⁺]i levels was precluded. Yet, during the experimental period for each cell (<30 min), there appeared to be minimal leakage of fluo 3, as indicated by the relatively stable baseline. Therefore, in β-escin-permeabilized TSM cells, only changes in [Ca²⁺]i relative to baseline level were assessed.

β-escin-permeabilized cell preparation. In previous studies on intact and β-escin-permeabilized TSM cells, we determined that the EC₅₀ for the [Ca²⁺]i response to ACh was ~1 µM. Therefore, a fixed concentration of 1 µM ACh was used in the present study. Intact TSM cells were first exposed to 1 µM ACh, and the initiation of [Ca²⁺]i oscillations was verified. The same cells were then washed in HBSS for 15 min and permeabilized by exposure to 25 µM β-escin (Sigma Chemical) in a pCa 9.0 solution for ~1 min. Adequate cell permeabilization was confirmed by a [Ca²⁺]i response to IP₃, which is excluded in intact cells. After permeabilization the cells were washed with pCa 9.0 solution for 2 min. The SR was then loaded by incubating the cells for 10–15 min in pCa 7.0 solution.

[Ca²⁺]i response to cADPR. In initial studies on 10 β-escin-permeabilized TSM cells we found that the [Ca²⁺]i response to 1 µM cADPR was not reproducible but displayed substantial decrement with repeated exposures. Therefore, it was not possible to analyze the concentration dependence of the [Ca²⁺]i response to cADPR within a single cell. Instead, cells were first exposed to one of four concentrations of cADPR (10 nM, 100 nM, 1 µM, or 10 µM), and the mean [Ca²⁺]i response for each cADPR concentration was determined.

Effects of RyR channel blockade on [Ca²⁺]i. Response to cADPR. β-escin-permeabilized cells were preexposed to 10 µM ruthenium red to inhibit RyR channels. The cells were then exposed to cADPR. In a second set of experiments, permeabilized TSM cells were preexposed to 10 µMryanodine to block RyR channels, and the cells were subsequently exposed to 1 µM cADPR. In both cases the efficacy of channel blockade was confirmed by exposing the same cells to 5 mM caffeine. In a third set of experiments, permeabilized TSM cells were preexposed to 5 mM caffeine to deplete caffeine-sensitive SR stores. The cells were then exposed to 1 µM cADPR.

Effects of IP₃ receptor channel blockade on [Ca²⁺]i response to cADPR. The [Ca²⁺]i response to 1 µM IP₃ was evaluated in one set of β-escin-permeabilized TSM cells. A second set of permeabilized cells was preexposed for 15 min to 0.5 mg/ml heparin to block IP₃ receptor channels. The cells were then exposed to 1 µM IP₃ to verify efficacy of heparin in blocking IP₃ receptor channels. The cells were finally exposed to 1 or 10 µM cADPR.

Effects of cADPR receptor antagonist on [Ca²⁺]i response to cADPR. β-escin-permeabilized TSM cells were preexposed for 15 min to 20 µM 8-amino-cADPR, a selective antagonist of the cADPR receptor. The [Ca²⁺]i response to cADPR was then evaluated. In the continued presence of cADPR and 8-amino-cADPR, the cells were finally exposed to 5 mM caffeine. In a second set of experiments, β-escin-permeabilized TSM cells were preexposed to 8-amino-cADPR and 10 µM ruthenium red or 10 µM ryanodine to block RyR channels. The cells were exposed to cADPR and finally to caffeine.

Effect of cADPR on ACh-induced [Ca²⁺]i oscillations. In intact TSM cells, [Ca²⁺]i oscillations were induced by exposure to 1 µM ACh. As previously described, these ACh-induced [Ca²⁺]i oscillations displayed an initial dynamic phase characterized by faster oscillation frequency and lower peak-to-trough amplitude because of an elevation of basal [Ca²⁺]. After ~90 s the ACh-induced [Ca²⁺]i oscillations reached a steady-state phase characterized by lower relatively constant oscillation frequency and higher peak-to-trough amplitude. The TSM cells were then permeabilized by exposure to β-escin, and [Ca²⁺]i oscillations were induced by exposure to 1 µM ACh and 10 µM GTP. After ~90 s the cells were exposed to different concentrations of cADPR in the continued presence of ACh and GTP.

In a second set of experiments, β-escin-permeabilized TSM cells were preexposed to different concentrations of cADPR, then exposed to 1 µM ACh and 10 µM GTP. In a third set of experiments, β-escin-permeabilized TSM cells were first exposed to 1 µM ACh and 10 µM GTP and then to 20 µM 8-amino-cADPR in the presence or absence of 0.5 mg/ml heparin. In a previous study we established that heparin does not block ongoing ACh-induced [Ca²⁺]i oscillations. According, heparin was added only to ensure that subsequent manipulations did not cause Ca²⁺ release through IP₃ receptor channels. The cells were then exposed to a maximal concentration of cADPR to verify the efficacy of 8-amino-cADPR in inhibiting the cADPR binding site. The cells were finally exposed to 5 mM caffeine to determine whether the RyR channel itself is inhibited.

Statistical analysis. [Ca²⁺]i responses were evaluated for a total of 216 TSM cells. The specific number of cells analyzed for each protocol is provided in RESULTS. Data were compared using Student's t-tests. Statistical significance was tested at a 0.05 level.

RESULTS

[Ca²⁺]i response to cADPR. In β-escin-permeabilized TSM cells, exposure to cADPR induced a [Ca²⁺]i response that was concentration dependent (Figs. 1 and 2). Exposure to 10 nM (n = 10) and 100 nM (n = 10) cADPR did not induce any appreciable change in [Ca²⁺] (4 ± 1% and 6 ± 1% increase, respectively). However, exposure to 1 µM (n = 10) and 10 µM (n = 10)
cADPR induced a robust transient [Ca\textsuperscript{2+}]i response (245 ± 24% and 419 ± 31% increase, respectively).

Effects of RyR channel blockade on [Ca\textsuperscript{2+}]i response to cADPR. The [Ca\textsuperscript{2+}]i response of β-escin-permeabilized TSM cells to cADPR was abolished by blocking RyR channels. For example, preexposing permeabilized TSM cells (n = 8) to 10 µM ruthenium red abolished the [Ca\textsuperscript{2+}]i response to 1 µM cADPR (Fig. 3A). Similarly, preexposing permeabilized TSM cells (n = 8) to 10 µM ryanodine abolished the [Ca\textsuperscript{2+}]i response to 1 µM cADPR (Fig. 3B). In both cases, the efficacy of RyR channel blockade was confirmed by the lack of a [Ca\textsuperscript{2+}]i response to 5 mM caffeine (Fig. 3, A and B).

In β-escin-permeabilized TSM cells (n = 8), preexposure to 5 mM caffeine induced a transient elevation in [Ca\textsuperscript{2+}] (Fig. 3C). In the continued presence of caffeine in these cells, thereby depleting the caffeine-sensitive SR Ca\textsuperscript{2+} store, subsequent exposure to cADPR failed to induce a [Ca\textsuperscript{2+}]i response (Fig. 3C).

Effects of IP\textsubscript{3} receptor channel blockade on [Ca\textsuperscript{2+}]i response to cADPR. In β-escin-permeabilized TSM cells (n = 8), exposure to 1 µM IP\textsubscript{3} induced a prolonged [Ca\textsuperscript{2+}]i response (Fig. 4A). Preexposing permeabilized TSM cells to 0.5 mg/ml heparin completely inhibited the [Ca\textsuperscript{2+}]i response to 1 µM IP\textsubscript{3} (Fig. 4B) but had no effect on the [Ca\textsuperscript{2+}]i response to 1 µM cADPR (n = 10; Fig. 4C) and 10 µM cADPR (n = 10; Fig. 4D).

Effects of cADPR receptor antagonist on [Ca\textsuperscript{2+}]i response to cADPR. The [Ca\textsuperscript{2+}]i response of β-escin-permeabilized TSM cells to cADPR was abolished by blocking cADPR binding sites with 20 µM 8-amino-cADPR (n = 22; Fig. 5A). However, 8-amino-cADPR did not inhibit the [Ca\textsuperscript{2+}]i response to a subsequent exposure to cADPR.
sure to 5 mM caffeine (Fig. 5A). On the other hand, in the presence of 20 µM 8-amino-cADPR, the [Ca^{2+}] response of permeabilized TSM cells to 5 mM caffeine was abolished by 10 µM ruthenium red (n = 10; Fig. 5B) or 10 µM ryanodine (n = 10; Fig. 5C).

Effect of cADPR on ACh-induced [Ca^{2+}] oscillations. After ß-escin permeabilization and SR reloading with pCa 7.0 solution, exposure to 1 µM ACh and 10 µM GTP induced propagating [Ca^{2+}] oscillations that were qualitatively similar to the steady-state oscillations observed in intact cells before permeabilization (n = 74). At the steady-state level the amplitude of ACh-induced [Ca^{2+}] oscillations in intact cells was between 100 and 700 nM (463 ± 11 nM). Assessment of nanomolar Ca^{2+} levels in ß-escin-permeabilized TSM cells was not possible because of uncontrolled leakage of fluo 3; however, substantial [Ca^{2+}] oscillations above baseline could be readily observed. The steady-state frequency of the ACh-induced [Ca^{2+}] oscillations in intact and permeabilized cells was 10–25 min⁻¹ (19 ± 2 min⁻¹).

During ongoing ACh-induced [Ca^{2+}] oscillations in ß-escin-permeabilized TSM cells, exposure to 100 nM (n = 11; Fig. 6A) or 1 µM (n = 15; Fig. 6B) cADPR resulted in an elevation of basal [Ca^{2+}], an increase in oscillation frequency (45 ± 8% increase with 1 µM cADPR), and a decrease in oscillation amplitude (62 ± 11% decrease with 1 µM cADPR). Exposure to 10 µM cADPR (n = 10; Fig. 6C) resulted in large transient [Ca^{2+}] elevations. After these [Ca^{2+}] transients, [Ca^{2+}] oscillations were inhibited, even in the continued presence of ACh (Fig. 6C).

Preexposure of ß-escin-permeabilized cells to 100 nM (n = 6; Fig. 7A) or 1 µM (n = 7; Fig. 7B) cADPR did not prevent the initiation of [Ca^{2+}] oscillations by ACh. However, preexposure of ß-escin-permeabilized cells to 10 µM cADPR (n = 9; Fig. 7C) resulted in a large [Ca^{2+}] transient and prevented the initiation of oscillations by ACh.

Fig. 5. Effect of cADPR receptor blockade on [Ca^{2+}] response to cADPR. In permeabilized TSM cells, preexposure to 8-amino-cADPR, a specific cADPR receptor antagonist, completely inhibited [Ca^{2+}] response to subsequent cADPR exposure (A). However, 8-amino-cADPR did not inhibit [Ca^{2+}] response to caffeine. Preexposure to ruthenium red (B) or ryanodine (C) inhibited [Ca^{2+}] response to cADPR and caffeine.
In a third set of experiments, exposing β-escin-permeabilized TSM cells to 8-amino-cADPR during ongoing ACh-induced [Ca^{2+}]_{i} oscillations completely inhibited the oscillations in the absence (n = 8; Fig. 8A) and presence of heparin (n = 16; Fig. 8B). Subsequent exposure to cADPR did not elicit any [Ca^{2+}]_{i} response (Fig. 8). However, exposure to caffeine induced a large [Ca^{2+}]_{i} transient (Fig. 8).

**DISCUSSION**

The present study demonstrates that cADPR induces SR Ca^{2+} release through RyR channels in porcine TSM cells. In previous studies, ACh-induced [Ca^{2+}]_{i} oscillations have been shown to occur through repetitive SR Ca^{2+} release through RyR channels. The present study suggests that the RyR channel is the mechanistic link between cADPR- and ACh-induced [Ca^{2+}]_{i} oscillations in porcine TSM cells.

In the present study, SR Ca^{2+} release under Ca^{2+}-clamped conditions was examined using β-escin-permeabilized TSM cells. This procedure has been previously used in intact smooth muscle strips and single smooth muscle cells to facilitate intracellular access to relatively high-molecular-weight substances, such as heparin, and to investigate receptor-signal transduction pathways. In the present study the integrity of the receptor-signal transduction pathways in β-escin-permeabilized TSM cells and the extent of permeabilization were confirmed by the [Ca^{2+}]_{i} response to IP3, which is excluded by intact cells.

There is considerable evidence from non-smooth muscle tissue for high-affinity cADPR binding sites in the SR membrane. Recently, Kuemmerle and Makhlouf (9) also demonstrated the existence of such high-affinity cADPR binding sites in intestinal smooth muscle. The present study did not directly examine whether cADPR binding sites also exist in porcine TSM cells. However, their existence is suggested by the concentration-dependent [Ca^{2+}]_{i} response to cADPR and the inhibition of the [Ca^{2+}]_{i} response by 8-amino-cADPR, a selective cADPR receptor antagonist.

In contrast to nonmuscle tissue such as sea urchin eggs, a relatively high concentration (micromolar) of cADPR was required to elicit [Ca^{2+}]_{i} responses in β-escin-permeabilized TSM cells. However, it must be...
Another important factor that may have influenced the concentration dependence of the [Ca\(^{2+}\)] response to cADPR is the presence of cADPR hydrolase as well as hydrolytic activity associated with CD38. These hydrolytic mechanisms are apparently ubiquitous, but the extent and rate of cADPR hydrolysis may vary across cell types. Accordingly, higher concentrations of exogenous cADPR may be required to achieve a given concentration at the level of the cADPR binding site.

Previous studies have suggested that cADPR mediates Ca\(^{2+}\) release through RyR channels. In agreement with these studies, the pCa\(^{2+}\) response of TSM cells to cADPR was blocked by ruthenium red and ryanodine, but not by heparin. Furthermore, depletion of caffeine-sensitive SR Ca\(^{2+}\) stores abolished the cADPR-induced [Ca\(^{2+}\)] response in TSM cells. However, it appears that RyR channel activation is not an exclusive mechanism underlying cADPR-induced Ca\(^{2+}\) release across cell types. For example, in a previous study in porcine coronary artery smooth muscle, we demonstrated that cADPR induced SR Ca\(^{2+}\) release even when RyR channels were blocked by ryanodine. Furthermore, in coronary artery smooth muscle cells, depletion of caffeine-sensitive SR Ca\(^{2+}\) stores did not inhibit the [Ca\(^{2+}\)] response to cADPR. Studies in skeletal muscle and canine cardiac SR vesicles have also demonstrated that specific blockers of RyR channels did not abolish cADPR-induced Ca\(^{2+}\) release. Lahouratet et al. (10) proposed that cADPR-mediated SR Ca\(^{2+}\) release in cardiac tissue does not involve RyR channels or caffeine-sensitive Ca\(^{2+}\) stores but that separate cADPR-sensitive channels and caffeine-insensitive Ca\(^{2+}\) stores exist. These discrepancies clearly indicate cell-specific differences in the mechanisms underlying cADPR-mediated [Ca\(^{2+}\)] regulation.

The results of the present study suggest that, in TSM cells, cADPR indirectly activates RyR channels. For example, in the presence of 8-amino-cADPR, caffeine still induced a [Ca\(^{2+}\)] response in TSM cells. This [Ca\(^{2+}\)] response to caffeine was blocked by ruthenium red and ryanodine. Although these results clearly suggest that cADPR indirectly activates RyR channels, it was not feasible to directly test this hypothesis with use of the current protocols. An alternate possibility is that cADPR directly activates RyR channels by binding to a site different from that of caffeine. However, previous reports in which photoaffinity labeling of cADPR receptors was used indicate cADPR binding sites that are distinct from the RyR channel itself. Furthermore, RyR channel activation by cADPR appears to require the involvement of other proteins, such as calmodulin. Lee and colleagues (12) suggested that cADPR receptors may be coupled to RyR in sea urchin eggs through an intermediate protein. It is unknown whether such intermediate proteins exist in TSM cells. Nonetheless, it appears likely that cADPR indirectly activates RyR channels in TSM cells, perhaps facilitating Ca\(^{2+}\)-induced Ca\(^{2+}\) release through these channels.

In porcine TSM cells, ACh induces repetitive [Ca\(^{2+}\)] oscillations that arise from SR Ca\(^{2+}\) release rather than Ca\(^{2+}\) influx. In β-escin-permeabilized TSM cells, ACh

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Fig. 8. Effect of 8-amino-cADPR on ACh-induced [Ca\(^{2+}\)] oscillations. Exposure to 8-amino-cADPR during ongoing ACh-induced [Ca\(^{2+}\)] oscillations, in absence (A) or presence (B) of heparin, inhibited oscillations. However, in either case, [Ca\(^{2+}\)] response to caffeine was not inhibited by 8-amino-cADPR.

Noted that previous studies in intact skeletal muscle and isolated cardiac SR vesicles have also reported the use of micromolar cADPR concentrations to elicit [Ca\(^{2+}\)] responses. The reasons for the vastly different cADPR concentrations required in different cell types are not clear. It is possible that different cADPR receptor subtypes exist in different cell types (akin to RyR channels) that vary in their binding affinity for cADPR. There is no information to support the presence of different cADPR receptor subtypes.

In several cell types it has been demonstrated that the [Ca\(^{2+}\)] response to cADPR depends on [Ca\(^{2+}\)]. For example, in intestinal smooth muscle, Kueemmerle and Makhlouf (9) also demonstrated that the cADPR-mediated Ca\(^{2+}\) release exhibits a "bell-shaped" dependence on [Ca\(^{2+}\)], much like the IP\(_3\) receptor channel, with a maximum activation at ~500 nM. The Ca\(^{2+}\) dependence of cADPR in TSM remains to be determined. However, it is possible that maximum cADPR binding in TSM cells also occurs at [Ca\(^{2+}\)], similar to those in intestinal smooth muscle. If so, this may explain, at least in part, the requirement for higher cADPR concentrations to elicit [Ca\(^{2+}\)] responses in TSM cells because of an apparently low affinity of cADPR binding at 100 nM Ca\(^{2+}\) (pCa 7.0) in the present study.

In sea urchin eggs, calmodulin increases the sensitivity of cADPR-mediated SR Ca\(^{2+}\) release by several orders of magnitude. If calmodulin is required for cADPR-induced Ca\(^{2+}\) release in TSM cells, the process of permeabilization may have led to some loss of calmodulin from the cell, thus decreasing the sensitivity of the [Ca\(^{2+}\)] response to cADPR.
also induced [Ca\(^{2+}\)] oscillations that were qualitatively similar to the steady-state phase of oscillations in intact TSM cells. In permeabilized TSM cells, [Ca\(^{2+}\)] oscillations were inhibited when RyR channels were blocked by ruthenium red, but not by heparin. Therefore, we concluded that ACh-induced [Ca\(^{2+}\)] oscillations in porcine TSM cells represent repetitive SR Ca\(^{2+}\) release through RyR channels. The results of the present study extend these previous observations by demonstrating that the cADPR receptor antagonist 8-amino-cADPR also inhibits ACh-induced [Ca\(^{2+}\)] oscillations in TSM cells. These results suggest that cADPR may play an important role in the initiation and maintenance of ACh-induced [Ca\(^{2+}\)] oscillations in TSM cells.

In a previous study we found that, although exposing β-escin-permeabilized TSM cells to heparin did not block ongoing ACh-induced [Ca\(^{2+}\)] oscillations, preexposure to heparin inhibited the initiation of oscillations. On the basis of our previous results, we concluded that there is a link between ACh-stimulated Ca\(^{2+}\) release through IP\(_3\) receptors and the initiation of [Ca\(^{2+}\)] oscillations mediated through RyR channels. ACh-induced SR Ca\(^{2+}\) release through IP\(_3\) receptor channels may elevate basal [Ca\(^{2+}\)]\(_i\) levels, thereby facilitating cADPR-mediated RyR activation. In support of this theory, it has been demonstrated that cADPR binding to its receptor and RyR activation are Ca\(^{2+}\) sensitive. In SR vesicles derived from rabbit intestinal smooth muscle, it was shown that maximal cADPR-induced Ca\(^{2+}\) release occurred at a basal Ca\(^{2+}\) concentration of ~500 nM. Thus, at resting basal [Ca\(^{2+}\)]\(_i\) within TSM cells (~100 nM), cADPR receptor binding and RyR channel activation are not maximally sensitized. IP\(_3\)-mediated SR Ca\(^{2+}\) release would elevate the local [Ca\(^{2+}\)]\(_i\) and thus sensitize cADPR receptor binding and RyR channel activation. The rapid rise time of the cADPR-induced [Ca\(^{2+}\)]\(_i\) response, as well as the ACh-induced [Ca\(^{2+}\)]\(_i\) oscillations, may reflect such a positive-feedback mechanism.

It is possible that ACh stimulation may regulate cADPR levels, akin to IP\(_3\) levels, and thus cADPR may act as a second messenger for RyR activation in TSM cells. Alternatively, it is possible that relatively high cADPR levels are present, even in the unstimulated cell, and that cADPR facilitates RyR channel activation once agonist stimulation elevates basal [Ca\(^{2+}\)]\(_i\) via other mechanisms, such as IP\(_3\)-induced SR Ca\(^{2+}\) release and Ca\(^{2+}\) influx. To discern among these possibilities, the effect of agonist stimulation on cADPR levels needs to be explored in TSM cells.

In conclusion, the present study clearly demonstrated that cADPR induces SR Ca\(^{2+}\) release through RyR channels in porcine TSM cells via a receptor-mediated mechanism. During ACh-induced [Ca\(^{2+}\)] oscillations, cADPR may serve to facilitate RyR activation. The precise mechanisms by which cADPR interacts with RyR channels in TSM cells remain to be explored.

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