Cyclic Adenosine Diphosphate Ribose Activates Ryanodine Receptors, whereas NAADP Activates Two-pore Domain Channels*

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The mechanism by which cyclic adenosine diphosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) mobilize intracellular Ca2+ stores remains controversial. It is open to question whether cADPR regulates ryanodine receptors (RyRs) directly, as originally proposed, or indirectly by promoting Ca2+ uptake into the sarco/endoplasmic reticulum (S/ER)2 has been considered to be the major releasable store. From this store, Ca2+ may be released through the opening of inositol 1,4,5-trisphosphate receptors (IP₃Rs) and/or ryanodine receptors (RyRs), the two groups of intracellular Ca2+ release channels located on S/ER membranes. It is generally accepted that of the recognized Ca2+-mobilizing second messengers, inositol 1,4,5-trisphosphate facilitates this process by activating IP₃Rs (1).

By contrast, the mechanism by which the pyridine nucleotides cyclic adenosine diphosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) (2) mobilize intracellular Ca2+ stores remains controversial. Although a wealth of evidence across a variety of cell types (3–9) supports the original proposal that cADPR activates RyRs (10), studies on reconstituted RyRs in lipid bilayers have failed to conclusively demonstrate direct regulation of these channels by cADPR (11), and it has been suggested that cADPR may initiate Ca2+ signals via RyRs and IP₃Rs by promoting Ca2+ uptake into the S/ER by S/ER Ca2+ ATPases (SERCA) (12–14). This proposal has not been effectively countered by studies on ventricular myocytes, which exclusively express RyR2. This is due to the fact that the principal regulatory effect of cADPR with respect to RyR2 is to increase the sensitivity of this RyR subtype to Ca2+–induced Ca2+ release (8, 9), and in light of the fact that the sensitivity of RyRs to Ca2+–induced Ca2+ release may be augmented by an increase in Ca2+ concentration within the cytoplasm and/or S/ER lumen (15).

The mechanism by which NAADP triggers intracellular Ca2+ release has also been hotly debated. We recently identified a family of two-pore domain channels (TPC1–3, TPCN1–3 for gene name) as endolysosome-targeted, NAADP-gated Ca2+ release channels (16, 17), and our findings have since been confirmed by others (18, 19). Nevertheless, recent investigations continue to provide support for the view (20) that NAADP may induce Ca2+ release from the S/ER by directly activating RyRs (20–22).

We therefore investigated the capacity of cADPR and NAADP, respectively, to induce Ca2+ signals via recombinant TPCs and RyRs stably expressed in HEK293 cells, which endog...

Intracellular Ca2+ signals are initiated by Ca2+ release from intracellular stores, and traditionally, the sarco/endoplasmic reticulum (S/ER) has been considered to be the major releasable store. From this store, Ca2+ may be released through the opening of inositol 1,4,5-trisphosphate receptors (IP₃Rs) and/or ryanodine receptors (RyRs), the two groups of intracellular Ca2+ release channels located on S/ER membranes. It is generally accepted that of the recognized Ca2+-mobilizing second messengers, inositol 1,4,5-trisphosphate facilitates this process by activating IP₃Rs (1).

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enously express very low levels of TPCs and do not contain endogenous RyRs. Our findings show conclusively that cADPR activates RyRs, whereas NAADP activates TPCs.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—HEK293 cell lines stably expressing either human TPC1, human TPC2 (16), rabbit RyR1, or mink RyR3 (23) were developed and cultured as described previously.

**Ca^{2+} Imaging**—Cells were incubated for 30 min with 5 μM Fura-2-AM in nominally Ca^{2+}-free PSS in an experimental chamber that was then placed on a Leica DMIRBE inverted microscope after washing with Ca^{2+}-containing, Fura-2-free PSS for at least 30 min prior to experimentation. PSS was of the following composition (mM): 130 NaCl, 5.2 KCl, 1 MgCl₂, 1.7 CaCl₂, 10 glucose, 10 Hepes, pH 7.45. Cytoplasmic Ca^{2+} concentration was reported by Fura-2 fluorescence ratio (F_{340}/F_{380}) excitation; emission 510 nm). Emitted fluorescence was recorded at 22 °C with a sampling frequency of 0.5 Hz, using a Hamamatsu 4880 CCD camera via a Zeiss Fluar ×40, 1.3 n.a. oil immersion objective. Background subtraction was performed on line. Analysis was via Openlab imaging software (Improvision, UK).

**Intracellular Dialysis**—NAADP (10 nM) and cADPR (100 μM), respectively, were applied intracellularly into single cells in the whole-cell configuration of the patch clamp technique (voltage clamp mode; holding potential = −40 mV). The pipette solution contained (mM): 140 KCl, 10 Hepes, 1 MgCl₂, and 5 μM Fura-2 (free acid), pH 7.4, nominally Ca^{2+}-free (−100 nM). The seal resistance was ≈3 gighms throughout each experiment. Series and pipette resistance was ≤10 and ≤3 megohms, respectively, as measured by an Axopatch 200B amplifier (Axon Instruments).

**Data Presentation and Statistical Analysis**—Data are presented as the mean ± S.E. Comparisons between the groups were carried out in MINITAB 14 using analysis of variance followed by a Tukey post hoc test. Probability values less than 0.05 were considered to be statistically significant.

**Drugs and Chemicals**—All compounds were from Sigma.

**RESULTS**

Clearly, further investigations into the mechanism of Ca^{2+} signaling by cADPR and NAADP are required to demonstrate the fundamental mechanism by which each messenger mobilizes intracellular Ca^{2+} stores. To this end, each messenger was applied to a variety of HEK293 cell lines by intracellular dialysis from a patch pipette, in the whole-cell configuration of the patch clamp technique and under voltage clamp conditions (holding potential = −40 mV). Changes in intracellular Ca^{2+} were reported by the fluorescence ratio (F_{340}/F_{380}) of the Ca^{2+} indicator Fura-2.

We first studied wild-type HEK293 cells, which express very low levels of TPC1 and TPC2 and do not express TPC3 (16) or RyRs (24). Neither cADPR (100 μM) nor NAADP (10 nM) evoked a change in the basal Fura-2 fluorescence ratio (F_{340}/F_{380}, Fig. 1). It therefore seems unlikely that either cADPR or NAADP mediate Ca^{2+} signals by facilitating Ca^{2+} uptake into the ER or for that matter acidic stores, as HEK293 cells express SERCA (25), V-H^{+}-ATPase (26), and IP_{3}Rs (23). We therefore investigated the capacity of cADPR and NAADP, respectively, to initiate Ca^{2+} signals in HEK293 cells that stably overexpress TPCs and RyRs.

A marked and transient increase in the Fura-2 fluorescence ratio was triggered by intracellular dialysis of 100 μM cADPR into HEK293 cells that stably expressed rabbit RyR1 (Fig. 2A) (23) or mink RyR3 (Fig. 2B) (23). Significantly, cADPR-induced Ca^{2+} signals remained unaffected upon depletion of acidic stores with 1 μM bafilomycin (Fig. 2, C and D) (16, 27) but were abolished upon depletion of ER stores by preincubation (40 min) with the SERCA inhibitor thapsigargin (1 μM; Fig. 2, E and F). That the cADPR-dependent Ca^{2+} transient was due to activation of either RyR1 or RyR3 was confirmed by preincubation of cells with dantrolene (10 μM), an RyR antagonist with selectivity for RyR1 and RyR3 over RyR2 (28). Under these conditions too, the basal Fura-2 fluorescence ratio remained virtually unchanged during intracellular dialysis of cADPR (Fig. 2, E and F).

In marked contrast to the effects of cADPR, intracellular dialysis of 10 nM NAADP failed to evoke a Ca^{2+} transient in HEK293 cells that stably expressed RyR1 (Fig. 3A) or RyR3 (Fig. 3B), with similar results obtained upon intracellular dialysis of 100 μM NAADP (Fig. 3). Therefore, it would appear that cADPR triggers Ca^{2+} release from the ER via both RyR1 and RyR3, whereas NAADP is ineffective as an agonist at either RyR subtype.

We next investigated the effects of NAADP and cADPR in cells that stably overexpressed TPC1 or TPC2, TPC3 being absent in humans/primates and some rodents (mice and rats) (16). Our findings in this respect were quite the opposite when compared with the outcomes with RyR-expressing cells. Thus, NAADP evoked, as reported previously (16), a Ca^{2+} transient in cells expressing either TPC1 (Fig. 4A) or TPC2 (Fig. 4B), and in each case NAADP-dependent Ca^{2+} signals were blocked following depletion of acidic stores by preincubation of cells with 1 μM bafilomycin (Fig. 4, A, panel ii, and B, panel ii). By contrast, cADPR failed to elicit a Ca^{2+} transient in HEK293 cells that stably overexpressed either TPC1 or TPC2 (Fig. 4, A, panel ii, and B, panel ii).

Our key findings are summarized in Fig. 5, which clearly shows that cADPR triggers intracellular Ca^{2+} signals by activating ER-targeted RyR1 and RyR3, whereas NAADP triggers intracellular Ca^{2+} signals by activating endolysosome-targeted TPC1 and TPC2.
DISCUSSION

This investigation sought to determine the relative importance of TPCs and RyRs to the mechanisms of intracellular Ca\(^{2+}\)/H\(^{+}\) release induced by cADPR and NAADP. We found that intracellular dialysis of NAADP and cADPR, respectively, failed to evoke a Ca\(^{2+}\)/H\(^{+}\) transient in wild-type HEK293 cells, which endogenously express very low levels of TPCs (16) and do not endogenously express RyRs (24). Because prolonged treatment with cADPR or NAADP did not alter the basal cytoplasmic Ca\(^{2+}\) levels, our data argue strongly against the possibility that either messenger initiates intracellular Ca\(^{2+}\) release by directly augmenting or inhibiting Ca\(^{2+}\) uptake into intracellular Ca\(^{2+}\) stores via the SERCA (25) and V-H\(^{+}\)/ATPase (26) expressed in HEK293 cells. This is clear from the fact that both RyRs (15) and IP3Rs (29), the latter of which are endogenously expressed in HEK293 cells (23), may be gated via an increase in Ca\(^{2+}\) concentration within the cytoplasm and/or S/ER lumen (16).

In HEK293 cells that stably express RyR1 or RyR3, intracellular dialysis of cADPR induced a robust Ca\(^{2+}\)/H\(^{+}\) transient that was abolished following depletion of ER Ca\(^{2+}\) stores by the SERCA inhibitor thapsigargin (1 \(\mu\)M; 40 min preincubation) and after blocking RyR1 with dantrolene (10 \(\mu\)M; 30 min preincubation), respectively; in the presence of thapsigargin and dantrolene, respectively, the Fura-2 fluorescence ratio measured 0.41 \(\pm\) 0.03 \((n = 4)\) and 0.45 \(\pm\) 0.04 \((n = 5)\) upon entering the whole-cell configuration and 0.43 \(\pm\) 0.03 and 0.46 \(\pm\) 0.05 after 120 s.
Serca inhibitor thapsigargin or by block of RyRs by dantrolene, but not by depletion of acidic Ca\(^{2+}\) stores by the \(V\)-\(H\)\(^{-}\)-ATPase inhibitor bafilomycin. These data provide direct support for the original proposal that cADPR is an endogenous regulator of ER-targeted RyRs (10). Therefore, that previous investigations on reconstituted RyRs in lipid bilayers have failed to conclusively demonstrate direct regulation of these channels by cADPR (11) does not argue in favor of an alternative mechanism to RyR activation by cADPR, but it simply provides further support for the view that gating of RyRs by cADPR may be mediated by an ancillary protein-binding partner such as FKBP 12.6 (30–32).

Contrary to our findings in relation to cADPR, NAADP failed to evoke a marked Ca\(^{2+}\) transient in HEK293 cells that stably express RyR1 or RyR3. Thus, NAADP does not appear to mediate intracellular Ca\(^{2+}\) signals by activating either RyR1 or RyR3, and previous studies on ventricular myocytes have demonstrated quite conclusively that NAADP does not directly activate RyR2 (33). It would therefore appear that NAADP is inca-

FIGURE 3. Intracellular dialysis of NAADP does not activate RyR1 or RyR3 stably expressed in HEK293 cells. A, record of the \(F_{340}/F_{380}\) ratio against time showing the effect of intracellular dialysis of 10 \(\mu M\) (black) or 100 \(\mu M\) (gray) NAADP into a HEK293 cell that stably expressed rabbit RyR1; for 10 \(\mu M\) NAADP across all cells, the \(F_{340}/F_{380}\) ratio measured 0.27 ± 0.03 \((n = 6)\) upon entering the whole-cell configuration and 0.29 ± 0.04 after 120 s. B, as in A but for a HEK293 cell that stably expressed mink RyR3; for 10 \(\mu M\) NAADP across all cells, the \(F_{340}/F_{380}\) ratio measured 0.37 ± 0.04 \((n = 4)\) upon entering the whole-cell configuration and 0.39 ± 0.05 after 120 s.

FIGURE 4. NAADP but not cADPR evokes a global Ca\(^{2+}\) transient in HEK293 cells stably overexpressing human TPC1 or human TPC2. A, panel i, upper panel shows a bright field image (BF) of a HEK293 cell that stably overexpressed human TPC1 and a series of pseudo-color images of the Fura-2 fluorescence ratio \(F_{340}/F_{380}\) recorded in the same cell (identified by red broken line) during intracellular dialysis from a patch pipette of 10 \(\mu M\) NAADP. The lower panel shows in black the corresponding record of the \(F_{340}/F_{380}\) ratio against time; across all cells studied, the Fura-2 fluorescence ratio increased from 0.35 ± 0.05 to 0.65 ± 0.07 \((n = 6)\). A, panel ii, green record shows the \(F_{340}/F_{380}\) ratio against time during intracellular dialysis of 10 \(\mu M\) NAADP into a HEK293 cell that stably overexpressed human TPC1 after depletion of acidic Ca\(^{2+}\) stores by bafilomycin (1 \(\mu M\); 1-h preincubation); across all cells, the \(F_{340}/F_{380}\) measured 0.5 ± 0.03 \((n = 4)\) immediately upon entering the whole-cell configuration and 0.53 ± 0.03 after 120 s. The blue record shows the effect of intracellular dialysis of cADPR into HEK293 cells stably expressing human TPC1; across all cells studied, the \(F_{340}/F_{380}\) measured 0.32 ± 0.05 \((n = 3)\) immediately upon entering the whole-cell configuration and 0.35 ± 0.05 after 120 s. B, as in A, panels i and ii, as in A, panels i and ii, but for HEK293 cells that stably expressed TPC2, across all cells NAADP induced an increase in the Fura-2 fluorescence ratio from 0.35 ± 0.05 to 0.99 ± 0.04 \((n = 10)\). In the presence of bafilomycin the \(F_{340}/F_{380}\) measured 0.43 ± 0.05 \((n = 3)\) immediately upon entering the whole-cell configuration and 0.45 ± 0.05 after 120 s. During intracellular dialysis of cADPR into TPC2-expressing cells, the \(F_{340}/F_{380}\) measured 0.37 ± 0.05 \((n = 4)\) immediately upon entering the whole-cell configuration and 0.39 ± 0.05 after 120 s.

FIGURE 5. Comparison of the peak of the Ca\(^{2+}\) transient induced by NAADP and cADPR in HEK293 cells that stably expressed TPC1, TPC2, RyR1, or RyR3. Bar chart compares, under all conditions studied (see key), the mean ± S.E. for the peak change in Fura-2 fluorescence ratio \(F_{340}/F_{380}\) against time in HEK293 cells stably expressing TPC1, TPC2, RyR1, or RyR3.
NAADP Activates TPCs, and cADPR Activates RyRs

...pable of activating RyRs, at least at physiologically relevant concentrations. Therefore, our findings argue against the contrary proposals of others (20–22) and suggest that previous indications of direct regulation of RyRs by NAADP may not have controlled for the amplification by Ca\(^{2+}\)-induced Ca\(^{2+}\) release via RyRs of an initial phase of NAADP-dependent Ca\(^{2+}\) release from acidic stores via endolysosome-targeted TPCs.

Our findings with respect to HEK293 cells that stably over-express TPC1 and TPC2 provided, when compared with RyRs, a mirror image in terms of the regulatory impact of pyridine nucleotides. Thus, as reported previously, NAADP was found capable of activating RyRs, at least at physiologically relevant concentrations. Therefore, our findings argue against the contrary proposals of others (20–22) and suggest that previous indications of direct regulation of RyRs by NAADP may not have controlled for the amplification by Ca\(^{2+}\)-induced Ca\(^{2+}\) release via RyRs of an initial phase of NAADP-dependent Ca\(^{2+}\) release from acidic stores via endolysosome-targeted TPCs.

In conclusion, our data suggest (Fig. 5) that TPCs are gated by transient in HEK293 cells that expressed either TPC1 or TPC2.

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