Cyclic ADP-Ribose Competes With ATP for the Adenine Nucleotide Binding Site on the Cardiac Ryanodine Receptor Ca\(^{2+}\)-Release Channel

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**Abstract** We have investigated the mechanism of action of the putative second messenger, cyclic ADP-ribose (cADPR), on the cardiac ryanodine-sensitive Ca\(^{2+}\)-release channel. Current fluctuations through single Ca\(^{2+}\)-release channels have been monitored after incorporation into planar phospholipid bilayers. We demonstrate that activation of the channel by cADPR is dependent on activating levels of cytosolic Ca\(^{2+}\) and lifetime analysis indicates that the mechanism of action may be sensitization of the channel to Ca\(^{2+}\). In the absence of ATP, cADPR activates the channel in a concentration-dependent manner in the presence of 10 \(\mu\)mol/L cytosolic Ca\(^{2+}\). However, in the presence of ATP, cADPR tends to decrease open probability, indicating that cADPR may be acting at the adenine nucleotide binding site. In addition, we demonstrate that the precursor of cADPR, \(\beta\)-NAD\(^{+}\), and the breakdown product, ADP-ribose, also activate the channel. As cADPR will have to compete with much higher concentrations of \(\beta\)-NAD\(^{+}\), ADP-ribose, and ATP, we suggest that cADPR does not act as a direct endogenous trigger for the opening of the cardiac Ca\(^{2+}\)-release channel. (Circ Res. 1994;75:596-600.)

**Key Words** • cyclic ADP-ribose • ryanodine receptor • Ca\(^{2+}\) release • cardiac excitation-contraction coupling

Cyclic ADP-ribose (cADPR) has been shown to cause release of Ca\(^{2+}\) from intracellular stores in sea urchin eggs,\(^1\)-\(^3\) and it has been suggested that the mechanism for the Ca\(^{2+}\) release is activation of ryanodine-sensitive channels. As cADPR is present in cells at between 20 and 600 nmol/L\(^4\) and as there are enzymes that synthesize and degrade cADPR,\(^5\),\(^6\) it has been suggested that cADPR is an endogenous activator of ryanodine-sensitive Ca\(^{2+}\)-release channels. Mészáros et al\(^7\) reported that cADPR increases the open probability (Po) of cardiac ryanodine-sensitive Ca\(^{2+}\)-release channels incorporated into planar phospholipid bilayers. On the basis of these results, it has been claimed that cADPR can trigger the release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR) in cardiac cells at resting (diastolic) [Ca\(^{2+}\)].\(^5\) In addition, it has become accepted dogma that cADPR directly activates either the cardiac or the brain isoform of the ryanodine receptor in sea urchin eggs and other systems.\(^5\),\(^6\) However, substantial evidence for these claims is lacking. To determine the mode of action (if any) of a direct effect of cADPR on the cardiac Ca\(^{2+}\)-release channel, the channel must be isolated and incorporated into bilayers. This will eliminate the possibility of cADPR activating the channel indirectly. Mészáros et al did carry out such experiments; however, they did not consider the mechanism of the cADPR action nor the possibility that the ability of cADPR to increase Po could be altered in the presence of agents such as ATP and Mg\(^{2+}\), which are thought to play an important role in controlling the Po of the channel in the cell.\(^7\),\(^8\) Given the structural similarity between cADPR and ATP, it is possible that they may act at, and compete for, the same activation sites on the Ca\(^{2+}\)-release channel. Additionally, consideration must be given as to whether the precursor of cADPR, \(\beta\)-NAD\(^{+}\), and its metabolite, ADP-ribose, may also compete with cADPR for its binding sites on the Ca\(^{2+}\)-release channel.

Our results show that cADPR, \(\beta\)-NAD\(^{+}\), and ADP-ribose all increase Po of the Ca\(^{2+}\)-release channel. We also show that cADPR is ineffective in the presence of ATP, suggesting that cADPR and ATP may act at the same site on the Ca\(^{2+}\)-release channel. Because cADPR will have to compete with much higher concentrations of \(\beta\)-NAD\(^{+}\), ADP-ribose, and ATP, it appears unlikely that cADPR can act as an endogenous regulator of SR Ca\(^{2+}\) release in cardiac muscle cells.

**Materials and Methods**

Heavy SR vesicles were isolated from sheep hearts and fused with planar phosphatidylethanolamine lipid bilayers, as previously described.\(^9\) The vesicles fused in a fixed orientation such that the cis chamber corresponded to the cytosolic space and the trans chamber corresponded to the SR lumen. The trans chamber was held at ground, and the cis chamber was held at potentials relative to ground. After fusion, the cis chamber was perfused with 250 mmol/L HEPES, 125 mmol/L Tris, and 10 \(\mu\)mol/L free Ca\(^{2+}\), pH 7.4, and the trans chamber was perfused with 250 mmol/L glutamic acid and 10 mmol/L HEPES, pH 7.4 with Ca(OH)\(_2\) (free Ca\(^{2+}\) = 60 mmol/L). The free [Ca\(^{2+}\)] on the cytosolic/cis side of the bilayer was altered by adding CaCl\(_2\) and EGTA.\(^9\) Current recordings were filtered at 1 kHz and digitized at 2 kHz. Events <1 millisecond in duration were not fully resolved and were excluded from lifetime analysis. Po and open and closed lifetimes were monitored by 50% threshold analysis. Po measurements were calculated from 3 minutes of steady-state recordings and are quoted as mean±SEM values. Probability density functions were fitted by the method of maximum likelihood\(^11\) according.

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cADPR activates the cardiac SR Ca²⁺-release channel in a concentration-dependent manner in the presence of activating cytosolic Ca²⁺. No effect on single-channel conductance was observed (data not shown). Significant increases in Po required concentrations of ≥1 μmol/L, which are higher than the physiological levels of cADPR in cardiac cells (20 to 200 nmol/L). For example, in the presence of 10 μmol/L Ca²⁺, Po was increased from 0.026±0.004 (n=8) to 0.112±0.062 (n=6) by 10 μmol/L cADPR, whereas at 0.1 μmol/L cADPR, the Po was 0.027±0.015 (n=4). The effect of cADPR is highly dependent on the activating cytosolic [Ca²⁺] as demonstrated in Fig 1. At subactivating (picomolar) levels of Ca²⁺, cADPR is unable to cause any channel openings even at concentrations up to 25 μmol/L (n=6). At 0.1 μmol/L Ca²⁺, which is the physiological resting free [Ca²⁺], 1 μmol/L cADPR increased Po from 0 to 0.018±0.004 (n=6), whereas at 10 μmol/L Ca²⁺, 1 μmol/L cADPR increased Po from 0.026±0.004 (n=8) to 0.051±0.025 (n=4). Fig 1D demonstrates the modification by ryanodine of a single channel activated by cADPR in the presence of 10 μmol/L Ca²⁺, indicating that cADPR is activating the ryanodine-sensitive Ca²⁺-release channel. Lifetime analysis (Fig 2) indicates that the primary mechanism for cADPR-induced increase in Po is an increase in the frequency of channel opening. A reduction in the duration of the events occurring to all three closed states is apparent, with little increase in the duration or the distribution of the open events. This mechanism is consistent with a sensitization of the channel to activating Ca²⁺ by cADPR. At high concentrations of cADPR (>10 μmol/L), Po values >0.3 can be achieved. Under these conditions, an increase in the duration of channel openings can also be observed. However, the increase in frequency of opening remains the major cause of the increase in Po.

A knowledge of the cADPR binding site is important in assessing the possible physiological relevance of cADPR as an activator of the cardiac SR Ca²⁺-release channel. The chemical structure of cADPR indicates that the receptor for cADPR binding could be the adenine nucleotide site on the Ca²⁺-release channel. Our experimental findings demonstrate that this may be the case. In the presence of 10 μmol/L Ca²⁺, a submaximal concentration of ATP (100 μmol/L) activates the channel to a Po of 0.538±0.175 (n=4). On subsequent addition of 1 or 10 μmol/L cADPR, Po fell to 0.437±0.154 (n=4) and 0.383±0.194 (n=4), respectively. A typical example of this effect of cADPR is shown in Fig 3. Clearly, there is no potentiation of the ATP effect by cADPR; in fact, Po declines, thus indicating that cADPR and ATP may be competing for the same site.

Mészáros et al. suggested that cADPR may be able to trigger the opening of the cardiac Ca²⁺-release channel as an alternative activator to Ca²⁺. We have therefore investigated whether cADPR can activate the channel in the presence of ATP and free Ca²⁺ and Mg²⁺ levels likely to be present in the cell. Cardiac diastolic free [Ca²⁺] is 0.1 μmol/L, free Mg²⁺ is 15 μmol/L, and the ATP content is 10 mmol/L. Fig 4 illustrates the effect of cADPR under these conditions. Concentrations of cADPR (1 and 10 μmol/L) that activate the channel in the presence of activating levels of Ca²⁺ are unable to open the channel in the presence of physiological levels of Mg²⁺ and ATP at resting free [Ca²⁺] (0.1 μmol/L) (Po=0, n=4). A subsequent increase in the free [Ca²⁺] to 100 μmol/L causes a rapid large increase in Po (0.326±0.224, n=4; see Fig 4D). If Ca²⁺ is increased to 100 μmol/L without prior addition of cADPR, similar Po levels are attained (0.271±0.127,
n=5), and addition of 1 and 10 μmol/L cADPR then has no effect on Po.

cADPR is a metabolite of β-NAD⁺ and is itself metabolized to ADP-ribose.² Both compounds are structurally very similar to cADPR and might be expected to interact with the adenine nucleotide binding site. We have therefore determined the effect of these agents on the cardiac Ca²⁺-release channel. We find that both β-NAD⁺ (n=3) and ADP-ribose (n=3) are potent activators of the channel. Fig 5 shows current fluctuations from two representative channels.

Discussion

We find that cADPR activation of the cardiac SR Ca²⁺-release channel is concentration dependent; however, unlike Meszários et al., we also find that the effect of cADPR is dependent on the cytosolic [Ca²⁺]. At subactivating cytosolic [Ca²⁺], cADPR is unable to open the channel. Lifetime analysis demonstrates that at the concentrations of cADPR likely to be present in cardiac cells (<1 μmol/L)⁴ the mechanism for an increase in Po will be an increase in the frequency of channel opening with no increase in the duration of the open lifetimes. This is the mechanism by which Ca²⁺ activates the channel and suggests that cADPR may be sensitizing the channel to Ca²⁺.⁸

In the presence of a submaximal concentration of ATP, the same concentrations of cADPR cause no increase in Po. The apparent reduction in Po caused by cADPR indicates that cADPR could be a partial agonist at the adenine nucleotide binding site. McGarry and Williams⁹ have demonstrated that the caffeine binding site on the sheep cardiac SR Ca²⁺-release channel is distinct from the adenine nucleotide binding site. We find that caffeine potentiates the effect of cADPR (n=3; results not shown) as would be expected if cADPR is exerting an effect via the adenine nucleotide receptor. The results of these experiments pose serious questions about the ability of cADPR to exert any significant effect on the cardiac SR Ca²⁺-release channel during excitation-contraction coupling. We have demonstrated that cADPR is unable to cause a significant increase in Po in the presence of 100 μmol/L ATP. However, the
physiological cytosolic concentration of ATP in cardiac cells is closer to 10 mmol/L.4 If cADPR is competing for the same receptor site as ATP, it is probable that the effect of cADPR will be completely swamped by the action of millimolar levels of ATP.

In the presence of Mg$^{2+}$, millimolar ATP, and diastolic free [Ca$^{2+}$], cADPR is unable to trigger channel openings (Fig 4). cADPR also fails to modify the Po of channels that have opened in response to elevating the cytosolic free [Ca$^{2+}$]. These results indicate that cADPR is unlikely to act as a trigger for the opening of cardiac SR Ca$^{2+}$-release channels under physiological conditions. Moreover, cADPR appears to have very little effect even as a modulator of Ca$^{2+}$-activated channels in the presence of Mg$^{2+}$ and ATP. Consideration of the mechanisms underlying the regulation of channel gating by Mg$^{2+}$ suggests that only Ca$^{2+}$ or another agent acting at the Ca$^{2+}$ binding site is likely to trigger channel opening. Experimental evidence indicates that Mg$^{2+}$ reduces Po by binding to the Ca$^{2+}$-activation site in a competitive manner. The Mg$^{2+}$-bound channel is closed and will not open until Mg$^{2+}$ is displaced by Ca$^{2+}$.

We have also demonstrated that β-NAD$^+$ and ADP-ribose can activate the cardiac Ca$^{2+}$-release channel. These results are particularly important because they suggest that cADPR not only has to compete with ATP for the adenine nucleotide binding site but also with β-NAD$^+$ and ADP-ribose. Since β-NAD$^+$ is at least as effective at increasing Po as cADPR but is also present in micromolar to millimolar quantities,17 it would appear unlikely that cADPR exerts any significant effect in cardiac excitation-contraction coupling by directly activating the Ca$^{2+}$-release channel. There has recently been much interest in the possible role of cADPR in Ca$^{2+}$-release mechanisms.3,5,19-22 However, the present study indicates that caution should be used when extrapolating from experimental results to events occurring physiologically. The magnitude of the cADPR-induced responses may be highly dependent on the experimental ATP concentration and/or Mg$^{2+}$ levels. This appears to be the case in the experiments of Walseth and Lee,20 who used sea urchin egg homogenates. The apparent affinity constant for cADPR binding was very different when derived from binding data or from Ca$^{2+}$-release data. The Ca$^{2+}$-release measurements were performed in the presence of ATP, and the apparent affinity constant was ~100 times greater than that of the binding data obtained in the absence of ATP. If experimental ATP or Mg$^{2+}$ levels are lower than those occurring in the cell, cADPR may exert a far greater effect than would be expected physiologically.

Our experiments suggest that Ca$^{2+}$ mobilization by cADPR in sea urchin eggs is not the result of direct activation of the cardiac isoform of the ryanodine receptor. Therefore, either another isoform is present in sea urchin eggs (which appears quite likely, since McPherson et al23 have identified a Ca$^{2+}$-release channel in sea urchin eggs with a molecular weight lower than that expected for the cardiac Ca$^{2+}$-release channel) or the mechanism is an indirect one.

In conclusion, we have demonstrated that cADPR can activate the sheep cardiac SR Ca$^{2+}$-release channel; however, cADPR appears to compete with ATP at the adenine nucleotide binding site on the channel protein. There appears to be no distinct cADPR binding site on the cardiac SR Ca$^{2+}$-release channel. Given the ~100 000-fold concentration difference in the cytosolic concentrations of cADPR and ATP, it is extremely unlikely that cADPR could exert any significant effect on channel gating in the cell. Our demonstration that in addition to cADPR, β-NAD$^+$ and ADP-ribose activate the cardiac SR Ca$^{2+}$-release channel lends support to this conclusion.

The data and arguments in the present study are entirely compatible with a mechanism for Ca$^{2+}$ release from the SR of cardiac muscle involving Ca$^{2+}$ as the physiological trigger.24,25 The results of earlier studies involving cADPR-induced release of Ca$^{2+}$ from intracellular stores of nonmuscle cells have been interpreted in terms of an action of this compound on a ryanodine-sensitive channel. The findings of the present study suggest that this effect is not the result of direct activation of the cardiac isoform of the ryanodine receptor by cADPR.

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References


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