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Cyclic ADP-ribose induced Ca^{2+} release in rabbit skeletal muscle sarcoplasmic reticulum

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The Ca^{2+}-mobilizing metabolite cyclic ADP-ribose (cADPR) has been shown to release Ca^{2+} from ryanodine-sensitive stores in many cells. We show that this metabolite at a concentration of 17 μM, but not its precursor β-NAD^+ nor non-cyclic ADPR at the same concentration, is active in releasing Ca^{2+} from rabbit skeletal muscle sarcoplasmic reticulum. The release was not sensitive to Ruthenium red (1 μM) nor to the ryanodine receptor-specific scorpion toxin *Buthotus*$_1$-1 (10 μM). In planar bilayer single channel recordings, concentrations up to 50 μM cADPR did not increase the open probability of Ruthenium red and toxin-sensitive Ca^{2+} release channels. Thus Ca^{2+} release induced by cADPR in skeletal muscle sarcoplasmic reticulum may not involve opening of ryanodine receptors

Cyclic ADP-ribose, Ryanodine receptor; Ca^{2+} release channel

1. INTRODUCTION

A well-known Ca^{2+} mobilizing cascade is initiated by the membrane-bound metabolite inositol 1,4,5 trisphosphate (IP_3) which, once released from the plasma membrane, binds to a receptor and opens a Ca^{2+} channel in intracellular Ca^{2+} stores [1]. Other Ca^{2+}-mobilizing agents, including the recently described NAD^+ metabolite cyclic ADP ribose [2–4], mobilize Ca^{2+} from intracellular stores but operate independently of the IP_3 pathway [5,6]. Therefore, it is highly likely that intracellular Ca^{2+} channels other than the IP_3 receptor participate in cell Ca^{2+} signalling mediated by cADPR. A separate class of intracellular Ca^{2+} channels, namely ryanodine receptors, has been described in muscle and brain cells where they are believed to mediate the ubiquitous Ca^{2+}-mobilizing mechanism known as Ca^{2+}-induced Ca^{2+} release [7]. In skeletal and cardiac muscle, ryanodine receptors are abundant in the junctional sarcoplasmic reticulum [8] and release Ca^{2+} during excitation–contraction coupling in response to cell membrane depolarization [9]. Ryanodine receptors are activated by Ca^{2+} at physiological concentrations, and are modulated by a variety of ligands that affect Ca^{2+}-induced Ca^{2+} release such as Mg^{2+}, adenine nucleotides and caffeine [10–14].

Lee [15] showed that cADPR like caffeine, potentiated Ca^{2+}-induced Ca^{2+} release in sea urchin eggs. This observation, plus the fact that cADPR-sensitive stores are also ryanodine-sensitive [5,6], and results from recent single channel recordings in cardiac SR [16], suggested that cADPR may be a naturally occurring endogenous ligand of the ryanodine receptor [5,16]. We tested this possibility in rabbit skeletal SR by performing Ca^{2+} release measurements in isolated junctional SR vesicles and single channel recordings in planar bilayers. While cADPR specifically released SR-stored Ca^{2+}, this release was not sensitive to ryanodine-receptor blockers. Furthermore, high concentrations of cADPR up to 50 μM failed to increase the open probability of skeletal ryanodine receptor channels in planar bilayers. We thus suggest that a non-ryanodine receptor release mechanism may be involved in the action of this novel ligand in skeletal muscle.

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2. MATERIALS AND METHODS

2.1. *Preparation of junctional SR*

SR was prepared from rabbit back and leg skeletal muscle [14]. Sucrose density-purified membranes sedimenting between 35% to 40% sucrose were used in all experiments. Fresh membranes or membranes that were stored in 0.3 M sucrose, 0.1 M KCl, 5 mM Na-PIPES pH 6.8 at −80°C for up to two weeks were used in all experiments.

2.2. *Planar bilayer recording of Ca^{2+} release channels*

Planar bilayer formation and recording was described previously [17]. Bilayers were composed of equal concentrations of brain phosphatidylethanolamine and phosphatidylserine dissolved in decane at 20 mg/ml. SR (100–200 μg) was added to the *cis* (cytosolic) solution composed of 240 mM Cs-methanesulfonate, 10 mM CsCl and 10 mM HEPES titrated with Tris to pH 7.2. The *trans* (lumenal) solution was 40 mM Cs-methanesulfonate, 10 mM CsCl, and 10 mM HEPES-Tris pH 7.2. The contaminant-free Ca^{2+} of the *cis* chamber

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was in the range of 1 to 3.6 μM and was measured by Ca^{2+} electrode. Recordings were filtered through a low-pass Bessel (Frequency Devices, Haverhill, MA) at 1 kHz and digitized at 4 kHz.

2.3. *Ca^{2+} release measurements*

Ca^{2+} release from SR vesicles was measured using the Ca^{2+} indicator dye Fura-2 (Molecular Probes, Eugene, OR) on a Hitachi F-2000 fluorescence spectrophotometer. Approximately 150 μg of SR vesicles were actively loaded with Ca^{2+} by the addition of 2 mM MgATP in a 300 μl cuvette containing 100 mM potassium gluconate, 5 mM phosphocreatine, 5 μg/ml creatine phosphokinase, 0.5 μM Fura-2 (free acid) and 20 mM HEPES-Tris pH 7.2 at 25°C. The free Ca^{2+} used for Ca^{2+} loading was 0.5 to 2 μM and was present in the loading solution as a contaminant. Ca^{2+} transients were quantified as described previously using built-in software [18].

2.4. *Synthesis of cADPR*

cADPR was synthesized from β-NAD^+ using the enzyme ADP ribosyl cyclase (also known as NADase) purified from the ovotestis of *Aplysia californica* as described by Hellmich and Strumwasser [19]. cADPR was purified as described by Lee et al. [20]. The concentration of cADPR in water at pH 6.0 was determined using an extinction coefficient ε_{254} = 14,300 [20]. Cyclic ADPR was homogeneous as assessed by C_{18} reverse-phase thin-layer chromatography in methanol/water, 1:1 (R_f = 0.87) and by PEI cellulose thin-layer chromatography (R_f = 0.73) in a system which was 0.2 M LiCl for 2 min, 1.0 M LiCl for 6 min, followed by 1.6 M LiCl.

3. RESULTS AND DISCUSSION

Following ATP-dependent sequestration of Ca^{2+} into junctional SR vesicles of rabbit skeletal muscle we tested the ability of cADPR to release the stored Ca^{2+} (Fig. 1). In preliminary experiments we found that 1 μM cADPR released approximately 5 nmol of stored Ca^{2+}, in agreement with measurements in brain and pancreas

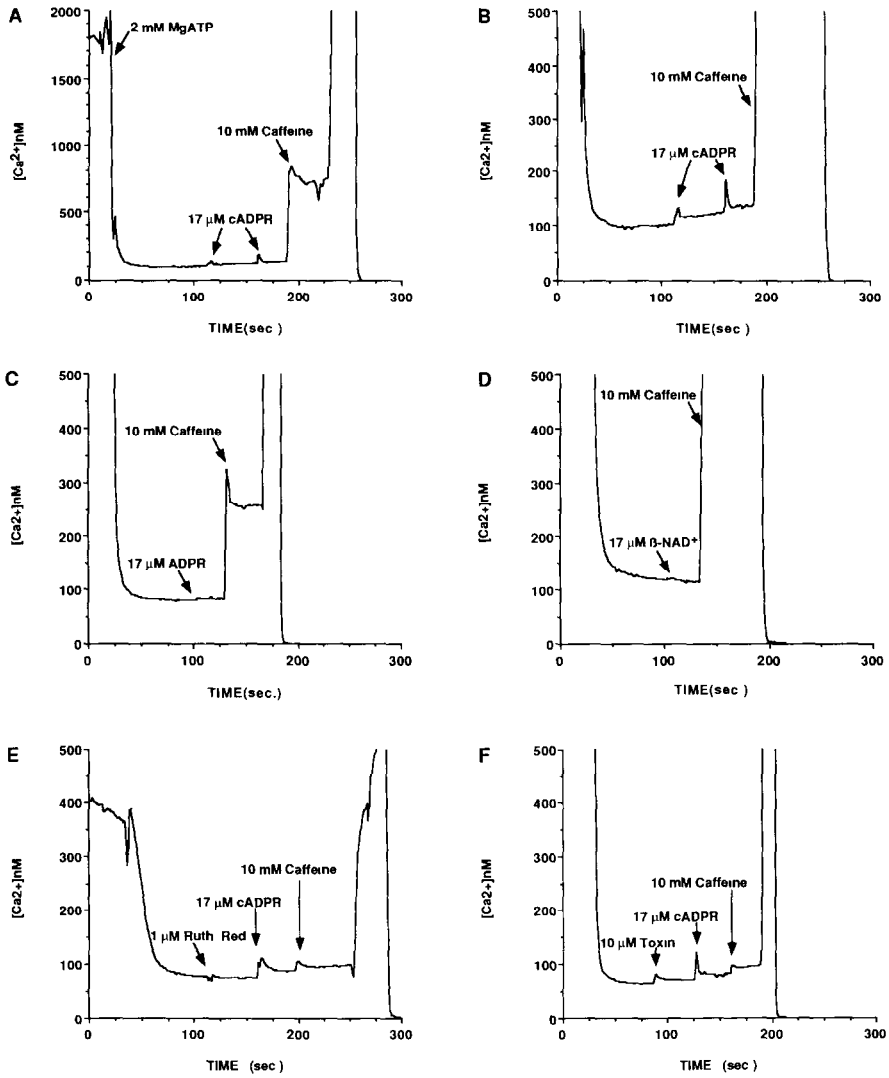

Fig. 1. cADPR-induced Ca^{2+} release from rabbit junctional SR measured by fluorescence of the Ca^{2+} indicator Fura-2. Vesicles (150 μg SR protein in a 300 μl cuvette volume) were actively loaded at the beginning of each experiment with the addition of 2 mM MgATP as described in Materials and Methods. (A) cADPR and caffeine elicit rapid Ca^{2+} release. (B) Same as (A) with expanded scale. (C) Non-cyclic ADPR fails to elicit Ca^{2+} release. (D) β-NAD^+ fails to elicit Ca^{2+} release. (E) Ruthenium red inhibits caffeine-induced but not cADPR-induced Ca^{2+} release. (F) *Buthotus*$_1$-1 toxin inhibits caffeine-induced but not cADPR-induced Ca^{2+} release.

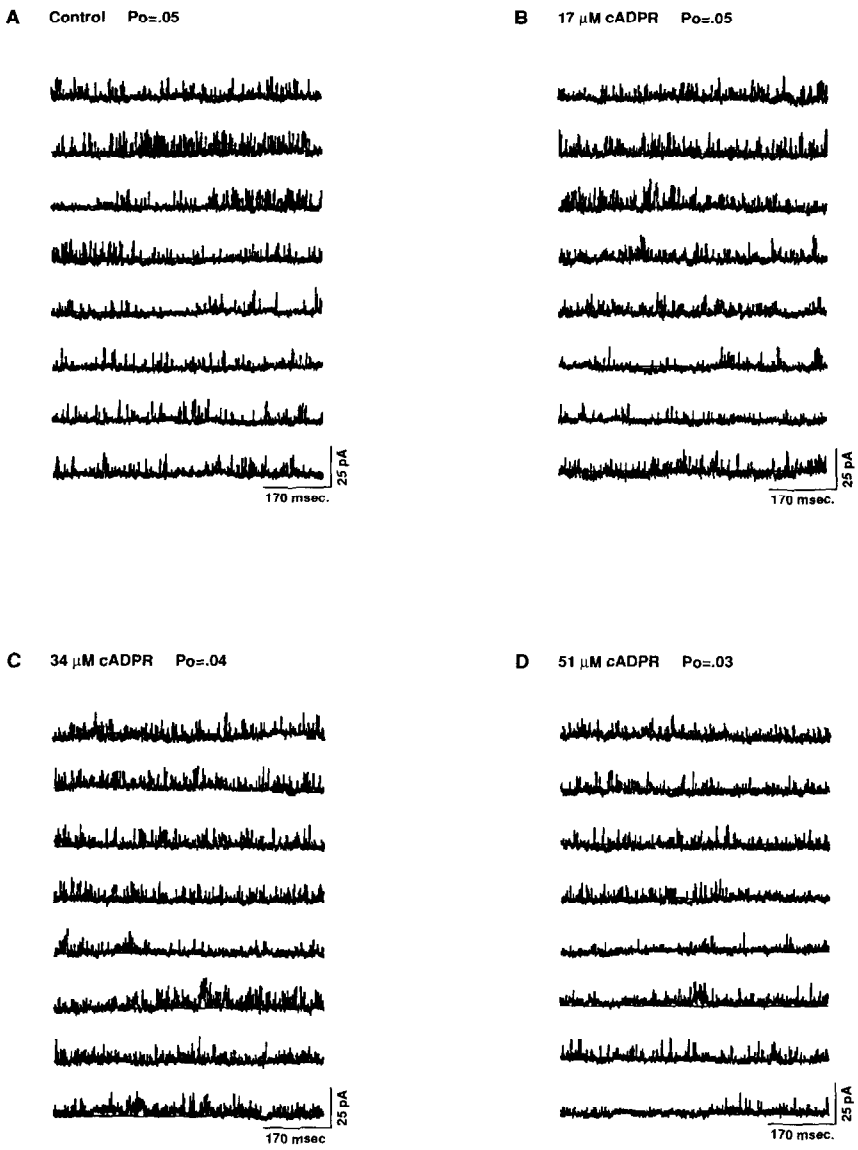

Fig. 2. cADPR fails to activate ryanodine receptor Ca^{2+} release channels incorporated in lipid bilayers. Single channel traces of a ryanodine receptor are shown with openings as upward deflections at a holding potential of 0 mV. The average open probability, P_o, during $\geq$ 90 s of activity in each condition is shown before (control, panel A) and after three additions of cADPR to the *cis* solution of the same channel (panels B,C,D). Average baseline is shown as a thin line under each trace.

microsomes [5,6]. Unlike in the previous studies however, Ca^{2+} release induced by 1 μM cADPR did not saturate with further increases in concentration. We thus decided to use higher concentrations of cADPR to improve the signal-to-noise ratio. At a concentration of 17 μM (Fig. 1A,B), cADPR released 25–50 nmol Ca^{2+} which is approximately 10 times larger than the Ca^{2+} released by saturating concentrations of this compound in brain or pancreas microsomes ($\approx$1 μM cADPR) at comparable protein concentrations [5,6]. cADPR did not interfere with the release induced by caffeine (10 mM) which typically mobilized 300 to 500 nmol of stored Ca^{2+}. The specificity for the cyclic analogue was demonstrated in Fig. 1C,D in which neither the non-cyclic analogue adenosine 5′-diphosphoribose (ADPR) nor the precursor β-NAD^+, at the same concentration, exhibited Ca^{2+} releasing activity. In other experiments (not shown) non-cyclic ADPR did not interfere with the ability of cADPR to release SR Ca^{2+}. To test if cADPR-induced release of Ca^{2+} occurred by opening of ryanodine receptors, we used the blocker Ruthenium red (1 μM) and *Buthotus*$_I$-1 (10 μM), a 13 kDa peptide toxin blocker purified from the venom of the scorpion *Buthotus hottentota* that is specific for ryanodine receptors [21]. As shown in Fig. 1E,F neither of the two ryanodine receptor blocking agents were effective in blocking the Ca^{2+} releasing ability of cADPR. At the same time, the responses to caffeine were almost com-

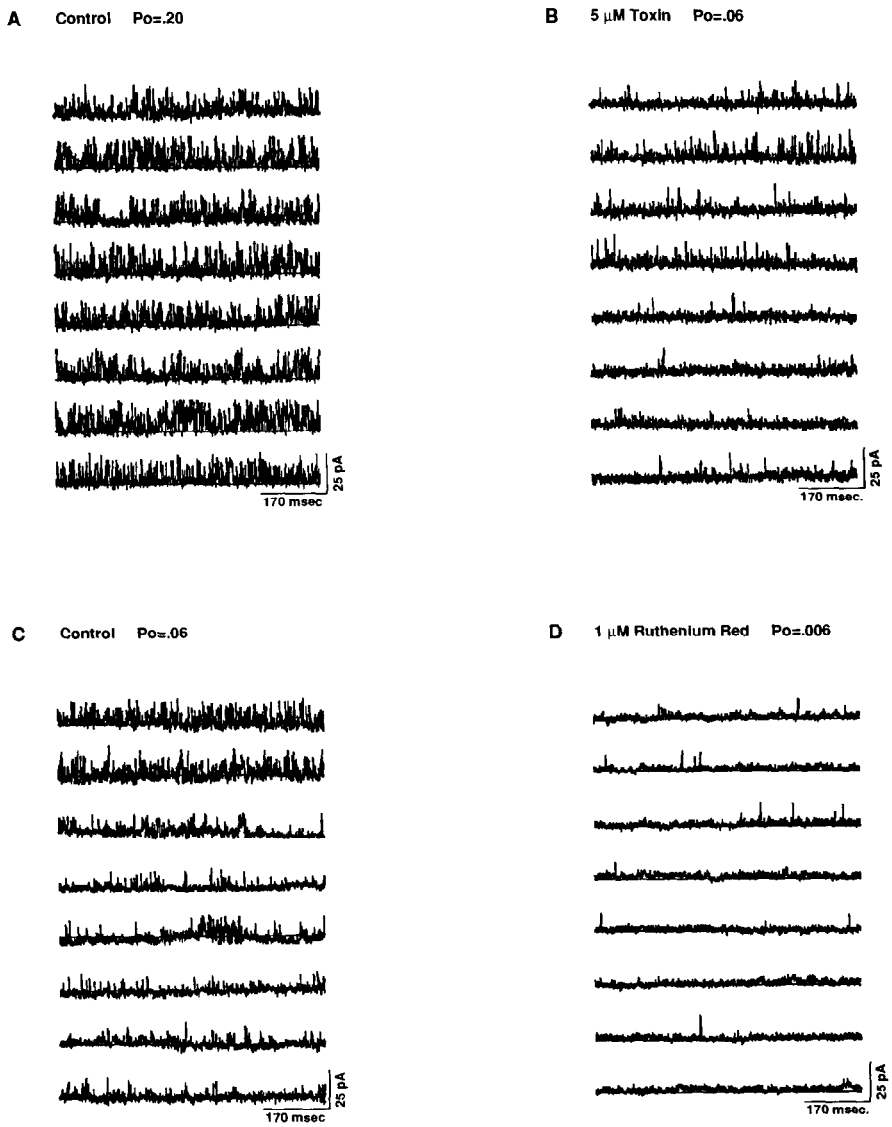

Fig. 3. *Buthotus*$_I$-1 toxin (5 μM) and Ruthenium red (10 μM) inhibit ryanodine receptor Ca^{2+} release channels incorporated in lipid bilayers. Single channel traces of two separate ryanodine receptors (A,B and C,D) are shown with openings as upward deflections at a holding potential of 0 mV. The average open probability, P_o, during $\geq$ 90 s of activity in each condition is shown before (control panel A,C) to the *cis* solution. Average baseline is indicated as a thin line under each trace.

pletely eliminated by Ruthenium red and *Buthotus*$_I$-1 toxin. Thus it was considered highly unlikely that cADPR may have activated ryanodine receptor channels under these conditions since caffeine, which is a much more potent Ca^{2+} releasing agent, clearly could not stimulate the blocked channel.

The lack of effect of cADPR on ryanodine receptor channels was further confirmed in Fig. 2 by fusion of rabbit junctional SR to a planar bilayer. The identification of this large unit conductance channel as the ryanodine receptor has been documented extensively [9,17,21]. In this case, the control channel activity at 0 mV was elicited by Ca^{2+} which is present in the myoplasmic-equivalent *cis* solution as a contaminant and is typically 1 to 3 μM free Ca^{2+}. Open probability in the control segment and following each of three separate additions of cADPR, was monitored for at least 90 s. The figure shows representative consecutive traces during 5.4 s following each addition of cADPR to the *cis* solution. The open probability, P_o, for the entire monitoring period ($\geq$ 90 s) is indicated at the top of each panel. There was no activation by cADPR at concentrations that clearly resulted in a release of stored Ca^{2+}, i.e. 17 μM (panel B), nor at higher doses, i.e. 34 (panel C) or 51 μM (panel D). The slight decrease in activity during the recording period in panel D may have been caused by channel rundown.

Fig. 3 shows that under the recording conditions used to test cADPR, ryanodine receptors remained sensitive to Ruthenium red and scorpion toxin. In the top left (A)

and right (B) panels, *Buthotus*$_1$-1 toxin at half the concentration used in Fig. 1, inhibited open probability 3-fold. In the bottom left (C) and right (D) panels, Ruthenium red at the same concentration used in Fig. 1 inhibited activity 10-fold. Thus, the ryanodine receptor block by scorpion toxin and Ruthenium red was totally consisten with the inhibition of the Ca^{2+} release response elicited by caffeine in Fig. 1. Based on these results, it becomes difficult to argue that ryanodine receptor channels in planar bilayers become desensitized to cADPR while at the same time remain sensitive to two other ligands.

In sea urchin egg microsomes, cADPR potentiates the caffeine-induced release of stored Ca^{2+} and conversely, caffeine potentiates the cADPR-induced release of stored Ca^{2+} [15]. This observation suggested both agents share a common caffeine and cADPR sensitive release mechanism [15]. Studies in pancreas and brain further suggested that cADPR-sensitive stores are sensitive to ryanodine but not to IP_3 [5,6], thus establishing the ryanodine receptor as a possible target of cADPR [5]. Mezaros et al. [16] conducted single channel recordings of ryanodine receptors in planar bilayers and concluded that the cardiac but not the skeletal receptor type was sensitive to cADPR. Our results are consistent with those of Mezaros and collaborators in that we found no activation of rabbit skeletal Ca^{2+} release channels by this compound even at an extremely high dosage. The lack of participation of skeletal ryanodine receptors in the response to cADPR in skeletal SR raises the possibility that a separate Ca^{2+} release channel type may be sensitive to cADPR in this tissue.

In conclusion, we describe a significant and specific release of Ca^{2+} from rabbit skeletal SR by cADPR occurring via a non-ryanodine receptor mechanism. This is supported by two pharmacological interventions and the lack of stimulatory effect of cADPR in single channel recordings of skeletal ryanodine receptors. It is important to mention however that in some respects, the response to cADPR in skeletal SR is different from that described in brain, pancreas, and invertebrate eggs [5,6,15]. Release in skeletal SR increased with concentration in the micromolar range of cADPR and unlike the release in brain and pancreas [5,6], it did not desensitize with consecutive additions of cADPR to the same vesicle suspension (Fig. 1B). Furthermore the cADPR-induced release in skeletal SR resulted in the mobilization of a much larger amount of stored Ca^{2+} than previously reported in non-muscle cells. Although not as an alternative explanation to our results with skeletal muscle, it is possible that the ryanodine receptor sensitivity to cADPR may indeed be tissue-specific, being high in the heart and brain receptors [5,16] but absent in the skeletal receptor type. The presence of proteins that may confer cADPR sensitivity to the ryanodine receptor then becomes an intersting possibility to consider, specially in the case of the sea urchin egg where the skeletal ryanodine receptor type has been described [22].

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