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Cyclic ADP-ribose does not affect cardiac or skeletal muscle ryanodine receptors

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Abstract The cardiac muscle isoform of the ryanodine receptor/Ca^{2+} release channel (RYR) has been proposed to be an important target of cyclic ADP-ribose (cADPR) action in mammalian cells. However, we now demonstrate that neither cADPR (0.1–5 μM), nor the related metabolites β-NAD^+ (0.1–30 mM) and ADP-ribose (0.1–5 μM), affected cardiac RYR activity as determined by [^{3}H]ryanodine binding to cardiac sarcoplasmic reticulum (SR) vesicles. Similarly, cADPR (1 μM) failed to activate single cardiac RYR channels in planar lipid bilayers. Skeletal muscle SR [^{3}H]ryanodine binding was also unaffected by cADPR (up to 30 μM). These results argue against a direct role for the well-characterized RYRs of cardiac or skeletal muscle in mediating cADPR-activated Ca^{2+} release.

Key words: Cyclic ADP-ribose; Ryanodine receptor; Ca^{2+} release channel; Sarcoplasmic reticulum

1. Introduction

Cyclic ADP-ribose (cADPR) is an endogenous metabolite of NAD^+ which releases Ca^{2+} from intracellular stores in sea urchin eggs and certain mammalian cell types [1–3]. Because cADPR-activated Ca^{2+} release in sea urchin eggs is sensitive to ryanodine, caffeine, and Ruthenium red, but insensitive to inositol 1,4,5 trisphosphate and heparin, it is considered that cADPR may operate through a member of the ryanodine receptor (RYR) family of Ca^{2+} release channels. Indeed, RYR Ca^{2+} release channels have now been described in a variety of vertebrate and invertebrate cell types [4]. However, to date the best characterized RYR isoforms are those expressed in mammalian cardiac and skeletal muscle, where they constitute the pathway for the rapid release of Ca^{2+} from the sarcoplasmic reticulum (SR) which triggers muscle contraction.

Recently, the possibility that cardiac and skeletal muscle RYR isoforms may be targets of cADPR action has been investigated. In particular, a report by Mészáros et al. concluded that the cardiac RYR isoform (which is also expressed in the brain) may be an important pathway for cADPR-activated Ca^{2+} release [5]. At concentrations of 1–2 μM, cADPR stimulated $^{45}Ca^{2+}$ efflux from both cardiac SR and brain microsomes, stimulated [^{3}H]ryanodine binding to cardiac SR, and increased the open probability of single cardiac RYR channels in planar lipid bilayers. As RYR channel activation was observed only at submicromolar Ca^{2+} concentrations, the authors proposed that cADPR-activated Ca^{2+} release may represent an alternative mechanism to Ca^{2+}-induced Ca^{2+} release for activation of the cardiac RYR channel [5].

The effect of cADPR on skeletal muscle RYR activity has been investigated by two laboratories. Mészáros et al. reported that cADPR had no effect on skeletal muscle SR Ca^{2+} efflux, yet showed a ~70% inhibition of [^{3}H]ryanodine binding to skeletal muscle SR in the presence of 2 μM cADPR [5]. From these data the authors concluded that cADPR produced a conformation change in the skeletal muscle RYR that inhibits ryanodine binding without affecting SR Ca^{2+} release. In contrast, Morrissette et al. reported that Ca^{2+} efflux from skeletal muscle SR vesicles was activated by 1–17 μM cADPR [6]. However, these authors also demonstrated that cADPR-activated Ca^{2+} efflux from skeletal muscle SR vesicles was insensitive to RYR channel inhibitors, and that cADPR had no effect on single skeletal muscle RYR channels in planar bilayers. They concluded that skeletal muscle SR does exhibit a cADPR-activated Ca^{2+} release pathway, but that this pathway operates through non-RYR channels [6].

To further investigate the potential role of RYRs in cADPR-activated Ca^{2+} release, we have examined the effect of cADPR on cardiac RYR [^{3}H]ryanodine binding and single channel activity in planar lipid bilayers. The effect of cADPR on skeletal muscle RYR [^{3}H]ryanodine binding was also examined. Our results fail to confirm the findings of Mészáros et al. and rather suggest that proposed effects of cADPR on intracellular Ca^{2+} release are not likely to be mediated via direct effects on cardiac or skeletal muscle RYR function.

2. Materials and methods

Cardiac muscle SR vesicles were prepared from porcine ventricular tissue as described [7], except as noted. Briefly, following homogenization in 10 mM $NaHCO_3$, membranes were pelleted and extracted in 0.6 M KCl, 20 mM Tris, (pH 6.8), resuspended in 10% sucrose, and stored frozen at −70°C. Porcine cardiac muscle SR vesicles were also prepared by the procedure [10] used by Mészáros et al. [5] to address possible differences between this preparation and our own. Skeletal muscle SR vesicles were prepared from porcine longissimus dorsi [8]. Briefly, membranes obtained by differential ultracentrifugation of a muscle homogenate were extracted with 0.6 M KCl and fractionated on discontinuous sucrose gradients. Membranes sedimenting between 36–40% sucrose were collected and stored frozen at −70°C. All isolation buffers contained 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) to minimize proteolysis.

[^{3}H]Ryanodine binding was determined in media containing 0.2 mg SR protein/ml, 0.1 M KCl, 10 mM PIPES (adjusted to pH 7.0 with Tris), 2 mM $CaCl_2$, 0.5 mM PMSF, 100 nM [^{3}H]ryanodine. EGTA and nitrilotriacetic acid were varied from 2.90–9.09 mM to achieve the desired range of ionized Ca^{2+} concentrations. Following 90 min incubations at 37°C, membranes were filtered onto Whatman GF/B filters and

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washed with 8 ml ice-cold 0.1 M KCl, 10 mM PIPES buffer (pH 7.0). For determinations of nonspecific binding, incubations were performed in the presence of 20 μM nonradioactive ryanodine.

The single channel activity of cardiac RYR channels in planar lipid bilayers was examined essentially as described [11]. Bilayers were formed by painting a lipid mixture comprised of synthetic palmitoyl-oleyl-phosphatidylethanolamine, palmitoyl-oleyl-phosphatidylserine, and palmitoyl-oleyl-phosphatidylcholine dissolved in *n*-decane (50 mg/ml) in a 5:3:2 ratio by weight, across a 250 μm aperture in a Delrin cup. The recording solution was symmetric 400 mM cesium methylsulfonate, 20 mM HEPES, pH 7.2. Analogue data from a custom-designed headstage and amplifier was filtered at 1,500 Hz through an 8-pole Bessel filter and digitized at 15,200 Hz for storage on a computer. Cardiac SR vesicles were added to the *cis* (cytoplasmic) chamber and incorporated into the bilayer using a pulsing protocol (−50 mV to +50 mV) in the presence of a gradient of 400 mM/200 mM cesium methanesulfonate, 20 mM HEPES, pH 7.2, 7 μM ionized Ca^{2+}. The gradient was dissipated after vesicle fusion by the addition concentrated cesium methanesulfonate to the *trans* chamber. RYR channels were identified by their characteristic Cs^+ conductance of approximately 560 pS and by their inactivation in the presence of nanomolar Ca^{2+}. Whenever possible, channels were also identified by a characteristic response to ryanodine, caffeine, or ATP added at the conclusion of an experiment. Single channel data was collected using a pulsing protocol in which the potential was held at +70 mV for 990 ms, then pulsed to −70 mV for 60 ms (CLAMPEX software, Axon Instruments). When two channels were present in the bilayer simultaneously (as indicated by current amplitudes two times the expected current amplitude) single channel percent open time (P_o) was calculated as:

$$\frac{P_{o_{level_1}} + (P_{o_{level_2}} \times 2)}{2}$$

cADPR was prepared and purified in the laboratory of Dr. H.C. Lee, University of Minnesota, following an established protocol (e.g. [2,5]). Briefly, cADPR was synthesized from NAD^+ using ADP-ribosyl cyclase from *Aplysia* ovotestis, and purified on an AG MP-1 column (Bio-Rad) using a gradient of trifluoroacetic acid. Concentrations of cADPR stock solutions were determined by absorbance at 254 nm using an extinction coefficient of 14,300.

3. Results and discussion

Ryanodine interacts with the open state of the RYR channel and thus its binding directly reflects RYR channel activity [9]. Accordingly, [^{3}H]ryanodine binding to cardiac SR was activated by 10 mM caffeine or by micromolar Ca^{2+}, and was inhibited by 10 μM Ruthenium red; in contrast 1 μM cADPR had no effect on either the Ca^{2+} dependence or the magnitude of [^{3}H]ryanodine binding to cardiac SR (Fig. 1A). Increasing cADPR concentrations up to 5 μM similarly had no significant effect on cardiac SR [^{3}H]ryanodine binding at either 100 nM or 10 μM Ca^{2+} (Fig. 1B). The lack of an effect of cADPR on [^{3}H]ryanodine binding cannot be explained by the hydrolysis of cADPR, because in control experiments, cADPR concentrations were unchanged following incubation with cardiac SR under our assay conditions (as determined by HPLC analysis, data not shown). Furthermore, neither the metabolic precursor of cADPR (NAD^+) nor the product of its hydrolysis (ADPR) affected [^{3}H]ryanodine binding to cardiac SR (Fig. 1B).

Table 1
Absence of cADPR effect on cardiac RYR single channel P_o

	P_o (%)		
	pCa 5	pCa 7	pCa 8
Control	23.0 ± 3.6 (9)	9.1 ± 1.6 (4)	0.2 ± 0.07 (5)
1 μM cADPR	–	8.0 ± 4.1 (4)	0.1 ± 0.03 (5)

Single channel percent open times were calculated from 215–400 s of total recording time for each condition. Data represent means ± S.E. of the number of experiments indicated in parenthesis. P_o values were not significantly different in the absence and presence of cADPR ($p = 0.3$ at pCa 8, student's *t*-test).

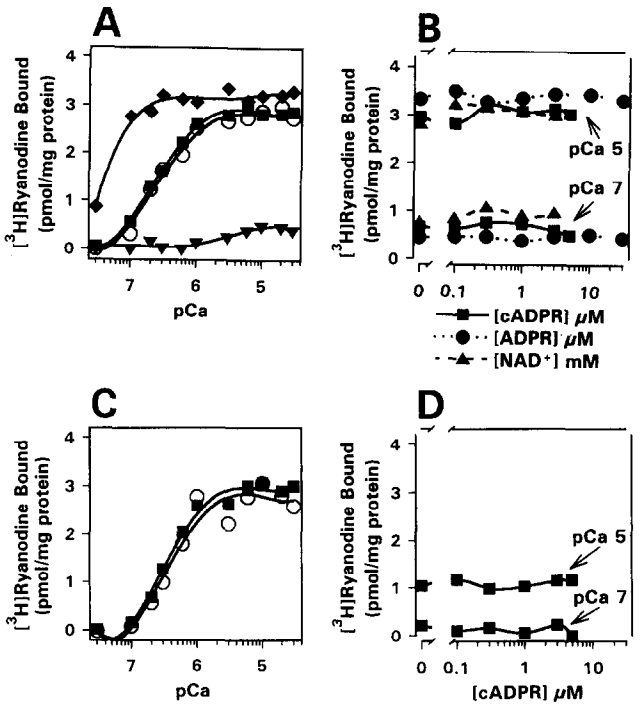

Fig. 1. Absence of cADPR effect on [^{3}H]ryanodine binding to cardiac muscle SR. (A) Ca^{2+} dependence of [^{3}H]ryanodine binding to cardiac SR in the absence (○) or presence of 10 mM caffeine (◆), 10 μM Ruthenium red (▼) or 1 μM cADPR (■). (B) Concentration dependence of cADPR and related metabolites on cardiac SR [^{3}H]ryanodine binding at pCa 5 and pCa 7. (C) [^{3}H]Ryanodine binding to cardiac SR determined essentially as described [5,9] in the presence of 10 nM ryanodine, 300 mM KCl, 20 mM PIPES, pH 7.0, in the absence (○) and presence (■) of 5 μM cADPR; 16 h incubations at 20°C. (D) Effect of cADPR on [^{3}H]ryanodine binding to cardiac SR prepared as described [5,9]. Data in panels (A) and (B) are means of at least four determinations performed in the presence of 100 nM ryanodine, 100 mM KCl, 10 mM PIPES buffer, pH 7.0 as described in section 2. Data in panels (C) and (D) are means of duplicate determinations.

The conditions of our [^{3}H]ryanodine binding assay differed somewhat from the assay conditions used by Mészáros et al. [5]. We therefore investigated the possibility that these differences may have contributed to our inability to demonstrate an activation of [^{3}H]ryanodine binding by cADPR. However, under conditions very similar to those used by Mészáros et al., cADPR (5 μM) again had no effect on cardiac SR [^{3}H]ryanodine binding (Fig. 1C). We also examined [^{3}H]ryanodine binding to cardiac SR prepared by the procedure [10] used by Mészáros et al. [5]. However, 1 μM cADPR again had no effect on [^{3}H]ryanodine binding to this preparation (Fig. 1D). Thus, our results fail to confirm the stimulation of [^{3}H]ryanodine binding to cardiac SR (~450% stimulation in the presence of 2 μM cADPR, 100 nM Ca^{2+}) reported by Mészáros et al. [5].

The failure of cADPR to activate the cardiac RYR was confirmed by examining the single-channel open probability (P_o) of cardiac RYR incorporated into planar lipid bilayers. The representative experiment shown indicates two channels in

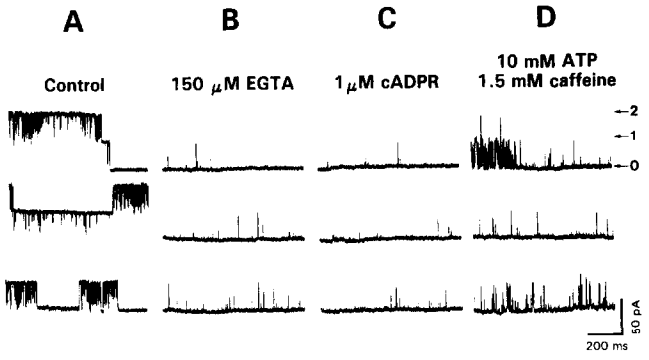

Fig. 2. Absence of cADPR effect on porcine cardiac RYR channels incorporated into lipid bilayers. Representive 858 ms traces are shown under control conditions (A, pCa 5; P_o = 22%) and following subsequent additions of 150 μM EGTA (B, pCa 8; P_o = 0.2%), 1 μM cADPR (C, P_o = 0.1%), and 1.5 mM caffeine and 10 mM ATP (D, P_o = 0.8%). Arrows at the upper right indicate current amplitudes for 0, 1, and 2 open channels. Records are from an experiment performed in the presence of symmetric 400 mM cesium methylsulfonate, 20 mM HEPES, pH 7.2, at a potential of +70 mV.

the bilayer, each with a unitary current amplitudes of 42 pA (Fig. 2). In the presence of 10 μM Ca^{2+}, channels were frequently in the open state as indicated by upward deflections (Fig. 2A; P_o = 22%). Addition of 150 μM EGTA to the *cis* and *trans* chambers decreased the free Ca^{2+} concentration to approximately 10 nM (as determined by a Ca^{2+} electrode) and decreased the single channel open probability to near zero (Fig. 2B; P_o = 0.2%). Symmetric addition of 1 μM cADPR failed to stimulate RYR channel activity (Fig. 2C; P_o = 0.1%). However, the subsequent addition of 1.5 mM caffeine and 10 mM ATP increased the single channel P_o and demonstrated the continued presence of two channels in the bilayer (Fig. 2D; P_o = 0.8%). A summary of similar experiments (Table 1) demonstrates no significant effect of cADPR on cardiac RYR single channel P_o in the presence of either 10 nM or 100 nM Ca^{2+}. These results contrast with the data of Mészáros et al. [5] who reported a 390% increase in open probability in the presence of 1 μM cADPR and 10 nM Ca^{2+}.

The effect of cADPR on [^{3}H]ryanodine binding to skeletal muscle SR was also examined. Skeletal muscle SR [^{3}H]ryanodine binding was maximally activated by 1–10 μM Ca^{2+} and inhibited by millimolar Ca^{2+}; cADPR (1–30 μM) had no significant effect on skeletal muscle SR [^{3}H]ryanodine binding at any Ca^{2+} concentration examined (Fig. 3). These results thus fail to confirm the inhibition by cADPR of skeletal muscle SR [^{3}H]ryanodine binding (~70% inhibition at 100 μM Ca^{2+} and ~46% inhibition at 10 μM Ca^{2+}, in the presence of 2 μM cADPR) reported by Mészáros et al. [5]. Our results, however, are consistent with those of Morrissette et al. [6] showing no effect of 1–17 μM cADPR on skeletal muscle RYR single channel activity.

Ryanodine receptors have been shown to be regulated by a variety of physiological ligands. For example, adenine nucleotides, Mg^{2+}, H^+, inorganic phosphate, sphingosine, acyl carnitines, and calmodulin, as well as Ca^{2+}, regulate the opening and/or closing of RYR channels in vitro [4,12,13,17]. RYR activity is similarly modulated by a variety of non-physiological ligands including ryanodine, caffeine, Ruthenium red, procaine, halothane, perchlorate, and certain peptide toxins [4,14,15,17]. The effects of these various ligands on RYR activity can be demonstrated by corresponding changes in $^{45}Ca^{2+}$ efflux, [^{3}H]ryanodine binding, and single RYR channel activity in planar lipid bilayers. Thus, it is difficult to reconcile our results showing no effect of cADPR on either [^{3}H]ryanodine binding or RYR channel activity in bilayers with the proposed activation of cardiac muscle RYRs by this metabolite [5].

In sea urchin eggs, cADPR-binding proteins with apparent molecular masses of 100 kDa and 140 kDa have been identified by photoaffinity labelling with 8-azido-cADPR [16]. As these proteins are considerably smaller than the ~565 kDa subunits which form cardiac and skeletal muscle RYRs, it is possible that they constitute a novel intracellular Ca^{2+} release pathway. Alternatively, it has been suggested [16] that the proteins identified by photoaffinity labelling may represent accessory proteins that interact with high molecular weight RYR channels after binding cADPR. Similarly, activation of cardiac RYRs by cADPR might be postulated to depend on interactions with as yet unidentified accessory proteins. In this view, our inability to demonstrate activation of cardiac RYRs by cADPR might be attributed to differences in our preparations or methods that somehow prevented the interaction of accessory proteins with RYRs. However, this explanation seems unlikely in that we were unable to demonstrate activation of cardiac RYRs by cADPR using SR preparations [7,10] and methods of monitoring RYR activity similar to those used by Mészáros et al. [5].

In conclusion, we have been unable to reproduce the effects of cADPR on [^{3}H]ryanodine binding to cardiac and skeletal muscle SR reported by Mészáros et al. [5]. In addition, we have failed to confirm the reported stimulation by cADPR of cardiac RYR single-channel activity in lipid bilayers [5]. Mészáros et al. also reported a significant effect of cADPR on $^{45}Ca^{2+}$ efflux from cardiac muscle SR and brain microsomes. However, these data by themselves do not prove that cADPR-activated Ca^{2+} efflux is mediated by cardiac RYR channels, or even demonstrate that this effect of cADPR can be blocked by known RYR inhibitors [5]. Therefore, based on the [^{3}H]ryanodine binding and single-channel activity determinations reported here, we conclude that cADPR has no direct effects on the functional activity of either cardiac or skeletal muscle RYR isoforms. This suggests that cADPR-activated Ca^{2+} release in muscle and other cell types may operate through pathways which are distinct from these well-characterized RYR channels.

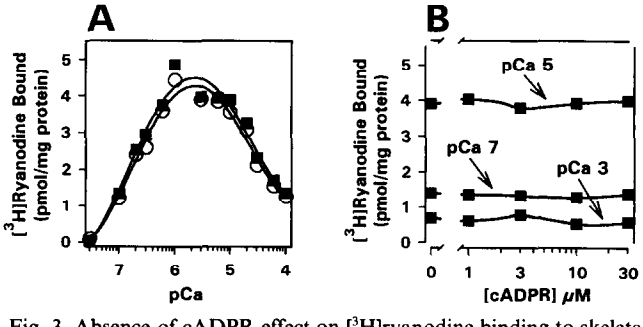

Fig. 3. Absence of cADPR effect on [^{3}H]ryanodine binding to skeletal muscle SR. (A) Ca^{2+} dependence of [^{3}H]ryanodine binding to skeletal muscle SR in the absence (○) or presence of 1 μM cADPR (■). (B) Effect of 1–30 μM cADPR on skeletal muscle SR [^{3}H]ryanodine binding at pCa 3, pCa 5 and pCa 7. Data represent means from two experiments (A) or triplicate determinations from a single experiment (B), performed as described in section 2.

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