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Sarcoplasmic Reticulum-Associated and Protein Kinase C-Regulated ADP-Ribosyl Cyclase in Cardiac Muscle

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Two types of ADP-ribosyl cyclase activity were distinguished in dog and rat cardiac muscles by measuring the enzymatic conversion of NGD (as an NAD analog) into the fluorescent product cyclic GDP-ribose in cardiac muscle subcellular fractions. Both types of activity were confined to membrane fractions isolated from microsomes by sucrose gradient centrifugation. One of the activities co-purified with fractions that were enriched in sarcolemma (SLM), as evidenced by immunodetection of the dihydropyridine receptor, while the other activity was found to co-precipitate with the sarcoplasmic reticulum (SR), that was identified on the basis of its immuno-staining with a ryanodine receptor monoclonal antibody. In certain aspects, the plasma membrane-bound ADP-ribosyl cyclase activity resembled the characteristics of CD38 or CD38-like proteins: it was sensitive to thiols and lectins and was recognized by a monoclonal anti CD38 antibody. The SR enzyme had apparently distinct properties, as it was insensitive to both thiols and lectins and was not recognized by the CD38 antibody. In addition, the SR-associated ADP-ribosyl cyclase was inhibited by endogenous protein kinase C (PKC)-dependent phosphorylation in both dog and rat cardiac SR. The PKC-modulated SR ADP-ribosyl cyclase we describe here might be a principal component of the signal transduction machinery that is responsible for regulation of the intracellular levels of cADPR. © 1997 Academic Press

Cyclic ADP-ribose (cADPR) has been recently discovered as a β-NAD metabolite that is capable of mobilizing Ca^{2+} from inositol 1,4,5-trisphosphate-insensitive intracellular Ca^{2+} stores (1) by either directly or indirectly activating the other intracellular Ca^{2+} release channel, the ryanodine receptor channel (2-4). The wide-spread occurrence of cADPR in various tissues of vertebrates (1) suggests that it might function as a ubiquitous second messenger in Ca^{2+} signaling. However, the notion of cADPR being a possible second messenger would imply that the enzymes that produce and eliminate cADPR are under the control of extracellular stimuli, whereas neither vertebrate enzymes which catalyze the production (and elimination) of cADPR intracellularly nor signaling pathways which modulate these enzymes have yet been identified.

Enzyme-catalyzed formation of cADPR from NAD has been found to take place in a variety of vertebrate tissues as well as in invertebrates (1). From *Aplysia* ovotestis, a 29 kD soluble protein (named ADP-ribosyl cyclase) that catalyzes the stoichiometric conversion of NAD into cADPR (5) has been isolated and its x-ray structure (6) as well as its amino-acid sequence determined (7). A high degree of sequence homology between the *Aplysia* enzyme and the human CD38 lymphocyte surface antigen has been recognized (8) and, as subsequent studies indicate, CD38 is in fact capable of catalyzing the NAD - cADPR conversion (9). Surprisingly however, CD38 was also shown to catalyze the hydrolysis of cADPR to form ADP-ribose (ADPR). A plasma membrane-bound NAD glycohydrolase (NADase) purified from canine spleen was also identified with catalytic activities very similar to those of CD38 (10), raising the possibility that the long-known NADases, that are localized on the outer surface of various cell-types, are identical or highly homologous to CD38. In fact, CD38 or homologous CD38-like proteins have been found in several mammalian tissues (11). However, since both CD38 and the spleen NADase are considered to be ectoenzymes with their active centers oriented outside the cell, it still remains to be determined whether they have any role in producing and/or eliminating cADPR intracellularly, or whether extracellularly produced cADPR can enter the cell and thus can exert any physiological effect intracellularly.

There are some data suggesting that intracellular cADPR production, which has been detected (12), might be under the control of extracellular stimuli. In sea urchin eggs, an increase in NADase activity, and (presumably, although not evidently) an increase in cADPR

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levels as well, has been shown to occur as a result of cGMP-dependent phosphorylation (13). In mammalian cells, increases in cellular levels of cADPR have been reported in response to cholecystokinin- (12) as well as to retinoic acid-challenge (14,15), although the signaling pathways involved in eliciting these cADPR rises are not yet known.

We have recently reported (16) that cardiac muscle possesses both NADase and ADP-ribosyl cyclase activities. Here we demonstrate that two distinct types of ADP-ribosyl cyclase activity are present in rat and dog cardiac muscles. One is confined to the plasma membrane and the other is associated with the sarcoplasmic reticulum (SR) membrane. In addition, we also present evidence that the SR-associated enzyme is regulated by protein kinase C-dependent phosphorylation. The SR-associated ADP-ribosyl cyclase we describe here may be a second messenger generating enzyme that is responsible for controlling the intracellular level of cADPR. Thus, this enzyme might establish a link between extracellular stimuli and cADPR production.

MATERIALS AND METHODS

Fractionation of cardiac muscle. Whole rat hearts or left ventricular muscle from dog hearts were homogenized on ice in a solution of 10 mM NaHCO3, pH 7.0, containing a mixture of protease inhibitors (17). The homogenate was first centrifuged at 7,600 × g to remove the nuclear and mitochondrial fractions, then the supernatant was spun at 130,00 × g to separate the membranous (microsomes) and the cytosolic fractions. The microsomes were further fractionated into 4 (in some cases 3) subfractions (M1 - M4, from top to bottom) on a sucrose gradient as described (18). The cytosolic fraction was used after overnight dialysis against 500 volume of KCl, MOPS, pH 6.85. The membrane fractions taken from the sucrose gradient were washed with 10 mM sucrose, 75 mM KCl and 20 mM MOPS, pH 6.8, then stored frozen at −80°C until use. Since fraction M1 was not present in significant quantities in each dog preparation, it was not characterized during the course of this study. The membrane fractions were identified on the basis of ouabain sensitive K-phosphatase (sarcolemma, SLM) and thapsigargine-sensitive Ca^{2+}-ATPase (sarcoplasmic reticulum, SR) marker enzyme activities which were determined in the presence of 20 mM K-MOPS, pH 6.8 as described (21) and by immunodetection of the dihydropyridine $\alpha 2$ subunit as sarcolemma marker and that of the ryanodine receptor as SR marker on western blots. Western blotting was performed with 10-120 μg membrane preparation denatured and separated on 4-15% SDS polyacrylamide gels. The proteins in gels were electrotransferred onto nitrocellulose membranes (ECL-Hybond C, Amersham) in 12 mM Tris pH 8.3, 192 mM glycine and 20% methanol. The membranes were blocked with 5% fat-free dry milk in a buffer consisting of 20 mM Tris-HCl, pH 7.6, 137 mM NaCl and 0.1% Tween-20 (TBST). After several washes with TBST, the membranes were incubated with the primary antibodies; mouse monoclonal anti-ryanodine receptor (RyR-2) antibody, MA3-916 (Affinity Bioreagents, Inc.) and mouse monoclonal anti-dihydropyridine receptor $\alpha 2$ subunit antibody (SWant, Bellinzona, Switzerland) in TBST. After extensive washing with TBST, the membranes were then incubated with the secondary peroxidase-conjugated antibody (anti mouse IgG, Amersham). Detection was achieved by the ECL procedure (Amersham) applied for 1 min at room temperature, with subsequent exposure to ECL-Hyperfilm.

Enzyme assays. Protein kinase C activity was detected on the basis of phorbol ester (PMA)-activated and staurosporine-sensitive γ-^{32}P-ATP incorporation carried out for 5 min in the presence of 3-12 μM free Ca^{2+}. The phosphorylation reaction was initiated by the addition of radiolabeled ATP (100 μM, containing 4×10^7 cpm) and quenched by boiling SDS-containing sample buffer. The incorporation of ^{32}P-radioactivity into the gel-electrophoretically separated SR proteins was evaluated by 256-gray-level scanning (Jandel Scientific) of autoradiographic films (Fuji-RX) that were previously exposed to the radiolabeled gels.

ADP-ribosyl cyclase activity was determined fluorometrically utilizing a technique (19,20) which is based on the measurement of the conversion of NGD into the fluorescent product cyclic GDP-ribose (cGDPR). The samples were excited at 300 nm and fluorescence emission was measured at 410 nm in a Perkin-Elmer LS-5B spectrofluorometer. Glycohydrolase activity was determined fluorimetrically (see above) by using cGDPR (as substrate) that was generated enzymatically from NGD by the *Aplysia* ADP-ribosyl cyclase enzyme (Sigma) in the reaction cuvette. The fluorescence intensity was calibrated to known concentrations of cGDPR prepared enzymatically *via* the *Aplysia* reaction and determined by HPLC as described earlier (16). Specific activity was calculated from the linear portion of the time course by fitting a linear function to the data points.

Miscellaneous. All chemicals were purchased from Sigma, except those specified otherwise. γ-^{32}P-ATP stock from ICN had a specific activity of 4,000 Ci/mmol. The significance in differences were evaluated by one-way ANOVA (Origin, Microcal Software, Northhampton, MA) at levels specified in the figure captions and marked with the symbol "*". Sequence analysis on human CD38 was performed by using the software PCGene (IntelliGenetics, Inc.)

RESULTS AND DISCUSSION

ADP-ribosyl cyclase activity associated with cardiac SR. Cardiac muscle possesses both ADP-ribosyl cyclase and NADase activities, i.e. the cleavage of b-NAD results in the formation of both cADPR and ADPR (16). The method that utilizes NGD as substrate (instead of NAD) is capable of distinguishing between cADPR-producing (resulted from either a CD38-type or an *Aplysia*-type activity, see also Introduction) and "classical" NADase activities, as only the CD38-type and the "pure" *Aplysia*-type ADP-ribosyl cyclase utilize NGD as substrate (19). Taking an advantage of the NGD technique, we determined the distribution of ADP-ribosyl cyclase (i.e. cGDPR-forming) activities in cardiac subcellular fractions (obtained as described in Materials and Methods) following the homogenization of whole rat hearts (Fig. 1). No significant activity was detected in cardiac cytosol (not shown), however, several cardiac membrane fractions (M1 - M4) contained enzymes that catalyzed cGDPR formation (Fig. 1A). As illustrated in Fig. 1B, the highest specific activity was detected in the fractions designated M1 and M2. However, more than 95 % of the total activity was present in the M3 and the M4 fractions. This type of distribution of activities suggests that there are most likely two forms of ADP-ribosyl cyclase present in cardiac muscle. The distribution of marker enzyme activities (ouabain sensitive K-phosphatase for SLM; and thapsigargine-sensitive Ca^{2+}-ATPase for SR, not shown but see ref. 21) suggested that the M1 and M2 fractions were enriched in membranes of SLM origin, while M3

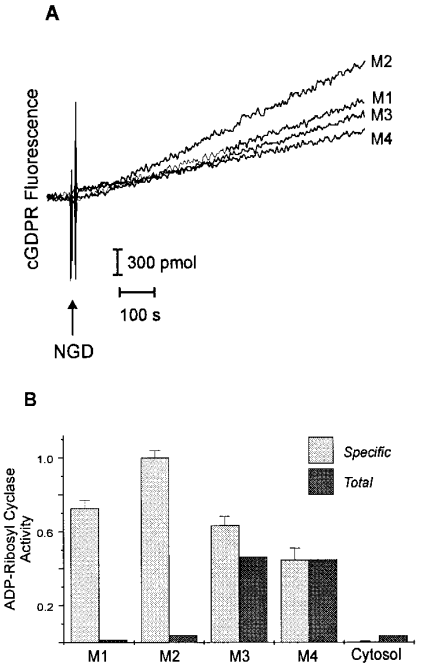

FIG. 1. The distribution of ADP-ribosyl cyclase activities in cellular fractions of rat cardiac muscle. Membrane fractions (M1 - M4) and cytosol were prepared and the time courses of cGDPR production were measured fluorimetrically as described in the Material and Methods section (A). To initiate the reaction, 60 μM NGD was added to the incubation solution of 150 mM KCl, 2 mM $MgCl_2$ and 20 mM MOPS, pH 6.8, also containing the cellular fractions of 120-250 μg protein. Normalized ADP-ribosyl cyclase activities averaged from the results of 3 membrane (M1 - M4) and cytosol preparations in *B*. The activities were normalized to fraction M2 whose specific activity was 1.22 nmol cGDPR produced/mg/min.

and M4 mostly originated from SR. The SLM preparations obtained by the procedure employed in this study mostly consist of sealed right-side-out vesicles (see ref. 21). Furthermore, the use of a hypotonic reaction solution (20 mM K-MOPS) did not significantly increase cGDPR forming activity in either M1 or M2 (not shown). Thus, it seems likely that, due to the unfettered accessibility to the substrate NGD, the plasma membrane-bound activity belongs to an ectoenzyme (either CD38 or a homologous NADase).

To reduce the contribution of possibly contaminating non-muscle cells, we repeated these experiments with fractions that were prepared from carefully dissected left ventricle muscles of dog hearts. Again, only negligible amount of cGDPR forming activity was detected in the cytosolic fraction (not shown), and most of the activity was found to co-purify with three microsomal membrane fractions (M1 - M3; a fraction that corresponds to M1 isolated from the rat microsomes was not present in significant quantities in most dog preparations, thus this fraction was not further studied). The fractions were designated according to their contents of dihydropyridine and ryanodine receptors (as SLM and SR markers, respectively), using immunodetection on Western blots (Fig. 2A and see Materials and Methods). Fraction M1 showed the presence of a small amount of RyR-2 (Fig. 2A, lane 1, top panel), but significantly higher level of the dihydropyridine receptor a2 subunit (Fig. 2A, lane 1, bottom panel). Factions M2 and M3 exhibited at least 10-fold stronger signal with RyR-2 antibody than M1 (Fig. 2A, lanes 2 and 3, top panel), and yielded a very weak or no signal when stained with the dihydropyridine receptor antibody (Fig. 2A, lanes

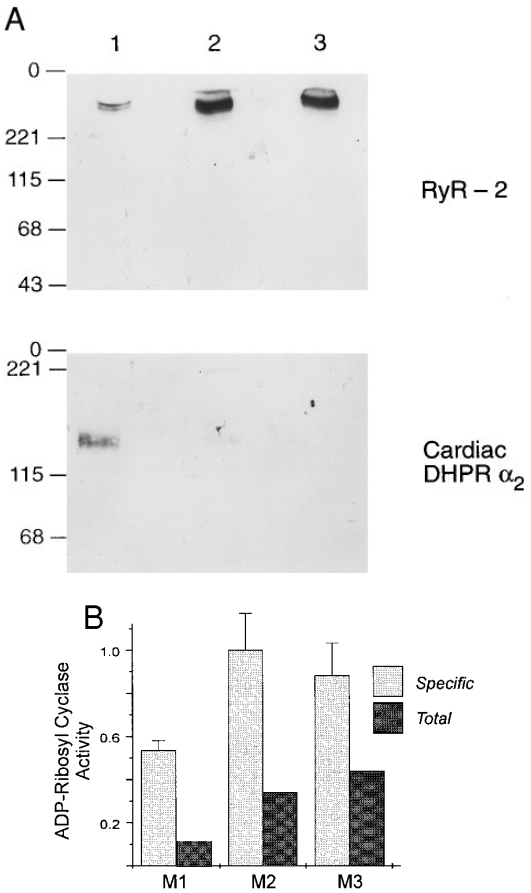

FIG. 2. The distribution of ADP-ribosyl cyclase activities in membrane fractions isolated from dog left ventricular muscle. The membrane fractions (M1 - M3) were prepared and cGDPR production was measured as described in the Materials and Methods section and the legend to Fig. 1. ***A:*** *Immuno-detection of RyR-2 and dihydropyridine receptor a2 subunit in ventricular membrane fractions. Top panel:* 20 μg of protein from each fraction was electrophoresed on 4-15% SDS-PAGE, then transferred to ECL-Hybond membranes. Primary antibody was applied at 1 μg/ml concentration. The secondary antibody (horseradish peroxidase-conjugated anti-mouse antibody) was used in 1:20,000 dilution. *Bottom panel:* fractions containing 100 μg protein were electrophoresed on a 7.5% SDS-Page and blotted as above. The primary antibody was used at a dilution of 1:500, and the secondary antibody at 1:10,000. Lanes 1,2 and 3 correspond to M1, M2 and M3, respectively. ***B:*** *ADP-ribosyl cyclase activities in membrane fractions M1- M3.* The activity values (the average of 4 preparations) were normalized to M2, which had a specific activity of 0.87 nmol cGDPR produced/mg/min.

2 and 3, bottom panel). Based on the distribution of the markers, M1 was designated as an SLM fraction, while M2 and M3 as SR fractions. Although fraction M2 was slightly contaminated with SLM, the distribution of cGDPR forming activities (Fig. 2B) again indicated that most of the activity was associated with the SR membrane, i.e. was present in fractions that stained positive for the ryanodine receptor (M2 and M3). The treatment of M3 with detergents (CHAPS or Triton X-100) resulted in the solubilization of 60-80% of the SR ADP-ribosyl cyclase activity (not shown), further supporting the notion of its membrane-association.

Since the CD38-type ADP-ribosyl cyclase activity is expressed in a wide variety of tissues (), thus presumably in the heart as well, and the enzymatic activity we detected in SLM-containing membrane fractions is likely the manifestation of the activity of an ectoenzyme (due to the orientation of the SLM vesicles, see above), we tried to make distinctions between cGDPR forming activities in SLM and SR fractions based on some known properties of CD38. Due to the presence of catalytically essential multiple cysteine residues (22), CD38 is inactivated by thiol reducing reagents (23). As illustrated in Fig. 3B and C, the activity in the M1 fraction was in fact inhibited by about 80% in the presence of millimolar dithiotreitol (DTT). However, the activity in fraction M3 was seemingly not influenced by DTT (trace not shown, see Fig. 3C), which resembles the DTT-insensitivity of the *Aplysia* enzyme (24). CD38 is a glycoprotein with identified glycosylation sites in its extracellular domain (25,26), suggesting that lectins might possibly affect its activity. We found (Fig. 3A and C) that the cGDPR forming activity in M1 was sensitive to wheat germ agglutinine, while the enzyme in M3 was apparently not. Another possibility to distinguish between the SLM- and the SR-containing fractions was presented by the availability of antibodies raised against CD38. We found that the monoclonal anti-CD38 antibody OKT-10 (see also the Materials and Methods section) significantly increased the rate of cGDPR formation in M1 (Fig. 3A and C), but was without any effect on the respective activity in M3 (Fig. 3C; this effect of OKT-10 was only observed in rat membrane preparations, but not in those from the dog). Taking all these observations together, it seems safe to suggest that cardiac muscle cells express an SR-associated ADP-ribosyl cyclase enzyme that is distinct from CD38 or CD38-type ADP-ribosyl cyclases. Since

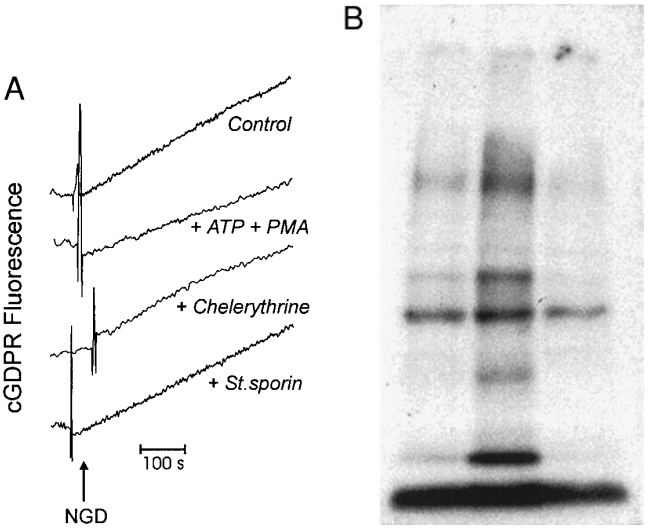

FIG. 4. The effect of PKC-dependent phosphorylation on the SR-associated ADP-ribosyl cyclase activity. ***A:*** Representative traces of cGDPR production in dog ventricular M3 fraction are shown. ATP, PMA, chelerythrine and staurosporine (where indicated) were added in final concentrations of 200, 1, 5 and 0.05 μM, respectively, prior to NGD addition. ***B:*** The phosphorylation reaction with the M3 fraction of the dog (50-100 mg protein) was initiated by the addition of γ-^{32}P-ATP (100 mM containing 10^7 cpm) and stopped by boiling in SDS sample buffer. The proteins were resolved by SDS-PAGE (7.5%), then visualized by autoradiography. Results shown are typical of three separate experiments. Incubations were carried out for 5 min in the presence (middle lane) or absence (left lane) of PMA (1 μM) and in the presence (right lane) or absence (left and middle lanes) of staurosporine (0.01 μM).

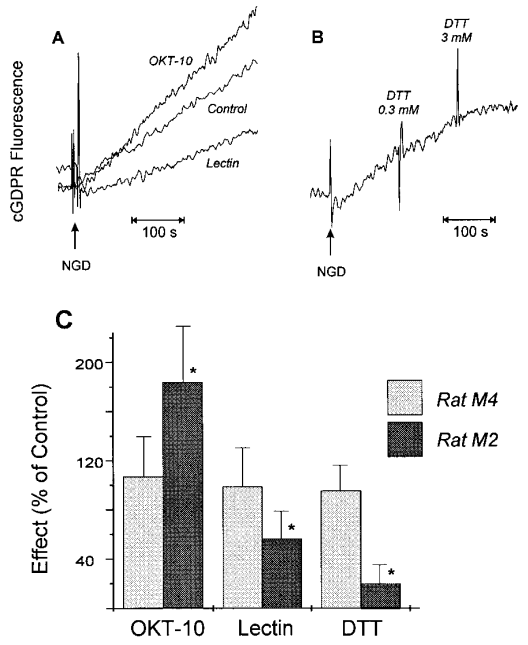

FIG. 3. The effects of a monoclonal CD38 antibody, wheat germ agglutinine and dithiotreitol (DTT) on cGDPR producing activities in M2 and M4 membrane fractions of rat cardiac muscle. cGDPR production was monitored as described in the legend to Fig. 1. ***A:*** *Representative time courses of cGDPR production.* The antibody OKT-10 and wheat germ agglutinine (lectin) were added in 1:100 dilution and 10 μg/ml, respectively, to the membrane samples 1 hour before the reaction was started by the addition of NGD. ***B:*** The averages of at least 3 independent determinations on 3 separate preparations are shown. Significance at the level of $P < 0.05$ (as compared to control) is denoted by the symbol "$_*$". The concentration of DTT was 4 mM.

the SLM enzyme appears to be an ectoenzyme (and our experiments with CD38 positive T lymphocytes do not suggest that cADPR would readily cross the plasma membrane, not shown), it is possible that the SR enzyme is chiefly responsible for intracellular production of cADPR.

SR-associated ADP-ribosyl cyclase influenced by protein kinase C-dependent phosphorylation. The analysis of the amino acid sequence of CD38 revealed the occurrence of 8 potential protein kinase C (PKC) phosphorylation sites (at amino acids 19,55,56,165,193,258,267 and 274). Based on this information, we tested whether PKC-dependent phosphorylation influences the SR-associated ADP-ribosyl cyclase activity in the M3 fraction isolated from the dog (Fig. 4) and in the M4 fraction of rat microsomes (not shown). We found for both the dog and the rat preparations that in the presence of Ca^{2+} and ATP, phorbol esters (PMA is shown) significantly (at $P < 0.02$) reduced cGDPR forming activity (Fig. 4A) by $32.4 \pm 9.1\%$ (average of 4 preparations), which effect was prevented by the addition of either chelerythrine or staurosporine, inhibitors of PKC activity (Fig. 4A). The experiments, that were performed to detect PKC-dependent protein phosphorylation using γ-^{32}P-ATP, confirmed the presence of endogenous PKC activity in both dog (Fig. 4B) and rat (not shown) cardiac SR: PMA enhanced the phosphorylation of several proteins, which was blocked by the inclusion of staurosporine. These results suggest that PKC-dependent phosphorylation of the SR-associated ADP-ribosyl cyclase might represent one of the potential signaling pathways that control the cellular level of cADPR. (Similar effects of PMA on the SLM enzyme was not observed; not shown.) Although the SR fractions we tested here possessed cAMP-, cGMP- and Ca/calmodulin-dependent phosphorylation activities, we found no evidence for any effect of either of these cyclic nucleotides or calcium ions on dog M3 or rat M4 ADP-ribosyl cyclase activity (data not shown).

The results we present here suggest the existence of a cardiac muscle SR-associated ADP-ribosyl cyclase enzyme which is, in many aspects, distinguishable from CD38-type proteins, as it is not sensitive to thiol reduction, to lectins and whose activity is regulated by protein kinase C-dependent phosphorylation. These results, for the first time, demonstrate the existence of a vertebrate enzyme that might be the second messenger enzyme that is chiefly responsible for the intracellular production of cADPR, a putative messenger molecule whose involvement in Ca^{2+} signaling in many mammalian cells, including cardiac muscle (27), is becoming more and more evident. In addition, the response of the SR-bound ADP-ribosyl cyclase to PKC-dependent phosphorylation might establish an important link between cADPR as intracellular messenger molecule and other signal transduction patways that involve the activation of PKC.

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