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Cyclic ADP-Ribose-Dependent Ca^{2+} Release Is Modulated by Free [Ca^{2+}] in the Scallop Sarcoplasmic Reticulum

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Cyclic ADP-ribose (cADPR) elicits calcium-induced calcium release (CICR) in a variety of cell types. We studied the effect of cADPR on Ca^{2+} release in muscle cells by incubating SR vesicles from scallop (*Pecten jacobaeus*) adductor muscle in the presence of the Ca^{2+} tracer fluo-3. Exposure of SR to cADPR (20 μM) produced Ca^{2+} release, which was a function of free [Ca^{2+}] in a range between about 150 and 1000 nM, indicating an involvement of ryanodine-sensitive Ca^{2+} channels. This Ca^{2+} release was not significantly enhanced by calmodulin (7 μg/ml), but it was enhanced by equimolar addition of noncyclic ADPR. Also, the Ca^{2+} release elicited by cADPR/ADPR was a function of free [Ca^{2+}] in a range between about 150 and 3000 nM, over which Ca^{2+} was inhibitory. cADPR self-inactivation was observed at low free [Ca^{2+}] (about 150 nM), but it tended to disappear upon [Ca^{2+}] elevation (about 250 nM). Caffeine or ryanodine induced a Ca^{2+} release which was ruthenium red (2.5 μM) sensitive at low [Ca^{2+}]. However, the Ca^{2+} release induced by either ryanodine or cADPR was no longer ruthenium red sensitive when free [Ca^{2+}] was increased. Based on these data, a model is proposed for Ca^{2+} signaling in muscle cells, where a steady-state cADPR level would trigger Ca^{2+} release when free [Ca^{2+}] does reach a threshold slightly above its resting level, hence producing cascade RyR recruitment along SR cisternae from initial Ca^{2+} signaling sites.

Key Words: **cADPR; ADPR; ryanodine receptor; caffeine; Ca^{2+}-induced Ca^{2+} release.**

Cyclic adenosine diphosphoribose (cADPR), a natural metabolite of NAD^+ is a Ca^{2+} releasing agent in a variety of cells (reviewed in 1, 2), including sea urchin eggs (3), pancreatic acinar and β cells (4, 5), cardiac myocytes (6), sensory neurons (7), cerebellar granule cells (8), cortical astrocytes (9), and brain cell microsomes (10). In recent years, evidence has been accumulating to support the notion that cADPR is the physiological ligand of ryanodine-sensitive Ca^{2+} channels (RyR) modulating calcium-induced calcium release (CICR) from intracellular stores (11, 12). RyR channels, together with inositol 1,4,5-trisphosphate (IP_3) receptors (13), are members of a unique family of receptors which display the process of CICR (14). CICR, first described in muscle cells (15, 16), has been subsequently shown to be a pivotal process accounting for cytosolic free Ca^{2+} spatiotemporal dynamics and Ca^{2+} signaling (17, 18). In muscle cells, voltage sensing and CICR are the main mechanisms for RyR activation. In skeletal muscle, membrane depolarization activates the dihydropyridine receptor (DHPR)–RyR1 complex to give the initial calcium burst from SR lumen, which in turn, through CICR, recruits those RyR1s which are not directly coupled to DHPR (19). In cardiac muscle, membrane depolarization causes Ca^{2+} entry through voltage-operated channels, which elicits a CICR process involving RyR2s (14).

Two kinds of enzymes responsible for the synthesis of cADPR were found, an ADP-ribosyl cyclase, converting NAD^+ to nicotinamide and cADPR (20), and endocellular bifunctional enzymes catalyzing a two-step reaction involving transient formation of cADPR followed by its hydrolysis to ADPR. CD38, a T-lymphocyte differentiation antigen, was demonstrated to be an ectocellular bifunctional enzyme catalyzing the synthesis of cyclic-ADP-ribose (cADPR) from NAD^+, its hydrolysis to ADP-ribose (ADPR) as well as the exchange

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Abbreviations used: ADPR, adenosine 5′-diphosphoribose; $ADPR_2$, dimeric adenosine 5′-diphosphoribose; cADPR, cyclic adenosine 5′-diphosphoribose; cGDPR, cyclic guanosine 5′-diphosphoribose; CICR, calcium-induced calcium release; IP_3, inositol 1,4,5-trisphosphate; NGD^+, nicotinamide guanine dinucleotide; RR, ruthenium red; RyR, ryanodine-sensitive Ca^{2+} channels; SR, sarcoplasmic reticulum.

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of ADPR with the nicotinammide group of NAD^+ resulting in a dimeric ADPR ($ADPR_2$) (21). In addition, an enzyme catalyzing the conversion of both cADPR and NAD^+ to ADPR as well as the synthesis of NAD^+ from cADPR and the reverse reaction, was found in canine spleen (22). Moreover, a possible novel Ca^{2+}-release mechanism activated by another pyridine nucleotide metabolite, nicotinate-adenine dinucleotide phosphate (NAADP) was described (23) which, however, does not seem to behave as a CICR system (24) and would not involve Ca^{2+} stores responsive to either IP_3 or cADPR (25).

In the present study, we investigated the mechanism of Ca^{2+} release by cADPR in SR vesicles of the scallop (*Pecten jacobaeus).* We found that SR is able to release Ca^{2+} in response to cADPR, and that free [Ca^{2+}] variations can modulate such a Ca^{2+} release process. Based on these data, a model for Ca^{2+} signaling in muscle cells is proposed.

MATERIALS AND METHODS

Chemicals. Adenosine 5′-triphosphate (ATP), ruthenium red (RR), cyclic adenosine 5′-diphosphoribose (cADPR), adenosine 5′-diphosphoribose (ADPR), nicotinamide guanine dinucleotide (NGD^+), cyclic guanosine 5′-diphosphoribose (cGDPR), thapsigargin, ryanodine, calmodulin, ruthenium red (RR) and the calcium ionophore A23187 were from Sigma Chemical Co. (St. Louis, MO). The fluorescent probe fluo-3 was from Molecular Probes Inc. (Eugene, USA). All other reagents were of analytical grade.

SR isolation and Ca^{2+} measurements. All procedures were performed according to Burlando *et al.* (26). Briefly, SR-containing fractions consisting of a 20,000 *g* supernatant were prepared from scallop (*Pecten jacobaeus*) adductor muscle and incubated at room temperature in the presence of 4 mM ATP and 2.5 μM fluo-3. Free Ca^{2+} fluorimetric recordings (ex. = 505 nm, em. = 534 nm) were done with a Perkin–Elmer LS 50B. [Ca^{2+}] quantitation was achieved by probe calibration at the end of each experiment.

cADPR treatments and Ca^{2+} background tuning. A preliminary set of experiments was devoted to find optimal conditions for SR responses to cADPR in terms of Ca^{2+} release from SR vesicles. These tests showed that the level of Ca^{2+} background resulted a critical factor. We therefore sought for an expedient allowing us to regulate the level of free Ca^{2+} in the incubation medium to different values (ranging from about 200 to over 3000 nM), without preventing the recording of ensuing peak Ca^{2+} releases. This was achieved by first allowing free Ca^{2+} to be driven to low values (about 150 nM) by Ca^{2+} ATPase-dependent uptake into SR vesicles (see Ref. 26), and then by performing a series of careful additions of the SR Ca^{2+} ATPase inhibitor thapsigargin (27) (up to a maximum concentration of 250 nM thapsigargin). This elicited moderate Ca^{2+} releases, thereby producing controlled [Ca^{2+}] rises in the medium, followed by a plateau. By this way, it was possible to drive the free Ca^{2+} level to a desired value, which was real-time inferred from the probe fluorescent signal by referring to previous probe calibrations, and eventually assessed by probe calibration at the end of the experiment.

Fluorometric assay of cGDPR synthesis. The putative enzyme activity responsible for cADPR synthesis was pointed out by spectrofluorometric assay, using nicotinamide guanine dinucleotide (NGD^+) as a substrate and then following the formation of cyclic GDP-ribose (cGDPR), which is a fluorescent compound. According to Graeff *et al.* (28), for fluorimetric assay (ex. = 300 nm, em. = 410 nm) suitable aliquots of sample were incubated at room temperature in the same reaction mixture as for Ca^{2+} measurements, containing 1 mM NGD^+. Fluorescence increases were converted to rates of cGDPR production by using a calibration curve obtained from known concentrations of commercially available cGDPR.

Protein assay. The protein content of the 20,000*g* supernatant was assayed by the method of Hartree (29), using bovine serum albumin as standard.

Statistics. Data were analyzed by using the Systat 5.2 software (Systat Inc., Evanston, IL).

RESULTS

Addition of the calcium-reporting dye fluo-3 to scallop SR-containing supernatant allowed to follow Ca^{2+} sequestering and releasing activities present in the samples. Similarly to what reported in Burlando *et al.* (26), at the beginning of experiments the free Ca^{2+} present in the incubation medium was rapidly sequestered into SR vesicles. After allowing the system to equilibrate at a Ca^{2+} background of about 170 nM, addition of cADPR (20 μM) elicited a minimal Ca^{2+} release (1.42 $\pm$ 0.17 pmol/ mg protein). However, by operating at different Ca^{2+} background levels obtained using suitable additions of thapsigargin (see Methods), it was demonstrated that the Ca^{2+} releasing effect of cADPR was strengthened by increasing free [Ca^{2+}] (Fig. 1A). In these and the following experiments, the quantification of cADPR effects was achieved by measuring peak Ca^{2+} releases occurring just after cADPR additions, in order to dissect the effects of Ca^{2+} per se from the inhibition of thapsigargin on Ca^{2+} sequestration. A plot of peak Ca^{2+} release data shows that the enhancing effect of free Ca^{2+} is strong from about 200 to 500 nM, followed by a tendency to saturation (Fig. 1B). Also, when free [Ca^{2+}] was driven above 3000 nM an inhibitory effect on Ca^{2+} release was found. However, these latter data were not plotted as [Ca^{2+}] estimation is less reliable when the probe fluorescent signal approaches saturation.

Preincubation with calmodulin (7 μg/ml) did not significantly increase the cADPR Ca^{2+} releasing effect (not shown), possibly due to the presence of endogenous calmodulin in the supernatant preparation. Conversely, the Ca^{2+} release was markedly enhanced by coaddition of an equimolar amount of the non-cyclic analogue ADPR. The use of thapsigargin allowed to find that, similarly to what detected for cADPR alone, also the Ca^{2+} releasing effect of cADPR plus ADPR was a function of free [Ca^{2+}] (Fig. 2). To quantitatively compare the Ca^{2+} releases induced by cADPR alone and by cADPR plus ADPR we fitted saturation kinetic-like curves to data. In the case of cADPR alone we obtained a K_{50} = 870 nM Ca^{2+} and an asymptotic Ca^{2+} release of 20 pmol/ mg $prot^{-1}$ (Fig. 1B), whereas due to the enhancing effect of ADPR these values raised up to

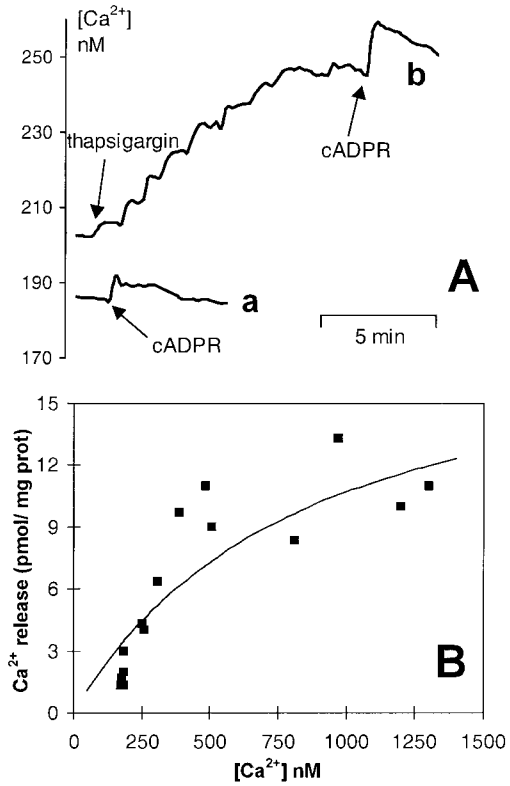

FIG. 1. cADPR evokes a $[Ca^{2+}]$-dependent Ca^{2+} release from scallop SR vesicles. (A) Fluorometric traces from typical experiments depicting free Ca^{2+} variations after incubation of SR vesicles from scallop adductor muscle with the Ca^{2+} tracer fluo-3 in the presence of ATP. cADPR (20 μM) elicits a Ca^{2+} release from SR vesicles, which is enhanced by increasing Ca^{2+} background levels obtained after careful SR exposure to thapsigargin. Minimal Ca^{2+} release is recorded after cADPR addition at a low Ca^{2+} level of about 180 nM (a), whereas the Ca^{2+} release is more than doubled after thapsigargin-induced free Ca^{2+} elevation up to about 240 nM (b). (B) Peak Ca^{2+} release data, plotted as a function of free Ca^{2+} levels, show a nonlinear trend ($R^2 = 0.93$) which is steeper at free $[Ca^{2+}]$ slightly above cell resting levels, followed by a tendency to saturation.

1520 nM and 142 pmol/ mg $prot^{-1}$, respectively (Fig. 2B).

To investigate the possibility of endogenous cADPR formation in the scallop muscle cells we assayed a putative ADP-ribosyl cyclase activity through spectrofluorometric detection of cGDPR synthesis, using NGD^+ as a substrate (28). Incubation of the SR-containing supernatant with NGD^+ was followed by cGDPR production, as revealed by fluorescence rise, thus allowing quantification of the cyclase activity (Fig. 3). Transformation of fluorescent data into cGDPR production rates yielded an average value of 2.75 $\pm$ 0.3 nmol min^{-1} mg $prot^{-1}$.

Given that free Ca^{2+} exerts a modulatory effect on the cADPR-dependent Ca^{2+} release from SR stores, we also investigated the effect of free Ca^{2+} levels on typical RyR functional properties. A set of experiments was aimed at studying cADPR self-desensitization. Subsequent cADPR additions produced decreasing Ca^{2+} releases when operating at a low free Ca^{2+} level of about 180 nM (Fig. 4, a). Conversely, upon free Ca^{2+} elevation (about 250 nM) cADPR produced higher Ca^{2+} releases, while desensitization was low (Fig. 4, b). Another set of experiments was devoted to explore the interaction between RyR agonists and a blocker. Both ryanodine (50 μM) and caffeine (1 mM) triggered Ca^{2+} release over basal (about 150 nM) Ca^{2+} levels (Fig. 4, c), while their action was respectively almost or completely blocked by sample preincubation with 2.5 μM RR (Fig. 4, d). Yet, the inhibitory action of RR on ryanodine was abolished when the Ca^{2+} background was elevated (Fig. 4, e). Accordingly, over the same elevated Ca^{2+} background, RR did not inhibit the cADPR/ADPR-induced Ca^{2+} release (Fig. 4, f).

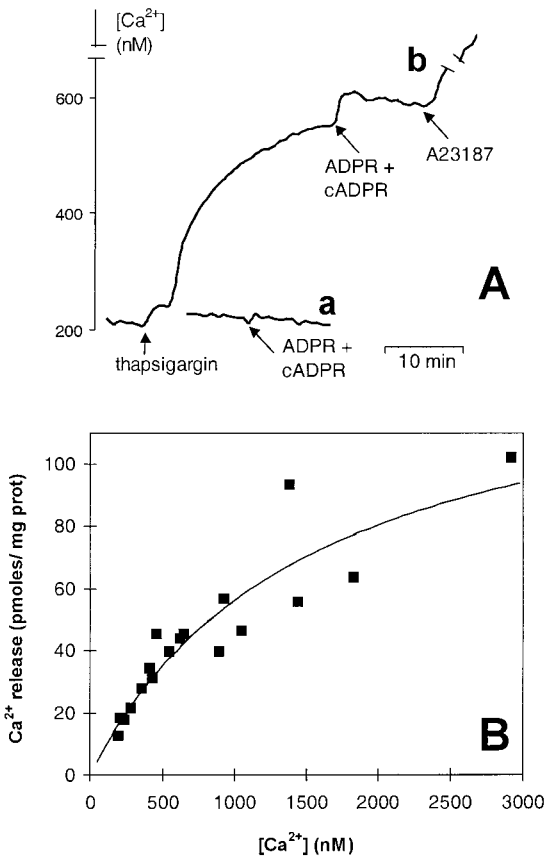

FIG. 2. Effect of ADPR on cADPR-induced Ca^{2+} release. (A) Fluorometric traces from typical experiments, showing a low Ca^{2+} release after ADPR/cADPR addition at a free Ca^{2+} level of about 200 nM (a), and a much higher response to ADPR/cADPR after free Ca^{2+} elevation by thapsigargin up to above 500 nM (b). Ionophore addition (1 mM A23187) at the end of the experiment produces massive Ca^{2+} release from SR stores (b). (B) Plot of ADPR/cADPR-dependent peak Ca^{2+} releases as a function of different Ca^{2+} background levels. Similarly to what reported in Fig. 1, data show a nonlinear trend ($R^2 = 0.96$) with a tendency to saturation.

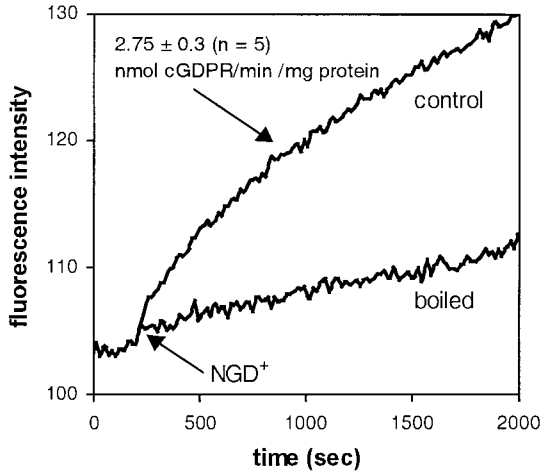

FIG. 3. Fluorometric assay of ADP-ribosyl cyclase activity in scallop SR preparation. The enzyme activity was assayed by incubating the SR-containing supernatant with NGD^+ and then following cGDPR production by spectrofluorometric analysis. In a typical experiment, the fluorimetric trace shows marked fluorescence rise above background signal after NGD^+ addition to control sample, indicating cGDPR synthesis by ADP-ribosyl cyclase. A boiled sample shows no background fluorescence variation following NGD^+ addition. By using a calibration curve, fluorescence data from different experiments were converted into cGDPR production rates, yielding average production ± SD.

DISCUSSION

The present data show that cADPR elicits a Ca^{2+} release from scallop SR vesicles. Moreover, the dependence of such a Ca^{2+} release on free [Ca^{2+}] provides evidence for an involvement of the RyR (2). The use of thapsigarging to drive up the free Ca^{2+} level to different plateaus allowed us to depict the cADPR-dependent, [Ca^{2+}]-modulated Ca^{2+} release mechanism in SR vesicles. The trend of the response to free [Ca^{2+}] in terms of Ca^{2+} release resulted in being steep at Ca^{2+} concentrations slightly above physiological resting levels, followed by a tendency to saturation in the low micromolar range. In addition, according to previous data (14), higher Ca^{2+} levels were inhibitory.

A dependency of Ca^{2+} release on free [Ca^{2+}] was also found when using combined additions of ADPR and cADPR, which clearly indicates that also in this case the observed Ca^{2+} release should be ascribed to the RyR. The enhancing effect of ADPR could be ascribed to an inhibition of the cADPR hydrolase activity, similarly to what reported for sea urchin eggs (30), although the absence of delay in the ADPR effect suggests a different mechanism. The RyR is known to possess a domain binding adenine nucleotides (31), and these latter compounds have been shown to amplify the effects of RyR agonists (32). Therefore, in our experiments, a direct modulatory effect of ADPR on the SR Ca^{2+} channel, possibly intensified by enhanced free

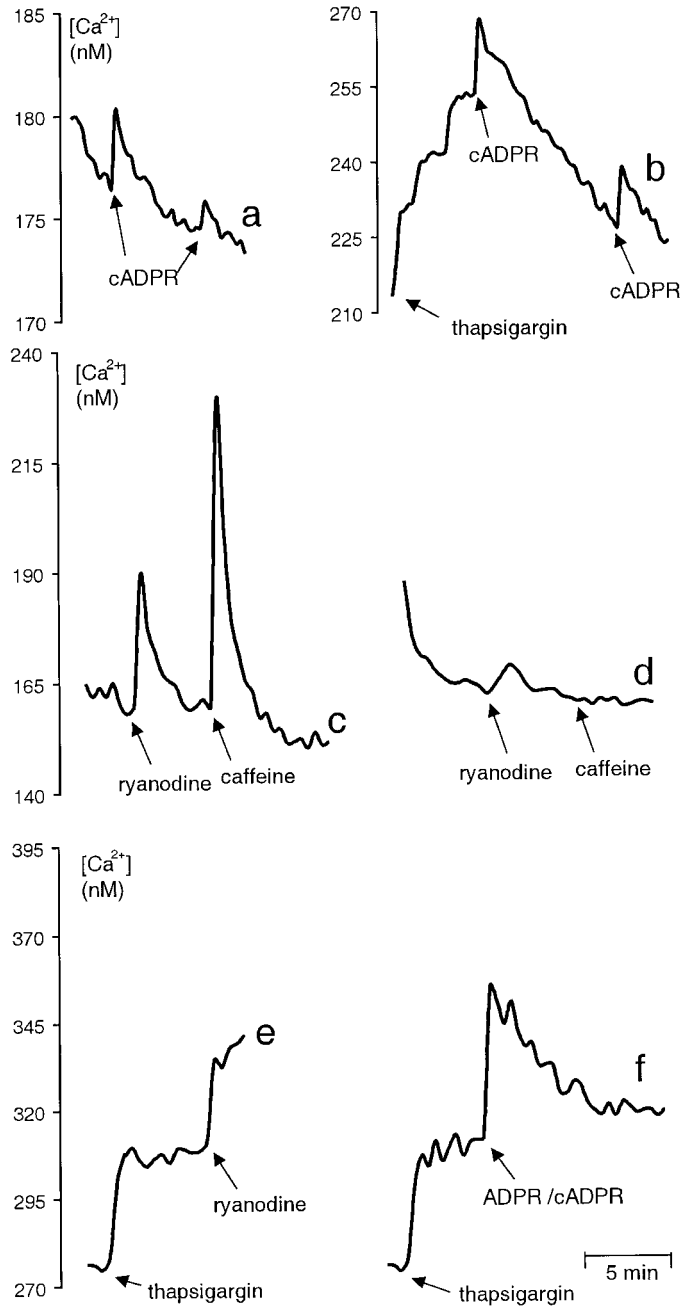

FIG. 4. cADPR self-inactivation and effect of ruthenium red (RR), and their reversal by elevated free Ca^{2+} level. (a) Subsequent additions of cADPR at low free Ca^{2+} level result in a marked decrease of ensuing Ca^{2+} releases. (b) Conversely, the same cADPR additions made at a higher free Ca^{2+} level produce negligible self-inactivation. (c) Distinct additions of ryanodine and caffeine produce Ca^{2+} releases from SR vesicles. (d) Pre-incubation with 2.5 μM RR almost completely inhibits the Ca^{2+} release induced by ryanodine and totally inhibits that induced by caffeine. (e) Pre-incubation with RR only slightly inhibits Ca^{2+} release response to ryanodine after thapsigargin-dependent free Ca^{2+} elevation. (f) Pre-incubation with RR does not inhibit Ca^{2+} release responses to ADPR/cADPR after thapsigargin-dependent free Ca^{2+} elevation. Each trace is representative of at least three experiments.

Ca^{2+} levels, should not be ruled out. For instance, our data could be related to the finding that dimeric ADPR $(ADPR)_2$ enhances Ca^{2+} release in sea urchin egg homogenates (21). Such a supposed effect of ADPR on the RyR could represent a means of tuning Ca^{2+}-induced channel desensitization to a higher cytosolic $[Ca^{2+}]$, thus allowing for larger Ca^{2+} release effects. As for the effect of ADPR itself, it was shown that it can release Ca^{2+}, but at a 10^4-fold higher concentration with respect to cADPR (33).

ADP-ribosyl cyclase assay in our 20.000 *g* supernatant from scallop muscle showed activity rates comparable to what reported for dog brain extracts (28). It should be noted that among the biochemical reactions pertaining to the NAD/cADPR/ADPR metabolic pathway, the enzymatic activity detected in our experiments is mainly devoted to cADPR synthesis (28). Hence, our data suggest the occurrence of fair cADPR production rates in scallop muscle cells, thus providing further insight about a physiological role for cADPR-dependent Ca^{2+} release.

It has been shown in different studies that subsequent additions of intracellular Ca^{2+} channel agonists, including cADPR, produce self-desensitization (e.g., 34). Moreover, it has been reported that in muscle cells Ca^{2+} release is enhanced by caffeine, ryanodine and divalent cations, and that ruthenium red blocks Ca^{2+} release in response to caffeine (34) and ryanodine (35). However, our data indicate that the Ca^{2+} level at which RyR agonists (ryanodine, cADPR) are added does have a primary importance for the occurrence of both self-inactivation and ruthenium red inhibition (see Fig. 4). The present results suggest that elevated free $[Ca^{2+}]$ drives RyR channels to an activated state over which desensitization and blockage are partially or completely ineffective, an effect that to our knowledge was never reported before. Such a possible behavior of the channel could also explain previous data about ruthenium-red insensitive, cADPR-dependent Ca^{2+} release in microsomes from rabbit muscle (36).

In conclusion, scallop muscle cells have been shown to possess Ca^{2+} stores operated by cADPR, a finding that confirms what reported in Berridge (14). In skeletal muscle cells of vertebrates these stores are possibly located in SR portions which do not participate in the triad complex. So the global calcium signal in skeletal muscle would derive from both depolarization- and ligand-activated events (14). cADPR has been thought either as a Ca^{2+} messenger operated through the activation of its synthetic enzyme, or as a modulator, which is present in cells at a low level (2). This latter view seems closer to the possible role of cADPR in muscle cells, where high-rate oscillatory behaviors need a direct connection between external stimuli and contraction, without delay due to signal transduction pathways. Evidence from our data provides further support to this hypothesis as (i) cADPR can elicit a $[Ca^{2+}]$-dependent Ca^{2+} release from SR vesicles at a free Ca^{2+} concentration slightly above cell resting levels, and (ii) fair basal levels of cADPR synthesis are present in muscle cells. A tentative model is therefore proposed for muscle cells, where cADPR-sensitive Ca^{2+} release channels would be maintained in an activatable state by a constant level of their physiological ligand. This recalls what proposed for the interaction between IP_3 and its receptor (37), with the difference that in muscle cells cADPR would act as a steady-state RyR ligand rather than as a messenger subject to fluctuations of its cellular concentration. Hence, according to our model the intracellular pacemaker of muscle cell contraction would depend primarily on cytosolic free Ca^{2+} dynamics in cell microdomains. cADPR would trigger a $[Ca^{2+}]$-dependent Ca^{2+} release when cytosolic free $[Ca^{2+}]$ rises above resting levels, thus producing cascade RyR recruitment along SR cisternae from initial Ca^{2+} signaling sites (see also Ref. 14). Such a model could account even for the fastest oscillatory activities observed in muscle cells.

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