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cADP-ribose activates reconstituted ryanodine receptors from coronary arterial smooth muscle

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Li, Pin-Lan, Wang-Xian Tang, Hector H. Valdivia, Ai-Ping Zou, and William B. Campbell. cADP-ribose activates reconstituted ryanodine receptors from coronary arterial smooth muscle. *Am J Physiol Heart Circ Physiol* 280: H208–H215, 2001.—The present study was designed to test the hypothesis that cADP-ribose (cADPR) increases Ca^{2+} release through activation of ryanodine receptors (RYR) on the sarcoplasmic reticulum (SR) in coronary arterial smooth muscle cells (CASMCs). We reconstituted RYR from the SR of CASMCs into planar lipid bilayers and examined the effect of cADPR on the activity of these Ca^{2+} release channels. In a symmetrical cesium methanesulfonate configuration, a 245 pS Cs^{+} current was recorded. This current was characterized by the formation of a subconductance and increase in the open probability (NP_o) of the channels in the presence of ryanodine (0.01–1 μM) and imperatoxin A (100 nM). A high concentration of ryanodine (50 μM) and ruthenium red (40–80 μM) substantially inhibited the activity of RYR/Ca^{2+} release channels. Caffeine (0.5–5 mM) markedly increased the NP_o of these Ca^{2+} release channels of the SR, but D-*myo*-inositol 1,4,5-trisphospate and heparin were without effect. Cyclic ADPR significantly increased the NP_o of these Ca^{2+} release channels of SR in a concentration-dependent manner. Addition of cADPR (0.01 μM) into the *cis* bath solution produced a 2.9-fold increase in the NP_o of these RYR/Ca^{2+} release channels. An eightfold increase in the NP_o of the RYR/Ca^{2+} release channels (0.0056 ± 0.001 vs. 0.048 ± 0.017) was observed at a concentration of cADPR of 1 μM. The effect of cADPR was completely abolished by ryanodine (50 μM). In the presence of cADPR, Ca^{2+}-induced activation of these channels was markedly enhanced. These results provide evidence that cADPR activates RYR/Ca^{2+} release channels on the SR of CASMCs. It is concluded that cADPR stimulates Ca^{2+} release through the activation of RYRs on the SR of these smooth mucle cells.

adenosine 3′,5′-cyclic diphosphate-ribose; calcium mobilization; vascular smooth muscle; coronary artery

CYCLIC ADP-RIBOSE (cADPR) serves as a second messenger to mediate intracellular Ca^{2+} mobilization independently of the D-*myo*-inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] signaling pathway in different tissues or cells (8–11, 22–26). This cADPR-mediated Ca^{2+} signaling participates in the regulation of a variety of cell functions or cellular activities such as the secretion of insulin from pancreatic β-cells, the fertilization of eggs, cell growth, neuronal activity, and the effects of nitric oxide in nonmuscle tissues (1, 11, 23, 40, 46). Recent studies in our laboratory and by others (12, 19, 30, 31, 47) demonstrate that cADPR induced Ca^{2+} release from intracellular stores of coronary arterial smooth muscle cells and that the inhibition of the production of cADPR results in the relaxation of coronary arteries. However, the mechanism by which cADPR activates Ca^{2+} mobilization is poorly understood. With the use of pharmacological approaches, cADPR has been found to activate or modulate ryanodine receptors (RYR) on the sarcoplasmic reticulum (SR) of nonvascular tissues (9, 10, 15, 22, 23, 28). [^{3}H]Ryanodine binding assays have indicated that cADPR may increase or decrease ryanodine binding on the SR depending on different tissues (7, 35). These results suggest that cADPR may be an endogenous regulator or modulator of the RYR. A recent study (37) has reported that cADPR may bind to an accessary protein of RYR, FK506 binding proteins, and thereby activate these receptors of the SR.

It is not known how cADPR produces Ca^{2+} mobilization in vascular smooth muscle cells. However, RYRs are found in arterial smooth muscle cells, and they can mediate Ca^{2+} release from these cells and participate in the control of vascular tone and vasomotor response (2, 15, 18, 34). The RYR is a homotetramer with molecular weight of ~550 kDa for each monomer. Four monomers comprise a high-conductance Ca^{2+} channel pore on the membrane of the SR, which is responsible for Ca^{2+} release. Three subtypes of the RYR with specific tissue distributions are now recognized and named as RYR1, RYR2, and RYR3. Cytosolic Ca^{2+} is a primary activating ligand of these RYR, and the binding of Ca^{2+} to the RYR activates Ca^{2+} release. Therefore, RYR has been proposed to mediate Ca^{2+}-induced Ca^{2+} release (CICR) (6, 15, 20, 21, 32, 44). Studies using ligand binding and molecular approaches dem-

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onstrate that RYR3 is a predominant form on the SR of vascular smooth muscle cells (6). It has yet to be determined whether cADPR directly activates these RYR/Ca^{2+} release channels.

The present study was designed to test the hypothesis that cADPR produces Ca^{2+} release through activation of RYR/Ca^{2+} release channels on the SR of coronary arterial smooth muscle cells (CASMCs). We reconstituted the RYR/Ca^{2+} release channels from the SR of CASMCs into a planar lipid bilayer and determined the biophysical and pharmacological characteristics of these channels. We then examined the effect of cADPR on the activity of these RYR/Ca^{2+} release channels. Because caffeine is a well-known activator of the RYR (21, 22), we also determined the effect of caffeine on the activity of these reconstituted RYR/Ca^{2+} release channels.

MATERIALS AND METHODS

Preparation of SR membrane from bovine coronary arteries. Coronary arteries were dissected from the bovine heart, and SR-enriched microsomes (SR membrane) of these arteries were prepared as described previously (14, 29, 31, 32). Briefly, the dissected coronary arteries (outer diameter 500–1,500 μm) were cut into small pieces and homogenized with a Tenbroeck glass tissue grinder in ice-cold MOPS buffer containing 0.9% NaCl, 10 mM MOPS (pH 7.0), 2 μM leupeptin, and 0.8 μM benzamidine. The homogenate was centrifuged at 4,000 *g* for 20 min at 4°C, and the supernatant was further centrifuged at another 8,000 *g* for 20 min at 4°C and then at 40,000 *g* for 30 min. The pellet, termed the SR membrane, was resuspended in a solution containing 0.9% NaCl, 0.3 M sucrose, and 0.1 μM phenylmethylsulfonyl fluoride, aliquoted, frozen in liquid N_2, and stored at −80°C until use (32).

Reconstitution of RYR into planar lipid bilayer. The SR membranes from CASMCs were reconstituted into planar lipid bilayers as described by Lokuta et al. (32, 33). Briefly, phosphatidylethanolamine and phosphatidyl-serine (1:1) were dissolved in decane (25 mg/ml) and used to form a planar lipid bilayer in a 250-μm aperture between two chambers filled with *cis* and *trans* solutions, respectively. The SR membranes (50–100 μg) were added into the *cis* solution, which corresponded to the cytosolic side of the SR channels. The *trans* solution represented the lumenal side of these SR channels. The recording solution in the *cis* chamber was 300 mM cesium (Cs^+) mechanesulfonate and 10 mM MOPS (pH 7.2). The *trans* solution was the same as the *cis* solution except that cesium methanesulfonate was 50 mM before fusion and 300 mM after fusion. In this configuration, Cs^+ flows from the lumenal (*trans*) to the cytosolic (*cis*) side at negative holding potentials. Cs^+, instead of Ca^{2+}, was chosen as the charge carrier to precisely control [Ca^{2+}] around the channel, to increase the channel conductance ($G_{Cs^+}/G_{Ca^{2+}} = \sim 2$), and to avoid interference from K^+ channels present in the SR membrane. Cl^- channels were blocked by replacing chloride with the impermeant anion methanesulfonate. The Ca^{2+} release channel activity was detected in a symmetrical cesium methanesulfonate solution (300 mM) in all experiments. To increase the channel activity, 1 μM free Ca^{2+} in the *cis* solution was adjusted by adding Ca^{2+} standard solution containing $CaCl_2$ and EGTA as described previously (32, 33).

Recordings of RYR/Ca^{2+} release channel currents. An integrating bilayer clamp amplifier (model BC-525C, Warner Instrument) was used to record single-channel currents. The amplifier output signals were filtered at 1 kHz with an eight-pole Bessel filter (Frequency Devices). Currents were digitized at a sampling rate of 10 kHz and stored on the hard disk of a Micron Pentium III computer for off-line analysis. Data acquisition and analysis were performed with pCLAMP software (version 7.01, Axon Instruments). Channel open probability (NP_o) in the lipid bilayer was determined from recordings of 3–5 min as described previously in our patch-clamp studies (30, 31). All lipid bilayer experiments were performed at room temperature (≈20°C).

To establish current-voltage relations of RYR/Ca^{2+} release channels, the reconstituted receptor/channel complex in the lipid bilayer was exposed to a symmetrical cesium in *cis* and *trans* solutions, and single-channel currents were recorded while holding potentials were varied from −40 to +40 mV in steps of 10 mV. Ryanodine (0.1–50 μM), caffeine (0.5–5 mM), imperatoxin A (0.01 μM), and ruthenium red (40–80 μM) were used as blockers or activators to characterize RYR/Ca^{2+} release channels in the lipid bilayer. Ins(1,4,5)P_3 (10 μM) and heparin (25 mg/ml), an Ins(1,4,5)P_3 receptor [Ins(1,4,5)P_3R] antagonist, were used to distinguish RYR/Ca^{2+} release channels from Ins(1,4,5)P_3-sensitive Ca^{2+}channels on the SR membrane. The effect of cADPR (0.01–1 μM) on Ca^{2+} release channels of the SR was defined in the absence or presence of ryanodine. To determine the effect of cADPR on the Ca^{2+} sensitivity of the channels, the concentration of ionized Ca^{2+} in the *cis* solution was varied from 10^{-7} to 10^{-5} mol/l in the absence or presence of cADPR. All of the compounds used in these experiments were added into the *cis* solution, and currents were recorded at holding potentials at −40 mV.

Statistics. Data are presented as means ± SE; the significance of the differences in mean values between and within multiple groups was examined using an analysis of variance for repeated measures followed by a Duncan's multiple range test. A Student's *t*-test was used to evaluate statistical significance of differences between two paired observations. $P < 0.05$ was considered statistically significant.

RESULTS

Recordings of reconstituted Ca^{2+} release channel currents of SR membranes from CASMCs. With symmetrical cesium in *cis* and *trans* solutions, a unitary Cs^+ current through the reconstituted receptor/channel complex in the lipid bilayer was detected at holding potentials from −40 to +40 mV (Fig. 1*A*). Mean slope conductance for these SR Cs^+ currents was 245 ± 4.5 pS with a reversal potential of ~0 mV (Fig. 1*B*). The NP_o of these Cs^+ currents increased when the bilayer holding potential increased from 0 to +40 mV or decreased from 0 to −40 mV, suggesting that the channel activity is dependent on the magnitude of holding potentials rather than the polarity of clamp voltage. The NP_o of these Cs^+ currents was 0.0056 ± 0.001 at a bilayer holding potential of +40 mV.

Pharmacological characteristics of reconstituted RYR/Ca^{2+} release channels. Several pharmacological approaches were used to further characterize and identify the Cs^+ currents in the lipid bilayer as ryanodine-sensitive Ca^{2+} release channels. First, the effects of ryanodine on the Cs^+ currents were examined. Figure 2*A* depicts the representative recordings of single-channel Cs^+ currents before and after the addition of ryanodine into the *cis* solution. Ryanodine increased

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channel activity and induced a subconductance state of Cs^+ currents, as showed by a 50 or 75% decrease in the amplitude of these Cs^+ currents. Ryanodine increased the NP_o of these currents at concentrations <20 μM. However, when a high concentration of ryanodine (50 μM) was administered into the *cis* solution, the channel openings were blocked. Figure 2*B* summarizes the results of these experiments. Ryanodine at concentrations of 0.1–10 μM increased the NP_o of these channels in a concentration-dependent manner. The NP_o was increased from 0.0042 ± 0.001 of control to 0.025 ± 0.001 in the presence of 10 μM ryanodine. However, ryanodine at concentrations >20 μM significantly inhibited the channel activity in this preparation.

The effect of imperatoxin A, a 33-amino acid peptide from the scorpion *Pandinus imperator*, on Cs^+ currents was then examined. Imperatoxin A has been widely used to characterize the RYR in other tissue preparations (13, 42, 48). It binds directly to the RYR and has similar pattern of action to ryanodine. Imperatoxin A at concentrations of 10 nM resulted in a subconductance state of the Cs^+ currents and increased the NP_o of these currents from 0.005 ± 0.001 of control to 0.024 ± 0.004 (Table 1). However, $Ins(1,4,5)P_3$ (10 μM) and the $Ins(1,4,5)P_3R$ antagonist heparin (25 mg/ml), which activate or inhibit $Ins(1,4,5)P_3$-sensitive Ca^{2+} channels at this concentration, respectively, had no significant effect on the activity of these Cs^+ currents. Thus these currents are not associated with the $Ins(1,4,5)P_3R$ (Table 1).

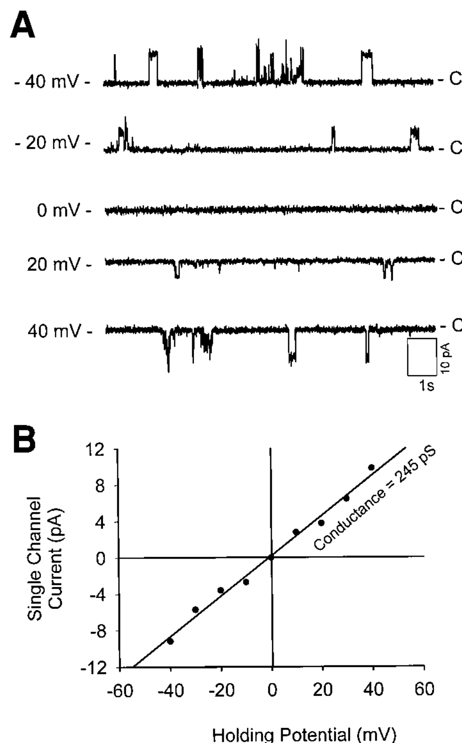

Fig. 1. Characterization of the reconstituted Ca^{2+} release channels of the sarcoplasmic reticulum (SR) from bovine coronary arterial smooth muscle in planar lipid bilayer. *A*: representative recording of ryanodine-sensitive Ca^{2+} channel currents (Cs^+ as charge carrier) at holding potentials ranging from −40 to +40 mV. *B*: current-voltage relations for the reconstituted Ca^{2+} release channel currents of the SR with symmetrical cesium methanesulfonate (300 mM) solution. C, channel closed.

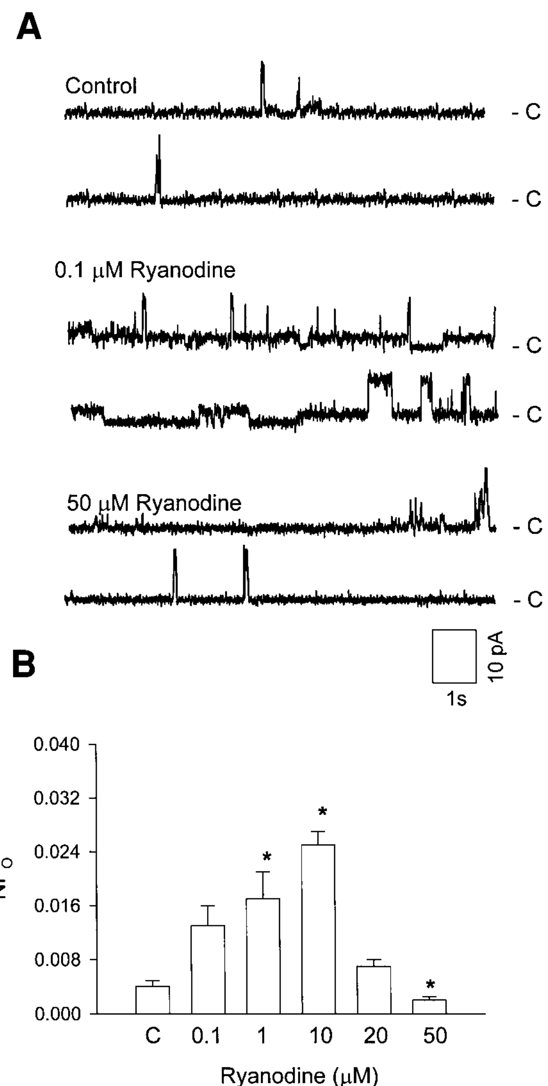

Fig. 2. Change in the activity of reconstituted Ca^{2+} release channel of the SR in the presence of ryanodine in the *cis* solution. *A*: representative recordings of reconstituted Ca^{2+} release channel currents under control conditions and after addition of ryanodine (0.1–50 μM) at a holding potential of +40 mV. *B*: summarized data showing the open probability (NP_o) of the reconstituted Ca^{2+} release channel of the SR in the absence or presence of ryanodine. *Significant difference from control ($P < 0.05$, n = 8–16 bilayers from 4–5 animals).

Effect of CICR inhibitor ruthenium red and activator caffeine on activity of reconstituted RYR/Ca^{2+} release channels. The effects of ruthenium red on the NP_o of reconstituted Cs^+ currents are also presented in Table 1. Addition of ruthenium red at concentrations of 40–80 μM markedly reduced the NP_o of these channels. A 68% inhibition was observed with 80 μM ruthenium red. The CICR activator caffeine significantly increased the activity of the reconstituted Ca^{2+} channels (Fig. 3). Figure 3*A* shows the representative recordings of Cs^+ currents under control condition and after addition of caffeine in the *cis* solution. Caffeine at a

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Table 1. *Effects of RYR and Ins(1,4,5)P_3 receptor modulators on NP_o of RYR/Ca^{2+} release channels*

Treatment	NP_o
Imperatoxin A, nM (n = 4)	
0	0.005 ± 0.0013
10	0.024 ± 0.0050*
Ruthenium red, μM (n = 5)	
0	0.0057 ± 0.0020
40	0.0025 ± 0.0005
80	0.0018 ± 0.0010*
Ins(1,4,5)P_3, μM (n = 8)	
0	0.0039 ± 0.0007
10	0.0041 ± 0.0007
Heparin, mg/ml (n = 11)	
0	0.0063 ± 0.0007
25	0.0061 ± 0.0014

Data are presented as means ± SE. RYR, ryanodine receptors; Ins(1,4,5)P_3, D-*myo*-inositol (1,4,5)-trisphosphate; NP_o, channel open probability. *Significant difference from control ($P < 0.05$).

concentration of 1 mM markedly increased the activity of these reconstituted channels. Figure 3*B* summarizes the effects of caffeine on the NP_o of the reconstituted Ca^{2+} release channels. Caffeine at concentrations of 0.5–5 mM produced a concentration-dependent increase in the NP_o of these channels. Maximal increase was observed at 1 mM caffeine added into the *cis* solution.

Effect of cADPR on activity of reconstituted RYR/Ca^{2+} release channels. The representative recordings depicting the effect of cADPR on the activity of reconstituted RYR/Ca^{2+} release channels of CASMCs are presented in Fig. 4*A*. These Cs^+ currents markedly increased when cADPR (1 μM) was added into the *cis* solution. In the presence of 50 μM ryanodine in the *cis* solution, cADPR-induced activation of the channels was blocked. Figure 4*B* summarizes the concentration-dependent effect of cADPR on the NP_o of the reconstituted RYR/Ca^{2+} release channels. cADPR at concentrations of 0.01 μM produced a 2.9-fold increase in the NP_o of these channels (0.006 ± 0.001 vs. 0.018 ± 0.002). An eightfold increase in the NP_o of the channels (0.006 ± 0.001 vs. 0.048 ± 0.01) was observed with 1 μM cADPR. This cADPR-induced increase in the channel NP_o was completely abolished by pretreatment of the bilayer membrane with 50 μM ryanodine (in the *cis* solution). ADP-ribose, a structural analog of cADPR, even at 10 μM had no effect on the activity of these channels.

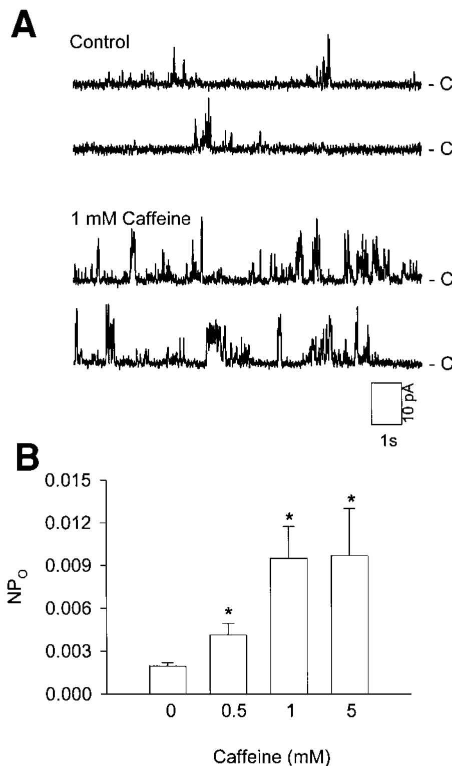

Fig. 3. Effect of caffeine on the activity of the reconstituted ryanodine receptor (RYR)/Ca^{2+} release channels in the planar lipid bilayer. *A*: representative recordings of reconstituted RYR/Ca^{2+} channel currents under control conditions and after addition of caffeine (1 mM) into the *cis* solution at a holding potential of +40 mV. *B*: summarized data showing the NP_o of reconstituted RYR/Ca^{2+} release channels in the absence or presence of caffeine. *Significant difference from control ($P < 0.05$, n = 8–18).

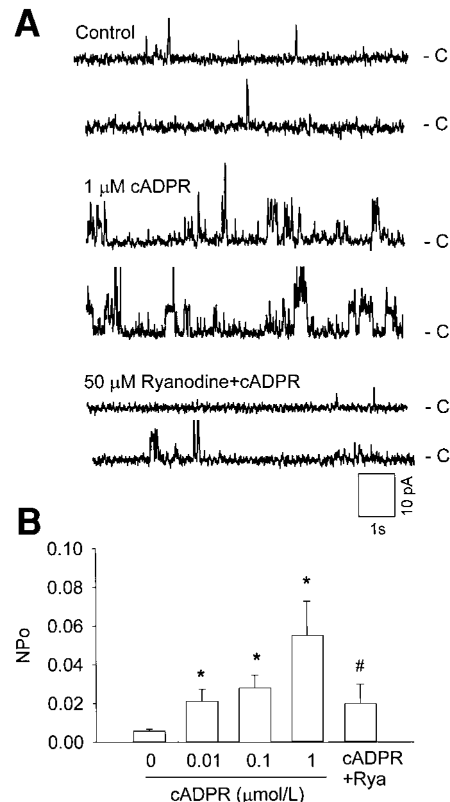

Fig. 4. Effect of cADPR on the activity of the reconstituted RYR/Ca^{2+} release channels in the planar lipid bilayer. *A*: representative recordings of reconstituted RYR/Ca^{2+} release channels under control conditions and after addition of cADP-ribose (cADPR) (1 μM) or ryanodine (Rya) (50 μM) and cADPR (1 μM) into the *cis* solution at a holding potential of +40 mV. *B*: summarized data showing the NP_o of the reconstituted RYR/Ca^{2+} release channels in the absence or presence of cADPR. *Significant difference from control ($P < 0.05$, n = 8). #Significant difference from cADPR alone.

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Effect of cADPR on Ca^{2+} sensitivity of reconstituted RYR/Ca^{2+} release channels. Because RYR mediate CICR, cytosolic Ca^{2+} should activate RYR/Ca^{2+} release channels. To test this hypothesis, the effect of Ca^{2+} on the activity of reconstituted RYR/Ca^{2+} release channels was examined. As shown in Fig. 5*A*, a $CaCl_2$ and EGTA mixture was added into the *cis* solution to adjust [Ca^{2+}] to 10^{-7}–10^{-5} M. $CaCl_2$ (10^{-5} M) significantly increased the activity of these RYR/Ca^{2+} release channels. In the presence of cADPR, Ca^{2+}-induced activation of these channels was markedly enhanced. As summarized in Fig. 5*B*, $CaCl_2$ (10^{-5} M) increased the NP_o of these RYR/Ca^{2+} release channels. Addition of cADPR into the *cis* solution significantly potentiated Ca^{2+} activation of these channels. At 10 μM, $CaCl_2$ produced a 2.5-fold increase in the NP_o of these channels and a 5-fold increase in the NP_o of these channels in the presence of cADPR in the *cis* solution.

DISCUSSION

The presence of the RYR has been documented in the vascular smooth muscle cells, and CICR, associated with activation of these SR receptors, participates in the control of vascular tone (6, 15, 20, 21, 32, 44).

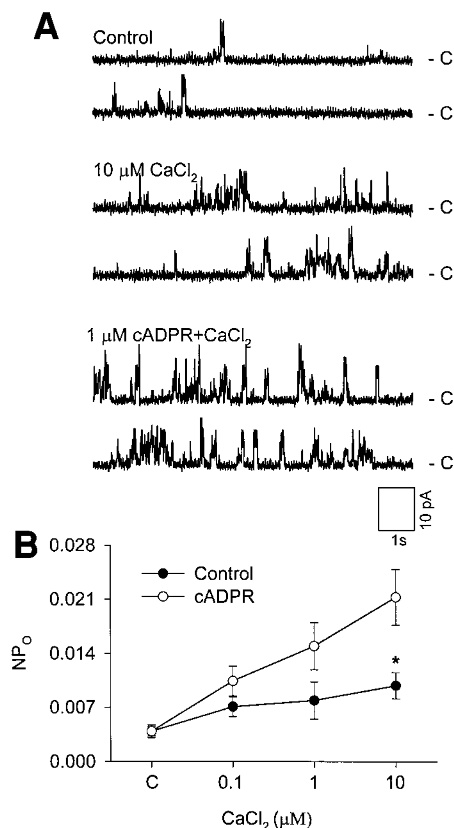

Fig. 5. Effect of cADPR on the sensitivity of the reconstituted RYR/Ca^{2+} release channels to [Ca^{2+}] in the *cis* solution. *A*: representative recordings of the reconstituted RYR/Ca^{2+} release channels under control conditions and after addition of cADPR (1 μM) and/or Ca^{2+} (10 μM) into the *cis* solution at a holding potential of +40 mV. *B*: summarized data showing the NP_o of the reconstituted RYR/Ca^{2+} release channels in the absence or presence of cADPR and/or Ca^{2+}. *Significant difference from control ($P < 0.05$, $n = 8$).

Numerous studies using pharmacological approaches have shown that the RYR mediates Ca^{2+} release independently of the Ins(1,4,5)P_3 pathway in vascular smooth muscle cells and thereby plays an important role in the regulation of vascular tone (6, 15, 20, 21, 32, 44). RYR-mediated CICR may contribute to the vasoconstrictor response to membrane depolarization, Ca^{2+} influx, caffeine, or some agonists such as endothelin, PG $F_{2\alpha}$ and histamine (16, 17, 19, 31, 34, 43, 45). Recent studies using molecular and electron microscopic approaches have demonstrated the localization of RYR in the SR of vascular smooth muscle cells (27). With the use of electrophysiological methods, the RYR/Ca^{2+} release channels currents were recorded across planar lipid bilayers reconstituted with aortic SR (14). These studies suggest that the RYR is the Ca^{2+}-gated Ca^{2+} channel on the SR (14). In the present study, we reconstituted RYRs from the SR of bovine coronary arterial smooth muscle into a planar lipid bilayer and examined their biophysical and pharmacological features and the regulatory effect of cADPR on the activity of these channels. With the use of cesium as the charge carrier, which is widely used to study the RYR in skeletal and cardiac muscles (32, 33, 44), a calcium channel with a conductance of 245 pS was detected in the SR of bovine CASMCs. In a recording configuration with symmetrical cesium, this Cs^+ current exhibited a reversal potential at ~0 mV, and its NP_o was dependent on the magnitude of the holding potential across the lipid bilayer. Compared with the RYR/Ca^{2+} release channels in skeletal and cardiac muscle, the conductance of RYR/Ca^{2+} release channels in coronary arterial smooth muscle was relatively small. It has been reported that the conductance of RYR/Ca^{2+} release channels was ~500–1,000 pS in either skeletal muscle or cardiac muscle (6, 33, 44). In addition, a higher concentration of the coronary arterial SR (100 μg) was needed for reconstitution and recording of these channels in the lipid bilayer compared with skeletal and cardiac muscle (~10–20 μg SR). This suggests that RYR may be less enriched in the SR of vascular smooth muscle than skeletal or cardiac muscle.

To further characterize RYR/Ca^{2+} release channels, we tested the effects of several specific RYR blockers or activators on the activity and conducting properties of these channels. Addition of the RYR ligand ryanodine at low concentrations into the *cis* solution significantly reduced the amplitude of Cs^+ currents and increased the NP_o, but a high concentration of ryanodine (50 μM) inactivated these channels. These results are consistent with previous studies indicating that ryanodine activates RYR/Ca^{2+} release channels and results in a subconductance state of these channels (6). Imperatoxin A has a similar action pattern to ryanodine and is widely used for characterization of RYR/Ca^{2+} release channels (13, 42, 48). It also resulted in a subconductance state of the Cs^+ currents and increased the NP_o of these currents. These results suggest that the Cs^+ currents recorded in our preparation are ryanodine sensitive and represent RYR/Ca^{2+} release channels from CASMCs. Because RYRs have been reported to

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mediate CICR, we examined the effects of the CICR inhibitor ruthenium red and CICR activator caffeine on the activity of these RYR channels. The NP_o of these channels was significantly decreased by ruthenium red and increased by caffeine. The amplitude of the currents was not altered by these drugs. These effects of ruthenium red and caffeine are similar to those described for the RYR/Ca^{2+} release channels of skeletal and cardiac muscles (6, 33) and aortic SR (14). These findings indicated that this reconstituted channel from CASMCs exhibits the major pharmacological property of the RYR/Ca^{2+} release channel of striated muscle.

The present study demonstrated that the Cs^+ currents recorded in the lipid bilayer were not altered by either Ins(1,4,5)P_3 or the Ins(1,4,5)P_3R antagonist heparin, suggesting that these channels are not Ins(1,4,5)P_3R-associated Ca^{2+} release channels. Previous studies reported that Ins(1,4,5)P_3R were abundantly expressed in the SR of CASMCs and also possessed the properties of receptor-operated ion channels. Like RYR, these Ins(1,4,5)P_3R allow the flow of Ca^{2+} from intracellular stores to the cytoplasm in response to Ins(1,4,5)P_3 binding (3, 5). However, the present study did not detect these Ins(1,4,5)P_3R-associated Ca^{2+} release channels. The reason for the lack of Ins(1,4,5)P_3 channels in our reconstitution remains unknown. It may be due to the biophysical properties of the Ins(1,4,5)P_3R or our recording configuration. In general, the conductance of Ins(1,4,5)P_3R/Ca^{2+} release currents is fourfold less than that of the RYR (3), and, therefore, a high gradient of the charge carrier or a high level of holding potential between the *cis* and *trans* solutions is needed for the recording of Ins(1,4,5)P_3R activity in the lipid bilayer. In our experiments with a symmetrical Cs^+ solution, if we increased the holding potential to more than 60 mV, we could record a small current (~1 pA, data not shown). However, under this condition, the large currents and active opening of the RYR/Ca^{2+} release channels often broke the lipid bilayer across the aperture. Considering that the present study mainly focused on the effect of cADPR on the activity of RYR/Ca^{2+} release channels, we did not characterize these small currents, which may represent Ins(1,4,5)P_3R-mediated Ca^{2+} channels. In addition, to activate and stabilize the channels in our bilayer, we routinely included 1 μM Ca^{2+} in the *cis* solution. This concentration of Ca^{2+} could completely block the Ins(1,4,5)P_3R activity. These receptors exhibited a bell-shaped Ca^{2+} dependence: the activity of the Ins(1,4,5)P_3R/Ca^{2+} release channels increased as the free Ca^{2+} concentration was elevated from 10 to 250 nM and decreased at Ca^{2+} concentrations above 250 nM (3). Thus the presence of 1 μM free Ca^{2+} in the *cis* solution may be another important reason for the lack of Ins(1,4,5)P_3R channel activity in our preparation.

An important finding in the present study is that cADPR increased the NP_o of the reconstituted RYR/Ca^{2+} release channels from CASMCs. Addition of cADPR to the *cis* solution markedly increased the NP_o of these Ca^{2+} channels of the SR at concentrations as low as 10 nM. In the presence of ryanodine (50 μM), the cADPR-mediated increase in the RYR/Ca^{2+} release channels was completely abolished. These results provide direct evidence that cADPR activates the RYR and may thereby produce Ca^{2+} release. This cADPR-mediated activation seems to be associated with increased Ca^{2+} sensitivity of RYR/Ca^{2+} channels, because the Ca^{2+} concentration-response curve of the channel activity was significantly shifted to the left in the presence of cADPR. Previous studies demonstrated that cADPR-mediated Ca^{2+} mobolization was blocked by pretreatment of the cells or the SR with ryanodine or RYR antagonists in a variety of vertebrate cells, including those from the brain and myocardium and sympathetic neurons, pancreatic acinar cells, and pituitary cells (35; see Ref. 39 for review), suggesting that RYRs mediate the effect of cADPR. The present study further supports this view. However, there is evidence indicating that cADPR releases Ca^{2+} independently of the RYR in some cells such as neurons and those from the myocardium and smooth muscle (19, 21). With the use of channel reconstitution techniques, Sitsapesan et al. (38) demonstrated that cADPR did not directly activate RYR in the SR from cardiac muscle. The reason for these discrepancies remains unknown. It is possible that there is a tissue-specific effect of cADPR on the RYR. This tissue-specific effect may be associated with the intermediate proteins or accessary proteins that regulate RYR activity. It has been reported that calmodulin is essential for the activation of cADPR-induced Ca^{2+} release in sea urchin eggs (23, 26, 41). A recent study (37) has demonstrated that a RYR accessary protein, FK506 binding protein, may have a binding site of cADPR and that cADPR activates RYRs through binding to this accessary protein. Further studies are needed to elucidate the role of these regulatory proteins in mediating the action of cADPR.

The present study did not attempt to address the physiological relevance of cADPR-mediated activation of RYR. It is possible that this RYR activation by cADPR plays a role in the control of basal vascular tone and vasomotor response to agonists. Under the resting condition, the intracellular [Ca^{2+}] in vascular smooth muscle is dependent on the Ca^{2+} influx, spontaneous brief bursts of calcium released from the SR into the cytoplasm, and CICR (2). cADPR may participate in the control of the resting Ca^{2+} levels in these smooth muscle cells through RYR or CICR (2, 23, 25). In regard to the agonist response, there is increasing evidence suggesting that cADPR may serve as a second messenger to mediate the Ca^{2+}-mobilizing effects of a number of agonists. Studies using pancreatic β-cells strongly suggested that cADPR mediates glucose-induced insulin secretion (40). It has been reported that cADPR may mediate the effects of acetylcholine receptors in adrenal chromaffin cells, 5-HT 2B receptors in arterial endothelial cells, and retinoic acid receptors in renal tubular cells and aortic smooth muscle (1, 4, 36, 43). Cholecystokinin and 5-HT may act through the cADPR pathway in longitudinal intestinal smooth muscle and tracheal smooth muscle (see Ref. 11 for review). It

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remains unknown which type of agonist acts through the cADPR signaling pathway in coronary arterial smooth muscle. However, to answer this question, considerable investment and effort will first be required to advance the technology for quantitation of basal level of intracellular cADPR and to develop more specific and potent cADPR antagonists.

In summary, the present study detected a RYR/Ca^{2+} release channel current of 254 pS in the planar lipid bilayer incorporated with the SR of coronary arterial smooth muscle. cADPR activated these reconstituted RYR/Ca^{2+} release channels and produced Cs^{+} flow across the bilayer. It is concluded that activation of the RYR/Ca^{2+} release channels is an important mechanism mediating cADPR-induced intracellular Ca^{2+} mobilization in CASMCs.

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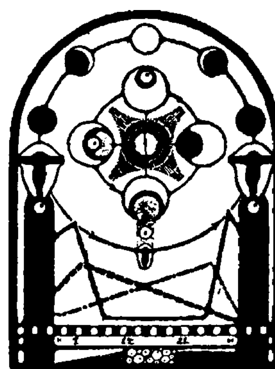

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