Cyclic ADP-ribose activates caffeine-sensitive calcium channels from sea urchin egg microsomes

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Pérez, Claudio F., Juan José Marengo, Ricardo Bull, and Cecilia Hidalgo. Cyclic ADP-ribose activates caffeine-sensitive calcium channels from sea urchin egg microsomes. Am. J. Physiol. 274 (Cell Physiol. 43): C430–C439, 1998.—Adenosine 5′-cyclic diphosphoribose (cyclic ADP-ribose (cADPR)), a metabolite of NAD+ that promotes Ca++ release from sea urchin egg homogenates and microsomal fractions, has been proposed to act as an endogenous agonist of Ca++ release in sea urchin eggs. We describe experiments showing that a microsomal fraction isolated from Tetrapagus nyger sea urchin eggs displayed Ca++-selective single channels with conductances of 155.0 ± 8.0 pS in asymmetric Cs+ solutions and 47.5 ± 1.1 pS in asymmetric Ca++ solutions. These channels were sensitive to stimulation by Ca++, ATP, and caffeine, but not inositol 1,4,5-trisphosphate, and were inhibited by ruthenium red. The channels were also activated by cADP-ribose in a Ca++-dependent fashion. Calmodulin and Mg2+ but not heparin, modulated channel activity in the presence of cADPR. We propose that these Ca++-channels constitute the intracellular Ca++-induced Ca++ release pathway that is activated by cADP-ribose in sea urchin eggs.

EXPERIMENTAL PROCEDURES

Isolation of sea urchin egg microsomes. Eggs were collected from seawater after electrostimulation of sea urchins (Tetrapagus nyger) into their coelomic cavity at room temperature. All subsequent steps were done at 4°C. After removal of jelly coats (10), eggs were washed once in Ca++-free artificial seawater. This first wash was followed by two brief low-speed sedimentations in sucrose buffer (0.3 M sucrose, 0.1 M KCl, 20 mM 3-(N-morpholino)propanesulfonic acid, pH 7.0, plus protease inhibitors: 10 µg/ml leupeptin, 2 µg/ml pepstatin A, 5 mM benzamidine, 100 µg/ml soybean trypsin inhibitor). Eggs were homogenized in a Dounce homogenizer and sedimented at 2,000 g for 20 min, and the resulting supernatant was sedimented at 12,000 g for 20 min. The pellet was discarded, and the supernatant was sedimented at 100,000 g for 60 min. For this purpose the isolated microsomes were added at a protein concentration of ~10 µg/ml and fractionated in an agarose column (Bio-Gel A-0.5 m, Bio-Rad) equilibrated with sucrose buffer. The column exclusion fraction, containing the microsomes, was sedimented at 100,000 g for 60 min, and the pellet was resuspended in sucrose buffer, quickly frozen in liquid nitrogen, and stored at −80°C.

Ca++ release studies. Ca++ release was measured in microsomes actively loaded with Ca++. For this purpose the isolated sea urchin egg microsomes were added at a protein concentration of 0.5 mg/ml to a fluorometer cuvette containing 1 ml of a solution of 1 mM Mg-ATP, 8 mM phosphocreatine, 4 U/ml of creatine kinase, 0.1 M KCl, and 20 mM 3-(N-morpholino)propanesulfonic acid-tris(hydroxymethyl)aminomethane (Tris),
calculated as follows

urchin egg microsomes were added to the solution, and fluo 3 fluorescence was determined using a Shimadzu RF-540 spectrofluorometer with excitation and emission wavelengths of 506 and 526 nm, respectively. Because after vesicle addition, external [Ca\textsuperscript{2+}] reached 5–10 μM, no extra Ca\textsuperscript{2+} was added to the incubation solution. After 30 min of incubation with Mg-ATP at 22°C, external [Ca\textsuperscript{2+}] decreased to 0.3–0.4 μM. At this point, different agonists of Ca\textsuperscript{2+} release channels were added, and the changes in fluo 3 fluorescence were followed as a function of time. Calibration curves of fluo 3 fluorescence vs. [Ca\textsuperscript{2+}] were done using solutions of known [Ca\textsuperscript{2+}], which was determined with a Ca\textsuperscript{2+} electrode.

Bilayer experiments. Planar phospholipid bilayers were formed from 5:3:2 palmitoyloleoyl phosphatidylethanolamine-phosphatidylserine-phosphatidylcholine. Fusion of vesicles to negatively charged Muller-Rudin membranes was performed as described previously (4), with slight modifications. Sea urchin egg microsomes were added to the cis compartment solution, containing 5 ml of 200 mM CsCl, 0.1 mM CaCl\textsubscript{2}, and 25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)-Tris, pH 7.4. The other (trans) compartment contained 25 mM HEPES-Tris, pH 7.4. After channel fusion, evidenced by the emergence of current fluctuations a few minutes after the addition of the microsomes, the cis compartment was perfused with five times the compartment volume (a total volume of 25 ml) of a solution containing 25 mM HEPES-Tris, pH 7.4. After perfusion, 200 mM Cs-methanesulfonate, 0.5 mM Ca-HEPES, and sufficient N-hydroxyethyl-ethylendiaminotriacetic acid or ethylene glycol-bis(β-aminoethyl ether)-N,N,N’,N’-tetraacetic acid to give the desired free [Ca\textsuperscript{2+}], which was always checked with a Ca\textsuperscript{2+} electrode, were added to the cis compartment. The trans compartment was supplemented with 50 mM Cs-methanesulfonate after fusion. Voltage was applied to the cis compartment, and the trans compartment was held at virtual ground through an operational amplifier in a current-to-voltage configuration. Unless indicated otherwise, most single channel records illustrated here were obtained in asymmetric Cs-methanesulfonate solutions (200 mM cis/50 mM trans) at room temperature (22–24°C), with membranes held at 0 mV. For analysis, data were filtered at 0.5 kHz using an eight-pole low-pass Bessel filter (model 902 LPF, Frequency Devices, Haverhill, MA) and subsequently digitized at 2 kHz with an acquisition system (Axotape, Axon Instruments, Foster City, CA).

Because the channels studied displayed four conductance levels, plus rapid and complex kinetics, the normalized mean current (P\textsubscript{o}) was used as an index of channel activity and was calculated as follows

\[ P_o = I_{\text{mean}} / I_{\text{max}} \]  

where \( I_{\text{max}} \) is the channel maximal current amplitude and \( I_{\text{mean}} \) is the mean current amplitude. Mean channel current and fractional times spent in each subconductance state (P\textsubscript{i}) were always calculated from steady-state records lasting ≥150 s. pClamp 6.0 (Axon Instruments) was used for the former calculations, and Transit software (Baylor College of Medicine, Houston, TX) was used for the latter. Furthermore, to establish that the four equal current levels observed corresponded to true subconductance states and not to four separate single channels, the following analysis was performed. For a binomial system made of four independent units, the frequency of occurrence of each conductance level is given by the following set of equations

\[ P_1 = 4 \cdot P_o \cdot (1 - P_o)^3 \] \[ P_2 = 6 \cdot P_o^2 \cdot (1 - P_o)^2 \] \[ P_3 = 4 \cdot P_o^3 \cdot (1 - P_o) \] \[ P_4 = P_o^4 \]

In Eqs. 2–6, \( P_o \) corresponds to the open probability of each unit and \( P_i \) to the probability of the closed state. To calculate \( P_o \), Eq. 7 was used

\[ P_o = \frac{\sum_{i=1}^{4} i \cdot P_i}{4} \]  

From the calculated \( P_o \), the binomial frequencies for the open conductance states were estimated, and the resulting theoretical frequency values were compared with the experimental distribution of frequencies using a \( \chi^2 \) test.

RESULTS

IP\textsubscript{3} and cADPR-induced Ca\textsuperscript{2+} release from sea urchin egg microsomes. The sea urchin egg microsomes isolated according to the procedures described above actively accumulated Ca\textsuperscript{2+} at 22°C after addition of 1 mM Mg-ATP (not shown). The uptake of Ca\textsuperscript{2+} ceased after 20–30 min of incubation, when the [Ca\textsuperscript{2+}] of the extravesicular solution reached 0.3–0.4 μM. At this point, addition of 5 μM IP\textsubscript{3} to the actively loaded vesicles produced transient Ca\textsuperscript{2+} release, and subsequent addition of 1 μM cADPR induced a new transient Ca\textsuperscript{2+} release of smaller magnitude (Fig. 1A). The inverse sequence of agonist addition, first cADPR and then IP\textsubscript{3}, produced a transient Ca\textsuperscript{2+} release after
addition of cADPR and a second release response to 5 µM IP3 smaller (Fig. 1B) than the release response obtained when 5 µM IP3 was added before cADPR (Fig. 1A). These results indicate that the isolated microsomes have release pathways responsive to IP3 and cADPR. Addition of the Ca2+ ionophore A-23187 at the end of the experiment produced in all cases a marked increase in extravesicular [Ca2+] (Fig. 1). This response to the ionophore indicated that the vesicles had actively accumulated significant amounts of Ca2+ and that the increase in external [Ca2+] observed after addition of cADPR or IP3 corresponded in effect to Ca2+ release through pathways in the microsomal membranes.

Sea urchin egg microsomes displayed caffeine-sensitive Ca2+ channels. Using planar lipid bilayers, we investigated the presence of Ca2+ channels in the isolated microsomes. For this purpose, we used experimental conditions similar to those routinely employed to detect ryanodine-sensitive Ca2+ channels in SR vesicles, such as pH 7.4 and high concentrations (up to 50 mM) of cations, Cs+ or Ca2+, in the trans compartment (see Experimental Procedures). In the standard recording conditions (200 mM Cs+ cis/50 mM Cs+ trans) and in the presence of 12 µM Ca2+ in the cis solution, only single channels that exhibited Imax of 4–4.2 pA, with a low P0 of 0.007, were observed (Fig. 2A, top trace). Channel activity increased to a P0 of 0.041 after addition of 2 mM caffeine to the cis solution (Fig. 2A, bottom trace). Consistent channel activation by caffeine was observed in all single channels tested.

In contrast to the consistent activation of channel activity by caffeine, not all channels were consistently activated by ryanodine. After addition of 50–100 µM ryanodine over 12 µM Ca2+ in the cis solution, a distinct

Fig. 2. Single channel records showing channel activation after addition of caffeine (A) or cADPR (B) to cis solution. Presence of 4 equal subconductance states (S1–S4) is shown in detail in bottom traces in B, plotted with a time scale amplified 5-fold. P0, normalized mean current.

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increase in channel $P_o$ was observed only in three of seven single channel experiments. Also, all our attempts to measure specific $[^3H]$ryanodine binding to sea urchin egg microsomes were unsuccessful. For these reasons, we have not included the effects of ryanodine on single channel activity in this report.

**Effect of cADPR on single Ca$^{2+}$ channel activity.**

Addition of cADPR to the cis solution containing 12 µM Ca$^{2+}$ produced more stimulation of channel activity than 2 mM caffeine. After cis addition of 2 and 5 µM cADPR, channel activity increased from a basal $P_o$ of 0.006 to a $P_o$ of 0.039 and 0.125, respectively (Fig. 2B).

At this level of activity it became evident that cADPR-activated channels exhibited a complex behavior. Channel activity presented bursting kinetics, and fluctuations among a single closed state and four near-equal subconductance open states were clearly discernible with an amplified time scale (Fig. 2B, bottom traces).

Subconductance states, observed as well in native ryanodine-sensitive Ca$^{2+}$ channels from SR (1), were consistently observed in all channels studied. Several channel experiments ($n = 4$) revealed that, in the presence of 12 µM Ca$^{2+}$ in the cis solution, $P_o$ increased as a function of cADPR concentration, reaching 0.095 ± 0.011 at 5 µM cADPR, the highest concentration tested. Significant channel activation over the control condition became noticeable at $0.5 \mu M$ cADPR (Fig. 3A, filled symbols).

To analyze in more detail the effects of cADPR on the distribution of subconductance open states, an experiment was performed using a higher Cs-methanesulfonate gradient (300 mM cis/50 mM trans) to increase channel current and thus increase the signal-to-noise ratio of the experimental records. An increase in $P_o$ by cADPR as low as 0.2 µM was observed when 300 mM Cs$^+$ was used in the cis solution (Fig. 3A, open circles). This observation suggests that increasing Cs$^+$ in the cis solution makes the channels more sensitive to stimulation by cADPR and is consistent with previous reports showing that the effect of agonists on mammalian ryanodine-sensitive Ca$^{2+}$ channels is modulated by ionic strength (8, 31).

Table 1. Comparison between experimental and predicted binomial frequencies for different conductance states of a cADPR-activated channel

<table>
<thead>
<tr>
<th>Conductance State</th>
<th>Control</th>
<th>+0.2 µM cADPR</th>
<th>+0.5 µM cADPR</th>
<th>+1 µM cADPR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>E</td>
<td>T</td>
<td>E</td>
<td>T</td>
</tr>
<tr>
<td>Closed</td>
<td>0.9884</td>
<td>0.9668</td>
<td>0.9765</td>
<td>0.9022</td>
</tr>
<tr>
<td>S 1</td>
<td>0.0018</td>
<td>0.0327</td>
<td>0.0033</td>
<td>0.0490</td>
</tr>
<tr>
<td>S 2</td>
<td>0.0019</td>
<td>0.0041</td>
<td>0.0047</td>
<td>0.0036</td>
</tr>
<tr>
<td>S 3</td>
<td>0.0036</td>
<td>2.35×10^-6</td>
<td>0.0091</td>
<td>6.38×10^-5</td>
</tr>
<tr>
<td>S 4</td>
<td>0.0043</td>
<td>4.97×10^-9</td>
<td>0.0154</td>
<td>4.16×10^-7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.0468</td>
<td>2.83×10^-5</td>
</tr>
</tbody>
</table>

Experimental (E) and theoretical (T) binomial frequency values are given for data shown in Fig. 3A. Differences between E and T values were significant, with $P < 0.005$. cADPR, adenosine 5'-cyclic diphosphoribose.

Fig. 4. A: current records showing 2 representative cADPR-activated channels conducting Ca$^{2+}$ or Cs$^+$. Single channel activity was recorded in asymmetric Ca-HEPES solutions (12 µM cis/43 mM trans) or asymmetric Cs-methanesulfonate solutions (200 mM cis/50 mM trans). B: current-voltage ($I_{max}$-V) plots for channels recorded in asymmetric Cs-methanesulfonate solutions (200 mM cis/50 mM trans; ●; $n = 4$) or asymmetric Ca-HEPES solutions (12 µM cis/43 mM trans; ○; $n = 2$). Values are means ± SE.
they did not follow the pattern expected for a binomial distribution. In fact, as shown in Table 1, a comparison of the experimentally determined open substate frequencies (data of Fig. 3B) with those predicted by Eqs. 2–6 revealed that in all these cases the $\chi^2$ test detected a significant difference between both frequencies. These findings indicate that the four current levels recorded in cADPR-activated channels are not due to a complex formed by four independent single channels.

Channels recorded in asymmetric Ca$^{2+}$ gradients (12 µM Ca$^{2+}$ cis/43 mM Ca$^{2+}$ trans) were likewise activated by cis addition of 1 µM cADPR (Fig. 4A). Because 43 mM Ca$^{2+}$, the concentration present in the trans compartment in these experiments, effectively blocks SR ryanodine-sensitive Ca$^{2+}$ channels at the cytoplasmic surface (8, 31), the observed channel activity most likely corresponds to channels fused with their cytosolic side oriented toward the cis compartment.

A plot of maximal current values vs. voltage, obtained in channels activated by cis addition of micromolar cADPR in 12 µM Ca$^{2+}$, revealed oocyte channel conductances of 47.5 ± 1.1 pS in asymmetric Ca$^{2+}$ solutions and 155.0 ± 8.0 pS in asymmetric Cs$^+$ solutions (Fig. 4B). The displacement of the $I_{\text{max}}$-voltage curves toward the right after trans addition of Ca$^{2+}$ to channels recorded in Cs$^+$ (data not shown) implies that the channels were more selective for Ca$^{2+}$ than for Cs$^+$. The higher selectivity for Ca$^{2+}$ than for monovalent cations such as Cs$^+$, K$^+$, or Na$^+$ is a well-known feature of mammalian ryanodine-sensitive Ca$^{2+}$ channels (8). For practical reasons, because higher currents were obtained in Cs$^+$, making readily visible the different subconductance states and increasing the differences between the lowest subconductance state and baseline noise (as shown in Fig. 4A), all subsequent channel experiments were done in asymmetric Cs$^+$ solutions (200 mM cis/50 mM trans).

Lack of effect of IP$_3$ on channel activity. Addition of 2 µM IP$_3$ to the cis solution containing 12 µM Ca$^{2+}$ had no effect on channel activity and did not interfere with subsequent channel activation by 5 µM cADPR (Fig. 5A, middle traces). In contrast, decreasing [Ca$^{2+}$] in the cis solution from 12 to 0.72 µM in the continuous presence of 2 µM IP$_3$ and 5 µM cADPR produced a significant reduction in channel activity, decreasing $P_o$ from 0.115 to 0.005 (Fig. 5A, bottom trace). This decrease in channel activity, which took place when [Ca$^{2+}$] in the cis solution was lowered to levels that optimize the Ca$^{2+}$ release response to IP$_3$ in sea urchin egg homogenates (5), indicates that these cADPR-activated channels are not responsive to IP$_3$. In addition, this reduction implies that stimulation by cADPR requires

Fig. 5. A: single channel records showing lack of channel activation by cis addition of 2 µM IP$_3$ and subsequent stimulation of channel activity by cis addition of 5 µM cADPR. Stimulatory effect of cADPR disappeared after cis [Ca$^{2+}$] was lowered to 0.72 µM. B: single channel records showing stimulation of channel activity by cis addition of 2 µM cADPR and lack of further stimulation by subsequent cis addition of 4 µg/ml calmodulin (CaM); channel activity decreased markedly after cis addition of 20 µM ruthenium red.
>0.72 µM Ca²⁺ in the cis solution, as reported in further detail below.

Effects of calmodulin, ruthenium red, and heparin on channel activity. In a separate experiment, addition of 4 µg/ml (0.24 µM) calmodulin to a channel already activated by 2 µM cADPR in 12 µM cis Ca²⁺ did not modify channel activity (Fig. 5B, 3rd trace). Yet, further addition of ruthenium red produced a marked inhibition, lowering P₆ from 0.063 to 0.005 (Fig. 5B, bottom trace). Likewise, no effect of cis addition of calmodulin on the activity of cADPR-activated channels was observed when the [Ca²⁺] of the cis compartment was lowered to 0.72 µM (records not shown). Because cADPR-activated Ca²⁺ release in sea urchin egg microsomes has an absolute requirement for calmodulin (22, 23, 27), endogenous calmodulin may be associated with the channels fused in the bilayers. That this is most likely the case is shown by the experiments described below.

Addition of heparin to the cis solution containing 12 µM Ca²⁺ plus 5 µM cADPR had a marginal stimulatory effect on channel activity and did not interfere with subsequent channel inhibition by ruthenium red. Thus channels (n = 2) that, after addition of 5 µM cADPR to the cis solution containing 12 µM Ca²⁺, increased their activity from a P₆ of 0.010 ± 0.001 to 0.083 ± 0.026, on addition of 300 µg/ml heparin increased their P₆ only to 0.125 ± 0.019. This small increase in channel activity brought about by 300 µg/ml heparin was not statistically significant. Further increasing heparin to 600 µg/ml had a negligible effect on channel activity, as reflected by a P₆ of 0.127 ± 0.028. Subsequent addition of 30 µM ruthenium red decreased channel activity to a P₆ of 0.008 ± 0.004.

Ca²⁺ dependence of channel activity. In the absence of cADPR, varying cis [Ca²⁺] from 0.7 to 270 µM had a modest effect on single channel activity (Fig. 6, open circles); P₆ increased from <0.003 in 0.7 µM Ca²⁺ to ~0.01 in 12 µM Ca²⁺. Channel P₆ remained constant at 12–30 µM cis Ca²⁺. Further increasing [Ca²⁺] to 270 µM produced some decrease in P₆ to an average of 0.006.

In contrast, in the presence of 1 µM cADPR, increasing cis [Ca²⁺] to 12 µM had a marked stimulatory effect on channel activity (Fig. 6, filled circles), as reflected by an increase in P₆ to ~0.035. As observed in the absence of cADPR, channel activity remained constant at 12–30 µM cis Ca²⁺. Further increasing cis [Ca²⁺] to 60 µM reduced P₆ to ~0.020.

In the experimental conditions used to record channel activity, 1 µM cADPR did not activate the channels at ≤0.7 µM Ca²⁺ (Fig. 6). Yet, as shown in Fig. 1, 1 µM cADPR was effective in eliciting Ca²⁺ release from sea urchin egg microsomes actively loaded with Ca²⁺ and bathed in solutions containing ~0.4 µM Ca²⁺. Furthermore, concentrations of cADPR as low as 10 nM have been reported to activate Ca²⁺ release from sea urchin egg homogenates actively loaded with Ca²⁺ (13). Because vesicular Ca²⁺ release and bilayer experiments were done in different conditions, such as the presence of millimolar Mg²⁺ and ATP in the external solution when vesicular release was measured, we investigated

Fig. 6. Effect of cis [Ca²⁺] on P₆. Experiments were done in absence (○) or presence (●) of 1 µM cADPR in cis solution. Values are means ± SE, with number of determinations in parentheses. Lines have no theoretical meaning.
whether cADPR stimulation of channel activity was modified by addition of ATP or Mg\(^{2+}\) to the cis solution.

Stimulation of channel activity by ATP. Addition of 2 mM ATP to the cis solution containing 12 µM Ca\(^{2+}\) produced a significant stimulation of \(P_o\) from 0.007 to 0.022 (Fig. 7). Subsequent addition of 0.5 µM cADPR did not stimulate further channel activity but produced a small reduction in channel \(P_o\) to 0.015 (not shown). Even increasing cADPR to 2 µM in the presence of ATP did not stimulate channel activity, because \(P_o\) remained at 0.015 (Fig. 7, top, 3rd trace). After extensive washing of the cis compartment (see EXPERIMENTAL PROCEDURES) and addition of 12 µM Ca\(^{2+}\) to the cis solution, the channel regained its low activity, with a \(P_o\) of 0.005. At this point, addition of 1 µM cADPR increased \(P_o\) to 0.032 (Fig. 7, bottom). These results indicate that the stimulatory effect of ATP on channel activity did not add to that of cADPR and was wholly reversible. In concordance with these results, several experiments revealed that cis addition of 1–2 mM ATP to cADPR-activated channels did not stimulate further channel activity (data not shown).

Stimulation of channel activity by cADPR plus Mg\(^{2+}\). To analyze the effects of Mg\(^{2+}\) on cADPR-activated Ca\(^{2+}\) channels, cis [Ca\(^{2+}\)] was held at 24 µM, a concentration that was within the range of [Ca\(^{2+}\)] that produced optimal channel activation. To avoid changes in [Ca\(^{2+}\)] after Mg\(^{2+}\) addition, ethylene glycol-bis[\(\beta\)-aminoethyl ether]-N,N,N',N'-tetraacetic acid was used as a buffer. Addition of 1 µM cADPR to a single Ca\(^{2+}\) channel increased \(P_o\) from 0.003 to 0.020 (Fig. 8A). Subsequent addition of 1 mM Mg\(^{2+}\) to the cis solution markedly enhanced channel activity, increasing \(P_o\) 12.8-fold, from 0.020 to 0.256 (Fig. 8A). Addition of Mg\(^{2+}\) in the absence of cADPR had no effect on channel activity (not shown).

Yet, not all channels studied displayed the same marked activation by Mg\(^{2+}\). In the example of the single channel illustrated in Fig. 8B, after addition of 1 µM cADPR in 24 µM cis Ca\(^{2+}\), the channel increased its activity from a \(P_o\) of 0.009 to 0.012 (Fig. 8B, top and middle traces). Subsequent addition of 1 mM Mg\(^{2+}\) produced only a 4.4-fold stimulation of channel activity, to a \(P_o\) of 0.053 (Fig. 8B, bottom trace).

The difference between these two types of responses is further illustrated in Fig. 9. All channels that presented a more marked activation by Mg\(^{2+}\) (Fig. 9, filled squares) were clearly activated by a cADPR concentration as low as 0.1 µM, with an apparent saturation at 1–2 µM cADPR. In contrast, the channels less activated by Mg\(^{2+}\) required >0.5 µM cADPR for discernible activation and did not seem to saturate at a cADPR concentration as high as 2 µM (Fig. 9, open circles). These results indicate that, in the combined presence of 24 µM cis Ca\(^{2+}\) and 1 mM Mg\(^{2+}\), some of the

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Fig. 8. Single channel records showing channel activation by cis addition of 1 µM cADPR and subsequent addition of 1 mM Mg\(^{2+}\). Channels similarly activated by 24 µM cis Ca\(^{2+}\) plus 1 µM cADPR responded differently after addition of 1 mM Mg\(^{2+}\) to cis solution. Channel in A was more activated after addition of 1 mM Mg\(^{2+}\), with an increase in \(P_o\) from 0.020 to 0.256, than channel in B, in which \(P_o\) was increased from 0.012 to 0.053.
channels markedly increased their sensitivity to activation by cADPR. This finding suggests that one of the additional components present in Ca^2+ release experiments makes the channels more responsive to cADPR. However, the differences in the requirements for higher cis [Ca^{2+}] to observe channel activation by cADPR in bilayers, as opposed to release experiments, cannot be attributed to Mg^2+. When cis [Ca^{2+}] was decreased to the submicromolar range, channels were not consistently activated by addition of cADPR, even in the presence of 1 mM Mg^2+ (data not shown).

A study of the response of the channels less sensitive to activation by Mg^2+ showed that concentrations of Mg^2+ >1 mM were less effective in enhancing channel activation by cADPR, and channel activity in 9 mM Mg^2+ was lower than in its absence (Fig. 9, inset). I_{\text{max}} was reduced as well by >7 mM Mg^2+ (data not shown).

Effects of calmodulin antagonists plus Mg^2+ on channel activity. Channels activated by cADPR were not affected by calmodulin addition (Fig. 5), even at a concentration (4 µg/ml) that induces near full activation of Ca^2+ release in sea urchin egg microsomes (22, 23, 27). Because calmodulin seems to be essential for activation of Ca^2+ release by cADPR in these microsomes (23), the lack of effect of calmodulin on single channel activity may reflect the presence of endogenous calmodulin tightly associated with the channel protein. To test this hypothesis, we examined the effects of the calmodulin antagonist W-7 (17) on channel activity. A single channel that displayed a P_o of 0.274 in the presence of 24 µM Ca^2+, 1 mM Mg^2+, and 2 µM cADPR in the cis solution (Fig. 10, top trace) decreased its activity to a P_o of 0.047 after cis addition of 10 µM W-7 (Fig. 10, middle trace). To test for the specificity of this effect, 10 µM calmodulin was subsequently added to the cis solution. After addition of this large excess of calmodulin, the channel increased its P_o to 0.223, which is similar to that measured before W-7 addition (Fig. 10, bottom trace). Inhibitory effects comparable to those obtained with W-7 were observed with use of compound R-24571 (calmidazolium), a different calmodulin antagonist (data not shown). These results support the above hypothesis that channels fused in the bilayer contain sufficient endogenous calmodulin to support channel activity.

**DISCUSSION**

The results described here demonstrate that microsomes isolated from T. nyger eggs contain Ca^{2+} channels that were activated by cis addition of caffeine, micromolar Ca^{2+}, or millimolar ATP. Channels were also activated by cis addition of micromolar cADPR, and stimulation by cADPR was modulated by cis [Ca^{2+}] and by Mg^2+.

Comparison with mammalian RyR-channels. Activation by caffeine, ATP, and cis [Ca^{2+}] in the micromolar range (8, 11, 12) is characteristic of vertebrate RyR-channels. Furthermore, as described for RyR-channels isolated from mammalian skeletal muscle (11), the egg channels displayed a bell-shaped Ca^{2+} dependence of P_o in the presence of cADPR. In addition, the egg
channels were inhibited by ruthenium red and displayed multiple subconductance states, behaviors displayed as well by the mammalian RyR-channels (1, 8). On the basis of these similarities, it is tempting to assign the cADPR-activated channels from sea urchin eggs to the ryanodine receptor family. Although the conductance of the cADPR-activated egg channels (50 pS) was lower than the conductances described for RyR-channels from mammalian skeletal and cardiac muscle (100–120 pS) (8), this difference may not be very significant, because variations in the conductance of the mammalian channels have been reported (2). Yet, in contrast to the well-reported effects of ryanodine on RyR-channels (8, 31), we did not observe consistent modulation of the sea urchin channels by ryanodine. We have no explanation for this lack of consistent effects of ryanodine on channel activity.

Channels activated by cADPR were not activated by IP$_3$. The egg channels activated by cADPR were not IP$_3$-gated channels, since addition of IP$_3$ (2 µM) to the cis chamber had no effect on channel activity. However, IP$_3$ addition to sea urchin microsomes actively loaded with Ca$^{2+}$ caused significant Ca$^{2+}$ release, indicating that the microsomes described in this study have release pathways responsive to IP$_3$. Accordingly, a straightforward interpretation of the lack of effect of IP$_3$ on channel activity is that the IP$_3$-gated channels of sea urchin eggs (not described so far in bilayers) are different from the Ca$^{2+}$ channels activated by cADPR studied in this work. It is pertinent to mention in this regard that there is significant evidence indicating that the cADPR- and IP$_3$-sensitive Ca$^{2+}$ release pathways of sea urchin eggs are different. 1) Ruthenium red and procaine, two antagonists of Ca$^{2+}$-induced Ca$^{2+}$ release in SR, inhibit cADPR-sensitive Ca$^{2+}$ release in egg homogenates without affecting IP$_3$-sensitive release (12, 13). 2) Even high concentrations of IP$_3$ do not alter the Ca$^{2+}$-releasing activity induced by cADPR (12). 3) cADPR-induced Ca$^{2+}$ release is insensitive to heparin (13, 24), a competitive inhibitor of IP$_3$ binding to its receptor. Furthermore, the lack of effect of heparin on Ca$^{2+}$ release is in agreement with the negligible effect of heparin on channel activity found in this work, even at heparin concentrations that completely inhibit IP$_3$-sensitive Ca$^{2+}$ release.

Ca$^{2+}$ dependence of channel activity in the presence of cADPR. Sea urchin egg Ca$^{2+}$ channels were moderately activated by increasing cis [Ca$^{2+}$] from 0.7 to 30 µM, but in the presence of micromolar concentrations of cADPR, channel activity by increasing cis [Ca$^{2+}$] in this range became more prominent. These results agree with a recent report describing significant enhancement of cADPR-gated Ca$^{2+}$ release rates in sea urchin eggs by increasing [Ca$^{2+}$] (16).

In the standard experimental conditions used to record channel activity, 1 µM cADPR did not activate the channels at ≤0.7 µM Ca$^{2+}$. In contrast, we found that 1 µM cADPR was effective in eliciting Ca$^{2+}$ release from microsomes actively loaded with Ca$^{2+}$ and bathed in solutions containing 0.3–0.4 µM Ca$^{2+}$. These results are in agreement with a previous report showing that cADPR induces Ca$^{2+}$ release from sea urchin egg homogenates at submicromolar [Ca$^{2+}$] (5). Nonetheless, it is important to note in this regard that Ca$^{2+}$ release from microsomal vesicles actively loaded with Ca$^{2+}$ is measured under experimental conditions quite different from those prevailing in bilayer experiments. In release experiments, 1) the vesicles have actively accumulated significant luminal Ca$^{2+}$, 2) they are bathed in solutions containing millimolar Mg-ATP, and 3) other vesicular proteins are present that may or may not fuse with the channels into the bilayers. Each one of these factors might in principle enhance the effect of cADPR at submicromolar [Ca$^{2+}$], since all of them regulate the activity of mammalian RyR-channels (8, 18, 31). However, even in the presence of 1 mM Mg$^{2+}$ channels were not consistently activated by cADPR in submicromolar [Ca$^{2+}$], making unlikely Mg$^{2+}$ by itself as the cause of the different Ca$^{2+}$ sensitivity. Whether the other factors mentioned above are responsible for the differences between bilayer and vesicular release experiments should be investigated. In particular, the effects of luminal Ca$^{2+}$ should be studied, since we have preliminary results (not shown) indicating that cADPR was more effective in stimulating channel activity when the trans compartment contained 50 µM Ca$^{2+}$ in addition to 50 mM Cs$^+$. Additionally, other factors that enhance the effects of cADPR on Ca$^{2+}$ release from sea urchin egg homogenates, such as the oxidation state of critical SH groups (13, 28) or the presence of palmitoyl-CoA (6), may enhance the channel response to cADPR at low [Ca$^{2+}$].

Effects of Mg$^{2+}$ on channel activity. It has been reported that 1 mM MgCl$_2$ is required for optimal activation of Ca$^{2+}$ release by cADPR (12–15) and that cADPR-activated Ca$^{2+}$ release from sea urchin egg homogenates is inhibited by millimolar concentrations of Mg$^{2+}$ (6, 15). In agreement with these reports, our results indicate that Mg$^{2+}$ presented a dual effect over the activity of cADPR-stimulated channels. Thus 1 mM Mg$^{2+}$ increased markedly the stimulatory effect of cADPR on channel activity, but at higher concentrations it was less effective and eventually became inhibitory. Furthermore, in the presence of 1 mM Mg$^{2+}$, the sea urchin egg channels displayed two types of responses toward activation by cADPR. Why channels presented these two different responses is not clear and should be the subject of future investigation.

Conclusions. The results of this work indicate that sea urchin egg microsomes possess Ca$^{2+}$ channels that share similar properties with vertebrate RyR channels, such as activation by Ca$^{2+}$, ATP, and caffeine and inhibition by ruthenium red. Because activation of oocyte channels by cADPR was enhanced by Ca$^{2+}$, we propose that these Ca$^{2+}$-channels of sea urchin eggs constitute an intracellular Ca$^{2+}$-induced Ca$^{2+}$ release pathway in these cells.

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