Cyclic ADP-Ribose-Induced Calcium Release in Sea Urchin Egg Homogenates Is a Cooperative Process†

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ABSTRACT: Cyclic ADP-ribose (cADPR), an enzymatic product formed from NAD^+ in sea urchin eggs and in several mammalian cell types, has been proposed as a second messenger for the regulation of cytosolic free Ca^{2+} concentration. Although cADPR induces Ca^{2+} release from an intracellular Ca^{2+} pool by a mechanism that differs from that used by inositol 1,4,5-trisphosphate, the precise action is unknown. We have analyzed the kinetics of cADPR-induced Ca^{2+} release at 15 °C in sea urchin egg homogenates using the calcium indicator fluo-3. In this system, Ca^{2+} release induced by cADPR was a positively cooperative process with a Hill coefficient of 1.8 ± 0.32 (mean ± SE, n = 15). We also examined the effect of caffeine, which potentiates the action of cADPR, on the kinetics of cADPR-induced Ca^{2+} release. Caffeine (3 mM) did not affect the degree of cooperativity induced by cADPR, but it did cause an increase in the apparent affinity of cADPR for its cellular target; the apparent K_d, derived from the kinetic analysis, decreased from 176 ± 50 to 68 ± 13 nM. We conclude that cADPR-induced Ca^{2+} release in sea urchin eggs is a cooperative process. A straightforward interpretation of the analysis is that opening of the Ca^{2+}-release channel requires the binding of two molecules of cADPR (Hill coefficient of about 2). However, it is not yet known whether cADPR binds directly to the release channel. It is possible that potentiation of the action of cADPR by newly released Ca^{2+} could also contribute to the observed cooperative effect.

The concentration of cytosolic free Ca^{2+}($[Ca^{2+}]_i$)[1] is a key effector in cellular regulation (Berridge & Irvine, 1989). Many extracellular signals stimulate the synthesis of the intracellular messenger inositol 1,4,5-trisphosphate (IP_3), which causes opening of Ca^{2+} channels in an intracellular storage compartment resulting in Ca^{2+} release into the cytosol (Berridge, 1993; Rana & Hoklin, 1990; Henzi & MacDermott, 1992; Ferris & Snyder, 1992). Reports of Ca^{2+} mobilization that are independent of the IP_3 mechanism indicate the presence of other second messengers involved in the regulation of intracellular Ca^{2+} homeostasis (Berridge & Irvine, 1989). One candidate for such an additional second messenger is cyclic ADP-ribose (cADPR), a metabolite of NAD^+. cADPR is a potent mediator of Ca^{2+} release in sea urchin egg microsomes (Clapper et al., 1987) and in some mammalian cells (Koshiyama et al., 1991; Takasawa et al., 1993; Mészáros et al., 1993). Specific binding of cADPR to sea urchin egg microsomes has been demonstrated, and the binding was shown to be unaffected by either IP_3 or heparin, an antagonist at the IP_3 receptor (Lee, 1991). cADPR is as potent as IP_3 in mobilizing sequestered Ca^{2+} in sea urchin eggs (Dargie et al., 1990), and both the IP_3- and cADPR-controlled Ca^{2+}-mobilizing systems are activated during fertilization (Lee et al., 1993; Galione et al., 1993a). Some evidence has been presented to suggest that cADPR may be an endogenous modulator of Ca^{2+}-induced Ca^{2+} release (CICR) (Galione et al., 1991; Galione, 1992; Lee, 1993), a process which is well characterized in muscle and may be responsible for oscillations in cytosolic Ca^{2+} and Ca^{2+} wave propagation (Berridge & Galione, 1988; Dupont et al., 1990; Galione et al., 1993a).

We have investigated the kinetics of cADPR-induced Ca^{2+} release from intracellular storage sites in sea urchin eggs to gain insights into the biochemical mechanism of action of cADPR. Our results demonstrate that cADPR-induced Ca^{2+} release is a positively cooperative process. Caffeine, a known potentiator of the action of cADPR (Lee, 1993), caused an increase in the rate of Ca^{2+} release induced by cADPR but had no effect on cooperativity. This cooperative property of cADPR-induced Ca^{2+} release would be expected to give rise to a steeper rate of rise in Ca^{2+} release as the concentration of cADPR is increased. This property was observed in our experimental preparations.

MATERIALS AND METHODS

Materials. Fluo-3 pentaamonium salt (fluo-3) and DTPA Polymetal Sponge B (DTPA polyacrylamide) were purchased from Molecular Probes (Eugene, OR). Ionomycin was from Calbiochem (La Jolla, CA) and Chelex 100 resin from Bio-Rad (Richmond, CA). Other chemicals were reagent grade and were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher (Pittsburgh, PA), unless otherwise noted. cADPR was a generous gift from Professor H. C. Lee (University of Minnesota School of Medicine) (Lee et al., 1989).

Preparation of Homogenates from Strongylocentrotus purpuratus Eggs. Egg homogenates were prepared as

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[1] Abbreviations: $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CICR, Ca^{2+}-induced Ca^{2+} release; cADPR, cyclic ADP-ribose; IP_3, inositol 1,4,5-trisphosphate.

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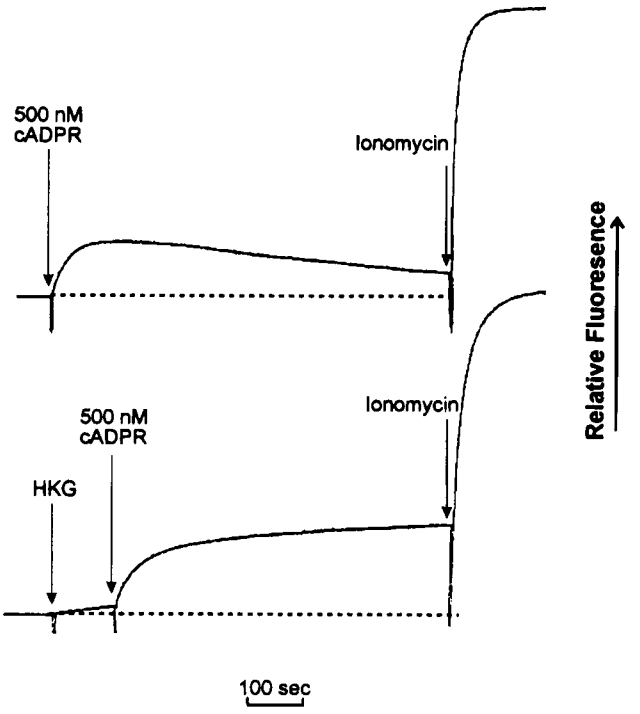

FIGURE 1: Time course of cADPR-induced Ca^{2+} release in sea urchin egg homogenates. (Top) cADPR (500 nM), a maximum concentration, was added to cause release of Ca^{2+} from the cADPR-responsive pool. Then, 200 nM ionomycin was added to release all residual sequestered Ca^{2+}. (Bottom) Hexokinase (10 units/mL) and glucose (10 mM) (HKG) were added to consume ATP and stop ATP-dependent calcium resequestration before addition of cADPR. In subsequent experiments, the rate of Ca^{2+} release induced by cADPR was corrected for the slow leak observed after ATP depletion by subtracting the leak rate from the rate of Ca^{2+} release induced by cADPR.

described by Clapper et al. (1987). Briefly, eggs from *S. purpuratus* were washed with artificial sea water (ASW), then with Ca^{2+}-free ASW containing 1 mM EGTA, once with Ca^{2+}-free seawater without EGTA, and once with the homogenization buffer (250 mM *N*-methylglucamine, 250 mM potassium gluconate, 20 mM HEPES, and 1 mM $MgCl_2$, pH 7.2) and resuspended (25%, v/v) in the same buffer containing 50 μg/mL leupeptin, 50 μg/mL aprotinin, 250 μg/mL soybean trypsin inhibitor, 10 units/mL creatine kinase, 20 mM phosphocreatine, and 2.5 mM ATP. The suspension was homogenized with 30−40 strokes of a Dounce-type glass homogenizer and centrifuged for 10 s (13000*g*). All procedures were performed at 4 °C. The supernatant fraction was stored at −70 °C until use. All solutions were pretreated with Chelex 100 resin to remove heavy metal ions.

Ca^{2+} Release Assay. Egg homogenates were thawed at 15 °C for 10 min and then diluted to 2.5% with homogenization medium containing 1 mM ATP and 2 μM fluo-3. After incubation at 15 °C for 1 h, 3 mL of the equilibrated homogenate was transferred to a cuvette maintained at 15 °C and stirred throughout the experiment. The Ca^{2+}-release response to cADPR was stable for more than 4 h at 15 °C. Ionomycin (200 nM final concentration) was added at the end of each experiment to determine the size of the residual Ca^{2+} storage compartment in each homogenate preparation. Ambient free calcium in the bulk medium was determined from the fluorescent signal measured with a Spex Fluorog F111A spectrofluorometer (excitation 492 nm; emission 535 nm). Ca^{2+} released from microsomes in the homogenate was calibrated by fitting the fluorescence measurements to a standard curve determined by addition of known amounts of Ca^{2+}. The increase in Ca^{2+} concentration as a function of fluorescence (F) was calculated as

$$[Ca^{2+}] = K_d(F - F_{min})/(F_{max} - F) + [Ca^{2+}]_0$$

where F was the observed fluorescence, F_{min} was the fluorescence in the absence of calcium, and F_{max} was the fluorescence of the calcium saturated in dye. $[Ca^{2+}]_0$ was the basal concentration of calcium. K_d was 450 nM. F_{max} and F_{min} were determined according to the method recommended by Molecular Probes, Inc.

The Ca^{2+}-release data were fitted to first-order kinetics using the standard curve-fitting functions of the Sigma Plot program Version 5.0 (Jandel Scientific, Corte Madera, CA) as given by

$$[Ca^{2+}] = A[1 - \exp(-k_{obs}t)] + bt + [Ca^{2+}]_0$$

where $[Ca^{2+}]$ was the calcium concentration at time t, $[Ca^{2+}]_0$ was the basal concentration of calcium, k_{obs} was the observed rate constant of the calcium-release process, and bt accounted for a slow Ca^{2+}-leak process due to ATP depletion (see Results). A and b are constants.

Data presented are those obtained from at least 10 independent experiments.

RESULTS

Rate of Ca^{2+} Release as a Function of cADPR Concentration. Addition of cADPR to the sea urchin egg homogenate caused a rapid release of Ca^{2+} followed by slow Ca^{2+} uptake (Figure 1, top trace). When hexokinase and glucose (HKG) were added, ATP in the homogenate was consumed, and a slow leak of Ca^{2+} from the microsomes was observed (Figure 1, bottom trace). There was no Ca^{2+} uptake after the cADPR-induced Ca^{2+} release in the presence of HKG (Figure 1, bottom trace). The rate of Ca^{2+} release induced by cADPR depended on its concentration (Figure 2). The initial rate of cADPR-induced Ca^{2+} release (in the absence of ATP) occurred by a first-order kinetic process after correction for the slow leak rate (after HKG). Two further examples of Ca^{2+} release data are shown in Figure 3, using 60 and 100 nM cADPR. The observed pseudo-first-order rate constants for cADPR-induced Ca^{2+} release had a positive cooperative dependence on the cADPR concentration with a Hill coefficient of 1.8 ± 0.32 (mean ± SE, n = 15).

Effect of Caffeine on the Kinetics of Ca^{2+} Release Induced by cADPR. Caffeine has been shown to potentiate the action of cADPR on Ca^{2+} release (Lee, 1993; Tanaka & Tashjian, 1994). In our experimental system, near maximum potentiation occurred at 3 mM caffeine in the presence of 20 nM cADPR (Figure 4). Caffeine caused an increase in the pseudo-first-order rate constant for cADPR (Figure 5). Kinetic analysis demonstrated that caffeine did not affect the Hill coefficient (1.8 ± 0.22, mean ± SE, n = 16) for cADPR-induced Ca^{2+} release, but it did cause an increase in the apparent affinity of cADPR for its target; the apparent K_d, derived from the kinetics analysis, decreased from 176 ± 50 to 68 ± 14 nM.

DISCUSSION

The results of our analysis reveal that cADPR-induced Ca^{2+} release in sea urchin egg homogenates occurs by a

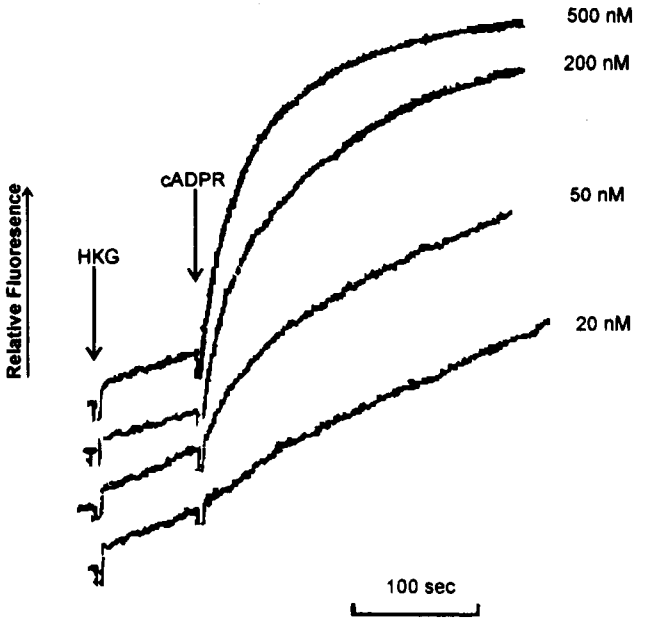

FIGURE 2: Concentration-dependent enhancement of the rate of Ca^{2+} release induced by cADPR. The time course of cADPR-induced calcium release in the sea urchin egg homogenate was monitored by the change in fluo-3 fluorescence intensity. Ca^{2+} release was induced by addition of cADPR (20−500 nM) about 80−100 s after addition of HKG.

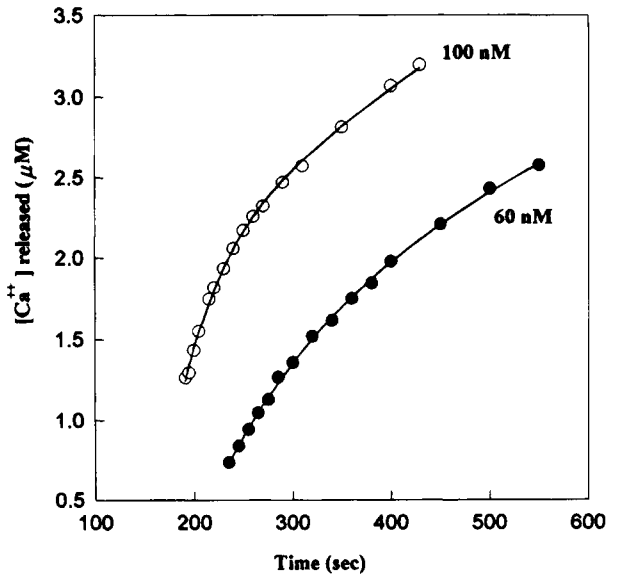

FIGURE 3: Time course of cADPR-induced Ca^{2+} release. Detailed time course of Ca^{2+} release at 60 and 100 nM cADPR is shown. Fluorescence changes were calibrated by addition of known amounts of Ca^{2+} to the egg homogenates. The smooth curves are nonlinear least-squares fits of the data to the first-order kinetics: at 60 nM cADPR, $[Ca^{2+}] = 0.95[1 - \exp(-0.0086(t - 238))] + 0.003t + 0.056$; at 100 nM cADPR, $[Ca^{2+}] = 0.94[1 - \exp(-0.0211(t - 191))] + 0.0042t + 0.445$.

positive cooperative process with a Hill coefficient of about 2. These findings suggest that opening of the Ca^{2+}-release channel requires the binding of at least two molecules of cADPR to its molecular target. From our experiments, it is not certain whether the cADPR target is the Ca^{2+} release channel itself or another protein, because we used whole egg homogenates and it has been proposed that cADPR may not bind directly to the release channel (Walseth et al., 1993).

It has been shown that a CICR mechanism is present in sea urchin egg microsomes (Galione et al., 1991) and that

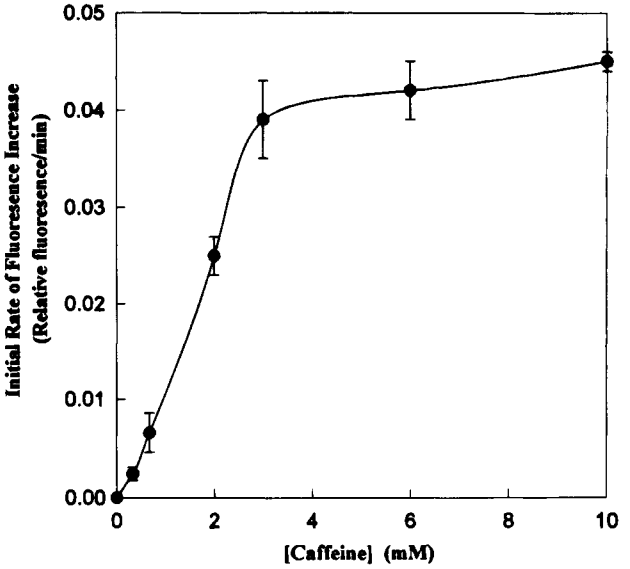

FIGURE 4: Concentration response for caffeine-enhanced Ca^{2+} release induced by cADPR. The initial rates of fluorescence change induced by various concentrations of caffeine in the presence of 20 nM cADPR are plotted. Each point gives the mean value, and the brackets indicate the standard deviation of triplicate samples.

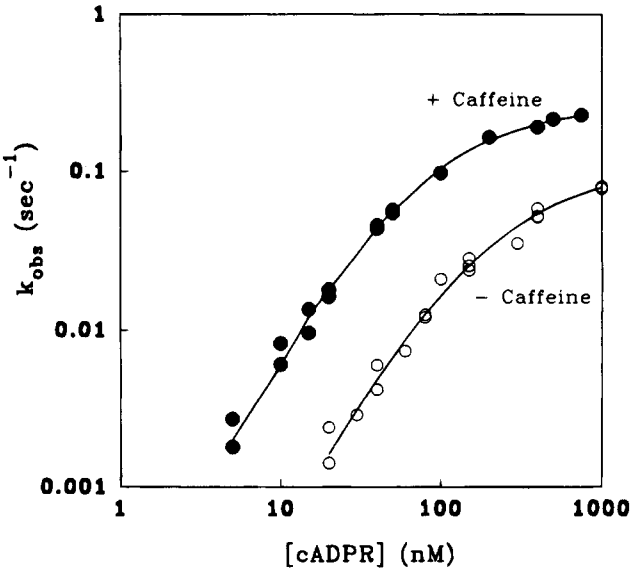

FIGURE 5: Observed rate constants for cADPR-induced Ca^{2+}release in the absence and presence of caffeine. Values of k_{obs} obtained from the best fits as shown in Figure 3 were plotted as a function of [cADPR]. The open circles are data obtained without caffeine, and the filled circles are data obtained in the presence of 3 mM caffeine. The smooth curves were calculated according to a simple model with independent and equal interaction with one of the regulatory sites: $k_{obs} = k(c/(c + K_d))^n$, where c = [cADPR], k is the observed rate constant of Ca^{2+} release when binding of cADPR to its target reached saturation, K_d is the apparent equilibrium dissociation constant for cADPR to its cellular target, and n is the Hill coefficient, which is the index for cooperativity of this kinetic process. The best fit for cADPR-induced Ca^{2+} release in the absence of caffeine was $k_{obs} = (0.106\ s^{-1})(c/(c + 176\ nM))^{1.83}$, and the best fit for cADPR-induced Ca^{2+} release in the presence of 3 mM caffeine was $k_{obs} = (0.268\ s^{-1})(c/(c + 68.45\ nM))^{1.82}$.

cADPR-induced Ca^{2+} release is potentiated dramatically by caffeine, a commonly used marker for CICR (Galione et al., 1991; Lee, 1993). We examined the kinetic properties of the caffeine effect on cADPR-induced Ca^{2+} release. The caffeine effect was dose dependent and showed near maximum potentiation for cADPR-induced Ca^{2+} release at

3 mM (Figure 4). In our experiments, caffeine enhanced both the apparent affinity of cADPR for its molecular target and the rate of opening of the Ca^{2+}-release channel induced by cADPR, but caffeine had no effect on cooperativity. Because the rate constant for cADPR-induced Ca^{2+} release is proportional to the fraction of channels which are open (Meyer et al., 1990), the potentiating action of caffeine may be due to an increase in the rate of channel opening or a decrease in the rate of channel closing, or both. The existence of an endogenous caffeine-like modulator of cADPR-induced Ca^{2+} release has been postulated (Lee et al., 1994; Tanaka & Tashjian, 1995). In the absence of such an endogenous modulator, the cADPR-sensitive Ca^{2+} channel requires relatively high concentrations of cADPR (100−200 nM) to open. Such concentrations could be achieved by enhanced enzymatic synthesis or decreased degradation of cADPR (Galione et al., 1993b). Thus, in the absence of an endogenous modulator, basal levels of cADPR (50−100 nM) (Lee, 1993) would not be expected to activate the Ca^{2+}-release channel. However, in the presence of an endogenous modulator such as calmodulin (Lee et al., 1994; Tanaka & Tashjian, 1995), the cADPR-sensitive Ca^{2+} channel would be switched to a more responsive state and release Ca^{2+} in the presence of low concentrations of cADPR. In addition, it is also possible that potentiation of cADPR-induced Ca^{2+} release occurs via newly released Ca^{2+} which contributes to the observed cooperative Ca^{2+}-release properties. However, because caffeine did not affect cooperativity but does enhance CICR, it is unlikely that CICR is the sole contributor to the observed cooperativity.

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