Cyclic ADP-ribose-gated Ca\(^{2+}\) Release in Sea Urchin Eggs Requires an Elevated [Ca\(^{2+}\)]*  

(Received for publication, March 11, 1997, and in revised form, April 30, 1997)

Xiaoqing Guo and Peter L. Becker‡

From the Department of Physiology, Emory University School of Medicine, Atlanta, Georgia 30322

Cyclic ADP-ribose (cADPr) has been shown to release intracellular Ca\(^{2+}\) from sea urchin eggs and a variety of vertebrate cell types, although its mechanism of action remains elusive. We employed the caged version of cADPr to study the [Ca\(^{2+}\)] transient kinetics in intact sea urchin eggs for insights into how cADPr gates Ca\(^{2+}\) release. Ca\(^{2+}\) release triggered by photolytic production of cADPr was initially slow, with an effective delay of several hundred milliseconds before the onset of a rapid Ca\(^{2+}\) release phase. In contrast, Ca\(^{2+}\) release induced by photolysis of caged inositol 1,4,5-trisphosphate was immediate in onset and roughly an order of magnitude faster. The delay before cADPr-induced Ca\(^{2+}\) release was eliminated when the [Ca\(^{2+}\)] was step-elevated coincident with the photoliberation of cADPr and greatly prolonged in the presence of exogenous Ca\(^{2+}\) buffers. Thus, the slow onset of Ca\(^{2+}\) release does not reflect an intrinsically slow rate by which cADPr gates release channels. Rather, a [Ca\(^{2+}\)] rise from resting levels is needed to achieve more than minimal cADPr activity. Full release of Ca\(^{2+}\) by cADPr in intact sea urchin eggs requires a positive Ca\(^{2+}\) feedback.

This paper is available online at http://www.jbc.org

* This study was supported in part by grants from the National Institutes of Health and the American Heart Association (Georgia affiliate). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Physiology, Emory University School of Medicine, 1648 Pierce Dr., Atlanta, GA 30322. Tel.: 404-727-3318; Fax: 404-727-2648; E-mail: plb@physio.emory.edu.

The abbreviations used are: cADPr, cyclic ADP-ribose; RyRC, ryanodine receptor/channel; IP\(_3\), inositol 1,4,5-trisphosphate; MOPS, 4-morpholinepropanesulfonic acid; FI, fluorescence intensity; CG-5N, Calcium Green 5N; NP-EGTA, nitrophenyl-EGTA.
previously described (21). Briefly, cells were illuminated with 470 nm excitation light for a 2.5-ms interval every 7.5 ms, and fluorescence emission light (500–550 nm) was detected with a photomultiplier tube/photom counter circuit. The longer wavelength, nonratiometric dyes fluo-3 and CG-SN were used to avoid photolysis of the caged compounds by the fluorescence excitation light.

Flash Photolysis—To photolyze caged compounds, the output of a xenon flashlamp (Hi-tech, UK) was passed through a UG-5 filter to select for ultraviolet light and merged into the excitation light path of the microfluorimeter with a dichroic beamsplitter. The nominal flash lamp energy was set to 350 J, producing a light burst with a duration of ~2 ms. Based on the [Ca$^{2+}$] response to repeated flashes, we estimate that a single flash photolyzes approximately 15% of intracellular NP-EGTA. The fraction of caged cADPr and caged IP$_3$ photolyzed by a single flash is unknown.

Calibration of Ca$^{2+}$ Dye Fluorescence—Fluorescence emission intensity (FI) was used as an index of the intracellular [Ca$^{2+}$]$, but no attempt was made to calibrate the fluorescence intensity to an actual concentration scale. In eggs injected with only minimal amounts of Ca$^{2+}$ chelators, we presume that the normal resting [Ca$^{2+}$] was ~150 nM based on estimates made by others in eggs studied under similar conditions (10, 22, 23). In eggs loaded with millimolar levels of the caged calcium NP-EGTA, the preflash [Ca$^{2+}$] presumably was somewhere between the normal resting [Ca$^{2+}$] and the free [Ca$^{2+}$] of the injection. NP-EGTA has a $K_d$ of 80 nM for Ca$^{2+}$ in a solution with an ionic strength of 0.10–0.15 M at pH 7.2 (24). Using this value, the injectate-free [Ca$^{2+}$] would be estimated to have been 120 and 53 nM for Ca$^{2+}$-NP-EGTA molar ratios of 0.6 and 0.4, respectively. However, most Ca$^{2+}$ chelators, including EGTA, BAPTA, fluo-3, and CG-SN, have much lower affinities at higher ionic strengths and lower pH levels (25–27). For example, the affinity of EGTA for Ca$^{2+}$ decreases 11-fold going from a 0.15 M ionic strength solution at pH 7.2 to one of 0.6 M ionic strength at pH 6.7 (the approximate cytosolic environment in sea urchin eggs (28)). If the affinity of the structurally similar NP-EGTA also decreases 11-fold under these conditions, the injectate-free [Ca$^{2+}$] would be estimated to have been 1320 and 587 nM for the 0.6 and 0.4 Ca$^{2+}$-NP-EGTA ratios, respectively.

Materials—Sea urchins were purchased from Marinus, Inc. (Long Beach, CA). Caged cADPr (29), NP-EGTA, fluo-3, and CG-SN were purchased from Molecular Probes (Eugene, OR). cADPr was purchased from Amersham Corp., and caged IP$_3$ was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). All other reagents were purchased from Sigma.

RESULTS

The kinetics of the Ca$^{2+}$ release activated by flash photolytic production of cADPr were studied in intact eggs from the sea urchin L. pictus. Eggs were microinjected with caged cADPr (29) and the Ca$^{2+}$-sensitive fluorescent dye fluo-3 to approximately 10 and 5 nM, respectively, and subjected to a single brief high intensity flashlight burst. As we have previously noted (21), the onset of rapid Ca$^{2+}$ release after a single flash occurred only after a several hundred millisecond delay (Fig. 1A and Table I). Peak [Ca$^{2+}$] was generally achieved in ~4 s (Table I), and the [Ca$^{2+}$] then gradually fell back to preflash levels over the next 60 s or so. The rise in [Ca$^{2+}$] was initially quite slow but accelerated during the first 1–2 s after the flash. In many cases there was no detectable change in the fluo-3 fluorescence over the first 200 ms, but even when the fluorescence did increase during this period, its rate of rise eventually increased more than 100-fold. Thus, the rate of Ca$^{2+}$ release appears to be less than 1% of its maximum for a significant period of time after the photoliberation of cADPr.

In contrast, [Ca$^{2+}$] transient induced by photolysis of caged IP$_3$ had a similar peak amplitude to those induced by cADPr (Fig. 1B) but strikingly different kinetics. By a number of criteria, the overall response was roughed in order of magnitude faster (Table I). In particular, the onset of Ca$^{2+}$ release occurred within 30 ms of the flash. Thus, the slow onset of Ca$^{2+}$ release appears to be a characteristic specific to the cADPr-activated release mechanism, not of Ca$^{2+}$ release in general.

Several experiments were performed to determine whether delayed Ca$^{2+}$ release was a consequence of our activation or detection methodology. The slow onset of Ca$^{2+}$ release was still observed when the amount of fluo-3 loaded in eggs was decreased 50-fold to approximately 100 nM (Fig. 1C), making it unlikely that this feature of the [Ca$^{2+}$] transient was due to the presence of the Ca$^{2+}$ dye. The characteristics of these cADPr-induced [Ca$^{2+}$] transients were not affected by coloading eggs with heparin (200 ng/ml egg volume; data not shown), ruling out the possibility that the more rapid IP$_3$-gated process was involved. Further, using an egg extract assay (30), we found that caged cADPr (5 µM) did not increase the ED$_{50}$ of cADPr to release Ca$^{2+}$ (data not shown), indicating that the caged compound does not interfere with cADPr binding.

To assess whether the kinetics of the [Ca$^{2+}$] transients were a consequence of submaximal or slow photoliberation of cADPr, we examined the dependence of the [Ca$^{2+}$] transient amplitude and kinetics on the amount of caged-cADPr loaded into eggs. Fig. 2 shows the peak amplitude and several kinetic descriptors of the [Ca$^{2+}$] transients induced by cADPr and IP$_3$, each plotted as a function of the estimated caged compound concentration. In eggs loaded with less than 5 µM of caged cADPr or less than 3 µM of caged IP$_3$, the [Ca$^{2+}$] transient kinetics and amplitude
progressively increasing rate of Ca^{2+} release could reflect, at least in part, the progressive enhancement of cADPr activity as the [Ca^{2+}] rises. Additionally, the slow onset of Ca^{2+} release might also reflect the intrinsic rate of the steps by which cADPr acts to open release channels, independent of Ca^{2+} facilitation. To determine the extent to which Ca^{2+} influenced the kinetics of cADPr-induced [Ca^{2+}] transients, we manipulated the [Ca^{2+}] in an attempt to alter Ca^{2+} feedback signals in situ. We first employed caged calcium to elevate the [Ca^{2+}] coincident with cADPr liberation. NP-EGTA and Ca^{2+} were added to the injectate at a Ca:NP-EGTA molar ratio of 0.6 and co.injected into eggs along with caged Ca^{2+}. Total NP-EGTA loading was estimated to be approximately 1 mM. Because initial experiments employing fluo-3 showed near-saturation of this dye after NP-EGTA photolysis, we switched to the lower affinity dye CG-5N, which was loaded into eggs to a concentration of ~20 µM. Under these conditions, a single flash produced a step [Ca^{2+}] jump followed by a kinetically distinct secondary rise that occurred without appreciable delay or acceleration (Fig. 3A). This secondary rise in [Ca^{2+}] was not observed in the absence of caged cADPr (Fig. 3B), indicating that it was not a result of direct Ca^{2+}-induced Ca^{2+} release. Thus, in the presence of a permissive [Ca^{2+}], cADPr can rapidly activate Ca^{2+} release without delay. Therefore, it is unlikely that delayed Ca^{2+} release under more physiological conditions is due to an intrinsically slow rate at which cADPr binds to and gates release channels.

Due to uncertainty regarding the preflash resting [Ca^{2+}] in the above experiments (see “Experimental Procedures”), we cannot rule out the possibility that a permissive [Ca^{2+}] existed prior to the flash. Thus, there remained the question of how rapidly Ca^{2+} could act to facilitate cADPr-activated release. To address this, additional eggs were injected with a similar solution having a lower Ca:NP-EGTA molar ratio of 0.4. We presume that under these conditions the preflash resting [Ca^{2+}] would be lower, as would the magnitude of the step change in [Ca^{2+}] resulting from a single flash. As expected, a single flash produced a smaller [Ca^{2+}] jump and only a slow cADPr-dependent secondary [Ca^{2+}] rise (Fig. 3C). However, a second flash ~2.5 s later jumped the [Ca^{2+}] further, and the Ca^{2+} release rate immediately increased. The cADPr produced by the first flash was unlikely to be significantly degraded during this 2.5-s interval, particularly because, as will be described below, the onset of rapid calcium release could occur more than 10 s after a single flash in the presence of excess calcium buffers. Thus, the rapid increase in the Ca^{2+} release rate after the second flash likely can be ascribed to the elevated [Ca^{2+}], not to a change in the cADPr concentration. We conclude that Ca^{2+} can also rapidly activate release if cADPr is present at a permissive concentration.

If the acceleration of Ca^{2+} release was Ca^{2+}-dependent, one would predict that the onset of rapid Ca^{2+} release could be prevented or postponed by attenuating the [Ca^{2+}] rise with exogenous Ca^{2+} buffers. In fact, this outcome was suggested by the slow, nonaccelerating Ca^{2+} release observed following the first flash in the set of experiments illustrated by Fig. 3C on eggs loaded with ~1 mM of the Ca^{2+} chelator NP-EGTA. No

---

**TABLE I**

<table>
<thead>
<tr>
<th>Peak F/F₀</th>
<th>Delay (ms)</th>
<th>Maximum dF/dt</th>
<th>Time to half peak (s)</th>
<th>Time to peak (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caged cADPr (n = 14)</td>
<td>4.3 ± 0.2</td>
<td>446 ± 20</td>
<td>2.7 ± 0.3</td>
<td>1.17 ± 0.11</td>
</tr>
<tr>
<td>Caged IP₃ (n = 11)</td>
<td>5.7 ± 0.4*</td>
<td>35 ± 3*</td>
<td>27.0 ± 2.4*</td>
<td>0.13 ± 0.01*</td>
</tr>
</tbody>
</table>

* Significantly different (p < 0.005) from caged cADPr.
conditions did not significantly alter the onset of IP3-induced Ca2+ release but does not significantly alter the kinetics of IP3-induced Ca2+ release. Eggs were loaded with approximately 120 μM CG-5N and >10 μM of either caged cADPr (A) or caged IP3 (B). In A, the maximum dF/dt was approximately 60-fold greater than its value over the initial 9 s following the flash. Arrows (UV) indicate the occurrence of the flashlamp burst. Responses are representative of six experiments each.

FIG. 4. Increasing the intracellular Ca2+ buffering capacity in eggs prolongs the delay before rapid cADPr-induced Ca2+ release but does not significantly alter the kinetics of IP3-induced Ca2+ release. Eggs were loaded with approximately 120 μM CG-5N and >10 μM of either caged cADPr (A) or caged IP3 (B). In A, the maximum dF/dt was approximately 60-fold greater than its value over the initial 9 s following the flash. Arrows (UV) indicate the occurrence of the flashlamp burst. Responses are representative of six experiments each.

The rapid appearance of a maximally effective concentration of cADPr.

In eggs loaded only with caged cADPr and fluo-3, the postflash rate of Ca2+ release was very low during the first 100–200 ms but then accelerated more than 100-fold over the next 1–2 s. Given that Ca2+ is known to facilitate cADPr-induced release (11, 20), it is reasonable to expect that positive Ca2+ feedback will contribute to this increase in release rate. The relatively slow initial rate of Ca2+ release could also be a consequence of the intrinsic rate of the cADPr and Ca2+ binding steps and/or of the gating process itself. However, we found that a step change in either the Ca2+ or cADPr concentration could lead to rapid and immediate activation of Ca2+ release when the other was present at a permissive concentration, indicating that the steps linking these messengers to release channel opening are not intrinsically slow. Thus, [Ca2+] appears to be the sole factor limiting Ca2+ release during the early postflash period, with acceleration of release reflecting positive feedback enhancement of cADPr activity by released Ca2+. This conclusion is reinforced by the finding that the onset of rapid Ca2+ release could be prevented or significantly delayed by the presence of excess exogenous Ca2+ buffers. Presumably calcium buffers are effective because they slow the [Ca2+] rise resulting from the initial release and perhaps limit the spatial range over which that Ca2+ can signal neighboring channels.

Based on the observed initial rate of Ca2+ release, we estimate that the ability of cADPr to gate release channels in the presence of normal resting [Ca2+] to be less than 1% of its maximum at higher [Ca2+]. The precise concentration range over which Ca2+ modulates cADPr activity remains uncertain, due largely to uncertainty about the affinity of the Ca2+ dyes and chelators we employed in intact eggs (see “Experimental Procedures”). Furthermore, a Ca2+ feedback site might be capable of sensing the locally higher [Ca2+] near open release channels and/or (in experiments employing NP-EGTA) the submillisecond transient [Ca2+] overshoots that immediately follow photolysis of caged calcium (32). Hence, even accurate calibration of the whole egg fluorescence signal might not allow the relevant [Ca2+] values to be determined. A more detailed description of the Ca2+ dependence of cADPr action will require a more appropriate model preparation.

Calmodulin, a required cofactor for cADPr-induced Ca2+ release in sea urchin eggs (18–20), is a possible candidate for mediating Ca2+-enhancement of cADPr activity. The very low activity of cADPr at normal resting [Ca2+], which suggests the involvement of a cooperative Ca2+ binding site, is a common feature of other processes regulated by this protein (33). How-
ever, there is evidence that at least one other divergent cation binding site is capable of enhancing cADPr activity in the absence of calmodulin (20). Thus, further investigation will be needed to establish the identity of the Ca$_2^{+}$ feedback site(s).

Recently, Chini and Doussa (34) compared IP$_2$- and cADPr-induced Ca$_2^{+}$ release from sea urchin egg extracts. They observed that both second messengers had a bell shaped [Ca$^{2+}$]$_i$ dependence, with IP$_2$ having greater relative activity at lower [Ca$^{2+}$]. Although they examined only the steady state Ca$^{2+}$-dependence and used a rather low resolution [Ca$^{2+}$]$_i$ range (full log unit intervals), their data strongly suggest the existence of both sensitization and desensitization Ca$^{2+}$-mediated Ca$^{2+}$ release. The actual Ca$^{2+}$ dependence of cADPr activity reported by this group (no activity in the absence of Ca$^{2+}$, maximum at 1 $\mu$M [Ca$^{2+}$], and about 60% of maximum activity at 100 nM [Ca$^{2+}$]) in egg extracts appears inconsistent with our estimate that cADPr activity in the intact egg was less than 1% of maximum at normal resting [Ca$^{2+}$]$_i$ (measured by others to be in the 150–200 nM range (22, 23)). Part of this discrepancy might reflect the experimental conditions Chini and Doussa employed in their study. Because cADPr action was determined in the steady state presence of [Ca$^{2+}$], the Ca$^{2+}$-dependent desensitization process likely would have attenuated the maximum release rate observed. In addition, the [Ca$^{2+}$]$_i$ range was examined only at full log unit intervals and so may have missed the optimal [Ca$^{2+}$]$_i$ having the greatest release rate. Underestimating the maximum Ca$^{2+}$ release rate will tend to inflate estimates of the relative release rate at lower [Ca$^{2+}$]. Nonetheless, full reconciliation of these independent estimates of cADPr activity at lower [Ca$^{2+}$]$_i$ will require further investigation.

Recently, Genazzani et al. (35) examined the kinetics of cADPr-induced Ca$^{2+}$ release in sea urchin egg homogenates as a function of the cADPr concentration using a stop flow apparatus with a 25-ms time resolution. They reported an apparent acceleration of release in response to low, submaximal levels of cADPr, which they attributed to a Ca$^{2+}$-dependent facilitation. However, in contrast to our observations in intact eggs, they observed no significant acceleration or delay of Ca$^{2+}$ release in response to near maximal cADPr concentrations. The reason for this important difference in the behavior of Ca$^{2+}$ release in these two models is not clear. A possible factor is that the homogenate extract is diluted 40-fold relative to its normal intracellular density, perhaps altering the normal spatial relations that permit efficient communication between neighboring channels. These relations might be critical if the [Ca$^{2+}$]$_i$ range needed for maximal cADPr action corresponds to local [Ca$^{2+}$]$_i$ levels achieved only near open release channels. We note that the rate of Ca$^{2+}$ release from homogenates observed by these authors were quite slow (although typical of those reported by others in this model), and peak [Ca$^{2+}$]$_i$ was achieved more than a minute after addition of cADPr. Thus, it is possible that Ca$^{2+}$ release in this model occurs with little or no calcium facilitation. An understanding of the basis for these differences in the [Ca$^{2+}$]$_i$ transient kinetics of these two models could reveal important insights into the mechanism by which cADPr gates release.

The sea urchin egg [Ca$^{2+}$]$_i$ transients we observed following photolysis of caged IP$_2$ were immediate in onset and were resistant to the levels of exogenous Ca$^{2+}$ buffers that compromised cADPr-induced release. Based on these qualitative assessments, IP$_2$-mediated release appears less critically dependent on Ca$^{2+}$ feedback. As noted previously, Chini and Doussa (34) found IP$_2$ to be more effective than cADPr at releasing Ca$^{2+}$ in egg extracts at lower [Ca$^{2+}$]. However, other factors could also contribute to this apparent difference. Despite releasing similar amounts of Ca$^{2+}$, the maximum rate of IP$_2$-induced Ca$^{2+}$ release was about 10-fold higher than that induced by cADPr (Table I). A 10-fold greater release flux, whether due to more channels or a larger conductance, would accelerate Ca$^{2+}$ feedback and help resist attenuation by exogenous Ca$^{2+}$ buffers, even if the initial fractional activity was the same.

Functionally, a maximal effective concentration of cADPr appears merely to enable a Ca$^{2+}$-induced Ca$^{2+}$ release pathway, with subsequent Ca$^{2+}$ release critically dependent on a facilitating [Ca$^{2+}$] signal. Lee (1) has proposed that the cADPr-gated release pathway can operate along a continuum between two extreme modes: a "modulator" mode at submaximal cADPr levels, where Ca$^{2+}$ release is a function of the ambient [Ca$^{2+}$] (thus permitting cADPr to amplify Ca$^{2+}$ signals generated by other mechanisms), and a "mesenger" mode at high cADPr levels, where full Ca$^{2+}$ release occurs independent of the ambient [Ca$^{2+}$]. Our findings challenge a strict mechanistic description of this dual mode hypothesis. Full activation of release, even in the presence of maximal cADPr levels, required a facilitating [Ca$^{2+}$] rise, and conditions that altered this Ca$^{2+}$ signal changed the characteristics of the [Ca$^{2+}$] transients. Nonetheless, from a functional perspective, our results are in accord with the dual mode hypothesis in sea urchin eggs. The initially slow postflush Ca$^{2+}$ release was always sufficient to ignite a positive Ca$^{2+}$ feedback that ensured eventual full activation of release.

However, the inevitability of full Ca$^{2+}$ release to a supramaximal cADPr signal in other cell types is not a given. In cells that have more active basal Ca$^{2+}$ uptake processes, a lower resting [Ca$^{2+}$], or a lower density of cADPr-gated channels, the initial release rate might be insufficient to initiate a positive Ca$^{2+}$ feedback before the cADPr is degraded. Indeed, a cADPr-gated release pathway might play an essential role in shaping and amplifying Ca$^{2+}$ signals induced by other mechanisms, yet go undetected under experimental conditions that interfere with or fail to provide a triggering Ca$^{2+}$ signal.

Acknowledgments—We thank H. Bindu Vanapalli and Drs. Ronald Abercrombie and J. Wylie Nichols for technical assistance and Michael A. Laflamme for helpful discussions.

REFERENCES

Ca\textsuperscript{2+} Dependence of cADPr-gated Ca\textsuperscript{2+} Release

Cyclic ADP-ribose-gated Ca^{2+} Release in Sea Urchin Eggs Requires an Elevated [Ca^{2+}]

Xiaoqing Guo and Peter L. Becker

doi: 10.1074/jbc.272.27.16984

Access the most updated version of this article at http://www.jbc.org/content/272/27/16984

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 19 of which can be accessed free at http://www.jbc.org/content/272/27/16984.full.html#ref-list-1