Cyclic ADP-ribose (cADPR) is a putative second messenger that has been demonstrated to mobilize Ca^{2+} in many cell types. Its postulated role as the endogenous regulator of ryanodine-sensitive Ca^{2+} release channels has been greatly supported by the advent and use of specific cADPR receptor antagonists such as 8-NH_{2}-cADPR (Walseth, T. F., and Lee, H. C. (1993) Biochim. Biophys. Acta 1178, 235–242). However, investigations of the role of cADPR in physiological responses, such as fertilization, stimulus-secretion coupling, and excitation-contraction coupling, have been hindered by the susceptibility of cADPR receptor antagonists to hydrolysis and the need to introduce these molecules into cells by microinjection or patch clamp techniques. We have recently reported on the discovery of a poorly hydrolyzable analogue of cADPR, 7-deaza-cADPR (Bailey, V. C., Sethi, J. K., Fortt, S. M., Galione, A., and Potter, B. V. L. (1997) Chem. Biol. 4, 41–51) but this, like cADPR, is an agonist of ryanodine-sensitive Ca^{2+} release channels. We therefore explored the possibility of combining antagonistic activity with that of hydrolytic resistance and now report on the biological properties of the first hydrolysis-resistant cADPR receptor antagonist, 7-deaza-8-bromo-cADPR. In addition this compound has the advantage of being membrane-permeable. Together these properties make this hybrid molecule the most powerful tool to date for studying cADPR-mediated Ca^{2+} signaling in intact cells.

7-Deaza-8-bromo-cyclic ADP-ribose, the First Membrane-permeant, Hydrolysis-resistant Cyclic ADP-ribose Antagonist*

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Cyclic adenosine diphosphate ribose (cADPR) is a ubiquitous Ca^{2+}-mobilizing metabolite of β-NAD^{+} (1, 2). It is reported to mediate Ca^{2+} release via ryanodine-sensitive channels in many cell types in both animal and plant kingdoms (1, 3–6). Endogenous levels of cADPR have been detected and reported to be equally widespread (7). This finding has led to the postulation that cADPR may be the endogenous/physiological regulator of ryanodine receptors (5, 8–10).

As is the case for the more established intracellular messengers (i.e. IP_{3}, cAMP, and cGMP), cADPR-metabolizing enzymes are also present that can modulate cADPR levels (11). The synthetic activity of ADP-ribosyl cyclase and catalytic activity of cADPR hydrolase are often co-localized on the same polypeptide. In these cases, the hydrolase activity often exceeds that of cyclase (1). However, one notable exception is Aplysia ADP-ribosyl cyclase, which is isolated and purified from soluble ovotestis extracts of the sea hare Aplysia californica. The exceptionally high level of cyclase activity exhibited by this enzyme (1) has been well exploited to synthesize large quantities of cADPR. In addition, the finding that this cyclase exhibits loose substrate specificity has allowed the development of a chemoenzymatic synthesis of a number of cADPR analogues (12).

The first series of pharmacologically useful cADPR analogues to be synthesized was the 8-substituted analogues (13). These differ from cADPR by a substitution at the 8-position of the adenine ring. This single modification abolishes the agonistic activity of these compounds and produces instead specific competitive antagonists of cADPR-sensitive Ca^{2+} release (13). Since its discovery, 8-NH_{2}-cADPR has been used successfully to demonstrate the involvement of cADPR-mediated Ca^{2+} signaling in sea urchin eggs during fertilization (14) and NO- and cGMP-induced Ca^{2+} release (15) in Purkinje neurons (16), hippocampal synaptic plasticity (36), permeabilized Jurkat T cells (6), intestinal smooth muscle during cholecystokinin-induced contractions (5), PC12 cells (17), and excitation-contraction coupling in cardiac myocytes (18). However, like the parent compound, cADPR, the 8-substituted analogues are prone to hydrolysis by endogenous enzymes (13). Indeed this may explain the absence of an inhibitory effect on secretogogue-induced Ca^{2+} release in rat pancreatic beta cells (19) during induction of long-term depression in Purkinje neurons (16) where a role for cADPR-mediated Ca^{2+} signaling remains controversial.

These observations underscore the need for a stable, hydrolysis-resistant cADPR antagonist. Recently, we reported on the synthesis of another analogue of cADPR, 7-deaza-cADPR, and demonstrated that it is more stable during heat-induced hydrolysis and is also a poor substrate for cADPR hydrolase (20). These changes in stability were also brought about by a single modification, a replacement of the 7-position nitrogen with carbon (Fig. 1A). These findings then raised an intriguing question: what would be the biological activity of a compound

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The abbreviations used are: cADPR, cyclic adenosine 5′-diphosphate ribose; IP_{3}, myo-inositol-(1,4,5)-trisphosphate; HPLC, high pressure liquid chromatography.
modified at both the 7- and 8-positions of the adenosine ring? A "hybrid" analogue was successfully synthesized, namely 7-deaza-8-bromo-cADPR (Fig. 1A). Its biological properties were examined and are reported herein. Our findings show that 7-deaza-8-bromo-cADPR retains useful pharmacological properties; i.e. it is a hydrolysis-resistant antagonist of cADPR-induced Ca\(^{2+}\) release. Furthermore, owing to the lipophilic nature of the bromo and CH moieties, we have explored its potential as a membrane-permeable analogue of cADPR, as has been established for 8-bromo-cGMP, cf. cGMP (21). A single molecular species exhibiting all three properties would be a very powerful pharmacological tool for investigations of cADPR-mediated Ca\(^{2+}\) signaling in intact cells. We report here that 7-deaza-8-bromo-cADPR could be such a tool.


eXPERIMENTAL PROCEDURES

Chemoenzymatic Synthesis of 7-Deaza-8-bromo-cADPR—The detailed synthesis and chemical analysis of 7-deaza-8-bromo-cADPR is discussed elsewhere (22). Briefly, 7-deazaadenosine (tubercidin) was brominated (23) and selectively phosphorylated with phosphorus oxychloride to yield 7-deaza-8-bromo-AMP using a general method (24). This was then coupled to nicotinamide mononucleotide to form 7-deaza-8-bromo-NAD using a general method (24, 25). The enzymatic cyclization of 7-deaza-8-bromo-NAD was catalyzed by crude Aplysia ADP-ribosyl cyclase, and the product was purified by ion-exchange chromatography (12, 20). The extinction coefficient for 7-deaza-8-bromo-cADPR was determined by total phosphate analysis to be \(\epsilon_{\text{max}} = 277 \text{ nm, } 10.85 \times 10^{3} \text{ M}^{-1} \text{ cm}^{-1}\), and this value was used to determine the concentrations of 7-deaza-8-bromo-cADPR used in the rest of the study.

In Vitro Ca\(^{2+}\) Release Assays—In vitro Ca\(^{2+}\) release assays were performed on sea urchin egg homogenates (2.5%, v/v) prepared from unfertilized Lytechinus pictus eggs according to the method of Clapper et al. (25) with modifications as described previously (26). Extramicrosomal Ca\(^{2+}\) was thus measured by monitoring changes in fluo-3 (3 \(\mu\)M) fluorescence (excitation 490 nm and emission 530 nm) in a Perkin-Elmer LS-50B fluorimeter. All additions (not exceeding 5 \(\mu\)M) were made to cuvettes (containing 500 \(\mu\)l of homogenate) in intracellular medium containing potassium gluconate, 250 mM; N-methylglucamine, 250 mM; Heps, 20 mM (pH 7.2); MgCl\(_{2}\), 1 mM; ATP, 0.5 mM; phosphocreatine, 10 mM; creatine phosphokinase, 10 units/ml; oligomycin, 1 \(\mu\)g/ml; antimycin, 1 \(\mu\)g/ml; sodium azide, 1 mM; EGTA, 10 \(\mu\)M.

Intracellular Free Ca\(^{2+}\) Measurements in Intact Eggs—Ca\(^{2+}\) imaging of intact cells was performed using unfertilized L. pictus eggs microinjected with 2 \(\mu\)M fura-2 and 250 \(\mu\)g/ml heparin as described previously (26, 27).

Materials—L. pictus sea urchins were from Marinus Inc. (Long Beach, CA). Fluo-3 and fura-2 were purchased from Molecular Probes, Inc. All other chemicals were from Sigma (London). Cyclic ADP-ribose, 8-bromo-cADPR, and 7-deaza-8-bromo-cADPR were synthesized as described previously (12, 20).
FIG. 2. Concentration dependent inhibition of cADPR-induced Ca\(^{2+}\) release by 8-bromo-cADPR and 7-deaza-8-bromo-cADPR. *L. pictus* egg homogenates (2.5%, v/v) containing the Ca\(^{2+}\)-sensitive dye, fluo-3 (3 \(\mu\)M), were prepared as described under "Experimental Procedures." Their sensitivity to cADPR-induced Ca\(^{2+}\) release was used to test the antagonistic action of 8-bromo-cADPR (A) as compared with that of 7-deaza-8-bromo-cADPR (B). This was done by pretreating homogenates with increasing concentrations of antagonist (in 5 \(\mu\)l of intracellular medium + EGTA) 3 min prior to challenge with 100 nM cADPR. Representative traces are shown in A and B where the addition artifact has been removed for clarity (gap in traces). The amount of Ca\(^{2+}\) released by cADPR application was determined and expressed as a percentage of control cADPR release (Fig. 2B). The effect of each modification on the antagonistic actions of both 7-deaza-8-bromo-cADPR (S.E., \(n = 3\)).

A single modification made at either the 7- or 8-position of the adenosine ring, 7-deaza-8-bromo-cADPR possesses both of these modifications. The effect of each modification on the Ca\(^{2+}\)-mobilizing (agonistic) ability was first tested using sea urchin egg homogenates (2.5%). Fig. 1B shows the Ca\(^{2+}\)-releasing action of 2 \(\mu\)M applications of 7-deaza-8-bromo-cADPR and related cyclic nucleotides. Unlike the agonists cADPR and 7-deaza-cADPR, 7-deaza-8-bromo-cADPR did not induce Ca\(^{2+}\) release from sea urchin egg microsomes even at concentrations up to 20 \(\mu\)M (a supra-maximal agonist concentration). In this respect 7-deaza-8-bromo-cADPR resembles the antagonist 8-bromo-cADPR.

Whether 7-deaza-8-bromo-cADPR, like 8-bromo-cADPR, was also an antagonist of cADPR-sensitive Ca\(^{2+}\) release was investigated next. Fig. 2 shows that this was indeed the case. Sea urchin egg homogenates pretreated with either 8-bromo-cADPR or 7-deaza-8-bromo-cADPR were markedly less responsive to 100 nM cADPR (Fig. 2A). These inhibitory actions were dependent on the antagonist concentration (Fig. 2B). Both 8-bromo-cADPR and 7-deaza-8-bromo-cADPR exhibited similar inhibition potencies with comparable IC\(_{50}\) values (IC\(_{50}\) = 0.97 ± 0.04 \(\mu\)M (S.E., \(n = 3\)) for 8-bromo-cADPR, IC\(_{50}\) = 0.73 ± 0.05 \(\mu\)M (S.E., \(n = 3\)) for 7-deaza-8-bromo-cADPR).

Lower concentrations (31 nM) of either antagonist were also significant in preventing Ca\(^{2+}\) release by 100 nM cADPR that did not exceed 85% of control values. Whether this is due to the presence of more than one population of receptors that exhibit different binding affinities and/or sensitizing properties remains to be investigated. Nonetheless, both 8-substituted analogues appeared to behave similarly with respect to these actions on the cADPR-induced Ca\(^{2+}\) release channel in sea urchin egg homogenates. Since previous studies have shown that 8-substituted cADPR analogues (13, 30) and 7-deaza-cADPR (20) are able to displace cADPR binding, it is likely that 7-deaza-8-bromo-cADPR also interacts at the cADPR receptor in the same specific manner.

Since a modification on the 8-position does not alter the stability of the molecule (13) but a substitution of N7 with a carbon has been shown to render the cyclic compound more resistant to hydrolysis (20), we investigated whether 7-deaza-8-bromo-cADPR could differ from 8-bromo-cADPR but resemble 7-deaza-cADPR in this respect. We subjected standard solutions of both antagonists to heat-induced hydrolysis. The treatment has previously been shown to strip unstable cyclic compounds such as cADPR and 8-NH\(_{2}\)-cADPR of their biological activity (13, 18, 20). Fig. 3 shows the effect of heat treatment on the antagonistic actions of both 7-deaza-8-bromo-cADPR and 8-bromo-cADPR. Whereas 8-bromo-cADPR is stripped of its antagonistic activity, 7-deaza-8-bromo-cADPR remains an effective antagonist of cADPR-induced Ca\(^{2+}\) release (Fig. 3, B compared with A). The antagonist activity of both 7-deaza-8-bromo-cADPR and 8-bromo-cADPR was markedly less responsive to 100 nM cADPR (Fig. 2A). These inhibitory actions were dependent on the antagonist concentration (Fig. 2B). Both 8-bromo-cADPR and 7-deaza-8-bromo-cADPR exhibited similar inhibition potencies with comparable IC\(_{50}\) values (IC\(_{50}\) = 0.97 ± 0.04 \(\mu\)M (S.E., \(n = 3\)) for 8-bromo-cADPR, IC\(_{50}\) = 0.73 ± 0.05 \(\mu\)M (S.E., \(n = 3\)) for 7-deaza-8-bromo-cADPR).

Since a modification on the 8-position does not alter the stability of the molecule (13) but a substitution of N7 with a carbon has been shown to render the cyclic compound more resistant to hydrolysis (20), we investigated whether 7-deaza-8-bromo-cADPR could differ from 8-bromo-cADPR but resemble 7-deaza-cADPR in this respect. We subjected standard solutions of both antagonists to heat-induced hydrolysis. The treatment has previously been shown to strip unstable cyclic compounds such as cADPR and 8-NH\(_{2}\)-cADPR of their biological activity (13, 18, 20). Fig. 3 shows the effect of heat treatment on the antagonistic actions of both 7-deaza-8-bromo-cADPR and 8-bromo-cADPR. Whereas 8-bromo-cADPR is stripped of its antagonistic activity, 7-deaza-8-bromo-cADPR remains an effective antagonist of cADPR-induced Ca\(^{2+}\) release (Fig. 3, B compared with A). HPLC analysis of the same samples confirmed that this loss of activity was due to degra-
membrane-permeant hydrolysis-resistant cADPR antagonist

FIG. 4. Antagonistic actions of extracellularly applied 7-deaza-8-bromo-cADPR and 8-bromo-cADPR on fertilization-induced Ca\(^{2+}\) transients in intact sea urchin eggs. A, control response showing a typical fertilization-induced Ca\(^{2+}\) transient in intact L. pictus eggs preinjected with heparin and fura-2 (final concentrations in an egg were approximately 250 µg/ml and 2 µM, respectively). Numbers refer to the points annotated in panel C. Despite the presence of heparin, an IP\(_3\) receptor antagonist, the wave properties of the sperm-induced Ca\(^{2+}\) rise were intact, and activation envelopes were formed (14, 33). The peak Ca\(^{2+}\) rise following the addition of sperm was 1705 ± 119 nM Ca\(^{2+}\) (S.E., n = 11). B, in the presence of 50 µM 7-deaza-8-bromo-cADPR in the bathing medium (5 min prior to sperm addition; similar results were observed whether preincubations lasted 5, 10, or 15 min), the amplitude of the Ca\(^{2+}\) transient was significantly reduced, and the propagation of the Ca\(^{2+}\) rise across the egg was significantly slowed compared with control. Numbers refer to the points annotated in panel C. Neither antagonist mobilized Ca\(^{2+}\) in the eggs during the 5–15-min preincubation period. C, accompanying data from panels A and B show the average rise in Ca\(^{2+}\) following sperm addition at t = 0. Note the slower increase in Ca\(^{2+}\) following sperm addition in eggs pretreated with 50 µM 7-deaza-8-bromo-cADPR and the reduced amplitude. Open squares represent control data, and the filled squares indicate the points represented by the images in panel A. Closed circles represent the response of an egg pretreated with 50 µM 7-deaza-8-bromo-cADPR, and open circles indicate the points represented by the images in panel B. Note also the presence of a small initial Ca\(^{2+}\) rise after sperm addition and prior to the full-blown Ca\(^{2+}\) rise associated with fertilization a feature often observed in eggs pretreated with 7-deaza-8-bromo-cADPR. At higher concentrations of 7-deaza-8-bromo-cADPR the fertilization-induced Ca\(^{2+}\) transient was completely abolished as shown by the crossed symbols.

When these antagonists were tested for resistance to enzyme-mediated hydrolysis (by cADPR hydrolases) a similar resistance emerged. Egg homogenates (2.5% containing fluo-3), were incubated overnight (at 17 °C) with 20 µM 8-bromo-cADPR or 7-deaza-8-bromo-cADPR. Samples (50 µl) were then taken and tested for antagonistic activity on cADPR-induced Ca\(^{2+}\) release (as described for Fig. 2) but where the cuvette concentration of the antagonists was initially 2 µM. Whereas the antagonistic activity of 8-bromo-cADPR in response to cADPR (100 nM) had dramatically reduced following the prolonged incubation with L. pictus cADPR hydrolase (65.8 ± 8.3% of control cADPR-induced Ca\(^{2+}\) release (S.E., n = 3)), the levels of 7-deaza-8-bromo-cADPR remained high thereby producing a greater antagonistic effect on cADPR-induced Ca\(^{2+}\) release (28.5 ± 11.4% of control cADPR-induced Ca\(^{2+}\) release (S.E., n = 3)). This was also confirmed by HPLC analysis of the same samples (data not shown).

It has been demonstrated that the presence of a lipophilic bromide moiety in cGMP affords greater membrane permeability to 8-bromo-cGMP (21), and the replacement of a nitrogen with a CH– group also offers greater hydrophobicity (32). Therefore, the novel cADPR analogue, 7-deaza-8-bromo-cADPR, should have greater hydrophobic character than any that were previously synthesized. We investigated this by testing the effect of extracellular applications of 7-deaza-8-bromo-cADPR on fertilization-induced Ca\(^{2+}\) mobilization in intact sea urchin eggs. Eggs were co-injected with the IP\(_3\) receptor antagonist, heparin (250 µg/ml), and Ca\(^{2+}\)-sensitive fluorochrome fura-2 (2 µM). Fig. 4A shows that upon sperm addition to control heparinized eggs, a propagating Ca\(^{2+}\) wave was produced (Fig. 4A, open squares). This Ca\(^{2+}\) wave had an average amplitude of 1705 ± 119 nM Ca\(^{2+}\) (S.E., n = 11) and took 41.9 ± 5.8 s (S.E., n = 11) to reach this peak. These data are consistent with previously reported observations of sperm-induced Ca\(^{2+}\) mobilization from IP\(_3\)-insensitive Ca\(^{2+}\) stores (14, 33), which suggests inhibition of the redundant cADPR-sensitive Ca\(^{2+}\) release mechanism (14, 33). At a concentration of 50 µM in the
heparinized controls ($p < 0.01$, Student’s $t$ test) as seen in Fig. 4C, open squares versus closed circles (mean value, 988 ± 81 nM Ca$^{2+}$ (S.E., $n = 6$)). As can also be observed in Fig. 4B, the propagation of the Ca$^{2+}$ wave across the egg was slowed significantly in the eggs treated with the 7-deaza-8-bromo-cADPR. The time to peak of the Ca$^{2+}$ rise at fertilization was also reduced in eggs pretreated with the antagonist compared with the heparinized controls ($p < 0.01$, Student’s $t$ test; mean value, 98.8 ± 12.1 s; S.E., $n = 6$). This is also apparent in Fig. 4C. At a higher concentration of 100 μM 7-deaza-8-bromo-cADPR in the bathing medium the sperm-induced intracellular Ca$^{2+}$ transients were completely abolished (Fig. 4C, crosses). The dose dependence of the effects of the 7-deaza-8-bromo-cADPR is shown in Fig. 5A, filled symbols, right-hand axis. Comparison with a preincubation with 8-bromo-cADPR showed that at 100 μM it also reduced the amplitude of the Ca$^{2+}$ transient following sperm addition and to a similar extent as observed with 50 μM 7-deaza-8-bromo-cADPR (see Fig. 5B cf. Fig. 5A, closed symbols). This result indicates that 7-deaza-8-bromo-cADPR appears to be the more effective antagonist. Neither antagonist released Ca$^{2+}$ in the eggs during a 5–15-min preincubation period (data not shown), which is consistent with the absence of agonistic activity observed in vitro (Fig. 1B).

Since intracellular Ca$^{2+}$ mobilization is a prerequisite for the cortical reaction (34, 35), we also monitored the formation of activation envelopes following fecundation and directly compared the actions of 7-deaza-8-bromo-cADPR and 8-bromo-cADPR in the bathing medium. Treatment of eggs with either antagonist prevented the cortical reaction (in heparinized eggs) in a concentration-dependent manner (Fig. 5, A and B, open symbols). This is in keeping with previous reports that demonstrate inhibition of the cortical reaction only when both redundant mechanisms have been blocked (14, 33). As indicated by the fertilization-induced Ca$^{2+}$ transients, we observed a greater effect of the 7-deaza-8-bromo-cADPR compared with the 8-bromo-cADPR at both 50 and 100 μM concentrations ($p < 0.05$, Student’s $t$ test in both cases). Eggs that were treated with antagonist only (i.e. not micro-injected with heparin) were also scored for activation envelopes in the same experiments. These consistently showed >95% activation as shown in Fig. 5, A and B, by the dotted lines and cross symbols. This suggested that neither 7-deaza-8-bromo-cADPR nor 8-bromo-cADPR acted as spermicides; rather they are able to permeate the sea urchin egg plasma membrane and specifically compete for endogenous cADPR-binding sites and inhibit agonist-induced Ca$^{2+}$ mobilization. Presumably, since the net charge on 7-deaza-8-bromo-cADPR is only 1 at physiological pH (Fig. 1A), this compares well with 8-bromo-cGMP, which has the same net charge and where 8-substitution confers membrane permeability. This property now eliminates the need for potentially disruptive protocols such as cell permeabilization or micro-injection methods to introduce cADPR antagonists into whole cells. This advancement should greatly aid investigations of the role of cADPR in physiological responses to extracellular stimuli.

In conclusion, we have demonstrated that, unlike 8-bromo-cADPR, 7-deaza-8-bromo-cADPR is a stable hydrolysis-resistant, cADPR antagonist. This is the first report of such a compound. In addition, we have exploited the lipophilic nature of the bromo moiety to produce a compound that is also sufficiently membrane-permeable. In all, this makes 7-deaza-8-bromo-cADPR a very powerful pharmacological tool for investigations of cADPR-mediated Ca$^{2+}$ signaling in intact cells.

**Fig. 5. Dose-dependent action of 7-deaza-8-bromo-cADPR and 8-bromo-cADPR on peak fertilization-induced Ca$^{2+}$ rise and egg activation.** For each concentration of both antagonists, 7-deaza-8-bromo-cADPR (A) and 8-bromo-cADPR (B), 4–9 eggs in a single dish were co-injected with heparin (250 μg/ml) and fura-2 (2 μM). Sperm were added following incubation in artificial sea water that contained either antagonist for 5 min, and the maximum change in intracellular free Ca$^{2+}$ (primary y axis, filled symbols) was monitored on 1 of the preinjected eggs. These data are taken from the experiments done in 1 day (1 egg at each concentration). Following recovery of the Ca$^{2+}$ transient we scored the presence or absence of an activation envelope in all injected eggs (secondary y axis, open symbols). Egg activation results represent the mean ± S.E. for 3–4 separate determinations of 4–12 eggs when concurrent Ca$^{2+}$ measurements were not always made. In separate fields within the same dish we scored the percentage of fertilization in un.injected, non-heparinized eggs (dotted lines). All were consistently activated, which indicated that sperm activity was unaffected by increasing concentrations of either antagonist. Note that the reduction of the fertilization-induced Ca$^{2+}$ transient with 100 μM 8-bromo-cADPR (panel B, filled triangles) was similar to that seen using 50 μM 7-deaza-8-bromo-cADPR (panel A, filled circle) supporting the greater effectiveness of the 7-deaza-8-bromo-cADPR compound. Further support for this comes from the far superior percent reduction in egg activation observed at 100 μM concentration of the 7-deaza-8-bromo-cADPR compared with the same concentration of 8-bromo-cADPR ($p = 0.02$, Student’s $t$ test).
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