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Cyclic ADP-ribose-induced Ca^{2+} release from rat brain microsomes

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Cyclic ADP-ribose (cADPR), an endogenous NAD^+ metabolite in many mammalian and invertebrate tissues, is a potent mediator of calcium mobilization in sea urchin eggs. Our results show that cADPR also stimulates calcium release from rat brain microsomes, marked release occurring over the concentration range 10–250 nM. This is not inhibited by concentrations of heparin which completely abolish inositol 1,4,5-trisphosphate (IP_3)-induced Ca^{2+} release. Ryanodine (100 μM) inhibits the cADPR response. Our results are consistent with cADPR being an endogenous messenger mediating Ca^{2+} release from ryanodine-sensitive pools in brain.

Cyclic ADP-ribose; Calcium; Rat brain microsome; Ryanodine receptor

1. INTRODUCTION

IP_3 is well established as a second messenger regulating Ca^{2+} release from intracellular stores [1]. Its receptor, which is itself a Ca^{2+} channel, has been purified to homogeneity from several tissues and has been cloned [2]. In addition to IP_3 receptors, a second class of intracellular Ca^{2+} channel has been identified the activity of which can be modulated by the plant alkaloid, ryanodine. Ryanodine receptors are the major release channels in muscle where they are important in excitation coupling [4]. They are also present in other tissues, notably brain [5–8]. They are often activated by caffeine but insensitive to IP_3. The physiological ligand for receptor activation is unknown, although they can be activated by Ca^{2+}, so-called Ca^{2+}-induced Ca^{2+} release (CICR) [4]. One candidate is an NAD^+ metabolite, cyclic ADP-ribose (cADPR), which has been shown to be as potent as IP_3 at releasing Ca^{2+} in sea-urchin eggs [9]. Recent work on homogenates prepared from sea-urchin eggs suggests that cADPR may be operating on a CICR mechanism mediated by a ryanodine receptor [10,11].

cADPR is a naturally occurring nucleotide, cytosolic concentrations in rat liver, brain and heart ranging between 20 and 100 nM [12]. The widespread occurrence of cADPR in mammalian tissues suggests that its efficacy as a Ca^{2+}-mobilizing agent might not be restricted to invertebrate eggs. Indeed, cADPR has been shown recently to release Ca^{2+} through an IP_3-insensitive mechanism in GH_4C1 cells and dorsal root ganglion cells [13,14]. The present study shows that cADPR induces Ca^{2+} release from rat brain microsomes and that this release is sensitive to inhibition by ryanodine. These results are consistent with a general role for cADPR as a physiological ligand for ryanodine receptors.

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2. MATERIALS AND METHODS

2.1. *Chemicals*

$^{45}CaCl_2$ was obtained from NEN Dupont. Fluo-3 was from Calbiochem. All other reagents were purchased from Sigma. Cyclic ADP-ribose was a generous gift from Dr. Hon Cheung Lee, University of Minnesota, USA.

2.2. *Microsome preparation*

Microsomes were prepared essentially as described in [15]. Whole brains from male Wistar rats (250–300 g) were homogenized gently (5 strokes of a glass homogeniser) in 9 vols. of ice-cold buffer consisting of *N*-methylglucamine (250 mM), potassium gluconate (250 mM), HEPES (20 mM), $MgCl_2$ (1 mM), soybean trypsin inhibitor (100 μg/ml), aprotinin (20 μg/ml), leupeptin (25 μg/ml) at pH 7.2. The homogenate was centrifuged (1,000 × *g*, 5 min) and the supernatant retained whilst the pellet was washed with 10 ml buffer. Supernatants were combined and centrifuged at 8,000 × *g* for 10 min. The supernatant was then centrifuged at 100,000 × *g* for 40 min. The microsomal pellet obtained was resuspended gently in an equal volume of homogenization buffer containing ATP (1 mM) and an ATP-regenerating system consisting of phosphocreatine (10 mM), creatine phosphokinase (10 U/ml), oligomycin (1 μg/ml), antimycin (1 μg/ml) and sodium azide (1 mM); it was diluted to a final protein concentration of 0.5 mg/ml for fluorimetry.

2.3. *Ca^{2+} release measurements*

Rat brain microsomes were always prepared on the day of the experiment. Loading of microsomes with Ca^{2+} took approximately 30 min at room temperature (Fig. 2a). Ca^{2+} uptake and release was followed by monitoring extra-microsomal Ca^{2+} using fluo-3 (1 μM). Fluorescence intensity of fluo-3 was measured at excitation and emission wavelengths of 490 nm and 535 nm, respectively. Fluorimetry was performed on 500 μl aliquots of microsomes using a Perkin-Elmer LS-3. Additions were made in volumes of 1–5 μl and all chemicals were added in incubation medium containing 10 μM EGTA. Ca^{2+} traces were calibrated by adding Ca^{2+} standard solution.

In addition to fluo-3 fluorimetry, Ca^{2+} release was measured directly by loading microsomes with $^{45}Ca^{2+}$. Microsomes were prepared as

described above but in the presence of 1 μCi/ml $^{45}CaCl_2$. Microsomal $^{45}Ca^{2+}$ was determined by removing 50 μl aliquots at appropriate time intervals and filtering rapidly through Whatman GF/B filters. Filters were washed with 2×2.5 ml ice-cold wash buffer consisting of *N*-methylglucamine (250 mM), potassium gluconate (250 mM) and 20 mM HEPES at pH 7.2. Radioactivity was determined by liquid scintillation counting.

3. RESULTS AND DISCUSSION

cADPR induced Ca^{2+} release from brain microsomes in a concentration-dependent manner with a threshold concentration of less than 10 nM (Fig. 1a). A maximally effective concentration of 250 nM cADPR elicited 2–4 nmol Ca^{2+} release and in several experiments at this concentration, Ca^{2+} was only slowly (>15 min) re-sequestered into the microsomes. Heating cADPR for 40 min at 95°C completely destroyed its Ca^{2+}-releasing activity (Fig. 1b). Heat treatment for 10 min caused only partial inactivation. The non-cyclic analogue, adenosine 5′-diphosphoribose (250 nM), did not cause Ca^{2+} release and did not inhibit cADPR-induced release of Ca^{2+} (Fig. 1c). β-NAD, the precursor of cADPR, exhibited no Ca^{2+}-releasing activity at concentrations as high as 50 μM (Fig. 1d). Fig. 1e shows that cADPR-induced Ca^{2+} release is inhibited by pretreatment with cADPR. This apparent desensitization is restricted to cADPR,

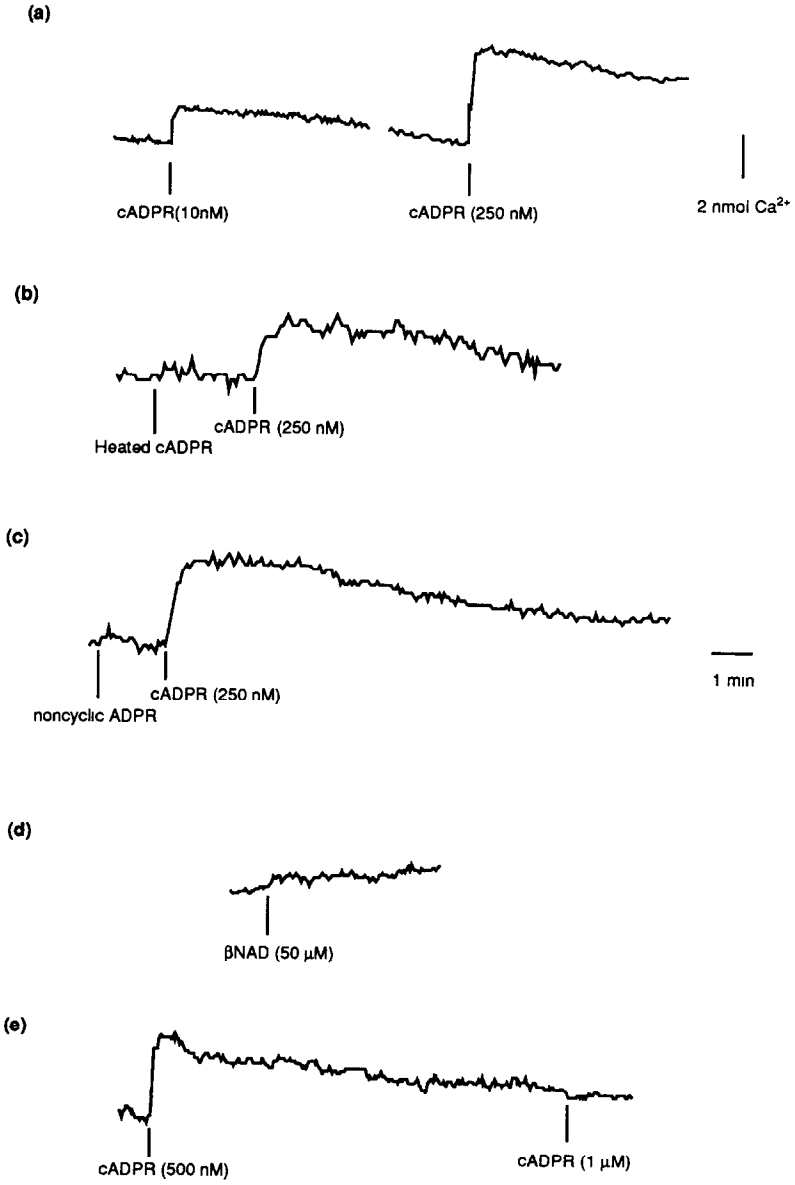

Fig. 1. cADPR-induced Ca^{2+} release from rat whole brain microsomes measured by fluo-3 fluorescence. Ca^{2+}-loaded microsomes were prepared as described in Materials and Methods and used at a final protein concentration of 0.5 mg/ml for fluorimetry. Representative traces of 2–3 experiments are shown. (a) cADPR elicits a rapid Ca^{2+} release. (b) 250 nM heat-treated cADPR (95°C, 40 min) does not release Ca^{2+} (c) The non-cyclic analogue, adenosine 5′-diphosphoribose, causes no increase in fluorescence. (d) 5 μM β-NAD causes no Ca^{2+} release. (e) Brain microsomes can be desensitized to cADPR-induced Ca^{2+} release by repeated cADPR additions.

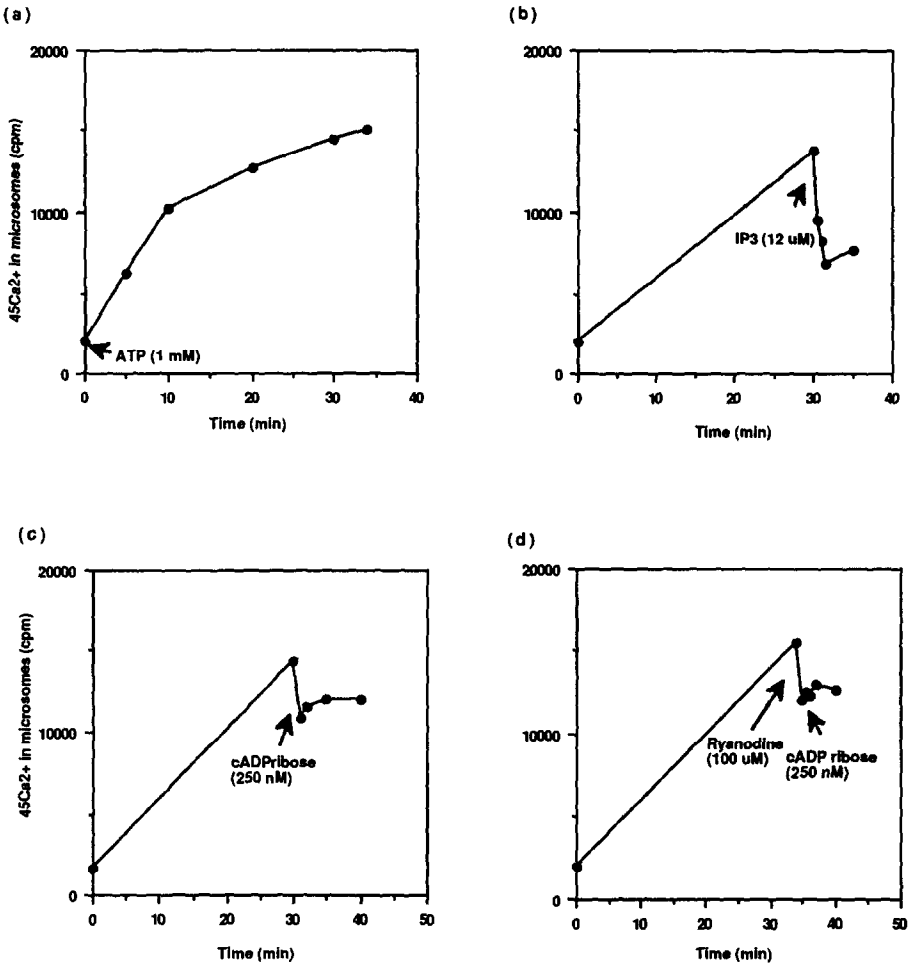

Fig. 2. $^{45}Ca^{2+}$ release from brain microsomes. (a) Microsomes (approx. protein concentration 0.5 mg/ml) were loaded with $^{45}Ca^{2+}$ (1 μCi/ml) for 30 min as described in Materials and Methods. (b) $^{45}Ca^{2+}$ release elicited by 12 μM IP_3. (c) $^{45}Ca^{2+}$ release elicited by 250 nM cADPR. (d) 100 μM ryanodine causes $^{45}Ca^{2+}$ release from microsomes. Subsequent addition of 250 nM cADPR has no effect.

the microsomes remaining responsive to IP_3. A similar desensitization has been observed in sea-urchin eggs [16]. These effects were corroborated by measuring $^{45}Ca^{2+}$ release from microsomes. Up to 60% of $^{45}Ca^{2+}$ could be released from microsomes by a maximal concentration of IP_3 (Fig. 2b). 250 nM cADPR caused an approximately 20% release of $^{45}Ca^{2+}$ release (Fig. 2c). 100 μM ryanodine caused a 20% release of sequestered $^{45}Ca^{2+}$ (Fig. 2d); cADPR elicited no further release.

To determine whether cADPR acted on the IP_3 receptor or at a site that was independent of IP_3, microsomes were pretreated with heparin, a competitive inhibitor of IP_3 binding. IP_3 (200 nM) elicited 2.5 nmol Ca^{2+} release (Fig. 3a). The presence of 600 μg/ml heparin completely blocked Ca^{2+} release by 200 nM IP_3, but had no effect on cADPR-induced Ca^{2+} release (Fig. 3b).

It has been suggested that cADPR may act through a Ca^{2+}-induced Ca^{2+} release (CICR) mechanism. Galione et al. [10] showed that treatment of sea-urchin egg homogenate with caffeine and ryanodine elicited a Ca^{2+} response which induced desensitization of the response to cADPR but not to IP_3. Figs. 2d and 3c show that pretreatment of rat brain microsomes with 100 μM ryanodine also inhibits release of Ca^{2+} by cADPR but not that induced by IP_3. This result is consistent with the demonstration that cADPR mimics caffeine but not IP_3 in inducing oscillations in a Ca^{2+}-dependent ion current known to reflect intracellular Ca^{2+} oscillations in rat dorsal root ganglion [14].

In conclusion, our results suggest that cADPR may be an important physiological modulator of IP_3-insensitive Ca^{2+} release in brain. This mechanism appears to involve ryanodine channels. The function of neuronal ryanodine receptors in contrast to their counterparts in muscle, is unclear. However, CICR through these receptors may be important in amplifying neuromodulatory Ca^{2+} signals in processes such as memory and learning [17].

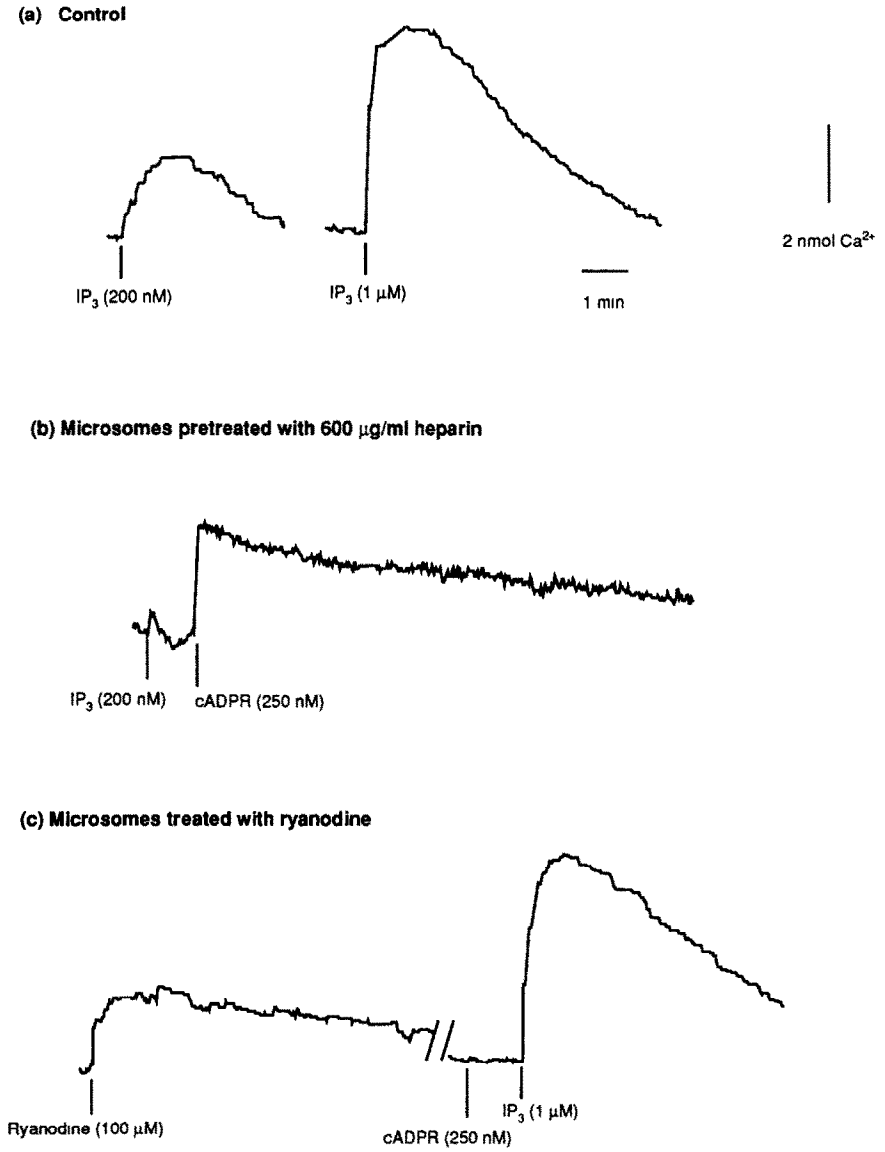

Fig. 3. Pretreatment with ryanodine inhibits Ca^{2+} release induced by cADPR but heparin has no effect. Ca^{2+} release from microsomes was performed as described in Fig. 1. (a) In the absence of heparin 200 nM IP_3 elicits 2.5 nmol Ca^{2+} release. (b) Pretreatment of microsomes with 600 μg/ml heparin completely blocks release of Ca^{2+} by IP_3 (200 nM) but does not affect cADPR-induced Ca^{2+} release. (c) 100 μM ryanodine causes rapid release of Ca^{2+} which is re-sequestered. Subsequent addition of cADPR (250 nM) causes no release, however, microsomes remain sensitive to IP_3.

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