RESEARCH COMMUNICATION

Nicotinate–adenine dinucleotide phosphate-induced Ca^{2+} release does not behave as a Ca^{2+}-induced Ca^{2+}-release system

Eduardo N. CHINI and Thomas P. DOUSA*

Renal Pathophysiology Laboratory, Departments of Physiology, Biophysics and Internal Medicine, Mayo Clinic and Foundation, Rochester, MN 55905, U.S.A.

We investigated the dependence of nicotinate–adenine dinucleotide phosphate (NAADP)-induced Ca^{2+} release from intracellular stores of sea urchin egg homogenates, upon extravesicular Ca^{2+}. In contrast to the Ca^{2+} release induced by inositol 1′,4′,5′-trisphosphate (IP_3) or cyclic ADP-ribose (cADPR), the Ca^{2+} release induced by NAADP was completely independent of the free extravesicular Ca^{2+} over a wide range of concentrations (0–0.1 mM). The Ca^{2+} release triggered by either cADPR or IP_3 was biphasically modulated by extravesicular Ca^{2+}, and the Ca^{2+} release by these agents was abolished when the extravesicular Ca^{2+} was removed by chelation with 2 mM EGTA. On the other hand, NAADP-triggered Ca^{2+} release was not influenced by EGTA. These data indicate that while both cADPR and IP_3 systems behave as functional Ca^{2+}-induced Ca^{2+} release mechanisms, NAADP activates a Ca^{2+} release mechanism which is independent of the presence of extravesicular Ca^{2+}. Therefore, the NAADP-sensitive Ca^{2+} release mechanisms may have a unique regulatory impact upon intracellular Ca^{2+} homoeostasis.

INTRODUCTION

In our recent studies we found that nicotinate–adenine dinucleotide phosphate (NAADP) serves as a potent stimulator of intracellular Ca^{2+} release in sea-urchin eggs [1–6]. NAADP-induced Ca^{2+} release was demonstrated in both homogenates and intact sea-urchin eggs [1–7]. NAADP, in nanomolar concentrations, triggers Ca^{2+} release via a mechanism which differs in many ways from those controlled by inositol(1′,4′,5′)trisphosphate (IP_3) and cyclic ADP-ribose (cADPR): (1) NAADP-induced Ca^{2+} release is not blocked by inhibitors of the IP_3- or cADPR-induced Ca^{2+} release systems [1–7]; and (2) Ca^{2+} release induced by NAADP is specifically inhibited by thionicotinamide-$NADP^+$ (thio-NADP) [1–7].

It is well established that agonist-triggered Ca^{2+} release through IP_3-sensitive receptor/Ca^{2+} channels and cADPR-sensitive ryanodine receptors (RyR) is dependent on the cytoplasmic Ca^{2+} concentration, and therefore both behave as regulated Ca^{2+}-induced Ca^{2+} release (CICR) systems [8–18]. The regulation of both the IP_3 channel/receptor and of the RyR channel by cytoplasmic Ca^{2+} is biphasic. At low concentration ranges ($< 1\ \mu M$) Ca^{2+} enhances the effect of agonists on Ca^{2+} release, whereas Ca^{2+} at concentrations higher than $1\ \mu M$ is inhibitory [15,17]. Thus, CICR is a common property of the two well-known intracellular mechanisms of Ca^{2+} release [8,15,17].

In view of these findings we explored whether or not the NAADP-induced Ca^{2+} release mechanism may also operate as a functional CICR. We found that, surprisingly, the NAADP-induced Ca^{2+} release mechanism was completely independent of the extravesicular Ca^{2+} concentration. Therefore, NAADP-induced Ca^{2+} release is unique in that it does not behave as a CICR. This fundamental functional property suggests that NAADP-trigged Ca^{2+} release occurs through a new type of channel, different from the IP_3 and cADPR/RyR channels.

MATERIALS AND METHODS

Homogenates from sea-urchin (*Lytechinus pictus*) eggs were prepared as described previously [1]. Frozen homogenates were thawed in a 17 °C-water bath and were diluted to 1.25% (v/v) with intracellular medium (IM) containing: 250 mM *N*-methyl-glucamine, 250 mM potassium gluconate, 20 mM Hepes buffer

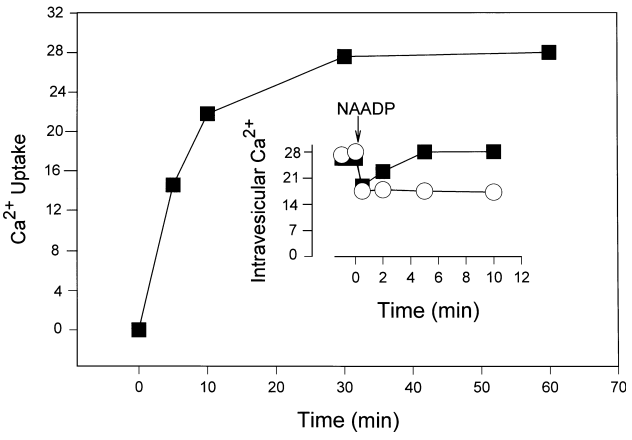

Figure 1 Ca^{2+} uptake by sea urchin egg homogenates

The time-course of Ca^{2+} uptake by 1.25% (v/v) sea urchin egg homogenates was determined using ^{45}Ca, as described in the Materials and methods section. Ca^{2+} uptake (■) was determined by filtration of 0.2 ml of egg homogenate. Inset: Ca^{2+} release induced by 1.0 μM NAADP in a homogenate preloaded with Ca^{2+} for 3 h. The Ca^{2+} release was initiated by addition of NAADP (■), or NAADP plus 2.2 μM thapsigargin (○). The data are representative of four experiments and the ^{45}Ca load of the vesicles varied from 28 to 22 nmol of Ca^{2+}/ml of 1.25% (v/v) sea urchin egg homogenate.

Abbreviations used: cADPR, cyclic ADP-ribose; NAADP, nicotinate–adenine dinucleotide phosphate; IP_3, inositol 1′,4′,5′-trisphosphate; RyR, ryanodine receptor/channel; CICR, Ca^{2+}-induced Ca^{2+} release; thio-$NADP^+$, thionicotinamide-NADP; Tg, thapsigargin; IM, intracellular medium.

* To whom correspondence should be addressed.

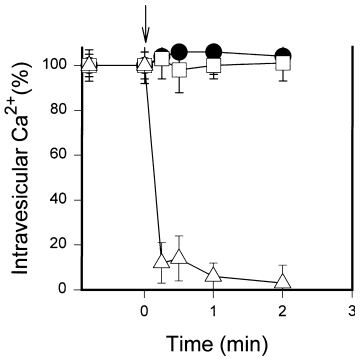

Figure 2 Effect of EGTA and thapsigargin on Ca^{2+} efflux by preloaded sea urchin egg homogenates

The remaining intravesicular Ca^{2+} was determined as described in the Materials and methods section. The 1.25% (v/v) sea urchin egg homogenate was preloaded with ^{45}Ca for 3 h and the effect of addition of 2 mM EGTA (●), 2 mM EGTA plus 2.2 μM Tg (□) or 10 μM A23187 (▽) upon intravesicular Ca^{2+} content was tested. The arrow indicates the time of addition of the compounds tested; the total intravesicular Ca^{2+} concentration was about 24 nmol. The data represent the means ± S.E.M. for three independent experiments.

(pH 7.2), 1 mM $MgCl_2$, 2 units/ml creatine kinase, 4 mM phosphocreatine, 1 mM ATP, 3 μg/ml oligomycin and 3 μg/ml antimycin, and Ca^{2+} uptake was determined by the addition of 50 μM $^{45}CaCl_2$ (105 c.p.m/nmol of Ca^{2+}). After 30 min incubation, about 48.5 ± 3% ($n = 10$) of the total quantum of added Ca^{2+} was taken up into the vesicles through a thapsigargin (Tg)-sensitive (Ca^{2+}/Mg^{2+})-ATPase; an observation compatible with the filling of the endoplasmic reticulum with ^{45}Ca (Figure 1). The calcium load of the vesicles varied from 28 to 22 nmol of Ca^{2+}/ml of 1.25% (v/v) sea urchin egg homogenate.

For the ^{45}Ca release experiments, the sea urchin egg homogenate was pre-loaded with $^{45}CaCl_2$, for 3 h, to achieve the steady-state level (Figure 1) as described above. The release of Ca^{2+} triggered by the different agonists investigated was determined in 1.25% (v/v) egg homogenates pretreated for 2 min with 2.2 μM Tg and with different ratios of added $CaCl_2$ and EGTA to provide a known concentration of free ionized Ca^{2+}. As shown in Figure 2, neither Tg nor 2 mM EGTA induced significant release of ^{45}Ca from preloaded vesicles by itself. In contrast, addition of the ionophore A23187 caused complete release of the intravesicular Ca^{2+} in a few seconds (Figure 2). Together, these observations indicate that the ^{45}Ca measured was taken up into the intravesicular space and was not only associated with the non-specific binding of ^{45}Ca on the membranes. In those experiments, Tg was included to block re-uptake of the released Ca^{2+} (Figure 1, inset), and to allow determination of the net rate of Ca^{2+} release caused by the Ca^{2+} release agents tested.

Ca^{2+} uptake and release were measured by an adaptation of the filtration method using ^{45}Ca and glass-fibre filters. The remaining intravesicular ^{45}Ca was determined by filtration of 0.2 ml of a 1.25% (v/v) egg homogenate through a prewashed GF/C glass filter (Whatman) under vacuum, followed by rapid washing three times with 1 ml of ice-cold IM containing 3 mM $LaCl_3$. The radioactivity retained on the filter was determined using standard scintillation counting. Similar results were obtained in preliminary experiments using Millipore filters. The free Ca^{2+} concentrations were calculated by using the apparent association constants of Ca^{2+} and EGTA using a computer program as described before [19].

NAADP and cADPR were synthesized as described previously [1,20]. The cADPR and NAADP used in all experiments were at least 97% pure, as determined by HPLC.

L. pictus and *Aplysia californica* were obtained from Marinus Inc., Long Beach, CA, U.S.A. IP_3, oligomycin, antimycin and Tg were from Calbiochem. All other reagents, of the highest purity grade available, were supplied by Sigma, St. Louis, MO, U.S.A.

The experiments were repeated at least 3–6 times, and when appropriate, the results were analysed statistically using Student's *t*-test.

RESULTS AND DISCUSSION

In the present study we compared the modulatory effect of different extravesicular free Ca^{2+} concentrations upon the Ca^{2+} release elicited by the three different Ca^{2+} release mechanisms present in the sea urchin egg homogenate (Figure 3). In the present study we determined how Ca^{2+} release induced by the three different agents (NAADP, IP_3 and cADPR) from ^{45}Ca-preloaded sea urchin egg homogenates is dependent upon various extravesicular Ca^{2+} concentrations achieved by different combinations of a Ca^{2+}/EGTA buffer. The details of the experimental protocol are described in the Materials and Methods section.

As shown in Figures 3(A) and 3(B), both IP_3- and cADPR-induced Ca^{2+} release were abolished when the free extravesicular Ca^{2+} was chelated by 2 mM EGTA. On the other hand, the Ca^{2+} release triggered by NAADP was not prevented by chelation of the Ca^{2+} (Figure 3C). In Figure 4 we demonstrate the effect of free extravesicular Ca^{2+} upon the Ca^{2+} release induced by NAADP, IP_3 and cADPR. Both cADPR- and IP_3-induced Ca^{2+} release systems are activated in the presence of low Ca^{2+} concentrations (< 1 μM) and inhibited in higher concentrations (> 1 μM). On the other hand, NAADP-induced Ca^{2+} release was totally independent of the presence of extravesicular free Ca^{2+} in the concentration range 0–0.1 mM (Figure 4, top), this was true for Ca^{2+} release induced by different concentrations of NAADP (Figure 4, bottom). Furthermore, the known pharmacological properties of NAADP-induced Ca^{2+} release were not modified by the extravesicular free Ca^{2+} concentrations. Further, we found that at all concentrations of free extravesicular Ca^{2+} tested, the Ca^{2+} release elicited by NAADP was inhibited by its specific antagonist thio-NADP and by the non-specific endocellular Ca^{2+}-

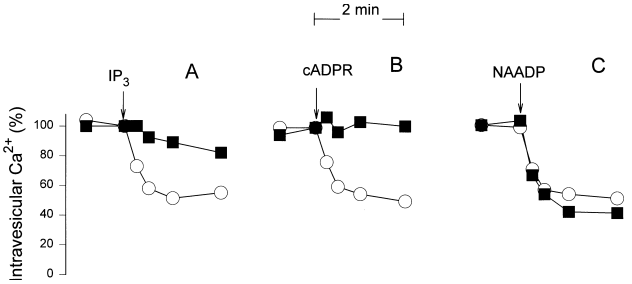

Figure 3 Effect of Ca^{2+} upon Ca^{2+} release induced by cADPR, IP_3 and NAADP

Sea urchin egg homogenates [1.25% (v/v)] were preloaded with ^{45}Ca for 3 h, as described in the Materials and methods section. The Ca^{2+} release was determined in the presence of 2 mM EGTA (■) or in the presence of 0.1 μM extravesicular (cytoplasmic) free Ca^{2+} (○). The Ca^{2+} release was initiated by addition of 1 μM of the different Ca^{2+}-releasing agents as shown in the Figure. The traces are representative of six experiments.

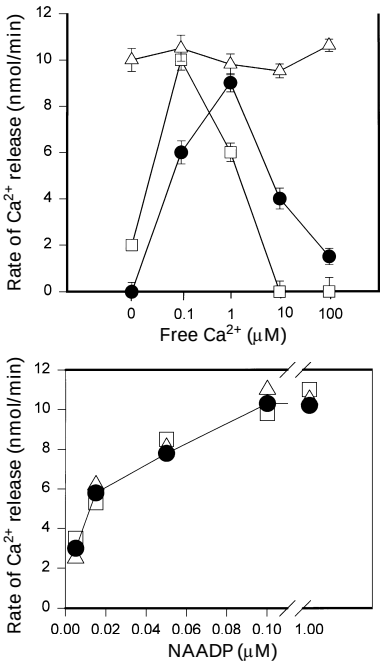

Figure 4 Effect of Ca^{2+} upon Ca^{2+} release

Top: the net rate of Ca^{2+} release induced by 1 μM IP_3 (□), 1 μM cADPR (●) and 1 μM NAADP (△) in the presence of different cytoplasmic free Ca^{2+} concentrations. Maximum Ca^{2+} release induced by Ca^{2+} itself was about ten times lower then the maximum release triggered in the presence of the compounds tested and was taken as the blank value for each free Ca^{2+} concentration studied. The data represent the means ± S.E.M.; $n = 4$. Bottom: the experiment was carried out as described in the Materials and methods section. The Ca^{2+} release induced by different NAADP concentrations was tested in the abscence of extravesicular Ca^{2+} (with 2 mM EGTA) (□), or in the presence of 0.1 μM extravesicular free Ca^{2+} (△), or with 100 μM extravesicular free Ca^{2+} (●). The data are representative of three independent experiments.

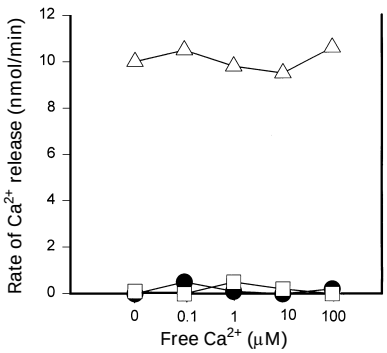

Figure 5 Inhibition of NAADP-induced Ca^{2+} release by thio-NADP and TMB-8

The Figure shows the net rate of Ca^{2+} release induced by 1 μM NAADP in control preloaded sea urchin egg homogenates (△), or in preloaded homogenates preincubated for 1 min with 50 μM thio-NADP (□), or 500 μM TMB-8 (●). The data are representative of four experiments.

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release antagonist TMB-8 (Figure 5). In contrast, neither heparin, an inhibitor of IP_3-induced Ca^{2+} release, nor Ruthenium Red, an inhibitor of the RyR, had any effect upon NAADP-induced Ca^{2+} (results not shown).

The IP_3 channel and RyR/channel display a CICR phenomenon that may have considerable regulatory significance [8,15–18]. In particular the effect of Ca^{2+} functioning as a feedback regulator of both IP_3 and RyR may have a very important physiological role [8,15,16], and CICR has been implicated as the basis for the generation of 'Ca^{2+} waves' in oocytes [15,16]. Here we demonstrate the existence of, to our knowledge, the first intracellular Ca^{2+} release system that appears to lack Ca^{2+} feedback and does not behave as a 'functional' CICR. Therefore the NAADP-triggered Ca^{2+} release system may contribute in a unique way to regulation of cytoplasmic Ca^{2+} homoeostasis. Further, the determination of this new Ca^{2+} release system led to the identification of a new type of intracellular Ca^{2+}-channel, that does not behave as a functional CICR. Determination of the molecular structure of NAADP-controlled Ca^{2+}-channels may reveal, in comparison with other channels, the molecular structure that is essential for CICR.

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REFERENCES

1 Chini, E. N., Beers, K. W. and Dousa, T. P. (1995) J. Biol. Chem. **270**, 3216–3223
2 Chini, E. N., Beers, K. W., Chini, C. S. and Dousa, T. P. (1995) Am. J. Physiol. **269**, C1042–C1047
3 Chini, E. N. and Dousa, T. P. (1995) Biochem. Biophys. Res. Commun. **270**, 167–174
4 Chini, EN. and Dousa, T. P. (1996) Am. J. Physiol. **270**, C530–C537
5 Chini, E. N., Perez, C., Beers, K., Clapham, D. and Dousa, T. P. (1995) Biophys. J. **68**, A229
6 Perez-Terzic, C. M., Chini, E. N., Shen, S. S., Dousa, T. P. and Clapham D. E. (1995) Biochem. J. **312**, 955–959
7 Lee, H. C. and Aarhus, R. A. (1995) J. Biol. Chem. **270**, 2152–2157
8 Berridge, M. J. (1994) Mol. Cell. Endocrinol. **98**, 119–124
9 Berridge, M. J. and Irvine, R. F. (1989) Nature (London) **341**, 197–205
10 Coronado, R., Morrissette, J., Sukhareva, M. and Vaughan, D. M. (1994) Am. J. Physiol. **266**, C1485–C1504
11 Galione, A., Lee, H. C. and Busa, W. B. (1991) Science. **253**, 1143–1146
12 Galione, A. and White, A. (1994) Trends Cell. Biol. **4**, 431–436
13 Lee, H. C. (1993) J. Biol. Chem. **268**, 293–299
14 Buck. W. R., Hoffmann, E. E., Rakow, T. L. and Shen, S. S. (1994) Dev. Biol. **163**, 1–10
15 Iino, M. and Tsukioka, M. (1994) Mol. Cell. Endocrinol. **98**, 141–146
16 Clapham, D. E. (1995) Cell **80**, 259–268
17 Endo, M. (1984) in Calcium Regulation in Biological Systems (Ebashi, S., Endo, M., Imahori, K., Kakiuchi, S. and Nishizuka, Y., eds.), pp. 197–209, Academic Press, Tokyo
18 Fabiato, A. (1983) Am. J. Physiol. **245**, C1–C14
19 Fabiato, A. and Fabiato, F. (1979) J. Physiol. (Paris) **75**, 463–505
20 Hellmich, M. R. and Strumwasser, F. (1991) Cell Regul. **2**, 193–202