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Pharmacological properties of the Ca^{2+}-release mechanism sensitive to NAADP in the sea urchin egg

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1 The sea urchin egg homogenate is an ideal model to characterize Ca^{2+}-release mechanisms because of its reliability and high signal-to-noise-ratio. Apart from the $InsP_3$- and ryanodine-sensitive Ca^{2+}-release mechanisms, it has been recently demonstrated that this model is responsive to a third independent mechanism, that has the pyridine nucleotide, nicotinic acid adenine dinucleotide phosphate (NAADP), as an endogenous agonist.

2 The sea urchin egg homogenate was used to characterize the pharmacological and biochemical characteristics of the novel Ca^{2+}-releasing agent, NAADP, compared to inositol trisphosphate ($InsP_3$) and cyclic ADP ribose (cyclic ADPR), an endogenous activator of ryanodine receptors.

3 NAADP-induced Ca^{2+}-release was blocked by L-type Ca^{2+}-channel blockers and by Bay K 8644, while $InsP_3$- and cyclic ADPR-induced Ca^{2+}-release were insensitive to these agents. L-type Ca^{2+}-channel blockers did not displace [^{32}P]-NAADP binding, suggesting that their binding site was different. Moreover, stopped-flow kinetic studies revealed that these agents blocked NAADP in a all-or-none fashion.

4 Similarly, a number of K^+-channel antagonists blocked NAADP-induced Ca^{2+}-release selectively over $InsP_3$- and cyclic ADPR-induced Ca^{2+}-release. Radioligand studies showed that these agents were not competitive antagonists.

5 As has been shown for $InsP_3$ and ryanodine receptors, NAADP receptors were sensitive to calmodulin antagonists, suggesting that this protein could be a common regulatory feature of intracellular Ca^{2+}-release mechanisms.

6 The presence of K^+ was not essential for NAADP-induced Ca^{2+}-release, since substitution of K^+ with other monovalent cations in the experimental media did not significantly alter Ca^{2+} release by NAADP. On the contrary, cyclic ADPR and $InsP_3$-sensitive mechanisms were affected profoundly, although to a different extent depending on the monovalent cation which substituted for K^+. Similarly, modifications of the pH in the experimental media from 7.2 to 6.7 or 8.0 only slightly affected NAADP-induced Ca^{2+}-release. While the alkaline condition permitted $InsP_3$ and cyclic ADPR-induced Ca^{2+}-release, the acidic condition completely hampered both Ca^{2+}-release mechanisms.

7 The present results characterize pharmacologically and biochemically the novel Ca^{2+}-release mechanism sensitive to NAADP. Such characterization will help future research aimed at understanding the role of NAADP in mammalian systems.

Keywords: NAADP; $InsP_3$; cyclic ADP ribose; Ca^{2+}-release; L-type Ca^{2+}-channel blockers; K^+-channel blockers; calmodulin antagonists

Introduction

The sea urchin egg homogenate is an ideal model to characterize Ca^{2+}-release mechanisms because of its reliability and low signal-to-noise-ratio. The intracellular stores of sea urchin eggs contain at least three independent Ca^{2+}-release mechanisms. Inositol 1,4,5-trisphosphate ($InsP_3$) and adenosine 3′: 5′-cyclic diphosphate ribose (cyclic ADPR) mobilize Ca^{2+} through $InsP_3$ and ryanodine receptors, respectively (Clapper & Lee, 1985; Lee *et al*., 1994b), while a third mechanism, which can be activated by nicotinic acid adenine dinucleotide phosphate (NAADP), has recently been described (Lee & Aarhus, 1995; Chini *et al*., 1995; for a review see Genazzani & Galione, 1997). The NAADP-sensitive Ca^{2+}-release mechanism is pharmacologically and kinetically different from those regulated by $InsP_3$ and cyclic ADPR. Heparin and 8-NH_2-cyclic ADPR, respective competitive antagonists of the $InsP_3$ and cyclic ADPR mechanisms, have no effect on NAADP-induced Ca^{2+}-release (Lee & Aarhus, 1995), while pretreatment of the sea urchin homogenates with thio-NADP, which has been shown to antagonize NAADP, has no effect on $InsP_3$- or cyclic ADPR-induced Ca^{2+}-release (Chini *et al*., 1995). Moreover, the intracellular organelles from which Ca^{2+} is released by NAADP appear to be different from those discharged by $InsP_3$ or cyclic ADPR (Lee & Aarhus, 1995; Genazzani & Galione, 1996). It is unlikely that Ca^{2+} release by NAADP is from the endoplasmic reticulum, since pretreatment with thapsigargin or cyclopiazonic acid, inhibitors of sarcoendoplasmic reticulum Ca^{2+}-pumps, abolishes Ca^{2+} release by cyclic ADPR and $InsP_3$, but does not affect NAADP-induced Ca^{2+}-release (Genazzani & Galione, 1996). However, the nature and distribution of the NAADP-sensitive store is unknown. Kinetically, NAADP-induced Ca^{2+}-release is biphasic, with a fast and a slow component, as is $InsP_3$-induced Ca^{2+}-release (Genazzani *et al*., 1997). NAADP releases in a slower manner, and Ca^{2+}-release induced by low concentrations of NAADP are preceded by a pronounced latency, which is absent at any concentration of $InsP_3$ (Genazzani *et al*., 1997).

The most striking feature of the NAADP-sensitive Ca^{2+}-release is in its inactivation properties (Aarhus *et al*., 1996; Genazzani *et al*., 1996). Concentrations as low as 5 fold less than those required to induce threshold Ca^{2+}-release irreversibly and fully block any further Ca^{2+}-release by NAADP, but do not affect $InsP_3$ or cyclic ADPR-induced Ca^{2+}-release. This

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inactivation is temperature-insensitive and is most likely due to full inactivation of a proportion of the receptors, as opposed to partial inactivation of all receptors (Genazzani *et al*., 1996; 1997). This unique property, which can be defined as 'quantal inactivation', precludes NAADP from releasing in a quantal manner, since sea urchin eggs will not respond to a second addition of NAADP, regardless of the amount of release induced by the first addition (Aarhus *et al*., 1996; Genazzani *et al*., 1996).

Although mammalian cells have not yet been shown to exhibit a NAADP-sensitive mechanism, synthesis from β-NADP and degradation of NAADP have been shown in rat tissues (Chini & Dousa, 1995. Moreover, it has been suggested that the same bifunctional enzyme which synthesizes and degrades cyclic ADPR is capable of metabolizing β-NADP as well (Aarhus *et al*., 1995). Mammalian cells therefore have the enzymatic machinery to produce and degrade NAADP.

The action of NAADP in a cell must represent the fine balance between activation and inactivation and it is possible that in systems, other than the sea urchin, the study of NAADP-induced responses might initially prove difficult because the mechanism will self-inactivate rapidly. To facilitate the task of identifying the role that NAADP plays in other systems, we have characterized the pharmacology of NAADP-induced Ca^{2+}-release in sea urchin egg homogenates. Indeed, in this system NAADP-induced Ca^{2+}-release was not only antagonized by L-type channel blockers, but also by some potassium channel blockers and by calmodulin antagonists. All these agents were unable to displace [^{32}P]-NAADP binding, indicating that their site of action was distinct from that of NAADP. These agents were found to be selective for NAADP-induced Ca^{2+}-release compared to $InsP_3$- and cyclic ADPR-induced Ca^{2+}-release.

Methods

Collection of sea urchin eggs

Eggs were obtained by stimulating ovulation of female *Lytechinus pictus* (Marinus, Inc., Long Beach, CA, U.S.A.) with a intracoelomic injection of KCl. These were then washed twice in artificial sea water (ASW) (composition in mM: NaCl 435, $MgCl_2$ 40, $MgSO_4$ 15, $CaCl_2$ 11, KCl 10, $NaHCO_3$ 2.5 and EDTA 1.0 at pH 8.0), and jelly removed by filtration through 90 mm nylon mesh.

Ca^{2+} release assays

Homogenates (2.5%) of unfertilized *Lytechinus pictus* eggs were prepared as described previously (Dargie *et al*., 1990) and Ca^{2+}-loading was achieved by incubation at room temperature for 3 h in an intracellular medium (IM) consisting of (in mM): potassium gluconate 250, N-methylglucamine 250, HEPES 20 (pH 7.2), $MgCl_2$ 1, adenosine 5′-triphosphate (ATP) 0.5, phosphocreatine 10; creatine phosphokinase 10 U ml^{-1}, oligomycin 1 mg ml^{-1}, antimycin 1 mg ml^{-1}, sodium azide 1 mM and fluo-3 3 μM. Free Ca^{2+} concentration was measured by monitoring fluorescence intensity at excitation and emission wavelengths of 490 nm and 535 nm, respectively. Fluorimetry was performed at 17°C with 500 μl of homogenate in a Perkin-Elmer LS-50B fluorimeter. Additions were made in 5 μl aliquots and all chemicals were added in IM containing 10 μM EGTA. Basal concentrations of Ca^{2+} were typically between 100 and 150 nM. Sequestered Ca^{2+} was determined by monitoring decrease in fluo-3 fluorescence during microsomal loading and by measuring Ca^{2+} release in response to ionomycin (5 μM) and was constant between experiments.

Stopped-flow measurements

Rapid kinetics of Ca^{2+}-release by NAADP was carried out on a 2.5% homogenate prepared as described above. Stopped-flow measurements were performed as described elsewhere (Champeil *et al*., 1988; Meyer *et al*., 1990; Mezna & Michelangeli, 1995). Ca^{2+}-loaded homogenate was introduced in a 2.5 ml syringe of a stopped flow fluorimeter (Applied Photophysics, Model SX17 MV), while a 250 μl syringe was filled with either IM, or NAADP (diluted in IM) at a concentration 10 times the concentration desired in the mixing chamber, as the mixing ratios of the two syringes were 10:1. Temperature of homogenate in either the syringes or mixing compartment was maintained at 17°C by a circulating water bath. Fluorescence changes of fluo-3 were monitored by exciting the sample at 490 nm and measuring the emission above 515 nm. Fluo-3 fluorescence was captured over 55 s in a split-time base mode, with 200 recordings taken in the first 5 s and 200 recordings taken in the remaining 50 s. Each experiment represents the average of at least 6 acquisitions.

The averaged traces were then analysed by use of non-linear regression analysis programs supplied by Applied Photophysics and Biosoft. The progress of Ca^{2+} release from at least three different homogenate preparations used in this study were shown to be biphasic and could be best fitted to a biexponential profile by use of the following equation:

$$[Ca^{2+}]\ \text{release} = A_1(1 - \exp^{-k_1 t}) + A_2(1 - \exp^{-k_2 t})$$

where A_1, A_2, k_1 and k_2 are the amplitudes and rate constants of Ca^{2+} release for the fast and slow phases respectively, and t is the time (s). The amplitudes A_1 and A_2 are expressed in arbitrary units and relate to the fluorescence intensity changes of fluo-3. Since the fluo-3 signal in these experiments was never saturating, these units are related to Ca^{2+} changes. In experiments where NAADP was used, Ca^{2+} release was preceded by a latency or lag phase. This latency was quantified and then subtracted before further analysis of the Ca^{2+} release process. In a control experiment the effect of the Ca^{2+} pumps responsible for the re-uptake of Ca^{2+} in these preparations was also quantified and the rate constants of the uptake were essentially negligible compared to Ca^{2+}-release by either $InsP_3$ or NAADP. In fact, the rate constant for Ca^{2+} uptake was $0.0008 \pm 0.000004\ s^{-1}$ ($n = 16$), while the rate constants observed for Ca^{2+} release were at least 30 times faster.

[^{32}P]-NAADP binding

Binding was performed on *Lytechinus pictus* egg homogenates that had been prepared as for the Ca^{2+}-release assay. The binding procedure and synthesis of [^{32}P]-NAADP were as described previously (Aarhus *et al*., 1996). Briefly, the homogenates were incubated with 1 nM [^{32}P]-NAADP (about 30000 c.p.m.) and various antagonists on ice in a final volume of 0.1 ml. The binding reactions were initiated by addition of homogenate to a final concentration of 2.5% to tubes containing [^{32}P]-NAADP and other additions. After being incubated for 20 m, the samples were vacuum filtered on GF/C filters. The filters were washed 2 times with 3 ml of ice-cold 10% polyethylene glycol in IM buffer. The radioactivity retained on the filter was determined by liquid scintillation counting. The effect of antagonists on the time-dependent self-inactivation property of NAADP binding and Ca^{2+}-release (Aarhus *et al*., 1996) was also analysed by determining the effect of the addition of 100 nM unlabelled NAADP either at zero time or 3 min after the initiation of the binding reaction. Dimethylsulphoxide (DMSO) was used as vehicle control since the antagonist stock solutions were made up in this solvent.

Materials

Fluo-3 was purchased from Molecular Probes and NAADP was from RBI or was synthesized as described in Aarhus *et al*. (1996). *N*-(6-aminohexyl)-5-iodo-1-naphtalenesulphonamide (J8) was kindly supplied by Prof. G.M. Blackburn (Sheffield University). All other chemicals were from Sigma.

Results

A pharmacological approach was used to characterize the NAADP-sensitive Ca^{2+}-release mechanism. In the present experiments, NAADP released Ca^{2+} potently with an EC_{50} of 20–30 nM on different experimental days (data not shown). Concentrations of NAADP as low as 1 nM were able to inactivate completely and irreversibly the NAADP-sensitive Ca^{2+}-release mechanism without affecting $InsP_3$ and cyclic ADPR-sensitive mechanisms (data not shown; see also Genazzani *et al.*, 1996).

As previously shown, μM concentrations of some dihydropyridines, verapamil and diltiazem blocked NAADP-induced Ca^{2+}-release in sea urchin egg homogenates without affecting $InsP_3$- and cyclic ADPR-induced Ca^{2+}-release in the sea-urchin egg homogenate (Genazzani *et al.*, 1996) (Figure 1a). Nifedipine, verapamil and diltiazem at a concentration of 100 μM had no effect on $InsP_3$- and cyclic ADPR-induced Ca^{2+}-release (data not shown; see also Genazzani *et al.*, 1996). Diltiazem appeared to be the most potent antagonist with an IC_{50} of 7.41 ± 0.33 μM, while the other two agents were equally capable of fully blocking NAADP-induced Ca^{2+}-release with IC_{50}s of 10.5 ± 0.45 (nifedipine) and 20.8 ± 3.02 (verapamil). Interestingly, the L-type Ca^{2+}-channel agonist Bay K 8644 (methyl 1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethyl-phenyl)-pyridine-5-carboxylate) in the present experiments fully blocked the NAADP-sensitive release mechanism with an IC_{50} of 30.2 ± 0.06 μM and possessed no intrinsic agonist activity. Potency within the dihydropyridine class was also evaluated, and nisoldipine, nimodipine and nicardipine appeared to be slightly more potent than nifedipine (Figure 1b).

To investigate the kinetic modifications of NAADP-sensitive CA^{2+}-release induced by these agents, the most potent of these agents, diltiazem, was assayed in a stopped-flow procedure. It has been previously demonstrated that NAADP releases CA^{2+} in a biphasic manner and that such release is preceded by a latency, which is inversely proportional to the concentration of NAADP used (Genazzani *et al.*, 1997). In the presence of diltiazem the total amplitude of Ca^{2+}-release was decreased in a concentration-dependent manner (Figure 1c) while the kinetic parameters were not affected (Figure 1d).

The possibility that Ca^{2+}-channel antagonists could competively displace or modulate NAADP binding to its site was also investigated. Diltiazem, verapamil and nifedipine were all unable to alter NAADP binding (Table 1). Moreover, these drugs were unable to modulate the time-dependent irreversibility of NAADP binding (Table 1), which is thought to be an important reflection of its peculiar inactivation properties (Aarhus *et al.*, 1996).

It has been shown that high concentrations of K^+-channel blockers are able to inhibit directly $InsP_3$-induced Ca^{2+}-release in a variety of tissues (Shah & Pant, 1988; Palade *et al.*, 1989; Michelangeli *et al.*, 1995). We therefore investigated whether some of these compounds can alter NAADP-induced Ca^{2+}-

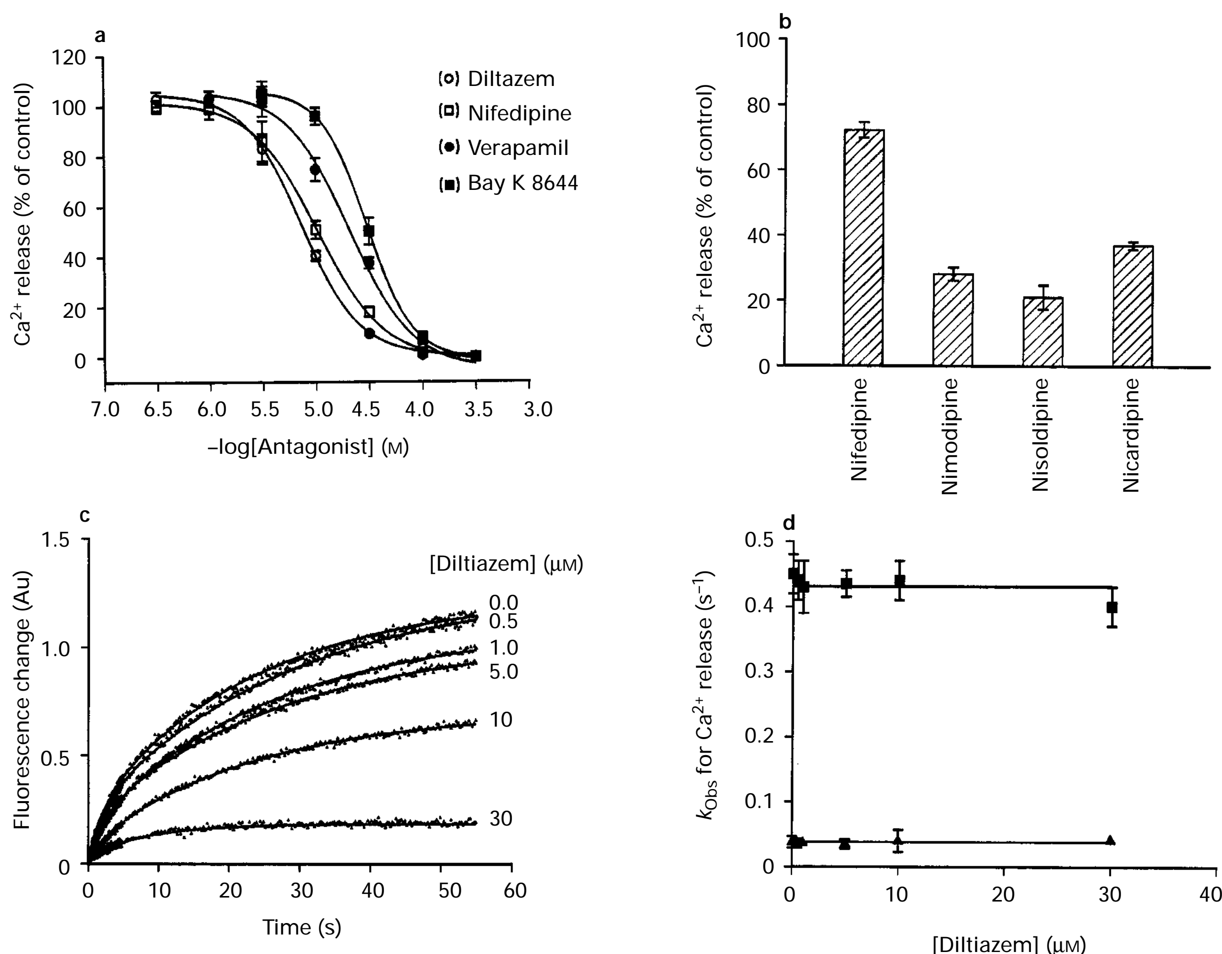

Figure 1 Effect of L-type Ca^{2+}-channel blockers on NAADP-induced Ca^{2+}-release (a) Dose-response curves of diltiazem, nifedipine, verapamil and Bay K 8644 on Ca^{2+}-release induced by 200 nM NAADP. Values are mean, and vertical lines show s.e.mean, of 6–12 determinations. (b) Effect of 20 μM of various dihydropyridines on Ca^{2+}-release by 200 nM NAADP. Values are mean $\pm$ s.e.mean of 6 determinations obtained on different experimental days from data in (a). (c) Stopped-flow representative traces of inhibition of 200 nM NAADP by diltiazem. (d) Rate constants of NAADP-induced Ca^{2+}-release in the presence of various concentrations of diltiazem. NAADP-induced Ca^{2+}-release, as previously described (Genazzani *et al.*, 1997), was composed of a fast phase (solid squares) and a slow phase (solid triangles).

Table 1 Effect of various agents that inhibit NAADP-induced Ca^{2+}-release on [^{32}P]-NAADP binding

		Agent alone	*Agent + NAADP (zero minutes)*	*Agent + NAADP (3 minutes)*
Control		7.7±0.3	1.3±0.3	7.2±1.0
Diltiazem	100 μM	8.5±0.9	1.9±0.7	8.0±0.8
Verapamil	100 μM	8.2±0.4	2.7±0.5	8.5±2.1
Nifedipine	100 μM	7.9±0.2	1.5±0.2	7.3±0.5
THA	10 μM	10.0±1.2	1.5±0.2	6.6±0.3
Quinine	500 μM	7.8±1.4	1.5±0.4	7.3±0.9
Glibenclamide	100 μM	7.7±0.9	1.4±0.3	6.7±1.2
W7	100 μM	8.8±0.6	1.5±0.4	10.3±1.4
Trifluoperazine	30 μM	7.6±0.7	1.2±0.1	7.7±1.8

Binding was performed as described in Methods. The first column shows the binding in the absence of unlabelled NAADP. The second column shows the binding in the presence of 100 nM unlabelled NAADP added at time zero. The third column represents the amount of binding when unlabelled NAADP was added three minutes after initiation of the binding reactions. Values are expressed as fmol NAADP bound mg^{-1} protein and are mean±s.e.mean of three determinations. Experiments performed on *Strongylocentrotus purpuratus* yielded similar results.

Table 2 Effect of various potassium channel blockers on agonist-mediated Ca^{2+}-release

		NAADP 200 nM	*$InsP_3$ 1 μM*	*Cyclic ADPR 200 nM*
Tetrahexylammonium	10 μM	24.1±2.3	76.1±3.1	101±5.0
Tetrabutylammonium	200 μM	23.7±1.4	102±7.0	99±0.6
Cromakalim	100 μM	81.8±2.1	ND	ND
Glibenclamide	100 μM	21.4±2.6	107±3.4	97.8±0.7
4-Amino-pyridine	100 μM	101±8.0	ND	ND
9-Amino-acridine	100 μM	77.2±2.1	ND	ND
Quinine	300 μM	36.1±2.8	94.4±2.0	96.8±6.3
Barium chloride	100 μM	84.9±8.1	92.9±11	92.5±2.6

Values are expressed as % of respective controls and represent mean±s.e.mean of 3–6 determinations. ND, not determined.

release as well. In the sea-urchin homogenate, $InsP_3$-induced Ca^{2+}-release was less sensitive than NAADP-induced Ca^{2+}-release to high concentrations of K^+-channel blockers, while cyclic ADPR-induced Ca^{2+}-release was insensitive (Table 2). Among the agents that affected NAADP-induced Ca^{2+}-release was cromakalim, a known activator of potassium channels. Tetrahexylammonium was the most potent antagonist among the agents tested with an IC_{50} of 4.4±0.5 μM (Figure 2), which is very similar to that found for $InsP_3$ antagonism in dog brain microsomes (Palade *et al*., 1989). It appears that in this system the potency of tetrahexylammonium in inhibiting $InsP_3$-induced Ca^{2+}-release is lower, since 10 μM could inhibit only around 25% of the Ca^{2+}-release induced by $InsP_3$ (Table 2).

It has been suggested that K^+, either as a counter ion or as a co-factor, is a requirement for $InsP_3$-induced Ca^{2+}-release (Mezna & Michelangeli, 1995). To test the effect of K^+ on NAADP-induced Ca^{2+}-release, various buffers were prepared with different monovalent cations in their chloride form substituted to potassium gluconate. Under such conditions, the sea urchin egg homogenate pumps worked properly and the amount of Ca^{2+} released by ionomycin or thapsigargin was similar to controls. Substitution of potassium gluconate with KCl did not significantly alter any of the Ca^{2+}-release mechanisms nor the reuptake system (data not shown). As previously shown, the absence of potassium in the experimental medium significantly decreased the amount of Ca^{2+} released by maximal concentrations of $InsP_3$ (Mezna & Michelangeli, 1995) (Table 3). Only Rb^+ and Li^+ appeared to substitute partially for the absence of potassium in the buffer. Cyclic ADPR action seemed also to be dependent on the presence of potassium, since in the presence of Li^+ and Cs^+ cyclic ADPR-induced Ca^{2+}-release was highly reduced. On the other hand, NAADP-sensitive Ca^{2+}-release channels appeared to be independent of monocation presence, and even the substitution of K^+ with Cs^+ still permitted Ca^+-release (Table 3). Only in the presence of NaCl was release reduced, although still largely present. Interestingly, the substitution of potassium gluconate with RbCl induced a small but significant increase of NAADP-induced Ca^{2+}-release.

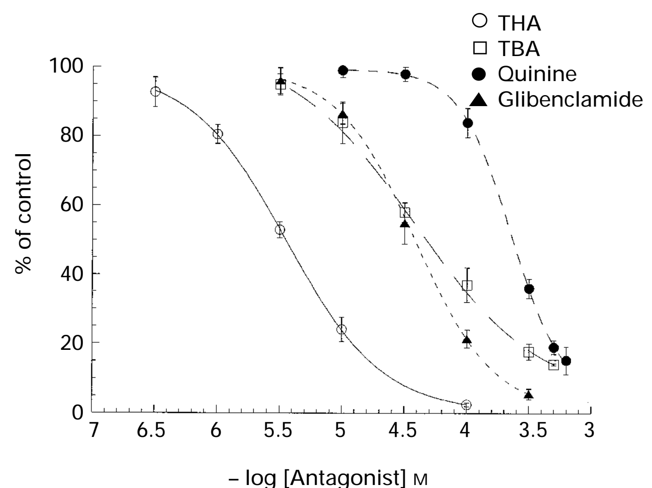

Figure 2 Effect of tetrahexylammonium (THA), tetrabutylammonium (TBA), quinine and glibenclamide on Ca^{2+}-release by 200 nM NAADP. Values are mean, and vertical lines show s.e.mean of 6–12 determinations.

To investigate whether K^+-channel blockers competed or modulated the NAADP-binding site, [^{32}P]-NAADP binding was performed in the presence of high concentrations of THA, quinine or glibenclamide (Table 1). All three substances did not affect NAADP binding nor did they alter the time-dependent irreversibility.

It has been previously demonstrated that calmodulin is able to modulate a number of ion channels, including the ryanodine and $InsP_3$ receptors (Saimi & Kung, 1994; Lee *et al*., 1994a;

Table 3 Effect of various monovalent cations on agonist-induced Ca^{2+}-release

		NAADP 200 nM	*Cyclic ADPR 200 μM*	*InsP$_3$ 1 μM*
Potassium gluconate	250 mM	65±2.5	42±1.0	35±1.3
Lithium chloride	250 mM	62±7.7	16±1.6	21±2.3
Sodium chloride	250 mM	44±2.3	28±0.2	8.3±1.0
Rubidium chloride	250 mM	78±5.5	42±1.0	24±0.6
Caesium chloride	250 mM	60±10	7.3±0.3	3.0±0.1

Values are mean±s.e.mean of 8–12 determinations and are expressed as % of ionomycin (10 μM)-induced Ca^{2+}-release. Substitution of potassium chloride for potassium gluconate did not affect Ca^{2+}-release by any of the three agents.

Michelangeli *et al*., 1995). In particular, calmodulin has been demonstrated to be a cofactor in cyclic ADPR-induced Ca^{2+}-release in sea-urchin eggs (Lee *et al*., 1994a). For this reason, calmodulin antagonists were tested as possible antagonists of NAADP-induced Ca^{2+}-release. Calmidazolium and *N*-(6-aminohexyl)-5-iodo-1-naphthalene sulfonamide (J8) (Caulfield *et al*., 1991) were found to be more potent than trifluoperazine and *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7), although full inhibition could not be achieved since at concentrations over 10 and 20 μM, respectively, these two agents had intrinsic Ca^{2+}-releasing activity (Figure 3).

Stopped-flow kinetic studies showed that J8, while decreasing the amplitude of Ca^{2+}-release by NAADP, did not affect its rate constant parameters (Figure 3) or alter the latency which precedes Ca^{2+}-release by NAADP (not shown). Similarly, addition of calmodulin to Percoll-purified sea urchin egg microsomes, where the concentration of calmodulin should be significantly reduced, greatly enhanced cyclic ADPR-induced Ca^{2+}-release but did not affect NAADP-induced Ca^{2+}-release (data not shown). The addition of calmodulin or of calmodulin antagonists was also unable to affect the inactivation of the NAADP-sensitive mechanism (data not shown; see also Table 1). Similarly, calmodulin antagonists did not modify [^{32}P]-NAADP binding (Table 1).

It has been previously suggested that binding and Ca^{2+}-release by InsP$_3$ is modulated by pH (Tsukioka *et al*., 1994; Mezna & Michelangeli, 1995). Thus, at acidic pH the rate and amount of Ca^{2+}-release by InsP$_3$ is strongly reduced compared to alkaline pH. To study this phenomenon, the pH of the IM buffer was altered from 7.2 to 6.7 and 8.0. This range was limited because of interference with the Ca^{2+}-pumps and the Ca^{2+}-dye fluo-3 (data not shown) by pH outside this range. Ca^{2+}-calibrations were done in all three buffers and ionomycin released similar amounts of Ca^{2+} in all three preparations. As previously found, InsP$_3$ was almost inactive at acidic pH, while pH 8.0 did not affect InsP$_3$-induced Ca^{2+}-release compared to control (Tsukioka *et al*., 1994) (Figure 4). In contrast, cyclic ADPR-induced Ca^{2+}-release was affected both at acidic and alkaline pH, although only to a minor extent in the latter condition. Surprisingly, NAADP-induced Ca^{2+}-release was markedly pH-insensitive within the range tested (Figure 4). Moreover, NAADP-induced NAADP-desensitization was not affected by differences in pH (data not shown).

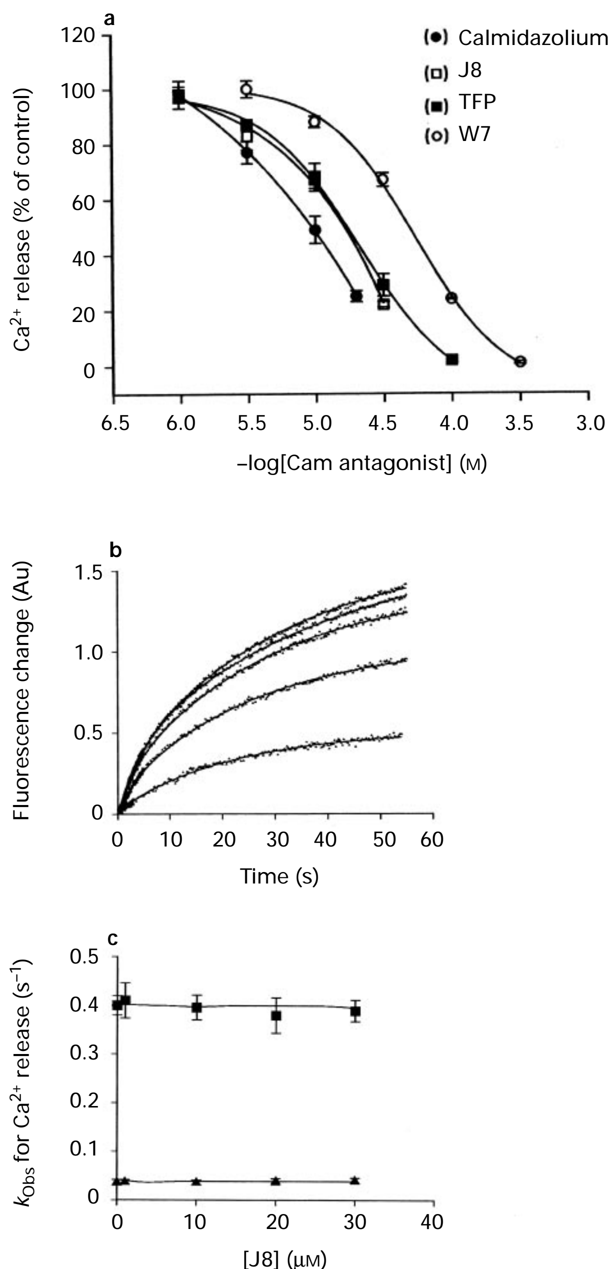

Figure 3 Effect of various calmodulin (CaM) antagonists on NAADP-induced Ca^{2+}-release. (a) Effect of calmidazolium, J8, trifluoperazine (TFP) and W7 on Ca^{2+}-release by 200 nM NAADP. Values are mean, and vertical lines show s.e.mean of 9–12 determinations. (b) Stopped-flow representative traces of inhibition of 200 nM NAADP by J8. (c) Rate constants for the fast (solid squares) and slow (solid triangles) phase of NAADP-induced Ca^{2+}-release in the presence of various concentrations of J8.

Discussion

In contrast to InsP$_3$ and cyclic ADPR, NAADP-induced Ca^{2+}-release in the present paper was shown to be sensitive to dihydropyridines, verapamil and diltiazem. Although L-type Ca^{2+}-channel antagonists are known to be non-specific at high concentrations, it is interesting concentrations in the low μM range of diltiazem, verapamil and nifedipine, which have been shown to bind to three distinct sites of the α_1 subunit of L-type Ca^{2+}-channels (Catterall & Striessnig, 1992), are all able to block NAADP-induced Ca^{2+}-release. These data provide a useful pharmacological distinction between NAADP-sensitive and InsP$_3$ and caffeine-sensitive Ca^{2+}-release systems, since it has been shown in a variety of systems that L-type Ca^{2+}-channel blockers do not modify Ca^{2+}-release by InsP$_3$ and ryanodine receptors (Michelangeli *et al*., 1995). A number of studies have shown that Ca^{2+}-antagonists of the dihydropyridine, phenylalkylamine and benzothiazepine classes interact with both L-type Ca^{2+}-channel structures and non-L-type

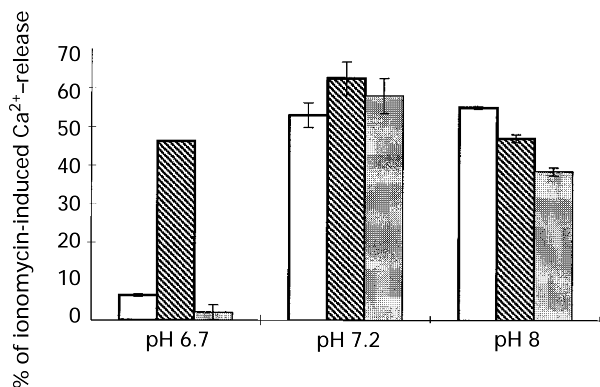

Figure 4 Effect of pH on Ca^{2+}-release by $InsP_3$ (open columns), NAADP (hatched columns) or cyclic ADPR (solid columns). Experiments were conducted in intracellular medium (IM) at the stated pH. More alkaline or acidic conditions could not be tested since Ca^{2+}-pumps and the fluorescent dye interfered with the interpretation of results. Values are mean±s.e.mean of 6 determinations.

Ca^{2+}-channel structures (for a review see Zernig, 1990). The latter are formed by a number of different structures, ranging from the nucleoside transporter to the multidrug-resistance-related-P-glycoprotein (Zernig, 1990). It is possible that among these structures is the site for NAADP action.

A similarity between NAADP-, $InsP_3$-, and cyclic ADPR-sensitive mechanisms appears to be the sensitivity to calmodulin antagonists. Although the agents tested appear to be more potent at inhibiting NAADP-induced Ca^{2+}-release than cyclic-ADPR-induced Ca^{2+}-release, which requires 3–5 times the concentration of the antagonists (Summerhill & Galione, unpublished results), it is likely that this inhibition is not calmodulin-dependent. It has been shown that when purified microsomes are prepared from sea urchin homogenate, ryanodine receptors lose their sensitivity to cyclic ADPR and that such sensitivity can be restored by the addition of calmodulin. These data strongly suggest that during the preparation of purified microsomes there is a loss of calmodulin, which is a requirement for cyclic ADPR action (Lee *et al*., 1994a). On the other hand, the NAADP-sensitive stores that copurify with the purified microsomes are not altered by this treatment, suggesting that calmodulin is not a requirement for this mechanism or that a minute concentration of calmodulin, that cannot be eliminated with this protocol, is sufficient.

To characterize further the NAADP-sensitive Ca^{2+}-release mechanism, we have tested its monovalent cation sensitivity and the pH dependency. It has been shown that K^+, either a counter ion or as a co-factor, is a requirement for $InsP_3$-induced Ca^{2+}-release in cerebellar microsomes (Mezna & Michelangeli, 1995). This was the case also in the sea urchin egg homogenate. Cyclic ADPR-induced Ca^{2+}-release was also highly dependent on the presence of K^+, whereas NAADP-induced Ca^{2+}-release was effective also when other monovalent cations were substituted for K^+. Moreover, the NAADP-sensitive Ca^{2+}-release mechanism, unlike $InsP_3$ and cyclic ADPR, was insensitive to pH changes from 6.7 to 8.0. In this context, it is interesting that ADP-ribosyl cyclase and CD38, the putative enzymes responsible for the synthesis of both cyclic ADPR and NAADP, are indeed able to catalyze the production of NAADP at acidic pH while at neutral pH only cyclic ADPR will be formed (Aarhus *et al*., 1995). Moreover, at alkaline pH, $NADP^+$ is non-enzymatically converted to NAADP (Lee & Aarhus, 1995).

Therefore, while intracellular pH changes can profoundly modulate $InsP_3$- and cyclic ADPR-induced Ca^{2+}-release, it appears that the NAADP-sensitive Ca^{2+}-release pathway is able to function in extreme conditions, making it a candidate for surrogate Ca^{2+}-release in conditions when the other two systems are hampered.

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