The EMBO Journal Vol. 20 No. 11 pp. 2666–2671, 2001

NAADP induces Ca^{2+} oscillations via a two-pool mechanism by priming IP_3- and cADPR-sensitive Ca^{2+} stores

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In sea urchin eggs, Ca^{2+} mobilization by nicotinic acid adenine dinucleotide phosphate (NAADP) potently self-inactivates but paradoxically induces long-term Ca^{2+} oscillations. We investigated whether NAADP-induced Ca^{2+} oscillations arise from the recruitment of other Ca^{2+} release pathways. NAADP, inositol trisphosphate (IP_3) and cyclic ADP-ribose (cADPR) all mobilized Ca^{2+} from internal stores but only NAADP consistently induced Ca^{2+} oscillations. NAADP-induced Ca^{2+} oscillations were partially inhibited by heparin or 8-amino-cADPR alone, but eliminated by the presence of both, indicating a requirement for both IP_3- and cADPR-dependent Ca^{2+} release. Thapsigargin completely blocked IP_3 and cADPR responses as well as NAADP-induced Ca^{2+} oscillations, but only reduced the NAADP-mediated Ca^{2+} transient. Following NAADP-mediated release from this Ca^{2+} pool, the amount of Ca^{2+} in the Ca^{2+}-induced Ca^{2+} release stores was increased. These results support a mechanism in which Ca^{2+} oscillations are initiated by Ca^{2+} release from NAADP-sensitive Ca^{2+} stores (pool 1) and perpetuated through cycles of Ca^{2+} uptake into and release from Ca^{2+}-induced Ca^{2+} release stores (pool 2). These results provide the first direct evidence in support of a two-pool model for Ca^{2+} oscillations.

Keywords: cADPR/calcium/IP_3/NAADP/oscillation

Introduction

Nicotinic acid adenine dinucleotide phosphate (NAADP) potently mobilizes Ca^{2+} in several cell types and species including sea urchin eggs (Lee and Aarhus, 1995), ascidian oocytes (Albrieux *et al*., 1998), starfish oocytes (Santella *et al*., 2000), mouse pancreatic acinar cells (Cancela *et al*., 1999), rat brain microsomes (Bak *et al*., 1999), plant microsomes (Navazio *et al*., 2000) and human T-lymphocytes (Berg *et al*., 2000). NAADP operates through a pathway that, although as yet undefined at the molecular level, is pharmacologically and physically distinct from the inositol 1,4,5-trisphosphate (IP_3)- and cyclic ADP-ribose (cADPR)-sensitive Ca^{2+} release pathway (Lee, 1997). In the sea urchin egg, NAADP is unique among the Ca^{2+}-releasing messengers in at least four respects (Genazzani and Galione, 1997; Lee, 1997; Galione *et al*., 2000). First, NAADP does not exhibit positive feedback by Ca^{2+}, termed Ca^{2+}-induced Ca^{2+} release (CICR) (Chini and Dousa, 1996). Secondly, NAADP potently self-inactivates even when present at concentrations less than those required to activate detectable Ca^{2+} release (Aarhus *et al*., 1996; Genazzani *et al*., 1996; Lee *et al*., 1997). Thirdly, once bound to its receptor, radiolabelled NAADP is occluded and cannot be displaced by non-labelled NAADP, which is thought to be related to its self-inactivation mechanism (Aarhus *et al*., 1996; Billington and Genazzani, 2000; Patel *et al*., 2000). Fourthly, NAADP mobilizes Ca^{2+} from a store that is distinct pharmacologically and physically from the IP_3- and cADPR-sensitive Ca^{2+} stores (Lee and Aarhus, 1995, 2000; Genazzani and Galione, 1996).

NAADP stimulates Ca^{2+} oscillations in both pancreatic acinar cells (Cancela *et al*., 1999) and sea urchin eggs (Aarhus *et al*., 1996; Genazzani *et al*., 1996; Lee *et al*., 1997). In pancreatic acinar cells, NAADP releases Ca^{2+}, which is then amplified by CICR through IP_3 and ryanodine receptors (Cancela *et al*., 1999). Amplification of NAADP-induced Ca^{2+} release through CICR pathways also occurs in starfish eggs (Santella *et al*., 2000) and sea urchin eggs (Churchill and Galione, 2000). In sea urchin eggs, however, a 'trigger' mechanism is unlikely to underlie the long-term Ca^{2+} oscillations induced by NAADP because of self-inactivation (Aarhus *et al*., 1996; Billington and Genazzani, 2000; Patel *et al*., 2000). Based on this, Lee and coworkers (Aarhus *et al*., 1996; Lee, 1997; Lee *et al*., 1997) suggested that these Ca^{2+} oscillations are more likely to involve overloading and spontaneous release from the IP_3- and/or cADPR-sensitive pathways. However, we demonstrated recently that NAADP self-inactivation is reversible in intact sea urchin eggs when NAADP release is submaximal and spatially localized (Churchill and Galione, 2001), raising the possibility that NAADP-induced Ca^{2+} oscillations are due to cycles of desensitization and re-sensitization.

Several theoretical models have been proposed to explain Ca^{2+} oscillations, and can be classified by the number of Ca^{2+} stores required (one or two) and whether the level of IP_3 must also oscillate (Berridge and Galione, 1988; Tsien and Tsien, 1990; Berridge, 1993). Depending on the cell type and experimental conditions, evidence exists for mechanisms requiring oscillating levels of IP_3 (Harootunian *et al*., 1991; Hirose *et al*., 1999) and control by positive and/or negative feedback of Ca^{2+} on Ca^{2+} release channels with a steady level of IP_3 (Wakui *et al*., 1989; Hajnoczky and Thomas, 1997). Direct evidence does not exist for the two-pool mechanism proposed by Berridge and colleagues (Berridge, 1988, 1991; Berridge and Galione, 1988), which envisages transfer of Ca^{2+} from IP_3-sensitive stores into CICR stores, which overload and spontaneously release Ca^{2+}.

The sea urchin egg is highly responsive to IP_3, cADPR and NAADP (Clapper and Lee, 1985; Clapper *et al*., 1987;

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Lee and Aarhus, 1995) and thus provides an excellent system in which to study the interaction between Ca^{2+} release pathways. Here we show that NAADP induces long-term Ca^{2+} oscillations by releasing Ca^{2+} from a separate store (pool 1) and is then taken up by CICR stores (pool 2). The shuttling of Ca^{2+} primes the CICR stores and results in cycles of Ca^{2+} overloading, release and re-uptake.

Results and discussion

NAADP but not IP_3 or cADPR induces long-term Ca^{2+} oscillations

Lee and coworkers (Aarhus *et al*., 1996; Lee, 1997; Lee *et al*., 1997) reported that NAADP but not IP_3 or cADPR resulted in Ca^{2+} oscillations in sea urchin eggs, and photorelease was much more effective than microinjection of NAADP (Aarhus *et al*., 1996). However, Swann and Whitaker (1986) reported that microinjection of IP_3 also resulted in a second Ca^{2+} increase that occurred several minutes after the primary Ca^{2+} increase. Therefore, to investigate the mechanism by which NAADP gives rise to Ca^{2+} oscillations, we first compared the Ca^{2+} responses of sea urchin eggs to NAADP, IP_3 and cADPR.

The photorelease of supramaximal amounts of messenger revealed that NAADP released more Ca^{2+} than cADPR, which in turn released more than IP_3 (Figures 1 and 6A). That NAADP evokes a larger increase than either IP_3 or cADPR supports the notion that NAADP is releasing Ca^{2+} from both a common store and a store responsive to only NAADP, as suggested by the data from sea urchin homogenates (Lee and Aarhus, 1995; Genazzani and Galione, 1996). NAADP induced long-lasting (at least 80 min) Ca^{2+} oscillations in 26 of 29 experiments (Figure 1), as demonstrated previously (Aarhus *et al*., 1996; Genazzani *et al*., 1996; Lee, 1997). NAADP-induced Ca^{2+} oscillations persisted in the absence of extracellular Ca^{2+} (data not shown), indicating that the oscillations involved mobilization from intracellular Ca^{2+} stores, as reported previously (Aarhus *et al*., 1996; Lee *et al*., 1997). In contrast to the high proportion of eggs exhibiting Ca^{2+} oscillations after photorelease of NAADP, Ca^{2+} oscillations were induced less frequently after photorelease of cADPR (eight of 17 experiments, $p = 0.004$) and IP_3 (one of nine experiments, $p < 0.0001$; Figure 1). Additionally, the magnitude of the Ca^{2+} oscillations was much larger after NAADP-mediated Ca^{2+} release than after either cADPR- or IP_3-mediated Ca^{2+} release (Figure 1). Taken together, it is clear that Ca^{2+} oscillations are more robust and frequent after a Ca^{2+} increase mediated by NAADP than after either cADPR or IP_3.

The Ca^{2+} increase that occurs 1–3 min after the NAADP-mediated Ca^{2+} increase occurs before Ca^{2+} recovers to baseline and appears as either a spike clearly separated from the primary increase or as only a shoulder during recovery from the primary increase (Figure 1) (Aarhus *et al*., 1996; Genazzani *et al*., 1996; Lee, 1997). To enable quantitative comparisons of data with and without a discernible Ca^{2+} spike, we determined the duration that Ca^{2+} was elevated above its half-maximal

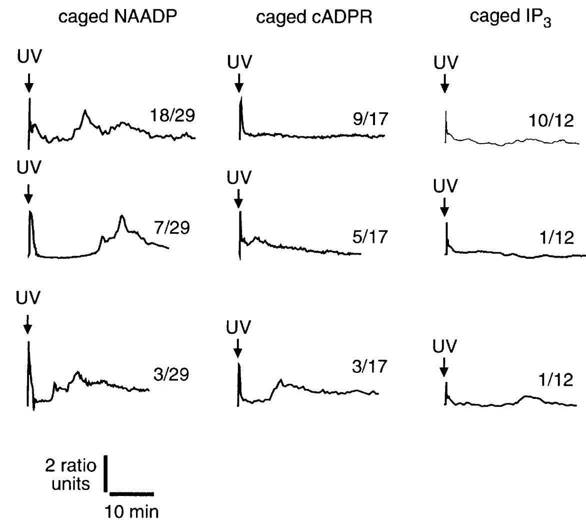

Fig. 1. Induction of Ca^{2+} oscillations by photorelease of NAADP, cADPR and IP_3. The number of similar responses (numerator) relative to the total number of experiments (denominator) is shown next to each trace. The final intracellular concentrations were: Oregon Green 488 BAPTA Dextran, 10 μM; caged NAADP, 0.5 μM; caged cADPR, 5 μM and caged IP_3, 5 μM.

concentration (Figure 6B). This measure validly described all treatments except for the effect of removing extracellular Ca^{2+} (Figure 6B), in which case it is an underestimate because the first oscillation is separated from the NAADP-induced Ca^{2+} increase to the extent that Ca^{2+} decreases below its half-maximal concentration before the first oscillation occurs (Aarhus *et al*., 1996; Lee *et al*., 1997). Duration data show that the initial Ca^{2+} increase was more prolonged when initiated by NAADP than when initiated by either IP_3 or cADPR (Figures 1 and 6B).

The later Ca^{2+} increases that occur 5–15 min after the NAADP-induced Ca^{2+} increase exhibit irregular patterns (Figure 1) (Aarhus *et al*., 1996; Genazzani *et al*., 1996; Lee, 1997). The time of onset, duration, amplitude and frequency of the Ca^{2+} increases is decidedly variable (Figure 1, NAADP). Often there are small Ca^{2+} transients of short duration (~1 min) superimposed on larger prolonged Ca^{2+} transients (~5–15 min). This suggests that there are at least two independent oscillatory mechanisms operating simultaneously. Ca^{2+} oscillations with a similar 'signature' occur after fertilization of sea urchin eggs with increases occurring before nuclear envelope breakdown, metaphase–anaphase transition and cytokinesis (Poenie *et al*., 1985; Whitaker and Larman, 2001).

In view of our recent data showing that NAADP desensitization is reversible in intact eggs (Churchill and Galione, 2001), we determined whether re-sensitization could explain NAADP-induced Ca^{2+} oscillations. Under the conditions used in the current study the eggs remained insensitive to NAADP for at least 2 h (data not shown), probably due to the use of supramaximal concentrations of NAADP. Thus, Lee and colleagues' original argument that the long-term Ca^{2+} oscillations cannot be due to NAADP because of desensitization (Aarhus *et al*., 1996; Lee, 1997; Lee *et al*., 1997) remains valid.

NAADP-induced Ca^{2+} oscillations require both IP_3- and cADPR-dependent Ca^{2+} release pathways

To determine the potential roles of IP_3- and cADPR-dependent Ca^{2+} release pathways in the later oscillations (⩾5 min after NAADP photorelease), the effects of IP_3 and cADPR antagonists on NAADP-induced Ca^{2+} oscillations were investigated. The involvement of IP_3 was probed with heparin, which inhibits IP_3 binding to its receptor (Taylor and Broad, 1998). NAADP induced oscillations in four of six experiments ($p = 0.20$) in the presence of 2.5 mg/ml heparin and in three of seven experiments ($p = 0.07$) in the presence of 5 mg/ml heparin (Figure 2). Heparin, however, inhibited the first Ca^{2+} oscillation after the NAADP-induced Ca^{2+} increase and shortened the duration of this Ca^{2+} transient (Figures 2 and 6B). The involvement of cADPR was probed with 8-amino-cADPR ($8NH_2$, Figure 6), which inhibits cADPR binding to its receptor (Walseth and Lee, 1993). The presence of 8-amino-cADPR (5 μM) did not block Ca^{2+} oscillations in six of six experiments (Figure 2, $p > 0.99$). Nevertheless, 8-amino-cADPR, as did heparin, decreased the amplitude and frequency of the Ca^{2+} oscillations (Figure 2), indicating a partially inhibitory effect. In contrast to the variable and partially inhibitory effects of either heparin or 8-amino-cADPR alone, the combination of both compounds eliminated NAADP-induced later Ca^{2+} oscillations in seven of seven experiments (Figure 2, $p < 0.0001$). In the presence of both inhibitors, NAADP released more Ca^{2+} than it did in the presence of either inhibitor alone (Figures 2 and 6A), possibly due to prevention of a basal leak of Ca^{2+} from a common store, which would lead to greater loading of this common store. These data demonstrate that both IP_3- and cADPR-mediated pathways for Ca^{2+} release are required for NAADP-induced Ca^{2+} oscillations.

That NAADP-induced Ca^{2+} oscillations could only be blocked completely by inhibition of both CICR pathways is similar to the findings reported for inhibition of fertilization-induced Ca^{2+} waves in sea urchin eggs (Galione *et al.*, 1993; Lee *et al.*, 1993), NAADP-induced Ca^{2+} release in starfish eggs (Santella *et al.*, 2000) and for amplification of NAADP-mediated Ca^{2+} waves in sea urchin eggs (Churchill and Galione, 2000). The situation is different from that in pancreatic acinar cells, where NAADP-induced Ca^{2+} oscillations can be blocked by inhibiting either the IP_3 or cADPR pathway (Cancela *et al.*, 1999). In the case of pancreatic acinar cells, the interaction was suggested to occur at the level of CICR, with Ca^{2+} released via the NAADP-mediated pathway serving as 'trigger Ca^{2+}', which feeds forward on to either IP_3 or ryanodine receptors thereby amplifying the response (Cancela *et al.*, 1999). Such a mechanism cannot explain the Ca^{2+} oscillations that occur after NAADP-induced Ca^{2+} mobilization because the NAADP receptors are completely desensitized to NAADP for at least 2 h after the initial release using this particular protocol (data not shown). Indeed, the first Ca^{2+} oscillation, which is sometimes distinct and at other times appears as a shoulder after the initial NAADP-mediated Ca^{2+} increase (Figure 1) (Aarhus *et al.*, 1996; Genazzani *et al.*, 1996; Lee, 1997), occurs during recovery from the NAADP-induced Ca^{2+} transient when Ca^{2+} is decreasing. This is at odds with a cytosol-based CICR mechanism, which would provide amplification during the increase in Ca^{2+}.

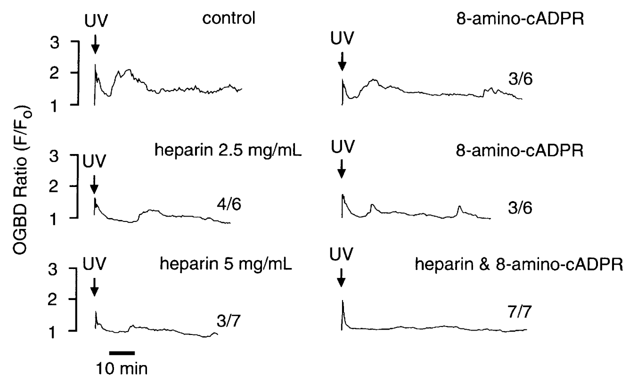

Fig. 2. Requirement of NAADP-induced Ca^{2+} oscillations on Ca^{2+} release pathways mediated by IP_3 and cADPR. The final intracellular concentrations were: Oregon Green 488 BAPTA Dextran, 10 μM; caged NAADP, 0.5 μM; heparin, 2.5 or 5 mg/ml; 8-amino-cADPR, 5 μM; and heparin and 8-amino-cADPR combined, 2.5 mg/ml and 5 μM, respectively. The number of similar responses relative to the total number of experiments is shown next to each trace. The positive control for NAADP-induced Ca^{2+} oscillations was from the same batch of eggs.

A Ca^{2+} store that is thapsigargin insensitive and NAADP sensitive is present in intact eggs

If NAADP-induced Ca^{2+} oscillations are due to overloading and spontaneous Ca^{2+} release from CICR stores, then Ca^{2+} would have to be moved into the CICR stores from an NAADP-sensitive Ca^{2+} store that is functionally and physically separate. The NAADP-sensitive Ca^{2+} store is pharmacologically and physically distinct in sea urchin egg homogenates (Lee and Aarhus, 1995; Genazzani and Galione, 1996). The presence of such a store in intact sea urchin eggs, however, was unknown until a recent report that demonstrated spatial separation of the NAADP-sensitive Ca^{2+} stores from the IP_3- and cADPR-sensitive Ca^{2+} stores in intact sea urchin eggs whose organelles were stratified by centrifugation (Lee and Aarhus, 2000). We used a pharmacological approach to determine whether NAADP-sensitive Ca^{2+} stores in intact sea urchin eggs were functionally distinct from IP_3- and cADPR-sensitive Ca^{2+} stores. Treatment of intact eggs with the Ca^{2+} pump inhibitor thapsigargin (2 μM) for 30 min or more completely eliminated the response to both IP_3 and cADPR (Figures 3A and 6A), as reported previously for homogenates (Genazzani and Galione, 1996). In contrast, thapsigargin reduced but did not eliminate the initial response to NAADP (Figures 3A and 6A). This result demonstrates that in the intact egg NAADP targets Ca^{2+} stores that are functionally separate from the CICR stores. This conclusion is consistent with that of Lee and Aarhus (2000), who demonstrated recently that the NAADP-sensitive store was separable from the endoplasmic reticulum and segregated with mitochondria. However, it is unlikely that mitochondria are the target for NAADP because NAADP-sensitive stores are distinct from cytochrome C oxidase, a mitochondrial marker, in fractionated sea urchin egg homogenates (Lee and Aarhus, 1995), and mitochondria are sinks not sources

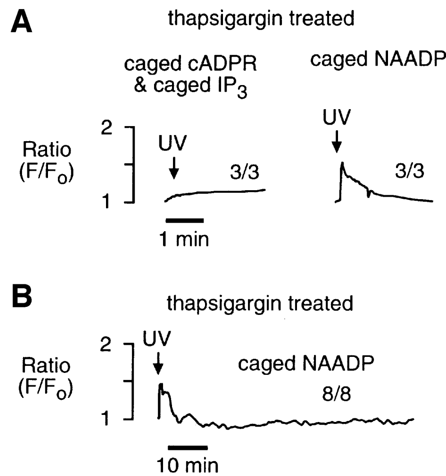

Fig. 3. NAADP-induced Ca^{2+} oscillations require thapsigargin-sensitive Ca^{2+} stores. (**A**) Effect of thapsigargin on the initial response to photorelease of an IP_3–cADPR mixture and NAADP. (**B**) Effect of thapsigargin on long-term NAADP-induced Ca^{2+} oscillations shown on a different time scale. Eggs were treated with thapsigargin (2 μM) for ⩾30 min and then exposed to UV. The final intracellular concentrations were: Oregon Green 488 BAPTA Dextran, 10 μM; caged NAADP, 0.5 μM, and both caged cADPR, 5 μM and caged IP_3, 5 μM.

for Ca^{2+} in non-fertilized eggs (Eisen and Reynolds, 1985). Therefore, NAADP probably targets an organelle that remains to be characterized.

NAADP-induced Ca^{2+} oscillations require thapsigargin-sensitive Ca^{2+} stores

Pretreatment with thapsigargin eliminated NAADP-induced Ca^{2+} oscillations in eight of eight experiments (Figure 3B; p <0.001 compared with the NAADP control), demonstrating a requirement for IP_3- and cADPR-sensitive Ca^{2+} stores. These data are consistent with the elimination of NAADP-induced Ca^{2+} oscillations obtained with heparin and 8-amino-cADPR (Figure 2), and consistent with a mechanism in which the CICR stores (IP_3- and cADPR-sensitive) play a role in the NAADP-induced Ca^{2+} oscillations.

CICR stores contain more Ca^{2+} after an NAADP-mediated Ca^{2+} transient

Next we directly assessed the effect of an NAADP-mediated Ca^{2+} release on the amount of Ca^{2+} in the CICR stores. As only the CICR stores are sensitive to thapsigargin (Genazzani and Galione, 1996 and Figure 3), the size of the Ca^{2+} transient induced by thapsigargin was used as a measure of CICR store loading. Figure 4 shows that thapsigargin (5 μM) induced a significantly (p = 0.01) larger Ca^{2+} transient after an NAADP-induced Ca^{2+} transient (maximum amplitude of 2.0 ± 0.27 ratio units, n = 10) than in control eggs (maximum amplitude of 1.3 ± 0.07 ratio units, n = 6). These data indicate that a substantial amount of the Ca^{2+} released by NAADP is taken up by the CICR stores. In contrast, the amplitude of Ca^{2+} release upon photorelease of NAADP was not increased after Ca^{2+} mobilization by either IP_3 (maximum amplitude of 2.2 ± 0.14 ratio units, t-test, p = 0.53) or both IP_3 and cADPR (maximum amplitude of 2.2 ± 0.13 ratio units, t-test, p = 0.62). The mechanism for the

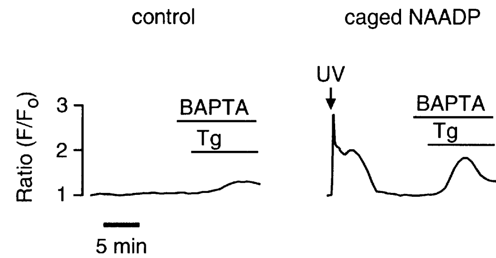

Fig. 4. Overloading of CICR stores in response to an NAADP-mediated Ca^{2+} release. The extracellular solution was changed from artificial sea water containing 10 mM Ca^{2+} to one containing no added Ca^{2+} and 2 mM BAPTA. Thapsigargin (5 μM) was added 2 min later. The final intracellular concentrations were: Oregon Green 488 BAPTA Dextran, 10 μM and caged NAADP, 0.5 μM.

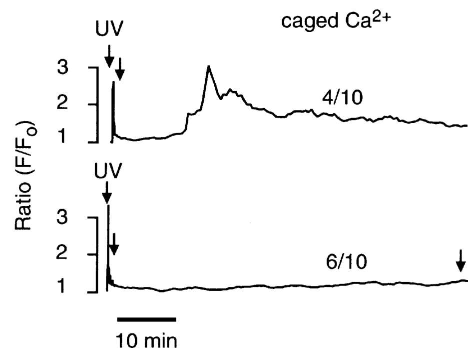

Fig. 5. Ca^{2+} oscillations induced by photorelease of Ca^{2+}. The final intracellular concentrations were: Oregon Green 488 BAPTA Dextran, 10 μM and NP-EGTA, 500 μM. The number of similar responses relative to the total number of experiments is shown next to each trace.

preferential accumulation of NAADP-released Ca^{2+} into the CICR stores is unknown. A possible mechanism could be faster removal of Ca^{2+} by thapsigargin-sensitive Ca^{2+} pumps than by thapsigargin-insensitive Ca^{2+} pumps.

Ca^{2+} oscillations can be induced by photorelease of Ca^{2+}

With a Ca^{2+}-overloading mechanism, any means of overloading Ca^{2+} stores should induce long-term Ca^{2+} oscillations (Berridge and Galione, 1988; Berridge, 1991). Photoreleasing Ca^{2+} (NP-EGTA) (Ellis-Davies and Kaplan, 1994) resulted in an increase equivalent to that induced by NAADP (Figures 5 and 6A), and induced long-term Ca^{2+} oscillations in four of 10 experiments (Figure 5). The pattern of the oscillations generated by photoreleasing Ca^{2+} (Figure 5) was similar but not identical to that generated by NAADP (Figure 1). The amount of caged Ca^{2+} remaining after the first period of photorelease was determined by two additional periods of photorelease, one immediately after recovery to basal levels and one 70 min after the first photorelease (Figure 5). These additional UV illuminations failed to increase Ca^{2+}, demonstrating that the first period of photorelease destroyed most of the NP-EGTA. The amount of Ca^{2+} can be approximated from this information. Given that NP-EGTA was at a concentration of 500 μM and that the resting Ca^{2+} (~100 nM) is approximately at the K_d (80 nM) for NP-EGTA (Ellis-Davies and Kaplan, 1994), there would be ~250 μM caged Ca^{2+} (and

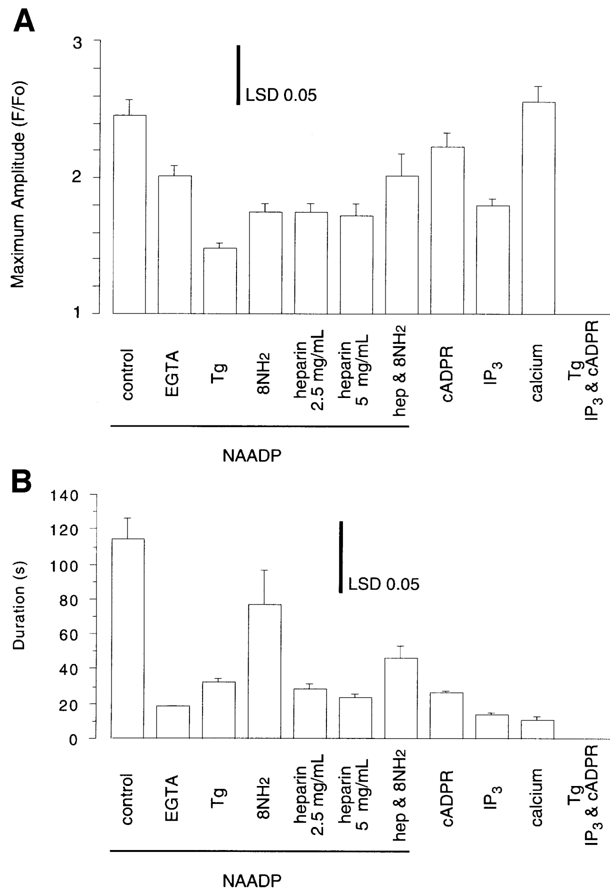

Fig. 6. Histograms of the maximal amplitude and duration of the Ca^{2+} transients elicited by the designated treatments. Maximal amplitude (**A**) was calculated as F/F_o and duration (**B**) was calculated as the time during which F/F_o remained above half the maximal amplitude. All data are from the Ca^{2+} transient elicited by photorelease of the messenger indicated. Histograms are the mean ± standard error of the mean for n = 3–29. The inset bar represents the least significant difference (LSD), which was calculated from the pooled error.

250 µM free NP-EGTA). Complete photorelease of the caged Ca^{2+} would liberate ~250 µM Ca^{2+}. Although an increase in Ca^{2+} is sufficient for inducing Ca^{2+} oscillations in sea urchin eggs about half the time, it does not induce Ca^{2+} oscillations as often as NAADP, and fails to induce the higher frequency component. Therefore, Ca^{2+} alone can partially, but not completely, mimic NAADP-induced Ca^{2+} oscillations.

Mechanism for NAADP-induced Ca^{2+} oscillations

The mechanism by which NAADP induces Ca^{2+} oscillations and a summary of the evidence for this mechanism are presented in Figure 7. The initial Ca^{2+} release mediated by NAADP is largely independent of any additional processes (pathway 1, Figure 7), except for an amplification by CICR pathways, as described previously (Churchill and Galione, 2000). A portion (approximately one-third) of the Ca^{2+} mobilized by NAADP comes from a thapsigargin-resistant store that is distinct from the CICR stores. This released Ca^{2+} is then taken up into CICR stores. Ca^{2+} uptake continues until the stores overload and spontaneously release Ca^{2+} as demonstrated previously (Galione *et al.*, 1991; Missiaen *et al.*, 1991). A necessary feature of this mechanism is that the released Ca^{2+} is taken up into thapsigargin-sensitive stores more quickly than into thapsigargin-insensitive (NAADP-sensitive) stores, but how this is accomplished is unknown. The first Ca^{2+} oscillation after the NAADP-induced transient requires IP_3-dependent Ca^{2+} release (pathway 2, Figure 7). Both IP_3- and cADPR-sensitive Ca^{2+} stores participate in the later cycles of Ca^{2+} uptake, overload and release (pathway 3, Figure 7). This Ca^{2+}-overloading mechanism can also explain the occasional induction of long-term Ca^{2+} oscillations by IP_3 (Figure 1 and Swann and Whitaker, 1986) and cADPR (Figure 1), in which overloading would occur via Ca^{2+} influx. This mechanism meets the criteria of a two-pool model for Ca^{2+} oscillations (Berridge, 1988; Berridge and Galione, 1988; Tsien and Tsien, 1990) because one Ca^{2+} store is the source of priming Ca^{2+} (NAADP sensitive) and a separate Ca^{2+} store mediates the oscillations (CICR), and oscillations can be triggered by any increase in Ca^{2+} that primes the CICR stores. Therefore, this mechanism represents the first direct demonstration of the two-pool model for Ca^{2+} oscillations.

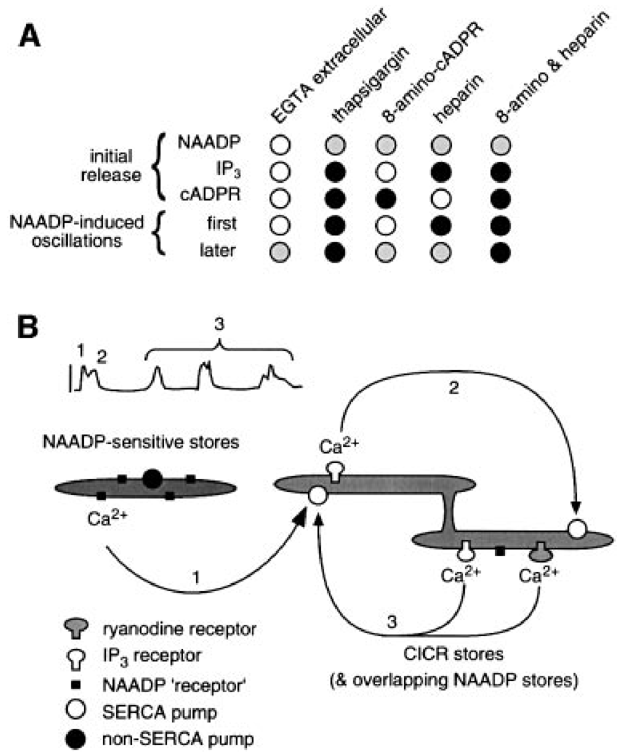

Fig. 7. NAADP induces Ca^{2+} oscillations by priming CICR stores. (**A**) The effect of each treatment is designated as having no effect (open circle), partial inhibition (grey circle) and complete inhibition (black circle). (**B**) Cartoon of the proposed mechanism. NAADP releases Ca^{2+} from a functionally separate Ca^{2+} store (designated 1). This Ca^{2+} is taken up into the CICR stores, thereby priming them. This results in overloading and spontaneous Ca^{2+} release initially from IP_3-sensitive Ca^{2+} stores (designated 2). Subsequently, Ca^{2+} is taken up into and released from both IP_3- and cADPR-sensitive Ca^{2+} stores (designated 3).

Materials and methods

Microinjection and Ca^{2+} imaging were performed as described previously (Churchill and Galione, 2000). Briefly, sea urchin (*Lytechinus pictus*) eggs (Marinus, Long Beach, CA) were obtained by intracoelomic injection of 0.5 M KCl, shed into artificial sea water (in mM, NaCl 435,

$MgCl_2$ 40, $MgSO_4$ 15, $CaCl_2$ 11, KCl 10, $NaHCO_3$ 2.5, EDTA 1), dejellied by passing through 90 μm nylon mesh, and then washed twice by centrifugation. Eggs were transferred to polylysine-coated glass coverslips, pressure microinjected (Picospritzer, World Precision Instruments) with Oregon Green 488 BAPTA Dextran (Molecular Probes) and caged compounds and/or inhibitors. The Ca^{2+}-sensitive dye was imaged by laser-scanning confocal microscopy (TCS NT, Leica) and caged compounds were photolysed with an ultraviolet laser. Images were processed with the software NIH Image to create a self-ratio by dividing each image by an image acquired before stimulation. Where appropriate, parametric data were subjected to analysis of variance with means separated by Fisher's least significant difference test with significance taken as $p \leq 0.05$. Proportions (e.g. number of eggs exhibiting oscillations versus number not) were analysed by creating a 2×2 contingency table, with NAADP compared with another treatment and then subjected to Fisher's exact probability test.

Acknowledgements

We thank F.Boittin, R.Masgrau, Y.Okada, S.Patel and J.Thomas for helpful discussion. This work was funded by the Wellcome Trust.

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Received February 13, 2001; revised and accepted March 29, 2001