

Kinetic Properties of Nicotinic Acid Adenine Dinucleotide Phosphate-induced Ca^{2+} Release*

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Three endogenous molecules have now been shown to release Ca^{2+} in the sea urchin egg: inositol trisphosphate (InsP_3), cyclic adenosine 5'-diphosphate ribose (cADPR), and nicotinic acid adenine dinucleotide phosphate (NAADP), a derivative of NADP. While the mechanism through which the first two molecules are able to release Ca^{2+} is established and well characterized with InsP_3 and cADPR-activating InsP_3 and ryanodine receptors, respectively, the newly described NAADP has been shown to release Ca^{2+} via an entirely different mechanism. The most striking feature of this novel Ca^{2+} release mechanism is its inactivation, since subthreshold concentrations of NAADP are able to fully and irreversibly desensitize the channel. In the present study we have investigated the fast kinetics of activation and inactivation of NAADP-induced Ca^{2+} release. NAADP was found to release Ca^{2+} in a biphasic manner, and such release was preceded by a pronounced latent period, which was inversely dependent on concentration. Moreover, the kinetic features of NAADP-induced Ca^{2+} release were not altered by pretreatment with low concentrations of NAADP, although the extent of Ca^{2+} release was greatly affected. Our data suggest that the inactivation of NAADP-induced Ca^{2+} release is an all-or-none phenomenon, and while some receptors have been fully inactivated, those that remain sensitive to NAADP do so without any change in kinetic features.

Intracellular Ca^{2+} stores in the sea urchin egg express both inositol trisphosphate (InsP_3)-sensitive Ca^{2+} channels (1) and ryanodine-sensitive Ca^{2+} channels (RyRs). The latter are activated by a pyridine nucleotide metabolite, cyclic adenosine 5'-diphosphate ribose (cADPR) (2). Recently, a third distinct Ca^{2+} release mechanism has been identified that is potently and selectively activated by a different pyridine nucleotide, nicotinic acid adenine dinucleotide phosphate (NAADP) (3, 4). NAADP-induced Ca^{2+} release can be induced both in the intact egg by microinjection and from microsomes of egg homogenates (4–6). Ca^{2+} release induced by NAADP is not blocked by hep-

arin, a selective inhibitor of InsP_3 receptors, nor by 8-amino-cADPR, a selective inhibitor of the cADPR-sensitive Ca^{2+} release (3, 4). Moreover, NAADP does not cross-desensitize with either InsP_3 nor cADPR, showing that it induces Ca^{2+} release via a mechanism independent of InsP_3 Rs and RyRs (3, 4, 6). This is also supported by the distinct pharmacological properties of the NAADP-sensitive mechanism. For example, NAADP-induced Ca^{2+} release is blocked by L-type Ca^{2+} channel blockers such as nifedipine and diltiazem, which have no effect on either of the other two Ca^{2+} release mechanisms in the sea urchin egg homogenates (6). The NAADP-sensitive Ca^{2+} store, unlike cADPR- and InsP_3 -sensitive stores, is insensitive to thapsigargin and cyclopiazoic acid, suggesting that the NAADP-sensitive Ca^{2+} release mechanism resides on a distinct intracellular organelle (7).

A unique feature of the NAADP-induced Ca^{2+} release is that it can be selectively inactivated by subthreshold concentrations of this molecule, which *per se* do not cause Ca^{2+} release (6, 8). The mechanism of this unusual form of desensitization is unclear. However, the binding of NAADP cannot be displaced when microsomes are pretreated with [³²P]NAADP and then challenged with a high concentration of NAADP after 3 min (8), perhaps indicating that treatment with nonstimulating concentrations of NAADP results in a conformational change of the receptor occluding further association and dissociation of NAADP binding.

Recent reports have indicated that mammalian cells possess the metabolic machinery to regulate intracellular levels of NAADP and therefore possibly utilize it as an intracellular messenger. NAADP can be synthesized in cell extracts from β -NADP in the presence of nicotinic acid in various rat tissues, including brain, liver, and spleen (9). Furthermore, it has been shown that ADP-ribosyl cyclase, the enzyme responsible for the cyclization of NAD^+ to cADPR, and CD38, a lymphocyte differentiation antigen, can also synthesize NAADP (10). The regulation of this class of enzymes is still unclear, but it appears that NAADP is synthesized in the presence of β -NADP and nicotinic acid at more acidic pH, while cADPR is produced in the presence of β -NAD at more neutral pH values (10).

In the present study we have investigated the transient kinetics of the NAADP-induced Ca^{2+} release mechanism in the sea urchin egg homogenates and compared it with the transient kinetics of the InsP_3 -sensitive and cADPR-sensitive Ca^{2+} release. Such approach can lead to further understanding of the similarities and differences between these release mechanisms and to provide information on the activation and inactivation mechanisms of NAADP-induced Ca^{2+} release. NAADP-induced Ca^{2+} release is composed of a fast and a slow phase, which can be described by two rate constants. Similar biexponential equations can also fit InsP_3 -induced and cADPR-induced Ca^{2+} release. NAADP triggers Ca^{2+} release after a pro-

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[¶] The abbreviations used are: InsP_3 , inositol trisphosphate; RyRs, ryanodine-sensitive Ca^{2+} channels; InsP_3 Rs, InsP_3 -sensitive Ca^{2+} channels; cADPR, cyclic adenosine 5'-diphosphate ribose; NAADP, nicotinic acid adenine dinucleotide phosphate.

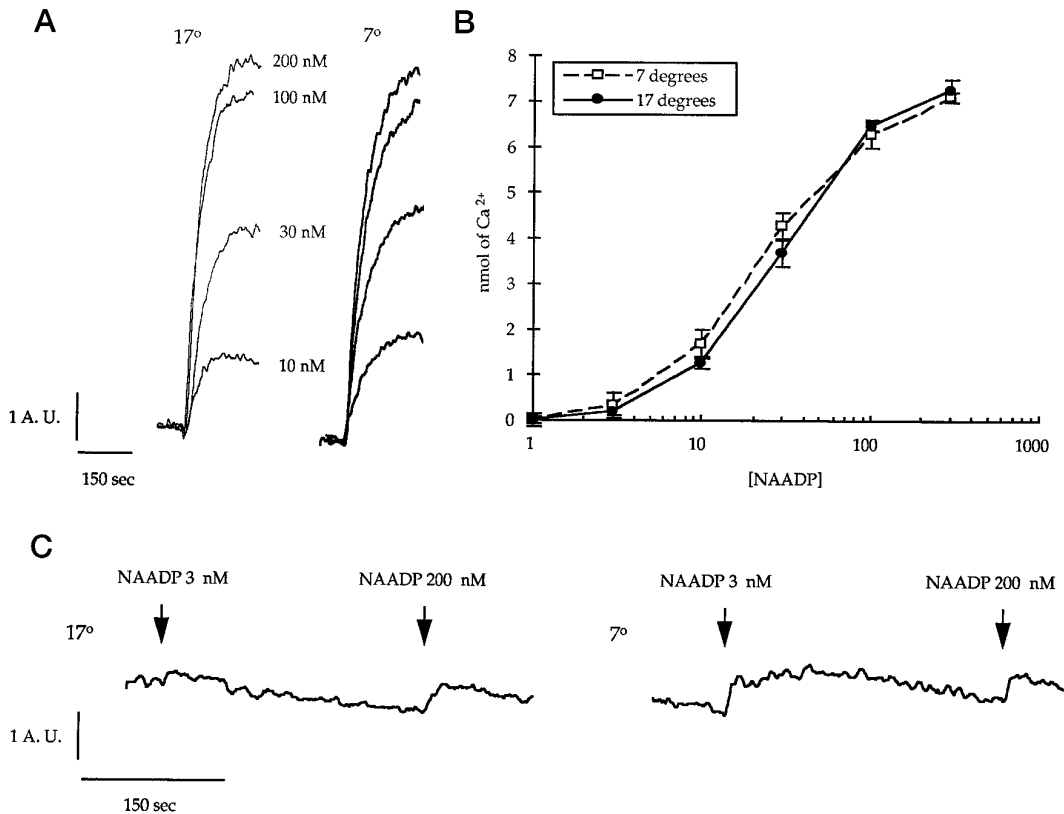


FIG. 1. *A*, representative fluorimetric traces of NAADP-induced Ca^{2+} release in sea urchin egg homogenates at 17 and 7 °C. *B*, concentration response curves of NAADP-induced Ca^{2+} release at 17 and 7 °C. Values are mean \pm S.E. of six to nine determinations from two to three separate experiments. *C*, desensitization of the NAADP-induced Ca^{2+} release at 17 and 7 °C. NAADP, 3 nM, was added 3 min prior to NAADP, 200 nM. A.U. = arbitrary fluorescence units.

nounced latency inversely dependent on the concentration of NAADP, while no latency is present before InsP_3 - and cADPR-induced Ca^{2+} release. Desensitization by subthreshold concentrations of NAADP does not affect either of the rate constants, but affects the magnitude of release. This finding suggests that NAADP desensitization proceeds in an all-or-none manner, with inactivation affecting a subpopulation of receptors while others are unaffected.

MATERIALS AND METHODS

Collection of Sea Urchin Eggs—Eggs were obtained by stimulating ovulation of female *Lytechinus pictus* (Marinus, Inc., Long Beach, CA) with an intracoelomic injection of KCl. These were then washed twice in artificial sea water (NaCl, 435 mM; MgCl_2 , 40 mM; MgSO_4 , 15 mM; CaCl_2 , 11 mM; KCl, 10 mM; NaHCO_3 , 2.5 mM; EDTA, 1.0 mM at pH 8.0) and jelly removed by filtration through 90- μm nylon mesh.

Ca^{2+} Release Assays—Homogenates (2.5%) of unfertilized *Lytechinus pictus* eggs were prepared as described previously (11), and Ca^{2+} loading was achieved by incubation at room temperature for 3 h in an intracellular medium (IM) consisting of potassium gluconate, 250 mM; *N*-methylglucamine, 250 mM; Hepes, 20 mM (pH 7.2); MgCl_2 , 1 mM; ATP, 0.5 mM; phosphocreatine, 10 mM; creatine phosphokinase, 10 units/ml; oligomycin, 1 mg/ml; antimycin, 1 mg/ml; sodium azide, 1 mM; fluo-3, 3 mM. Free Ca^{2+} concentration was measured by monitoring fluorescence intensity at excitation and emission wavelengths of 490 and 535 nm, respectively. Fluorimetry was performed at 17 °C using 500 μl of homogenate in a Perkin-Elmer LS-50B fluorimeter. Additions were made in 5- μl volume, 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.

Preparation of Purified Microsomes and Supernatant Fractions—Microsomes were purified from 50% egg homogenates by the Percoll density centrifugation method described previously (11). Briefly, a fractionating buffer was prepared by diluting Percoll stock to 25% in a

modified intracellular medium (333 mM *N*-methylglucamine, 333 mM potassium acetate, 27 mM Hepes, 1.3 mM MgCl_2 , pH titrated to 7.2 with acetic acid). This was then supplemented with 0.5 mM ATP, 2 units/ml creatine phosphokinase, 4 mM phosphocreatine, 10 μM EGTA. 1 ml of 50% egg homogenate was layered on 9 ml of this solution and centrifuged at $27,000 \times g$ for 30 min at 15 °C. The top fraction (1 ml) was collected and represented the supernatant fraction. The Ca^{2+} -storing, cADPR- and InsP_3 -sensitive microsomes formed a distinct tight band half way down the tube. 1 ml of this was collected using a disposable syringe to puncture the vessel wall. The fractions were aliquoted and stored at -70 °C until use.

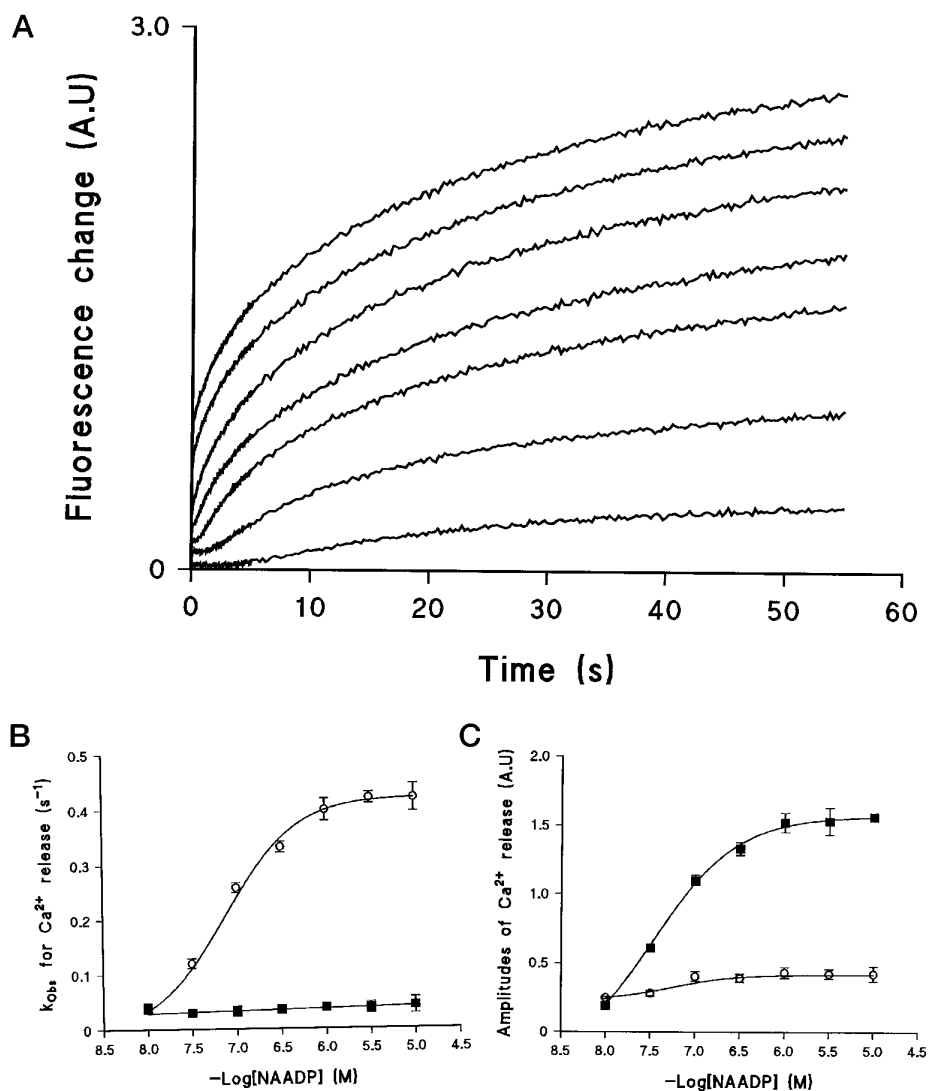
Stopped-flow Measurements—Rapid kinetics of Ca^{2+} release by InsP_3 , cADPR, and NAADP were carried out on 2.5% homogenate prepared as described above. Stopped-flow measurements were performed as described elsewhere (12–14). Ca^{2+} -loaded homogenate was introduced in a 2.5-ml syringe of a stopped-flow fluorimeter (Applied Photophysics, model SX17 MV), while a 250- μl syringe was filled with either IM, InsP_3 , 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 with an 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 six acquisitions.

The averaged traces were then analyzed using nonlinear 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 using the following equation,

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

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 (seconds). The amplitudes A_1 and A_2 are expressed in arbitrary units and relate to the fluorescence intensity changes of fluo-3. Since the

FIG. 2. Kinetics of NAADP-induced Ca^{2+} release. The top panel shows representative stopped-flow fluorimetric traces, which have been shifted upwards to show the latent period by lower concentrations of NAADP. NAADP concentrations used from top to bottom were: 10 μM , 3 μM , 1 μM , 300 nM, 100 nM, 30 nM, 10 nM. Traces were recorded with a 200- μs filter. The bottom panels show the rate constants and amplitudes of the slow (filled squares) and fast (open circles) phases of Ca^{2+} release. A.U. = arbitrary fluorescence units.



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 prior to 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 were also quantified, and the rate constants of the uptake were essentially negligible compared with Ca^{2+} release by either InsP_3 , cADPR, or NAADP. In fact, the rate constant for Ca^{2+} was $0.0008 \pm 0.000004 \text{ s}^{-1}$, while the rate constants observed for Ca^{2+} release were at least 30 times faster.

Materials—NAADP was either purchased from Research Biochemicals International (St. Albans, United Kingdom (UK)) or was a kind gift of Prof. T. F. Walseth (University of Minnesota), fluo-3 was from Molecular Probes (Cambridge, UK). All other chemicals were from Sigma (Poole, UK).

RESULTS AND DISCUSSION

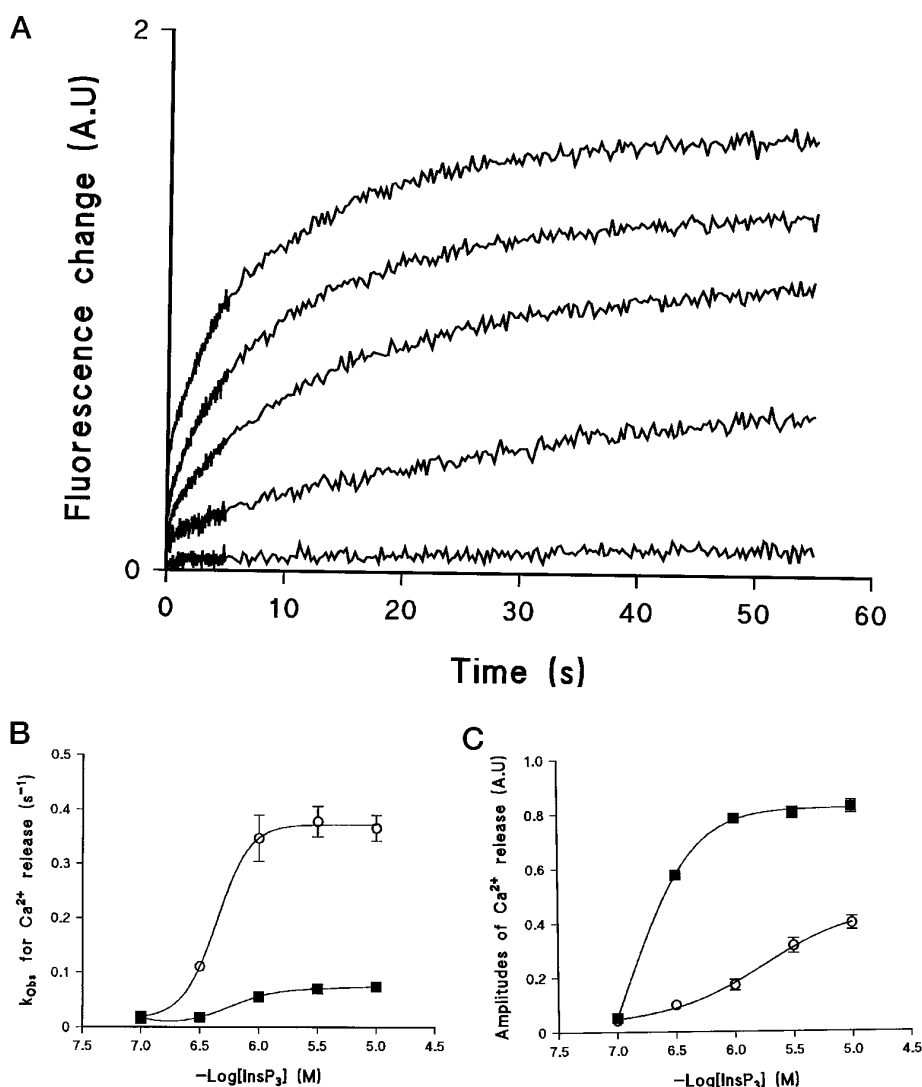
NAADP released Ca^{2+} dose-dependently with an EC_{50} of $25 \pm 5 \text{ nM}$ (Fig. 1, A and B). Furthermore, NAADP, 3 nM, which *per se* induced only a negligible release, was able to desensitize the mechanism to a subsequent addition of a maximal concentration of NAADP (200 nM) (Fig. 1C; see also Refs. 6 and 8). Performing experiments at 7 $^{\circ}\text{C}$ did not alter significantly the extent of Ca^{2+} release nor the extent of inactivation (Fig. 1).

If quantal release, first described for the InsP_3 -induced Ca^{2+} release, is defined by the observation that submaximal concentrations of mobilizing agent can rapidly release Ca^{2+} from the Ca^{2+} stores without affecting their ability to respond to further maximal concentrations of the same agent (15–17) (incremen-

tal detection), NAADP does not release Ca^{2+} in a quantal manner. It therefore appears either quantal Ca^{2+} release, which has been also described for the ryanodine receptors (18), is not central in the operation of all intracellular Ca^{2+} channels or that incremental detection is not necessarily associated with quantal release.

To investigate the fast kinetics of the Ca^{2+} fluxes activated by NAADP, InsP_3 , or cADPR, we have employed stopped-flow analysis, with a temporal resolution of 25 ms. In the present experiments Ca^{2+} pump inhibitors have not been used for two reasons: (i) the rate of Ca^{2+} uptake in our preparations was at least 30 times slower than the slower Ca^{2+} release parameter examined and therefore negligible (see also “Materials and Methods”); (ii) NAADP-induced Ca^{2+} release is known to be affected differently from cADPR and InsP_3 by Ca^{2+} uptake inhibitors (*e.g.* it is insensitive to thapsigargin and cyclopiazonic acid) (7). When NAADP, InsP_3 , or cADPR were added to the homogenate, it resulted in an increased fluorescence signal representing Ca^{2+} release (Figs. 2–4). Supramaximal concentrations of NAADP (1 μM) (6) released Ca^{2+} with a $t_{1/2}$ of $11.2 \pm 1.0 \text{ s}$. Supramaximal concentrations of cADPR (3 μM) (6) released Ca^{2+} with a $t_{1/2}$ of $6.5 \pm 0.9 \text{ s}$. In comparison, addition of maximal InsP_3 concentrations (10 μM) (6) resulted in a more rapid Ca^{2+} release, with a $t_{1/2}$ of $2.7 \pm 0.8 \text{ s}$. InsP_3 -induced Ca^{2+} release appears slower to that observed in cerebellar microsomes, where maximal InsP_3 concentrations release Ca^{2+} with

FIG. 3. Kinetics of InsP_3 -induced Ca^{2+} release. The top panel shows representative stopped-flow fluorimetric traces, which have been shifted upwards to show the absence of a latent period by lower concentrations of InsP_3 . Concentrations of InsP_3 used from top to bottom were: 10 μM , 3 μM , 1 μM , 300 nM, 100 nM. The bottom panels show the rate constants and amplitudes of the slow (filled squares) and fast (open circles) phases of Ca^{2+} release. A.U. = arbitrary fluorescence units.



a $t_{1/2}$ of less than a second (14). This difference may well reflect the relative abundance of InsP_3 -sensitive channels in the two systems.

An important and consistent feature of the NAADP-induced Ca^{2+} release was that low concentrations of NAADP did not release Ca^{2+} instantly, but rather a pronounced latency period was observed prior to release (Figs. 2 and 5). This latent period had a duration of up to several seconds and was found to be inversely dependent on the concentration of NAADP used (Fig. 5). However, at higher NAADP concentrations (3 and 10 μM), Ca^{2+} release was not preceded by a detectable latency (Figs. 2 and 5). In contrast, no lag phase was observed with any concentration of InsP_3 (Fig. 3). This is in agreement with most reports on the fast kinetics of the InsP_3 receptor (14) and consistent with direct gating of InsP_3Rs by InsP_3 , although a lag phase has been reported for InsP_3 -induced Ca^{2+} release in rat basophilic leukemia cells (13). cADPR was also not preceded by a lag phase. To investigate whether this lag phase was due to the conversion of NAADP to an active metabolite, stopped-flow experiments were performed at 7 $^\circ\text{C}$. If an enzymatic conversion was responsible for the latency, then lowering the temperature should prolong the lag phase. The lag phases at both 7 and 17 $^\circ\text{C}$ did not markedly differ at all concentrations tested (Fig. 4). Moreover, NAADP was able to release Ca^{2+} to the same extent in both conditions (Fig. 1). It therefore seems unlikely that an enzymatic conversion is responsible for the observed latency and that Ca^{2+} pumps affect release in this

system.

The marked lag phase observed could be dependent on the requirement of a modulatory protein to bind the NAADP receptor. To investigate this possibility, fluorimetric determinations of NAADP-induced Ca^{2+} release under various conditions were performed. In Percoll-purified microsomes, which should lack soluble cytosolic proteins, NAADP still released Ca^{2+} , although to a minor extent in respect to the 2.5% homogenate. Unlike cADPR -sensitive Ca^{2+} stores, NAADP-sensitive Ca^{2+} stores are not all contained in this microsomal fraction (4), and the minor extent of release observed is most likely due to this observation. Addition of calmodulin (1 μM) did not affect NAADP-induced Ca^{2+} release in microsomes, in contrast to the potentiating effect that it has on cADPR -induced Ca^{2+} release (19). Addition of FK506 (10 μM) and rapamycin (10 μM), which are known to affect Ca^{2+} -releasing mechanisms by inhibiting modulatory proteins associated with RyRs and InsP_3Rs (20), did not affect NAADP-induced Ca^{2+} release significantly. Moreover, the reconstitution of the preparation with 2.5% supernatant, only slightly enhanced the total amount of Ca^{2+} released by NAADP ($120 \pm 12\%$ compared with controls). Ca^{2+} release by NAADP is also unaffected by preincubation of 2.5% homogenate with cholesteryl hemisuccinate (5 mM), an agent which rigidifies membranes and slows down protein movement in the membrane (21, 22). Taken together, these observations suggest that modulatory proteins are unlikely to be involved in the opening of the channel, although the possibility of a closely

FIG. 4. **Kinetics of cADPR-induced Ca^{2+} release.** The top panel shows representative stopped-flow fluorimetric traces. The bottom panel shows the rate of the slow (filled squares) and fast (open circles) phases of Ca^{2+} release. Below 50 nM cADPR Ca^{2+} release traces could not be fitted to any standard mathematical fit routinely employed (see "Results and Discussion"). A.U. = arbitrary fluorescence units.

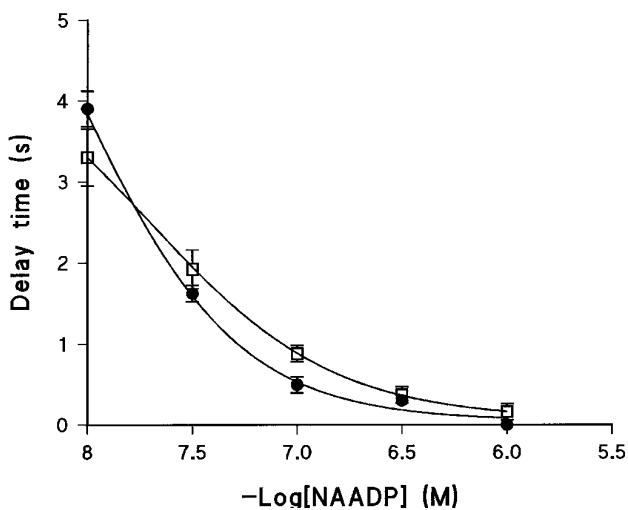
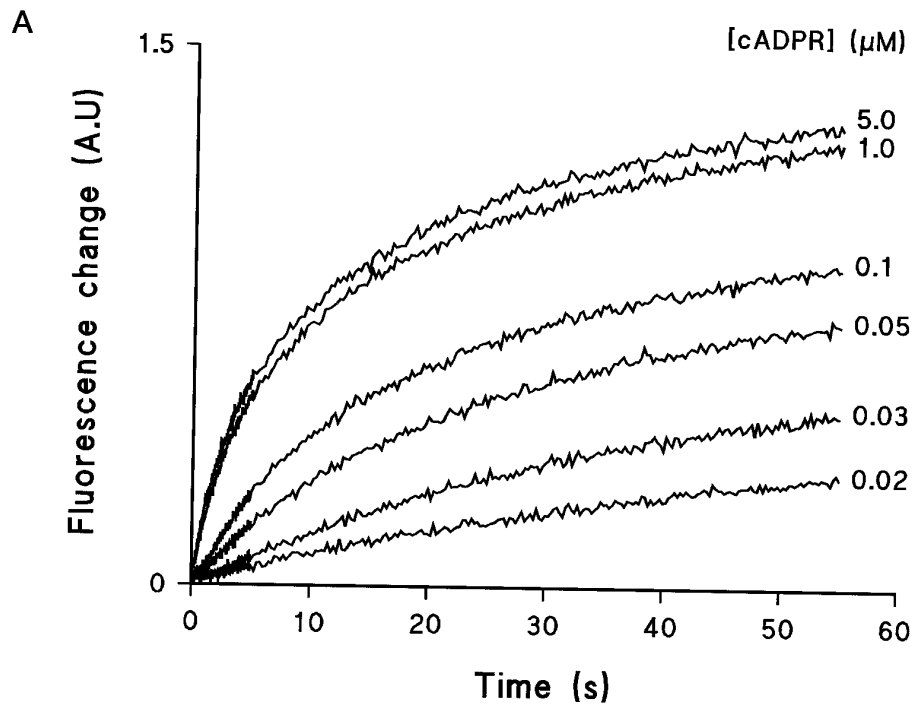


FIG. 5. **Latencies preceding NAADP-induced Ca^{2+} release at 17°C (filled circles) and 7°C (open squares).** Values from one representative experiment ($n = 8$) out of three performed.

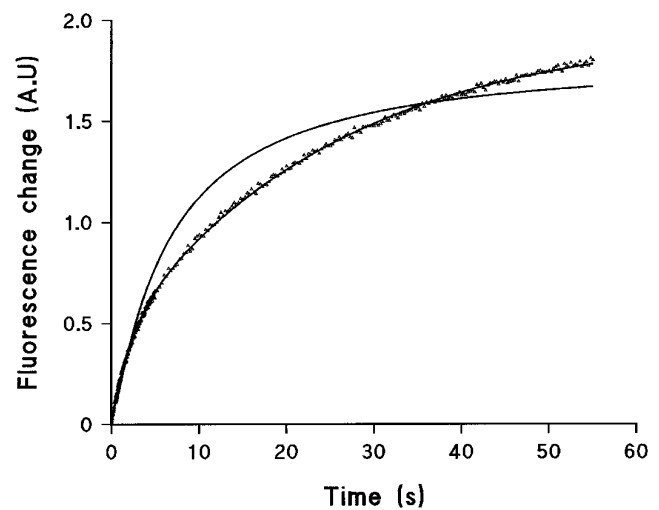


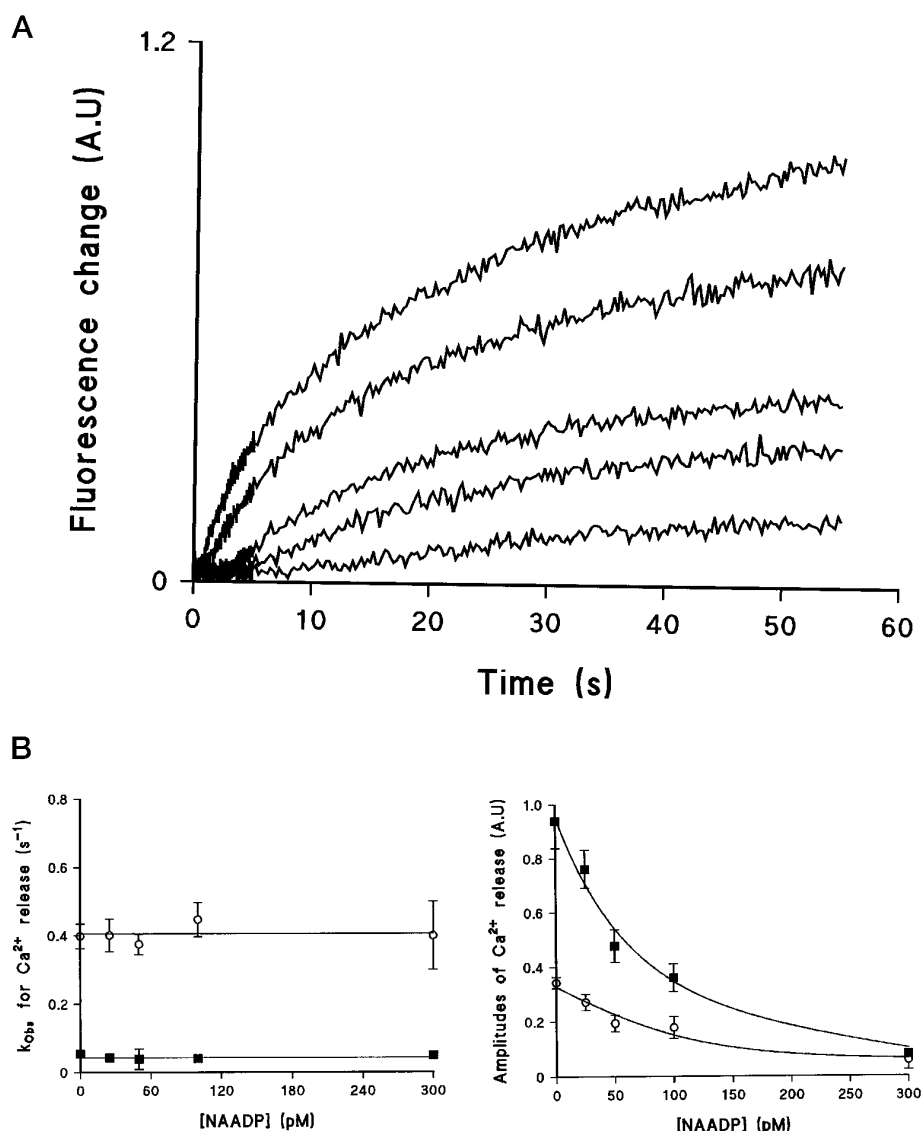
FIG. 6. **Best possible fitting of NAADP-induced Ca^{2+} release by monoexponential and biexponential equations** (see "Materials and Methods"). Similar good fits with biexponential equations were obtained with InsP_3 -induced Ca^{2+} release.

associated uncharacterized modulatory protein cannot presently be ruled out. The observed lag phase could also be due to the presence of one or more slow temperature-insensitive lim-

iting steps prior to channel opening.

NAADP-induced Ca^{2+} release was best fitted to a biexponential function (see Fig. 6 for an example) as defined by the

FIG. 7. Kinetics of the inactivation of NAADP-induced Ca^{2+} release by minute concentrations of NAADP. The top panel shows representative stopped-flow fluorimetric traces. Homogenates were pretreated for 15 min with picomolar concentrations of NAADP, placed in the stopped-flow syringe, and challenged with NAADP, 200 nM. Pretreatment NAADP concentrations were from top to bottom: 0, 25 pM, 50 pM, 100 pM, 300 pM. The bottom panels show the rate constants and amplitudes of the slow (filled squares) and fast (open circles) phases of Ca^{2+} release. A.U. = arbitrary fluorescence units.



equation given above as were InsP_3 - and cADPR-induced Ca^{2+} release. A monoexponential process proved to be completely unsatisfactory in all three cases. On the other hand, the Ca^{2+} release process that followed addition of low concentrations of cADPR (below 50 nM) could not be fitted to any standard mathematical fit routinely employed. The pattern of Ca^{2+} release induced by low concentrations of cADPR appeared sigmoidal, with the rate of release slowly increasing throughout the first few seconds of release. This can be explained by the regulatory role that cADPR plays on Ca^{2+} -induced Ca^{2+} release (2). It is possible that at low concentrations of cADPR, the early events of Ca^{2+} release potentiate further Ca^{2+} release. Rate constants for both the fast and slow phases of Ca^{2+} release induced by NAADP, InsP_3 , and cADPR were comparable in value at saturating concentrations of agonists used (for 10 μM NAADP the fast rate constant was 0.42 s^{-1} and the slow rate constant was 0.04 s^{-1} ; for 10 μM InsP_3 the fast and slow rate constants were 0.40 and 0.08 s^{-1} , respectively, and for 5 μM cADPR the fast and slow rate constants were 0.45 and 0.05 s^{-1} , respectively; Figs. 2–4). The InsP_3 - and cADPR-induced Ca^{2+} release rate constants increased with concentration reaching a maximum at around 1 μM (Figs. 3 and 4). The increases in the rate constants appear to closely follow the trend in increase in amplitude. Unlike in cerebellar microsomes (14), the slow rate constant for InsP_3 was responsible for

most Ca^{2+} release. This was also observed for NAADP-induced Ca^{2+} release (Fig. 2). However, for NAADP-induced Ca^{2+} release, the slow rate constant was concentration-insensitive, although the extent of Ca^{2+} release by this phase increased steadily with increasing concentrations of NAADP. This finding is difficult to rationalize. The most likely explanation is that a fine balance exists between the activation and inactivation of this receptor. Since the EC_{50} for inactivation is ~ 100 -fold lower than for activation, at low concentrations a high proportion of receptors will be desensitized without being activated. Increasing concentrations of NAADP will increase the probability that the receptors will activate before inactivating and increase the amplitude of release. As shown in Fig. 7 (for text see below), partial inactivation modifies the amplitude but not the kinetic parameters and therefore this model is plausible. In contrast, the fast rate constant increased with increasing concentrations of NAADP, but the extent of Ca^{2+} release appeared to plateau at 100 nM and contributed no more than 25% of the overall release. Experiments conducted at 7 $^{\circ}\text{C}$ did not substantially alter NAADP-induced Ca^{2+} release, although the slow rate constant decreased by approximately 30% (data not shown). The presence of two kinetic components can suggest that NAADP releases Ca^{2+} from two populations of Ca^{2+} stores with different Ca^{2+} -accumulating capacities, which would account for the two phases. Another hypothesis is that in

our homogenate preparation the majority of vesicles sensitive to NAADP have a low proportion of NAADP channels, which account for the slow phase of Ca²⁺ release, but a few vesicles contain a high proportion of NAADP receptors. With increasing concentrations of NAADP a higher proportion of receptors on this latter population of vesicles is activated and therefore Ca²⁺ fluxes are faster.

The cooperativity of NAADP-induced Ca²⁺ release was assessed for the fast rate constants and found to have a Hill coefficient of 1.1, suggesting that either only one binding site is involved in Ca²⁺ release, or that if multiple sites are present they act independently of each other in influencing Ca²⁺ channel activity. In comparison, it has been demonstrated previously that cADPR-induced Ca²⁺ release in sea urchin eggs is a cooperative process with a Hill coefficient of approximately 1.8 (23).

We then examined the kinetic properties of NAADP-induced Ca²⁺ release after partial desensitization with lower concentrations of NAADP. When this was performed, the magnitude of Ca²⁺ release by maximal concentrations of NAADP was reduced in a concentration-dependent manner (Fig. 7). Interestingly, both the fast and slow rate constants were unaffected by the pretreatment with low desensitizing concentrations of NAADP (Fig. 7). One possibility is that inactivation occurs in an all-or-none manner, with submaximal inactivating concentrations of NAADP causing the full inactivation of a proportion of receptors. Increasing the magnitude of the pretreatment, this proportion of inactivated receptors would progressively increase. In such a way, while a proportion of the receptors has been shifted to the desensitized form by the pretreatment, the active ones preserved the kinetic characteristics expressed by the control.

In conclusion, NAADP-induced Ca²⁺ release, although distinct, shows some common kinetic features with the established Ca²⁺-releasing molecule InsP₃. Both release Ca²⁺ in a biphasic manner and with similar rate constants. In both cases

the slow phase of release is responsible for the majority of Ca²⁺ release. NAADP, unlike InsP₃, releases Ca²⁺ after a latency. Furthermore, NAADP-induced Ca²⁺ release appears not to be cooperative. Moreover, NAADP inactivation is most likely an intrinsic all-or-none property of the receptor. In fact, partial inactivation does not alter the kinetic properties of the release, although it alters the amplitude, which suggests that the number of receptors responding has diminished but the active receptors have not been altered by pretreatment.

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REFERENCES

1. Clapper, D. L. & Lee, H. C. (1985) *J. Biol. Chem.* **260**, 13947–13954
2. Galione, A., Lee, H. C. & Busa, W. B. (1991) *Science* **253**, 1143–1146
3. Chini, E. N., Beers, K. W. & Dousa, T. P. (1995) *J. Biol. Chem.* **270**, 3216–3223
4. Lee, H. C. & Aarhus, R. (1995) *J. Biol. Chem.* **270**, 2152–2157
5. Perez-Terzic, C. M., Chini, E. N., Shen, S. S., Dousa, T. P. & Clapham, D. E. (1995) *Biochem. J.* **312**, 955–959
6. Genazzani, A. A., Empson, R. M. & Galione, A. (1996) *J. Biol. Chem.* **271**, 11599–11602
7. Genazzani, A. A. & Galione, A. (1996) *Biochem. J.* **315**, 721–725
8. Aarhus, R., Dickey, D. M., Graeff, R. M., Gee, K. R., Walseth, T. F., and Lee, H. C. (1996) *J. Biol. Chem.* **271**, 8513–8516
9. Chini, E. N. & Dousa, T. P. (1995) *Biochem. Biophys. Res. Commun.* **209**, 167–174
10. Aarhus, R., Graeff, R. M., Dickey, D. M., Walseth, T. F. & Lee, H. C. (1995) *J. Biol. Chem.* **270**, 30327–30333
11. Dargie, P. J., Agre, M. C. & Lee, H. C. (1990) *Cell Regul.* **1**, 279–290
12. Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orłowski, S., and Claret, M. (1988) *J. Biol. Chem.* **264**, 17665–17673
13. Meyer, T., Wensel, T. & Stryer, L. (1990) *Biochemistry* **29**, 32–37
14. Mezna, M. & Michelangeli, F. (1995) *J. Biol. Chem.* **270**, 28097–28102
15. Bootman, M. (1994) *Curr. Biol.* **4**, 169–172
16. Muallem, S., Pandol, S. J. & Beeker, T. G. (1989) *J. Biol. Chem.* **264**, 205–212
17. Combettes, L., Cheek, T. R. & Taylor, C. W. (1996) *EMBO J.* **15**, 2086–2093
18. Cheek, T. R., Moreton, R. B., Berridge, M. J., Stauderman, K. A., Murawsky, M. M., and Bootman, M. D. (1993) *J. Biol. Chem.* **268**, 27076–27083
19. Lee, H. C., Aarhus, R. & Graeff, R. M. (1995) *J. Biol. Chem.* **270**, 9060–9066
20. Jayaraman, T., Brillantes, A.-M., Timerman, A. P., Fleischer, S., Erdjument-Bromage, H., Tempst, P., and Marks, A. R. (1992) *J. Biol. Chem.* **267**, 9474–9477
21. Lazar, D. F. & Medzihradsky, F. (1993) *J. Neurochem.* **61**, 1135–1140
22. Tovey, S., Mezna, M. & Michelangeli, F. (1995) *Biochem. Soc. Trans.* **23**, 429S
23. Xu, Y., and Tashjian, A. H., Jr. (1995) *Biochemistry* **34**, 2815–2818