NAADP mobilizes Ca\(^{2+}\) from a thapsigargin-sensitive store in the nuclear envelope by activating ryanodine receptors

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Ca\(^{2+}\) release from the envelope of isolated pancreatic acinar nuclei could be activated by nicotinic acid adenine dinucleotide phosphate (NAADP) as well as by inositol 1,4,5-trisphosphate (IP\(_3\)) and cyclic ADP-ribose (cADPR). Each of these agents reduced the Ca\(^{2+}\) concentration inside the nuclear envelope, and this was associated with a transient rise in the nucleoplasmic Ca\(^{2+}\) concentration. NAADP released Ca\(^{2+}\) from the same thapsigargin-sensitive pool as IPs. The NAADP action was specific because, for example, nicotinamide adenine dinucleotide phosphate (NAADP), possessing two separate types of Ca\(^{2+}\) release from intracellular stores plays an important role in cytosolic Ca\(^{2+}\) signal generation in many different cell types (Berridge, 1993; Alvarez et al., 1999; Berridge et al., 2003). The ER is the key organelle (Meldolesi and Pozzan, 1998), possessing two separate types of Ca\(^{2+}\) release channels, namely inositol 1,4,5-trisphosphate (IP\(_3\)) and ryanodine receptors (Berridge, 1993; Petersen et al., 1994; Pozzan et al., 1994; Ashby and Tepikin, 2002; Bootman et al., 2002). However, several other organelles also have the capacity for storing and releasing Ca\(^{2+}\). The function of the mitochondria and their special role in cellular Ca\(^{2+}\) homeostasis have become increasingly clear in recent years (Pozzan et al., 2000; Gilabert et al., 2001; Collins et al., 2002; Villalobos et al., 2002), whereas the function and importance of Ca\(^{2+}\) release from the nuclear envelope (Malviya et al., 1990; Nicotera et al., 1990; Gerasimenko et al., 1995), the Golgi apparatus (Pinton et al., 1998), the secretory granules (Yoo, 2000), and the endosomes (Gerasimenko et al., 1998) are less clear.

The nucleus sits in an ER socket and the outer nuclear membrane is continuous with the ER membrane. Because the lumen of the nuclear envelope is continuous with the ER lumen, the nuclear Ca\(^{2+}\) store could be regarded as part of the ER Ca\(^{2+}\) store (Petersen et al., 1998). However, the distribution of Ca\(^{2+}\) transport proteins in the ER is very nonuniform. In the polarized pancreatic acinar cells, IP\(_3\) receptors are concentrated in the apical secretory pole (Thorn et al., 1993; Lee et al., 1997), Ca\(^{2+}\)-induced Ca\(^{2+}\) release can only be initiated in this part of the cell (Ashby et al., 2002), and selective activation of muscarinic receptors on the basal membrane initiates cytosolic Ca\(^{2+}\) signals in the apical pole (Ashby et al., 2003). To understand nuclear Ca\(^{2+}\) homeostasis, it is therefore not possible simply to extrapolate from the general knowledge of ER properties.

Abbreviations used in this paper: 2-APB, 2-aminoethyldiphenyl borate; AM, acetoxymethyl ester; β-NADP, β-nicotinamide adenine dinucleotide phosphate; cADPR, cyclic ADP-ribose; IP\(_3\), inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; NADP, nicotinamide adenine dinucleotide phosphate.
but the highly dynamic characteristics of the nucleus (Lyman and Gerace, 2001) have to be investigated directly. Previous work has shown that both IP$_3$ and cyclic ADP-ribose (cADPR) can release Ca$^{2+}$ from the nuclear envelope into the nucleoplasm, suggesting that the IP$_3$ and ryanodine receptors may be localized predominantly in the inner nuclear membrane (Gerasimenko et al., 1995; Hennager et al., 1995; Humbert et al., 1996; Santella and Kyozuka, 1997; Adebajo et al., 1999, 2000).

The Ca$^{2+}$-releasing agent nicotinic acid adenine dinucleotide phosphate (NAADP) was discovered in experiments on sea urchin eggs (Chini et al., 1995; Lee and Aarbus, 1995) and has since been shown to release Ca$^{2+}$ from internal stores in several cell types (Genazzani and Galione, 1997), including normal pancreatic acinar (Cancela et al., 1999; Petersen and Cancela, 1999; Cancela, 2001) and insulin-secreting $\beta$ cells (Masgrau et al., 2003; Mitchell et al., 2003). However, the mechanism of action is unclear. In sea urchin eggs, it would appear that NAADP, unlike IP$_3$, is abolished by ryanodine and ruthenium red because it is abolished by ryanodine and ruthenium red at a very high NAADP concentration (10$^{-3}$ M) to 10$^{-5}$ M NaADP evoked a reduction in Ca$^{2+}$ release from the nuclear envelope elicited by NAADP, cADPR or IP$_3$. 200 nM NAADP evoked a reduction in Ca$^{2+}$ concentration inside the nuclear envelope, which was irreversible upon removal of the agent (Fig. 1, H and I, n = 7). The action of NAADP was specific because two close NAADP analogs, $\beta$-NADP and nicotinic acid adenine dinucleotide (NAAD; Chini and De Toledo, 2002), were ineffective. Application of 200 nM $\beta$-NADP failed to elicit any Ca$^{2+}$ release from the envelope (n = 7), and similar results were obtained with 200 nM NAAD (n = 7). 20 $\mu$M IP$_3$ (n = 5) and 10 $\mu$M cADPR (n = 5) had effects very similar to those elicited by 200 nM NAADP. The dose–response curve for the action of NAADP is shown in Fig. 1 (J and K; n > 6 for each concentration). The lowest NAADP concentration capable of eliciting Ca$^{2+}$ release was 50 nM, whereas the optimal concentration was in the range of 200 nM to 1 $\mu$M. There was no response at a very high NAADP concentration (10 $\mu$M). This is in qualitative agreement with work on intact pancreatic acinar cells, where it has been shown that high NAADP concentrations do not evoke any measurable Ca$^{2+}$ release, presumably due to a very rapid desensitization process (Petersen and Cancela, 1999; Cancela et al., 2000). The maximal stimulus (200 nM NAADP) decreased the nuclear envelope Ca$^{2+}$ concentration from ~100–150 $\mu$M to ~30–60 $\mu$M (see legend to Fig. 1 for more details).

In the experiments described so far, 1 mM ATP was present and the free Ca$^{2+}$ concentration in the external solution, representing the cytosol, had been adjusted to 100 nM with a Ca$^{2+}$-EGTA buffer (low buffer concentration). Under these conditions, Ca$^{2+}$ reuptake into the envelope store

Results

Structural and functional characterization of the nuclear envelope

We stained isolated pancreatic acinar nuclei with the low affinity Ca$^{2+}$-sensitive fluorescent dye Mag-Fura Red™ (Fig. 1 A) or with BODIPY® FL thapsigargin, a fluorescent marker for ER-type Ca$^{2+}$ pumps (Fig. 1 B). The distributions of these two fluorescent markers were similar, as seen in the overlay picture (Fig. 1 C). The probes were clearly localized in the nuclear envelope, as can be seen by comparison with the transmitted light picture (Fig. 1 D). There was no staining of the nuclei with a mitochondrial marker (MitoTracker® Green) or a marker for acidic organelles (LysoTracker® Red; not depicted). The distribution of fluorescent ryanodine (BODIPY® FL ryanodine), a fluorescent marker for ryanodine receptors (Fig. 1 E), was also similar to the distribution of fluorescent thapsigargin (i.e., most of the staining was localized in the nuclear envelope). BODIPY® FL ryanodine staining could be effectively washed away by 10 $\mu$M “cold” nonfluorescent ryanodine, confirming the specificity of the staining (Fig. 1 F).

Isolation of nuclei inevitably involves breaking links with the major part of the ER, which is very widely distributed in the pancreatic acinar cells and is very tightly packed in the basolateral part of the cells surrounding the nucleus (Gerasimenko et al., 2002). As seen in electron microscopical images from intact acinar cells, the ER is indeed very tightly packed around the nucleus, and what appears in transmitted light images as a thin envelope (Fig. 1 D) most likely consists of multiple layers of ER (Fig. 1 G). It can also be seen that the structure of the nucleoplasm is nonuniform, with an apparently patchy coverage of the inner nuclear membrane by chromatin (Fig. 1 G).
did not occur (Fig. 1, H–K). To carry out experiments with multiple messenger applications, which would make it possible to compare control and test conditions in the same preparation, we searched for a protocol allowing recovery after a stimulation-elicted release of Ca\(^{2+}\) from the nuclear envelope. We found that an increase in the Ca\(^{2+}\) concentration in the solution bathing the isolated nuclei to \(\sim 300\, \text{nM}\) made the releasing effects of the various messengers reversible (Fig. 2). The effects of 200 nM NAADP, 10 \(\mu\text{M}\) cADPR, 20 \(\mu\text{M}\) IP\(_3\), or 10 \(\mu\text{M}\) caffeine at the elevated external Ca\(^{2+}\) concentration (300 nM) are shown in Fig. 2 (A–E). In all cases, addition of the Ca\(^{2+}\)-releasing agent caused a transient reduction in the Ca\(^{2+}\) concentration inside the nuclear envelope (\(n > 10\) for each messenger).

Caffeine is an established activator of ryanodine receptors and can thereby elicit substantial Ca\(^{2+}\) release from both sarcoplasmic reticulum and ER stores (Fabiato, 1985; Solovyova et al., 2002). However, caffeine is also an effective inhibitor of IP\(_3\) receptors and can completely block IP\(_3\)-elicited Ca\(^{2+}\) release (Wakui et al., 1990; Bezprozvanny et al., 1994). In intact pancreatic acinar cells, the effect of caffeine stimulation was very similar to that elicited by NAADP, cADPR, or IP\(_3\). However, caffeine stimulation was essentially inhibitory, and caffeine-induced Ca\(^{2+}\) release (Wakui et al., 1990; Bezprozvanny et al., 1994). In intact pancreatic acinar cells, the effect of caffeine stimulation was very similar to that elicited by NAADP, cADPR, or IP\(_3\).

In the experiments represented by Fig. 2, the Ca\(^{2+}\) concentration in the solution surrounding the nuclei was \(\sim 300\, \text{nM}\), but the concentration of the Ca\(^{2+}\) buffer (EGTA) was relatively low (100 \(\mu\text{M}\); Fig. 2, all traces except B). This would allow some changes in the Ca\(^{2+}\) concentration near the Ca\(^{2+}\) release channels, and therefore, we also tested the
The effect of clamping the external Ca²⁺ concentration to ~300 nM by using a high concentration of a Ca²⁺/BAPTA mixture (10 mM). In such experiments, the effect of 200 nM NAADP was not markedly different from that seen in experiments with low Ca²⁺ buffer concentration.

Fig. 2 (F and G) shows the effects of NAADP in the presence and absence of ATP (n > 3 for each experiment). From these data, it would appear that the transient nature of the NAADP-elicited response is due to ATP-dependent Ca²⁺ reuptake into the nuclear envelope. This is further supported by the experiment illustrated in Fig. 2 H, in which it is seen that thapsigargin blocks the restoration of the nuclear envelope Ca²⁺ concentration normally occurring during prolonged stimulation.

The experiments described so far were all performed with a relatively slow stimulation protocol in which the control solution flowing into the bath was simply replaced by one containing the stimulant (e.g., IP₃, cADPR, or NAADP). It also seemed desirable to carry out experiments in which more immediate effects of stimulation could be investigated. We used two techniques; local uncaging of caged IP₃ and local ionophoretic pipette application of IP₃ or cADPR. Fig. 3 (A–E) shows traces representing Ca²⁺ concentration inside the nuclear envelope obtained in response to local uncaging of caged IP₃ at various positions around one isolated nuclear envelope. It is seen that the IP₃-elicited reduction in the nuclear envelope Ca²⁺ concentration occurs much faster with this protocol than in experiments with simple bath exchange (Fig. 2 D). Ca²⁺ reuptake is also much faster, but this is most likely due to the short-lasting nature of the IP₃ stimulus. Ionophoretic IP₃ application affords the opportunity to produce short or long stimulation pulses. As seen in Fig. 3 F, Ca²⁺ reuptake does occur during prolonged IP₃ stimulation. This is also the case during cADPR stimulation (Fig. 3 G).

The nuclear envelope Ca²⁺ store could be one unified space or could consist of several distinct noncommunicating compartments. We attempted to give at least a partial answer to this question by conducting bleaching-recovery experiments. Mag-Fura Red™ in one region of the envelope was bleached, and thereafter substantial recovery, presumably due to diffusion of nonbleached dye from neighboring regions, was observed (Fig. 3 H). This type of experiment
demonstrates substantial communication between different parts of the nuclear envelope store, but does not completely rule out a degree of subcompartmentalization.

The recovery of the resting (prestimulation) Ca\(^{2+}\) concentration inside the nuclear envelope during sustained messenger stimulation may seem puzzling because it implies that Ca\(^{2+}\) pump-mediated movement can have an impact on the Ca\(^{2+}\) balance when the Ca\(^{2+}\) release channels might be expected to be open. It would normally be expected that ion movements through channels should be much faster than through pumps. In intact pancreatic acinar cells, Ca\(^{2+}\) reuptake into the ER only occurs after removal of the stimulus producing the Ca\(^{2+}\) release (Mogami et al., 1998). To clarify whether our data were in fact contradicting current model concepts, we attempted to model mathematically the nuclear envelope Ca\(^{2+}\) concentration changes in response to continued IP\(_3\) stimulation using values for the IP\(_3\) and cytosolic Ca\(^{2+}\) concentrations relevant to our experiments. Fig. 3 (I a) shows the time course of the open-state probability of the IP\(_3\) type 2 receptor according to Sneyd’s model (Sneyd and Dufour, 2002), whereas Fig. 3 (I b) illustrates the result of a similar model for the probably more relevant IP\(_3\) type 3 receptor, based on the data from Swatton and Taylor (2002). Finally, we also took into account the data from Mogami et al. (1998), with regard to the rate of SERCA-mediated Ca\(^{2+}\) uptake into the ER as a function of the Ca\(^{2+}\) concentration in the ER lumen in intact pancreatic acinar cells, to model the time course of the nuclear envelope Ca\(^{2+}\) concentration changes during continuous IP\(_3\) application (for further details see supplemental material, available at http://www.jcb.org/cgi/content/full/jcb.200306134/DC1).
The two separate Ca\(^{2+}\) release channels can be activated independently

Results of the type shown in Fig. 2 (F and G) indicate that the different Ca\(^{2+}\) channel activators release Ca\(^{2+}\) from a common pool in the nuclear envelope. Thus, IP\(_3\) can release Ca\(^{2+}\) from the envelope after NAADP stimulation, but only if Ca\(^{2+}\) reuptake has occurred before the IP\(_3\) application. This is different from the situation in sea urchin eggs, where it would appear that NAADP releases Ca\(^{2+}\) from a pool that is separate from the one IP\(_3\) acts on (Churchill et al., 2002). In intact pancreatic acinar cells, the local Ca\(^{2+}\)-spiking responses in the apical granular pole require interactions between IP\(_3\) and ryanodine receptors (Cancela et al., 2000, 2002). Therefore, we tested pharmacologically the different Ca\(^{2+}\) release channels and investigated possible interactions between IP\(_3\) and ryanodine receptors in the nuclear envelope.

The borate compound 2-aminoethyldiphenyl borate (2-APB) has been used to inhibit IP\(_3\) receptors in different cell types (Ma et al., 2000), but is clearly not a specific IP\(_3\) receptor antagonist (Bakowski et al., 2001; Prakriya and Lewis, 2001; Harks et al., 2003). Nevertheless, we tested the ability of 2-APB to influence nuclear Ca\(^{2+}\) release elicited by NAADP (Fig. 4 A), cADPR (Fig. 4 B), and IP\(_3\) (Fig. 4 C). 100 \(\mu\)M 2-APB abolished IP\(_3\)-elicited Ca\(^{2+}\) release (Fig. 4 C; \(n = 7\)), but failed to inhibit the responses to cADPR (Fig. 4 B; \(n = 3\)) or NAADP (Fig. 4 A; \(n = 6\)). During sustained caffeine stimulation in the presence of ATP, there was almost a full recovery of the prestimulation Ca\(^{2+}\) concentration inside the nuclear envelope. Subsequent addition of NAADP (\(n = 6\)) or cADPR (\(n = 5\)) induced a second Ca\(^{2+}\) release. In contrast, IP\(_3\) (\(n = 6\)) failed to elicit any response in the presence of 10 mM caffeine, consistent with caffeine’s known action as a blocker of IP\(_3\) receptors (Wakui et al., 1990; Bezprozvanny et al., 1994; Ehrlich et al., 1994). Heparin, the classical IP\(_3\) receptor antagonist (Ehrlich et al., 1994), also blocked IP\(_3\)-induced Ca\(^{2+}\) release (\(n = 4\)), but failed to block responses to NAADP (\(n = 7\)) and cADPR (\(n = 3\)).

We also used ryanodine which, at high concentrations (>10 \(\mu\)M), is an established inhibitor of ryanodine receptors (Surko et al., 1997). 100 \(\mu\)M ryanodine did not inhibit the effect of IP\(_3\) (Fig. 4 D; \(n = 7\)), indicating that the response to IP\(_3\) does not, in this preparation, depend on cooperation between IP\(_3\) and ryanodine receptors. However, the same concentration of ryanodine (100 \(\mu\)M) abolished the Ca\(^{2+}\) release normally elicited by caffeine (Fig. 4 E; \(n = 8\)), NAADP (Fig. 4 F; \(n = 6\)), and cADPR (Fig. 4 G).
G; \(n = 6\). 10 \(\mu M\) ruthenium red, an inhibitor of ryanodine receptors (Thorn et al., 1994; Hohenegger et al., 2002), also completely blocked NAADP-induced \(Ca^{2+}\) release from the nuclear envelope (Fig. 4 H; \(n = 7\)). These data indicate that both NAADP and cADPR interact functionally with the ryanodine receptors, but most likely via two separate primary receptors (as explained in a later section; see Fig. 6 B), and that the opening of the ryanodine receptors alone can cause \(Ca^{2+}\) release without any need for cooperation with functional IP\(_3\) receptor channels.

**\(Ca^{2+}\) release into the nucleoplasm elicited by NAADP, cADPR, or IP\(_3\)**

Previously, we have shown that accumulation (in the internal part of isolated nuclei) of \(Ca^{2+}\)-sensitive fluorescent indicators labeled with dextrans is a useful way of monitoring \(Ca^{2+}\) concentration changes in the nucleoplasm (Gerasimenko et al., 1995). Using Fluo-4 dextran (MW = 10,000) accumulated inside the isolated nuclei (Fig. 5 A), we found that 200 nM NAADP, 10 \(\mu M\) cADPR, and 20 \(\mu M\) IP\(_3\) each elicited a transient \(Ca^{2+}\) concentration rise in the nucleoplasm (Fig. 5 B–D; \(n > 10\) for each messenger). The maximal rise of the nucleoplasmic \(Ca^{2+}\) concentration was \(\sim 0.5 \mu M\).

**\(Ca^{2+}\) permeability of the nuclear pores after messenger-elicited \(Ca^{2+}\) release**

The transient nature of the nucleoplasmic \(Ca^{2+}\) concentration rise in response to messenger stimulation could be due to \(Ca^{2+}\) reuptake into the nuclear envelope store after the release, or it could be explained by movement of \(Ca^{2+}\) from the nucleoplasm through the nuclear pore complexes into the bathing solution outside the nucleus. Because ATP was not added to the solutions used for the experiments represented by Fig. 5 (E and F), it seems unlikely that the first explanation could apply (see Fig. 2). Because it has been reported that the permeability of the nuclear pore complex could be markedly reduced after depleting the nuclear envelope of \(Ca^{2+}\) (Greber and Gerace, 1995, Lee et al., 1998), we tested the ability of external \(Ca^{2+}\) changes to make an impact on the nucleoplasmic \(Ca^{2+}\) concentration after messenger-induced \(Ca^{2+}\) release. After NAADP stimulation, external application of initially a high \(Ca^{2+}\) concentration (0.5 mM) followed by a \(Ca^{2+}\) chelator (2 mM EGTA) induced a large rise and thereafter a fast decrease in the nucleoplasmic \(Ca^{2+}\) concentration (Fig. 5 E; \(n = 7\)). This indicates rapid movement of \(Ca^{2+}\) across the nuclear envelope, most likely through the nuclear pore complexes, in agreement with pre-

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**Figure 5.** The distribution of Fluo-4 dextran in a single isolated nucleus and the effects of messenger stimulation on \(Ca^{2+}\) concentration in the nucleoplasm. (A) Fluorescence image showing uniform distribution of Fluo-4 dextran throughout the nucleoplasm of a single isolated nucleus. 200 nM NAADP (B), 10 \(\mu M\) cADPR (C), and 20 \(\mu M\) IP\(_3\) (D) induced transient elevations of the nucleoplasmic \(Ca^{2+}\) concentration. After stimulation with 200 nM NAADP (E) or 20 \(\mu M\) IP\(_3\) (F), sequential exposure to a high external \(Ca^{2+}\) concentration (0.5 mM) and then to 2 mM EGTA caused corresponding changes in the nucleoplasmic \(Ca^{2+}\) concentration.
vious work (Brini et al., 1993; Gerasimenko et al., 1995; Lipp et al., 1997). The same protocol was used after stimulation with 20 mM IP3, and very similar results were obtained (Fig. 5F; n = 6). These results indicate that the nuclear pore complexes are permeable to Ca2+ even after depletion of the nuclear envelope Ca2+ stores.

**Interaction between Ca2+-releasing agents**

As already described, a high concentration (10 μM) of NAADP did not evoke any Ca2+ release from the nuclear envelope, most likely due to rapid auto-desensitization (Fig. 1). Subsequent application of IP3 (Fig. 6A; n = 6) or cADPR (Fig. 6B; n = 6), during continued exposure to NAADP, elicited normal Ca2+ release responses, indicating that the activation of IP3 or ryanodine receptors does not have an obligatory requirement for operational NAADP receptors. The fact that cADPR can evoke Ca2+ release in the presence of a high desensitizing NAADP concentration (10 μM; Fig. 6B) indicates that cADPR and NAADP most likely act on two separate receptors, although both agents cause Ca2+ release via opening of ryanodine receptors (Fig. 4).

We investigated the nature of the pool from which Ca2+ could be released by NAADP and the other messengers. When ATP was present, and reaccumulation of lost Ca2+ therefore was possible, thapsigargin was able to elicit renewed Ca2+ release after an NAADP-induced Ca2+ rise in the nucleoplasm (Fig. 6C; n = 13). This was also the case after application of the triple messenger mixture cADPR + IP3 + NAADP (Fig. 6D; n = 12).

In the absence of ATP, application of thapsigargin induced a markedly reduced Ca2+ release after exposure to the messenger mixture, suggesting that most of the intranuclear Ca2+ had already been liberated (Fig. 6E; n = 5). Pretreatment of nuclei with thapsigargin abolished the responses to NAADP (Fig. 6F; n = 5) or the triple messenger mixture (Fig. 6G; n = 6), indicating that NAADP and the other messengers release Ca2+ from a thapsigargin-sensitive store and that the whole of the thapsigargin-sensitive Ca2+ store can be released by the messengers.

**Does NAADP release Ca2+ from acid compartments?**

Recent work on sea urchin eggs indicates that NAADP mobilizes Ca2+ from an acid thapsigargin–insensitive pool, with lysosomal properties, that is separate from those sensitive to IP3 and cADPR (Churchill et al., 2002). Therefore, it seemed important to test whether the NAADP-elicited Ca2+ release from the nuclear envelope is dependent on acidic pools. One way of interfering with organellar acidification is to pretreat with bafilomycin, which is a blocker of the vacuolar type H+ ATPase (Bowman et al., 1988). In the presence of bafilomycin A1, at a near-optimal concen-
tration of 50 nM, we found that NAADP elicited an entirely normal Ca\(^{2+}\) release response (Fig. 7 A; \(n = 6\)). We also used 10 \(\mu M\) brefeldin A, a membrane transport blocker that disrupts the Golgi apparatus (Donaldson et al., 1992), and observed that NAADP evoked normal Ca\(^{2+}\) release (Fig. 7 B; \(n = 6\)). Finally, we used 7 \(\mu M\) of the protonophore nigericin (Camello-Almaraz et al., 2000), but we failed, also in this case, to find any evidence for a reduction in the magnitude of the NAADP-elicited Ca\(^{2+}\) release (Fig. 7 C; \(n = 6\)). These data indicate that the Ca\(^{2+}\) release from pancreatic nuclei elicited by NAADP is unlikely to come from acid compartments.

**Discussion**

By direct measurements of the Ca\(^{2+}\) concentrations both inside the nuclear envelope store and in the nucleoplasm, we have demonstrated that NAADP has a specific Ca\(^{2+}\)-releasing action on isolated pancreatic nuclei (Figs. 1, 2, 4, and 5). Ca\(^{2+}\) is liberated from a thapsigargin-sensitive pool in the nuclear envelope and moves into the nucleoplasm to generate a Ca\(^{2+}\) signal in that compartment. Because the action of NAADP is abolished by ryanodine, but not by blockade of IP\(_3\) receptors (Fig. 4), the simplest explanation for its effect is activation of ryanodine receptors. The new experiments described here demonstrate that release of Ca\(^{2+}\) from the nuclear envelope, most likely including adhering ER elements (Fig. 1), is associated with a rise in the nucleoplasmic Ca\(^{2+}\) concentration, confirming our earlier work on liver nuclei (Gerasimenko et al., 1995). We and several other groups (for review see Petersen et al., 1998) have provided evidence indicating that the Ca\(^{2+}\) release channels may at least in part be present in the inner nuclear membrane. It is also clearly possible that Ca\(^{2+}\) released from the ER just outside the outer nuclear membrane can diffuse into the nucleoplasm via open nuclear pore complexes (Fig. 8; Gerasimenko et al., 1995; Lipp et al., 1997).

**Ryanodine receptors in the nuclear envelope**

In isolated liver nuclei, we have previously demonstrated activation of Ca\(^{2+}\) release by a low concentration of ryanodine and by cADPR (Gerasimenko et al., 1995), indicating the presence of functional ryanodine receptors. In intact pancre-
tronic, and maximal at 100–300 nM NAADP. Hohenegger et al. (2002) conclude that NAADP most likely directly activates type 1 ryanodine receptors, although an action on a protein tightly coupled to the ryanodine receptor might be regarded as more likely.

Functional NAADP receptors are required specifically for the cytosolic Ca\(^{2+}\) signal generation normally elicited by physiological cholecystokinin concentrations in pancreatic acinar cells (Cancela et al., 2000). In addition to the local Ca\(^{2+}\) spikes in the apical (granular) pole, cholecystokinin also elicits longer lasting global Ca\(^{2+}\) transients that invade the nucleus (Osipchuk et al., 1990; Petersen et al., 1991; Thorn et al., 1993). Ca\(^{2+}\) signal globalization is helped by cooperation between activated IP\(_3\), cADPR, and NAADP receptors (Cancela et al., 2002). It seems likely that the NAADP-elicted nuclear Ca\(^{2+}\) release, revealed in this report on isolated nuclei, plays a role in Ca\(^{2+}\) signal globalization. However, we do not yet understand how the CD38/ADP-ribosyl cyclase may be regulated in the pancreatic acinar cells. This enzyme is responsible for the production of both cADPR and NAADP (Cancela, 2001) and also exists in the nucleus, where it has its catalytic site within the nucleoplasm (Adebanjo et al., 1999). There is also evidence for the existence of the polyphosphoinositol cycle inside the nucleus (Divecha et al., 1991). Therefore, the various Ca\(^{2+}\)-releasing messengers could be produced inside the nucleus to regulate release of Ca\(^{2+}\) from the nuclear envelope. In the intact cell, the Ca\(^{2+}\) store in the nuclear envelope is part of the unified and lumenally continuous ER store (Petersen et al., 2001), but this report on isolated nuclei reveals that the local control of Ca\(^{2+}\) release can operate in a distinct manner. Although the local Ca\(^{2+}\) spiking in the apical secretory pole region of the pancreatic acinar cells (as well as the global Ca\(^{2+}\)-induced Ca\(^{2+}\) waves) depends on cooperative interaction of IP\(_3\) and ryanodine receptors (Cancela et al., 2000; Ashby et al., 2002), these receptors can function independently in the nucleus to release Ca\(^{2+}\) into the nucleoplasm.

**Materials and methods**

**Materials**

Mg-Fura Red™ acetoxymethyl ester (AM), Rhod 5N AM, Fluo-4 dextran, BODIPY® FL thapsigargin, MitoTracker® Green, LysoTracker® Red, and caged IP\(_3\) were obtained from Molecular Probes, Inc. The protease inhibitor cocktail was obtained from Roche. The rest of the chemicals were purchased from Sigma-Aldrich.

**Experimental procedures**

Single pancreatic acinar cells or small clusters were acutely isolated from CD1 mouse pancreas as described previously (Thorn et al., 1993). Single nuclei were isolated from pancreatic acinar cells by homogenization and by centrifugation as described in Gerasimenko et al. (1995) with some modifications (Maruyama et al., 1995). The buffer for homogenization contained 140 mM KCl, 10 mM Hepes, 1 mM MgCl\(_2\), 100 μM EGTA, 1 mM ATP, and protease cocktail inhibitor (1 tablet per 10 ml of buffer; pH 7.2 adjusted with KOH). The final pellet of nuclei was resuspended in standard buffer (140 mM KCl, 10 mM Hepes, 1 mM MgCl\(_2\), 100 μM EGTA [low calcium buffer], 75 μM CaCl\(_2\), and 1 mM ATP [pH 7.2 adjusted with KOH]). We have also used the same standard buffer, but with a reduced concentration of MgCl\(_2\) (0.1 mM) to check the Mg\(^{2+}\) dependence of the Ca\(^{2+}\) release responses. However, the messenger-induced Ca\(^{2+}\) release from Mg-Fura Red™-loaded nuclear envelopes was not altered by this reduction in the external Mg\(^{2+}\) concentration. In some experiments, we used the standard buffer with the composition given above, but increased the buffering of Ca\(^{2+}\) by using a mixture of 10 mM BAPTA and 7 mM CaCl\(_2\).
Isolated nuclei were loaded with 20 μM Mag-Fura Red™ in AM form, with 5 μM Rhod 5N in AM form, or with 20 μM Fluo-4 dextran by incubation for 30–45 min at 4°C. Loaded nuclei were washed by centrifugation. All experiments were performed with single isolated nuclei at RT (20–21°C) in an experimental chamber with a perfusion system that allowed washing of nuclei with standard buffer for several minutes before each experiment. The Ca²⁺ concentration in the nuclear envelope was assessed by Mag-Fura Red™/Rhod 5N fluorescence measurements (excitation 488 nm, emission 550–650 nm) or by Rhod 5N fluorescence measurements (excitation 543 nm, emission 555–630 nm). The nucleoplasmic Ca²⁺ concentration changes were assessed by Fluo-4 dextran (MW = 10,000) fluorescence measurements (excitation 488 nm, emission 500–550 nm). Nuclear preparation were stained with 0.2 μM BODIPY FL Rh123, 1 μM BODIPY FL rhodamine, 0.5 μM MitoTracker® Green, or 0.2 μM Lysotracker® Red by incubation with those dyes for 5 min in standard buffer, and were then washed using the perfusion system. EM was performed on a transmission electron microscope (model H-600; Hitachi) as described previously (Johnson et al., 2003).

For flash photolysis experiments, caged IP₃ in a concentration of 100 μM was added to the nuclear preparation loaded with Mag-Fura Red™ in AM form. Uncaging was performed using the “regions of interest” option of the Leica confocal two-photon system with water immersion objective (63×). Images were acquired continuously at 74–998 ms intervals before and after uncaging. A similar protocol was used for bleaching-recovery experiments, but 100% of the laser power at 488 nm was used for bleaching waves.

Online supplemental material
Mathematical model of IP₃-induced Ca²⁺ release and uptake in an isolated nucleus. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200306134/DC1.

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