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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 IP_3. The NAADP action was specific because, for example, nicotineamide adenine dinucleotide phosphate was ineffective. The Ca^{2+} release was unaffected by procedures interfering with acidic organelles (bafilomycin, brefeldin, and nigericin). Ryanodine blocked the Ca^{2+}-releasing effects of NAADP, cADPR, and caffeine, but not IP_3. Ruthenium red also blocked the NAADP-elicited Ca^{2+} release. IP_3 receptor blockade did not inhibit the Ca^{2+} release elicited by NAADP or cADPR. The nuclear envelope contains ryanodine and IP_3 receptors that can be activated separately and independently; the ryanodine receptors by either NAADP or cADPR, and the IP_3 receptors by IP_3.

Introduction

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., 2003a). To understand nuclear Ca^{2+} homeostasis, it is therefore not possible simply to extrapolate from the general knowledge of ER properties,

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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; Adebanjo 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 Aarhus, 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 β 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 or cADPR, mobilizes Ca^{2+} from a pool that does not have ER characteristics (Genazzani and Galione, 1996). The source may be the so-called reserve granules, the functional equivalent of lysosomes (Churchill et al., 2002). However, in pancreatic acinar cells, the NAADP-elicited Ca^{2+} spikes occur specifically in the apical granular pole (Cancela et al., 2002), exactly as the spikes elicited by IP_3 or cADPR (Thorn et al., 1993, 1994). NAADP does not only elicit cytosolic Ca^{2+} spiking in the apical granular pole, which is the crucial region for regulation of exocytosis (Nemoto et al., 2001) and fluid secretion (Park et al., 2001), but also plays an important role, together with IP_3 and cADPR, in spreading the Ca^{2+} signal throughout the cell (Cancela et al., 2002). This globalizing action of NAADP must depend on interaction between NAADP and its receptors in the basal part of the acinar cell, which contains the nucleus.

To study specifically the action of NAADP on the nucleus, we have investigated the Ca^{2+}-releasing function of this agent in isolated nuclei from pancreatic acinar cells. We have compared the effects of stimulating with NAADP, cADPR, and IP_3, and have also studied interactions between these agents. We find that NAADP is an effective releaser of Ca^{2+} from the nuclear envelope and, like IP_3 and cADPR, causes a reduction in the Ca^{2+} concentration inside the nuclear envelope as well as increasing the Ca^{2+} concentration in the nucleoplasm. NAADP releases Ca^{2+} from the same thapsigargin-sensitive pool that is also the target for IP_3 and cADPR. The action of NAADP is specific because β-nicotinamide adenine dinucleotide phosphate (β-NADP) in the same concentration has no effect. The NAADP action is most simply explained by activation of ryanodine receptors because it is abolished by ryanodine and ruthenium red (agents that do not affect the IP_3-elicited Ca^{2+} release), but not by blockade of the IP_3 receptors.

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 μM "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).

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 effect of NAADP was specific because two close NAADP analogs, β-NADP and nicotinic acid adenine dinucleotide (NAAD; Chini and De Toledo, 2002), were ineffective. Application of 200 nM β-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 μM IP_3 ($n = 5$) and 10 μM 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 μM. There was no response at a very high NAADP concentration (10 μM). 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 μM to ~30–60 μM (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

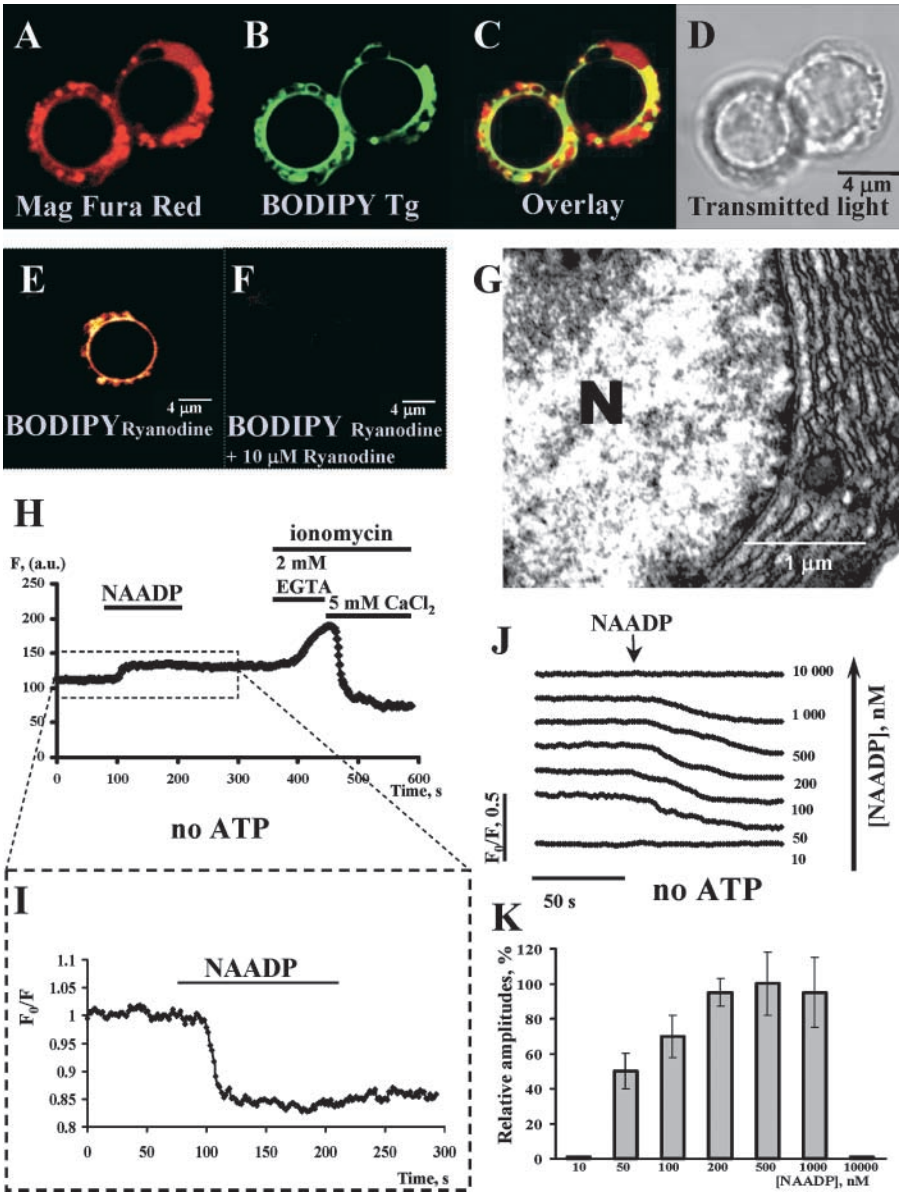

Figure 1. **Isolated nuclei and the effect of NAADP on the Ca^{2+} concentration inside the nuclear envelope.** (A) Distribution of Mag-Fura Red™ (loaded in AM form) in the nuclear envelope of two nuclei. (B) Distribution of BODIPY® thapsigargin in the nuclear envelope of isolated nuclei. Most of the staining with BODIPY® FL thapsigargin could be washed out with 10 μM "cold" thapsigargin (not depicted). (C) Overlay of images shown in A and B. (D) Transmitted light picture of the two isolated nuclei also shown in A and B. (E) Nucleus stained with BODIPY® FL ryanodine. (F) Most of the staining with BODIPY® FL ryanodine had disappeared after washing with 10 μM cold ryanodine. Bar, 4 μm. (G) Electron microscopical image of part of the nucleus (N) in an intact pancreatic acinar cell. Bar, 1 μm. (H–K) Effect of 200 nM NAADP on the Ca^{2+} concentration in the nuclear envelope. The raw fluorescence trace (Mag-Fura Red™ fluorescence intensity increases when the Ca^{2+} concentration is reduced) is shown in H. The average free Ca^{2+} concentration in the nuclear envelope before stimulation was 138 ± 16 μM (n = 16), using Mag-Fura Red™, (estimated K_d = 105 ± 21 μM; n = 6) or 127 ± 20 μM (n = 10) using Rhod 5N, (estimated K_d = 206 ± 56 μM; n = 5). During maximal stimulation, the Ca^{2+} concentration in the nuclear envelope was 53 ± 21 μM and 42 ± 19 μM, respectively. After removal of NAADP, the effects of changes in the external Ca^{2+} concentration (in the presence of 10 μM ionomycin) on the fluorescence level were monitored. The normalized fluorescence trace (F_o/F; where F_o is the initial fluorescence level) is shown in I. (J) Effects of different concentrations of NAADP (10 nM, 50 nM, 100 nM, 200 nM, 500 nM, 1 μM, and 10 μM) on the nuclear envelope Ca^{2+} concentration. (K) Averaged normalized amplitudes of Mag-Fura Red™ fluorescence changes (relative amplitudes, $F_0/\Delta F$*100%) due to the NAADP-induced reduction in the nuclear envelope Ca^{2+} concentration at different NAADP concentrations. In all these experiments, the external Ca^{2+} concentration was ~100 nM (low buffer concentration).

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-elicited 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 ~300 nM made the releasing effects of the various messengers reversible (Fig. 2). The effects of 200 nM NAADP, 10 μM cADPR, 20 μM IP_3, or 10 mM 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 is essentially inhibitory, and caffeine-induced Ca^{2+} release has only been observed under very special conditions (Osipchuk et al., 1990; Wakui et al., 1990). However, in the isolated nuclei, we found that caffeine itself can induce Ca^{2+} release (Fig. 2 E; n = 25). As seen in Fig. 2, the response to 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 ~300 nM, but the concentration of the Ca^{2+} buffer (EGTA) was relatively low (100 μM; 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

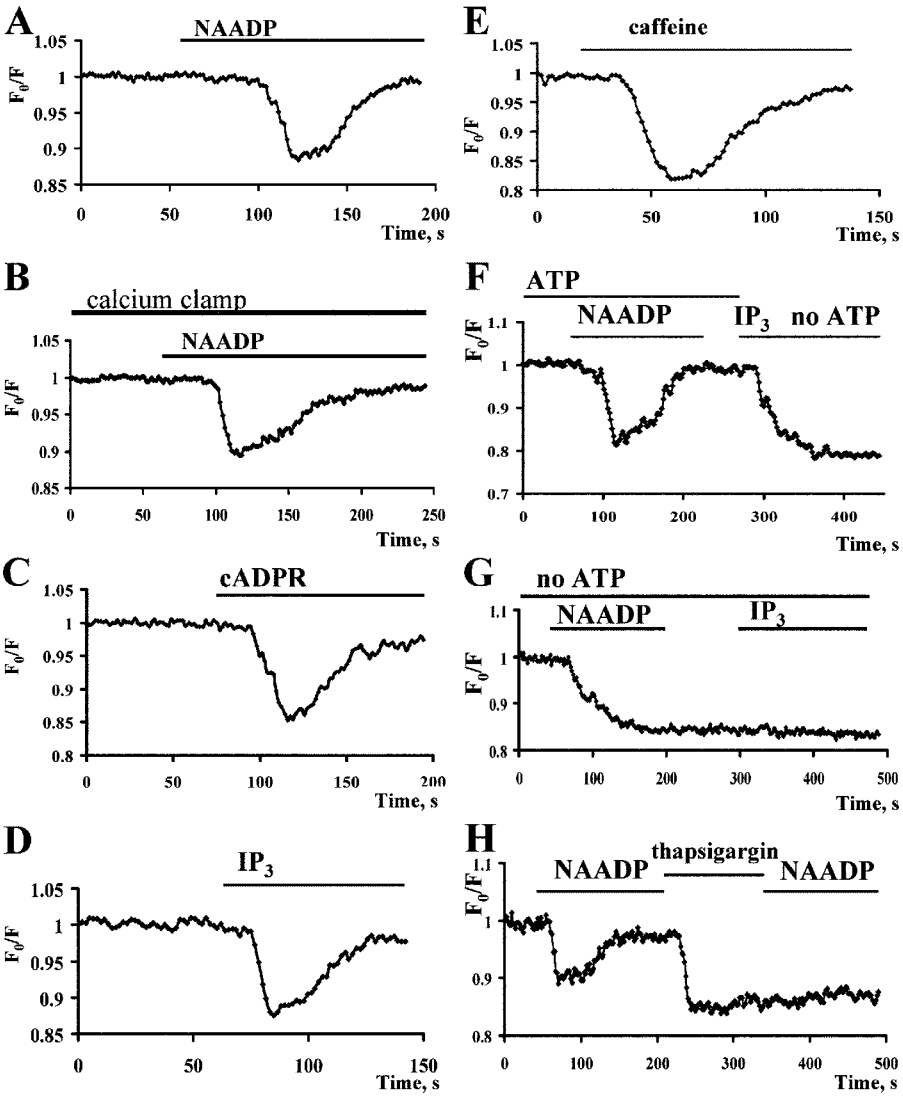

Figure 2. **Comparison of the effects of NAADP, cADPR, IP_3, and caffeine.** The external Ca^{2+} concentration was adjusted to ~300 nM (low buffer concentration) in all experiments, except in B, where the Ca^{2+} concentration was clamped (~300 nM) by using a high concentration of a Ca^{2+}/BAPTA mixture. Application of 200 nM NAADP (A and B), 10 μM cADPR (C), and 20 μM IP_3 (D), all in the presence of 1 mM ATP, induced a transient reduction in the Ca^{2+} concentration of the nuclear envelope. (E) Effect of 20 mM caffeine on the nuclear envelope Ca^{2+} concentration. (F) 20 μM IP_3 elicited a reduction in the nuclear envelope Ca^{2+} concentration when applied after a 200-nM NAADP stimulation period with 1 mM ATP present, which allowed Ca^{2+} reuptake. (G) When ATP was absent and there was no Ca^{2+} reuptake after the 200-nM NAADP-induced release, then a subsequent 20-μM IP_3 stimulus failed to elicit Ca^{2+} release. (H) In the presence of ATP, 10 μM thapsigargin abolished NAADP-induced Ca^{2+} release.

effect of clamping the external Ca^{2+} concentration to ~300 nM by using a high concentration of a Ca^{2+}/BAPTA mixture (10 mM). In such experiments, (Fig. 2 B; $n = 10$), the effect of 200 nM NAADP was not markedly different from that seen in experiments with low Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} 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., IP_3, 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 IP_3 and local ionophoretic pipette application of IP_3 or cADPR. Fig. 3 (A–E) shows traces representing Ca^{2+} concentration inside the nuclear envelope obtained in response to local uncaging of caged IP_3 at various positions around one isolated nuclear envelope. It is seen that the IP_3-elicited reduction in the nuclear envelope Ca^{2+} concentration occurs much faster with this protocol than in experiments with simple bath exchange (Fig. 2 D). Ca^{2+} reuptake is also much faster, but this is most likely due to the short-lasting nature of the IP_3 stimulus. Ionophoretic IP_3 application affords the opportunity to produce short or long stimulation pulses. As seen in Fig. 3 F, Ca^{2+} reuptake does occur during prolonged IP_3 stimulation. This is also the case during cADPR stimulation (Fig. 3 G).

The nuclear envelope Ca^{2+} 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

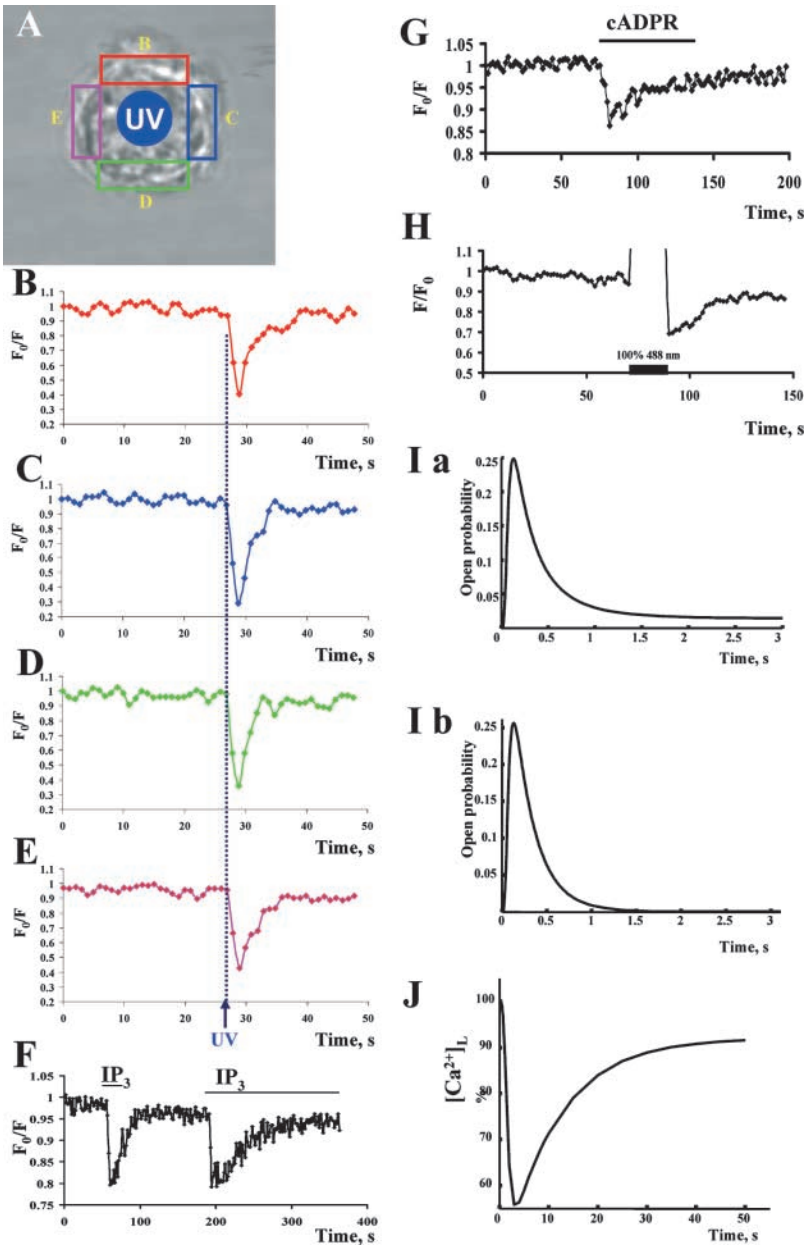

Figure 3. **Effects of rapid applications of IP_3 and cADPR to isolated nuclei loaded with Mag-Fura Red™.** (A) Arrangement for experiments with uncaging of IP_3 inside the nucleus (see Materials and methods). The colored boxes correspond to the colored traces in B–E, representing the Ca^{2+} concentrations in the indicated parts of the nuclear envelope, during uncaging of IP_3 in the center of the nucleus (UV). (F) Effects of ionophoretic IP_3 application. (G) Effect of ionophoretic cADPR application. (H) Bleaching-recovery experiment in isolated nucleus. Bleaching by laser light at 488 nm caused marked reduction in fluorescence intensity in affected area followed by fluorescence recovery due to diffusion from nonbleached areas. (I) Simulated time course of the open probability of (a) type 2 and (b) type 3 IP_3 receptors in response to 20 μM IP_3. (J) Simulation of the release and reuptake of Ca^{2+} in the nuclear envelope in response to IP_3 stimulation. $[IP_3] = 20/(1 + e^{(-(t-10)/2)})$ (μM) in this simulation, to model the gradual increase of IP_3 by perfusion (for further details see supplemental information, available at http://www.jcb.org/cgi/content/full/jcb.200306134/DC1).

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 result is shown in Fig. 3 J. It is clear that the IP_3-elicited reduction in Ca^{2+} concentration in the ER lumen is largely transient, in agreement with our experimental data (Fig. 2 D and Fig. 3 F).

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 μM 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 μM), is an established inhibitor of ryanodine receptors (Sutko et al., 1997). 100 μM 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 μM) abolished the Ca^{2+} release normally elicited by caffeine (Fig. 4 E; $n = 8$), NAADP (Fig. 4 F; $n = 6$), and cADPR (Fig. 4

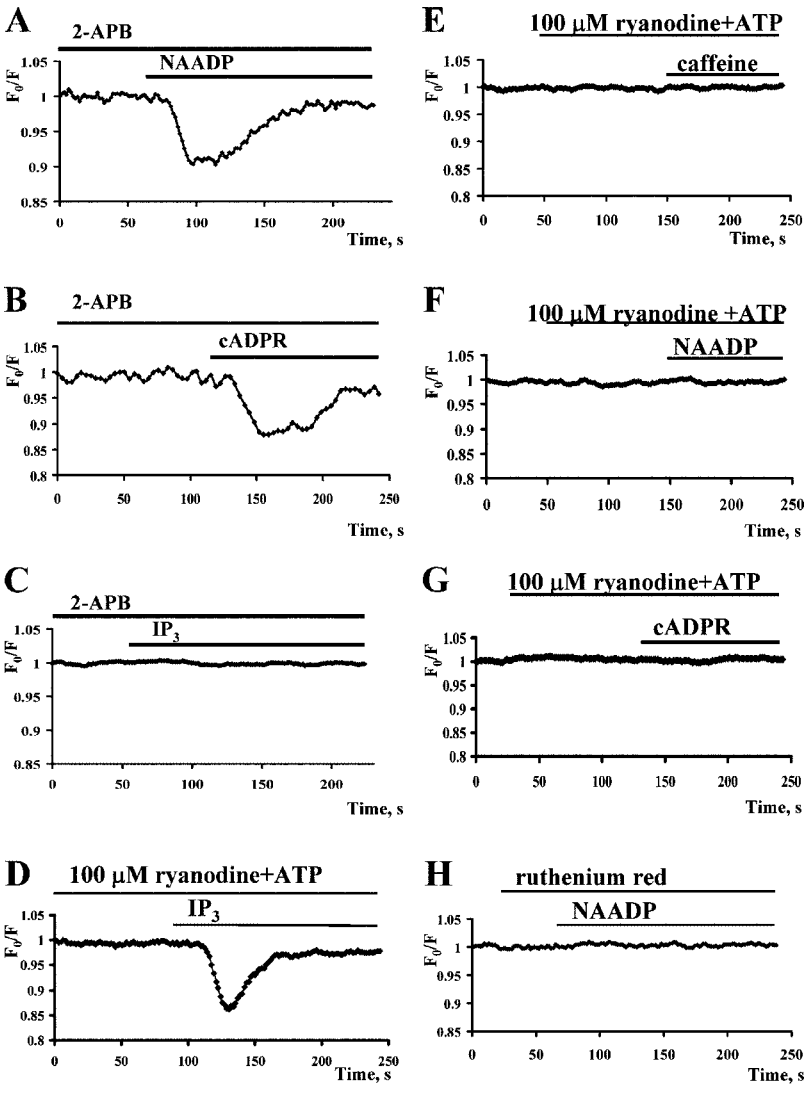

Figure 4. **Effects of 2-APB and ryanodine on messenger-elicited changes in the nuclear envelope Ca^{2+} concentration in the presence of 1 μM ATP.** (A) In the presence of 100 μM 2-APB, 200 nM NAADP induced a transient reduction in the envelope Ca^{2+} concentration. (B) In the presence of 100 μM 2-APB, 10 μM cADPR elicited a transient response. (C) 20 μM IP_3 failed to elicit any change in the nuclear envelope Ca^{2+} concentration in the presence of 100 μM 2-APB. (D) In the presence of 100 μM ryanodine, 20 μM IP_3 elicited a normal Ca^{2+} concentration change, whereas responses to 10 mM caffeine (E), 200 nM NAADP (F), and 10 μM cADPR (G) were abolished. (H) 10 μM ruthenium red completely abolished response to 200 nM NAADP.

G; $n = 6$). 10 μM 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 μM cADPR, and 20 μM 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 ~0.5 μM.

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-

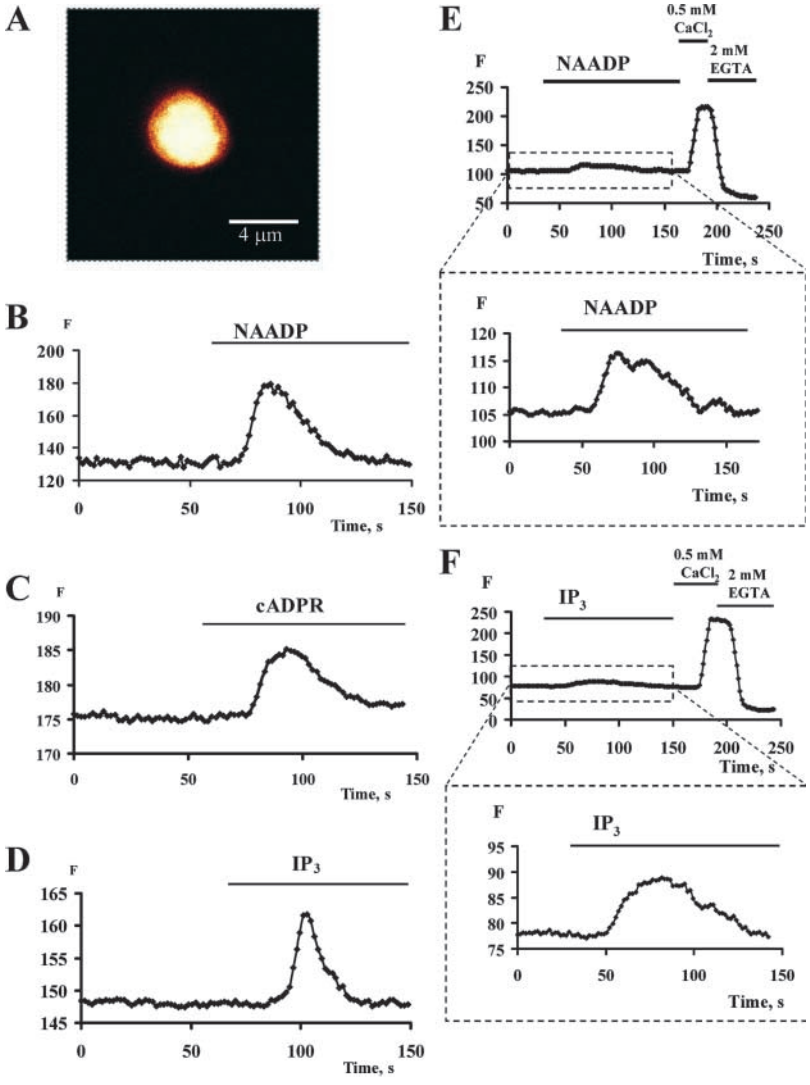

Figure 5. **The distribution of Fluo-4 dextran in a single isolated nucleus and the effects of messenger stimulation on Ca^{2+} concentration in the nucleoplasm.** (A) Fluorescence image showing uniform distribution of Fluo-4 dextran throughout the nucleoplasm of a single isolated nucleus. 200 nM NAADP (B), 10 μM cADPR (C), and 20 μM IP_3 (D) induced transient elevations of the nucleoplasmic Ca^{2+} concentration. After stimulation with 200 nM NAADP (E) or 20 μM 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.

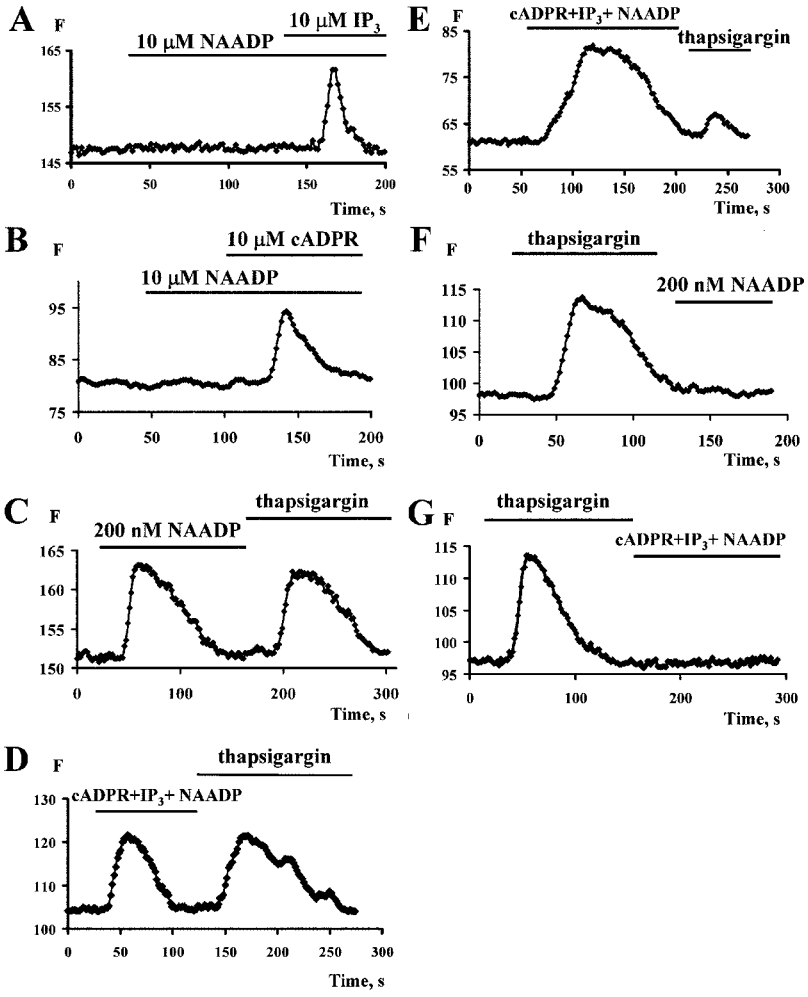

Figure 6. **The effects of Ca^{2+}-releasing messengers and thapsigargin on the nucleoplasmic Ca^{2+} concentration.** Both 10 μM IP_3 (A) and 10 μM cADPR (B) elicited a normal transient rise in the nucleoplasmic Ca^{2+} concentration in the presence of a high desensitizing NAADP concentration (10 μM), which did not itself induce any response. 10 μM thapsigargin elicited a substantial renewed rise in the nucleoplasmic Ca^{2+} concentration after stimulation, in the presence of 1 mM ATP, with 200 nM NAADP (C) or a mixture of 200 nM NAADP, 20 μM IP_3, and 10 μM cADPR (D), but not in the absence of ATP (E). After 10 μM thapsigargin had elicited a transient rise in the nucleoplasmic Ca^{2+} concentration, 200 nM NAADP (F) or a mixture of 10 μM cADPR, 20 μM IP_3, and 200 nM NAADP (G) failed to evoke any further nucleoplasmic Ca^{2+} rise.

vious work (Brini et al., 1993; Gerasimenko et al., 1995; Lipp et al., 1997). The same protocol was used after stimulation with 20 μM IP_3, and very similar results were obtained (Fig. 5 F; $n = 6$). These results indicate that the nuclear pore complexes are permeable to Ca^{2+} even after depletion of the nuclear envelope Ca^{2+} stores.

Interaction between Ca^{2+}-releasing agents

As already described, a high concentration (10 μM) of NAADP did not evoke any Ca^{2+} release from the nuclear envelope, most likely due to rapid auto-desensitization (Fig. 1). Subsequent application of IP_3 (Fig. 6 A; $n = 6$) or cADPR (Fig. 6 B; $n = 6$), during continued exposure to NAADP, elicited normal Ca^{2+} release responses, indicating that the activation of IP_3 or ryanodine receptors does not have an obligatory requirement for operational NAADP receptors. The fact that cADPR can evoke Ca^{2+} release in the presence of a high desensitizing NAADP concentration (10 μM; Fig. 6 B) indicates that cADPR and NAADP most likely act on two separate receptors, although both agents cause Ca^{2+} release via opening of ryanodine receptors (Fig. 4).

We investigated the nature of the pool from which Ca^{2+} could be released by NAADP and the other messengers. When ATP was present, and reaccumulation of lost Ca^{2+} therefore was possible, thapsigargin was able to elicit renewed Ca^{2+} release after an NAADP-induced Ca^{2+} rise in the nucleoplasm (Fig. 6 C; $n = 13$). This was also the case after application of the triple messenger mixture cADPR + IP_3 + NAADP (Fig. 6 D; $n = 12$).

In the absence of ATP, application of thapsigargin induced a markedly reduced Ca^{2+} release after exposure to the messenger mixture, suggesting that most of the intranuclear Ca^{2+} had already been liberated (Fig. 6 E; $n = 5$). Pretreatment of nuclei with thapsigargin abolished the responses to NAADP (Fig. 6 F; $n = 5$) or the triple messenger mixture (Fig. 6 G; $n = 6$), indicating that NAADP and the other messengers release Ca^{2+} from a thapsigargin-sensitive store and that the whole of the thapsigargin-sensitive Ca^{2+} store can be released by the messengers.

Does NAADP release Ca^{2+} from acid compartments?

Recent work on sea urchin eggs indicates that NAADP mobilizes Ca^{2+} from an acid thapsigargin–insensitive pool, with lysosomal properties, that is separate from those sensitive to IP_3 and cADPR (Churchill et al., 2002). Therefore, it seemed important to test whether the NAADP-elicited Ca^{2+} 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-

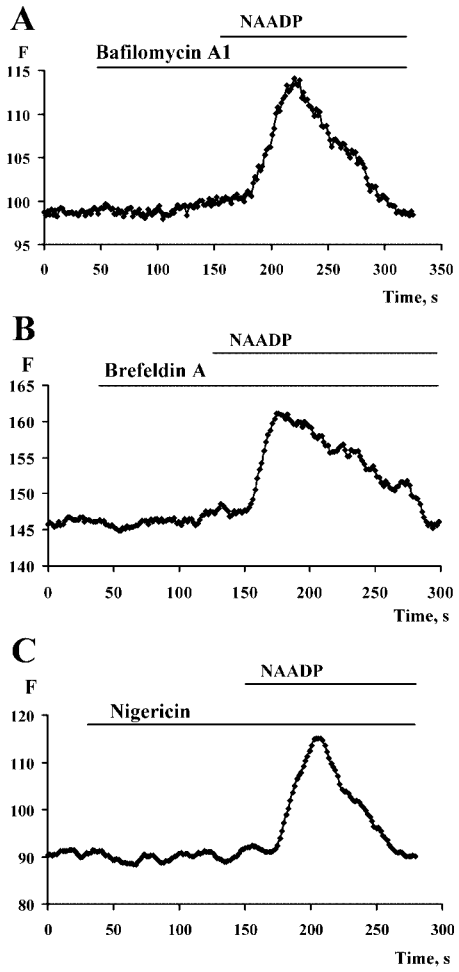

Figure 7. **Interference with acidic compartments does not inhibit nucleoplasmic Ca^{2+} responses to stimulation with NAADP.** 200 nM NAADP evokes a normal transient rise in the nucleoplasmic Ca^{2+} concentration during exposure to 50 nM bafilomycin A1 (A), 10 μM brefeldin A (B), or 7 μM nigericin (C).

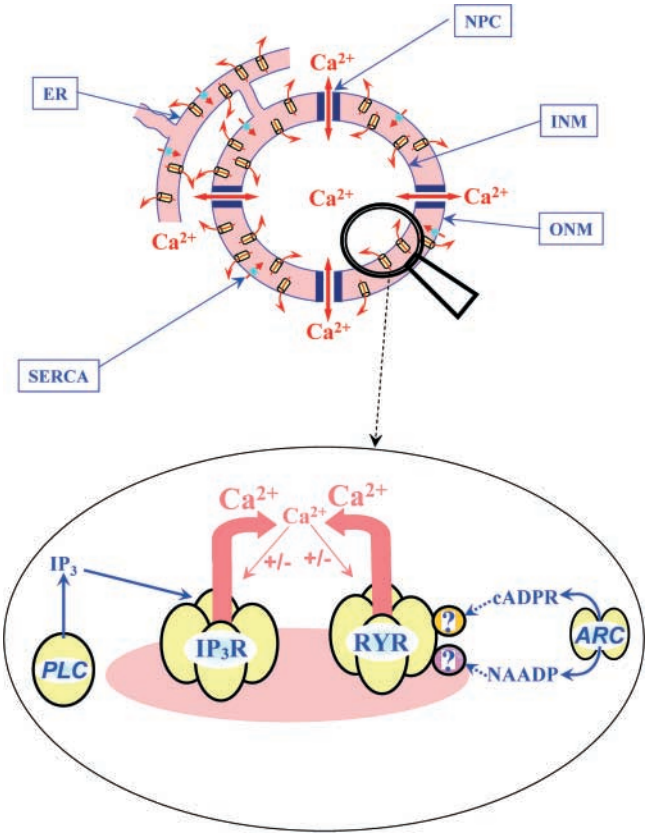

Figure 8. **Simplified model of Ca^{2+} release through two separate release channels from one unified store in the nuclear envelope and surrounding ER.** Ca^{2+} liberated from the store enters the nucleoplasm either directly through channels in the inner nuclear membrane (INM) or via the nuclear pore complexes (NPC) when released through channels in the outer nuclear membrane (ONM) or the surrounding ER. Messengers, IP_3 generated by phospholipase C (PLC) or cADPR and NAADP generated by the ADP ribosyl cyclase (ARC), can be produced either in the nucleoplasm or in the cytosol and then enter the nucleoplasm via the NPCs. Ca^{2+} is pumped into the nuclear envelope and ER by the sarco-ER Ca^{2+}-activated ATPase (SERCA).

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 μM 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 μM 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-

atic acinar cells, there is evidence for cADPR-elicited Ca^{2+} spiking (Thorn et al., 1994), and Ca^{2+} spiking evoked by the physiological agonists acetylcholine and cholecystokinin, as well as by the Ca^{2+}-releasing agents IP_3, cADPR, and NAADP, are all inhibited not only by IP_3 receptor antagonists, but also by a high ryanodine concentration (Cancela et al., 2000; Cancela, 2001). A high ryanodine concentration inhibits Ca^{2+}-induced Ca^{2+} release waves initiated in the apical pole of pancreatic acinar cells (Ashby et al., 2002, 2003b). Here, we have provided fresh evidence for the presence of ryanodine receptors in the nuclear envelope by using specific staining with fluorescent ryanodine (Fig. 1, E and F) and by demonstrating that both NAADP and cADPR elicit Ca^{2+} release by opening ryanodine-sensitive pathways (Fig. 4). NAADP and cADPR most likely bind to different receptors, which then directly or indirectly activate ryanodine receptors, as cADPR can elicit marked Ca^{2+} release in the presence of a high desensitizing NAADP concentration (Fig. 6 B).

In view of the evidence for operational ryanodine receptors in the pancreatic cells, it has always been puzzling that caffeine, a well-established activator of ryanodine receptors (Ehrlich et al., 1994; Solovyova et al., 2002), generally fails to evoke Ca^{2+} release in these cells (Petersen and Cancela, 1999). However, caffeine effectively blocks agonist- and messenger-elicited cytosolic Ca^{2+} signal generation due to its inhibitory effect on the IP_3 receptors (Wakui et al., 1990; Bezprozvanny et al., 1994; Ehrlich et al., 1994; Petersen and Cancela, 1999). In isolated nuclei, we have now obtained the first clear evidence for caffeine-elicited Ca^{2+} release in pancreatic acinar cells (Fig. 2) which, as expected, is blocked by a high ryanodine concentration (Fig. 4).

The mechanism of action of NAADP

The pioneering work on the Ca^{2+}-releasing effect of NAADP was performed on sea urchin eggs (Chini et al., 1995; Lee and Aarhus, 1995), and in this preparation, the store from which NAADP releases Ca^{2+} does not appear to have ER properties and is separate from the stores on which IP_3 and cADPR act (Churchill et al., 2002). Because NAADP-induced Ca^{2+} release in the sea urchin eggs does not behave as a Ca^{2+}-induced Ca^{2+}-release system (Chini and Dousa, 1996), it has generally been assumed that NAADP does not act on IP_3 or ryanodine receptors, but activates a separate type of Ca^{2+} release channel that has not yet been characterized (Cancela, 2001). Our new data do not fit this concept. In the pancreatic nucleus, NAADP releases Ca^{2+} from the same thapsigargin-sensitive store as IP_3 (Fig. 2, F–H; Fig. 6; Fig. 8). Furthermore, the Ca^{2+} release normally elicited by NAADP (but not by IP_3) is blocked by a high ryanodine concentration (Fig. 4, D and F) as well as by ruthenium red (Fig. 4 H). Finally, we have shown that interference with acid compartments has no effect on the NAADP-evoked Ca^{2+} release from the nuclear envelope (Fig. 7). The simplest interpretation of these data suggests that NAADP activates ryanodine receptors. Hohenegger et al. (2002) have recently described NAADP activation of single purified and reconstituted type 1 ryanodine receptors from skeletal muscle. This action, like the one in the pancreatic nuclei, is specific (no effect of NADP), ryanodine sensitive, 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-elicited 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 polyphosphoinositide 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

Mag-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 Mag-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 Ca^{2+} concentration in the nuclear envelope was assessed by Mag-Fura Red™ fluorescence measurements (excitation 488 nm, emission 550–650 nm) or by Rhod 5N fluorescence measurements (excitation 543 nm, emission 555–630 nm). The nucleoplasmic Ca^{2+} concentration changes were assessed by Fluo-4 dextran (MW = 10,000) fluorescence measurements (excitation 488 nm, emission 500–550 nm). Nuclear preparations were stained with 0.2 μM BODIPY® FL thapsigargin, 1 μM BODIPY® FL ryanodine, 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 IP_3 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 software in combination with custom-written macros. Fluorescence of Mag-Fura Red™ was excited at 488 nm, and a second laser (Coherent™) provided light for uncaging in the area of interest at wavelengths of 351 and 364 nm for the duration of the uncaging (74–998 ms). 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 Mag-Fura Red™ in a small area of the nuclear envelope.

IP_3 and cADPR were also applied by ionophoretic ejection from a microelectrode filled with a solution containing 1 mM IP_3 or cADPR (the ejecting current was 50–100 nA, retaining current 5–20 nA). An injection system (HVCS 02; NPI Electronics) was used for these experiments. All experiments were performed using a Leica confocal two-photon system with water immersion objective (63×, NA 1.2).

Online supplemental material

Mathematical model of IP_3-induced Ca^{2+} 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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