Regulation of Nuclear Ca\(^{2+}\) Signaling by Translocation of the Ca\(^{2+}\) Messenger Synthesizing Enzyme ADP-ribosyl Cyclase during Neuronal Depolarization*\\[S]

Received for publication, June 19, 2008 Published, JBC Papers in Press, July 16, 2008, DOI 10.1074/jbc.M804701200

Stéphanie Bezin\(^1\), Gilles Charpentier\(^1\)\(^,\)\(^5\), Hon Cheung Lee\(^4\), Gérard Baux\(^1\), Philippe Fossier\(^\dagger\), and José-Manuel Cancela\(^1\)

From the \(^1\)Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, UPR 9040, 1, Avenue de la Terrasse, 91198 Gif-Sur-Yvette Cedex, France, the \(^5\)Université Bordeaux 1 Laboratoire DMPFCS, IECB, 2, Rue Robert Escarpit, 33607 Pessac, France, and the \(^4\)Department of Physiology, University of Hong Kong, 4/F Lab Block, Faculty of Medicine Building, 21 Sassoon Road, Hong Kong

In neurons, voltage-gated Ca\(^{2+}\) channels and nuclear Ca\(^{2+}\) signaling play important roles, such as in the regulation of gene expression. However, the link between electrical activity and biochemical cascade activation involved in the generation of the nuclear Ca\(^{2+}\) signaling is poorly understood. Here we show that depolarization of *Aplysia* neurons induces the translocation of ADP-ribosyl cyclase, a Ca\(^{2+}\) messenger synthesizing enzyme, from the cytosol into the nucleus. The translocation is dependent on Ca\(^{2+}\) influx mainly through the voltage-dependent L-type Ca\(^{2+}\) channels. We report also that specific nucleoplasmic Ca\(^{2+}\) signals can be induced by three different calcium messengers, cyclic ADP-ribose, nicotinic acid adenine dinucleotide phosphate (NAADP), both produced by the ADP-ribosyl cyclase, and inositol 1,4,5-trisphosphate (IP\(_3\)). Moreover, our pharmacological data show that NAADP acts on its own receptor, which cooperates with the IP\(_3\) and the ryanodine receptors to generate nucleoplasmic Ca\(^{2+}\) oscillations. We propose a new model where voltage-dependent L-type Ca\(^{2+}\) channel-induced nuclear translocation of the cytosolic cyclase is a crucial step in the fine tuning of nuclear Ca\(^{2+}\) signals in neurons.

Ca\(^{2+}\) is one of the most important intracellular messengers in living cells and is involved in many cellular functions such as egg fertilization, synaptic plasticity, muscle cell contraction, gene expression, secretion, and cell proliferation (1–5). Many Ca\(^{2+}\) signals are not all-or-none events; they vary greatly in amplitude, duration, and localization (1, 6). In neurons, Ca\(^{2+}\) entry through voltage-dependent L-type Ca\(^{2+}\) channels and N-methyl-D-aspartic acid receptors has been shown to be important in nuclear Ca\(^{2+}\) signaling generation and, for example, in Ca\(^{2+}\)-dependent regulation of gene expression (7, 8). Although Ca\(^{2+}\) signaling in the nucleus has been shown to be a key element in the regulation of numerous neuronal functions such as gene transcription and synaptic plasticity, the mechanism involved in its generation remains largely unexplored (1, 9–11).

Nucleoplasmic Ca\(^{2+}\) changes can result either from passive diffusion through the nuclear pore following cytosolic Ca\(^{2+}\) increases or from mobilization of Ca\(^{2+}\) sequestered into the nuclear envelope by various intracellular messengers (12, 13). In the latter process, cytosolic and nucleoplasmic Ca\(^{2+}\) can be independently regulated (12, 14, 15). The differential control of the cytosolic and nuclear Ca\(^{2+}\) involves, in principle, several factors including cytosolic Ca\(^{2+}\) buffering and Ca\(^{2+}\) sequestration by the endoplasmic reticulum and the mitochondria (12, 14–18). The nucleus is equipped with all the necessary machinery for generating nuclear Ca\(^{2+}\) signaling, including Ca\(^{2+}\) pumps and release channels (12). Several groups have identified functional inositol 1,4,5-trisphosphate (IP\(_3\))\(^2\) and/or ryanodine receptors in the inner membrane of the nuclear envelope (19–23) or in the outer membrane of the contiguous nucleoplasmic reticulum (23–25).

NAADP and cADPR, two Ca\(^{2+}\) releasing messengers are produced by multifunctional enzymes of the ADP-ribosyl cyclase family (26–28). Several of these enzymes have been purified and cloned, including the ectoenzymes CD38 and CD157, and a soluble cyclase from the sea mollusk *Aplysia* (28). The surface antigen CD38 has been mostly found at the plasma membrane with the catalytic site exposed to the extracellular space (29). To solve this topological paradox, several authors have proposed that CD38 could generate the synthesis of extracellular cADPR, which then may enter into the cell interior possibly through nucleoside transporters (30). In addition, intracellular locations of CD38 have been reported such as in nuclei where no links with physiological stimuli were reported and no evidence for a nuclear role for NAADP (19, 22). The synthesis of NAADP through the base-exchange reaction by this enzyme requires exclusively acidic conditions. In the case of the *Aplysia* cyclase, although the base-exchange reaction depends critically on the pH, it appears that NAADP synthesis is possible at more neutral pH (26). In mammals, additional enzymes exist and CD38 may not be regarded as the principal

---

*The work was supported by grants from the Association Française contre les Myopathies and the Association pour la Recherche sur le Cancer (to J.-M.C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†Online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

‡To whom correspondence should be addressed. Tel.: 33-01-69-82-41-69; Fax: 33-01-69-82-41-41; E-mail: jose.cancela@nbcm.cnrs-gif.fr.

§The abbreviations used are: IP\(_3\), inositol 1,4,5-trisphosphate; NAADP, nicotinic acid adenine dinucleotide phosphate; cADPR, cyclic ADP-ribose; ASW, artificial seawater; BAPTA, 1,2-bis(aminophenoxy)ethane-N,N,N',N’-tetraacetic acid.
Nuclear Translocation of the ADP-ribosyl Cyclase

cyclase (31–33). For example, a novel ADP-ribosyl cyclase for cADPR synthesis has recently been detected in the brain (31) and one for NAADP synthesis in myometrial cells (34). Recently a soluble form has been purified in sea urchin (35). Clearly, our understanding of the physiological role of the expanding ADP-ribosyl cyclase family remains poor and the cyclase from Aplysia remains the only prototypical form for the soluble enzyme characterized in neurons so far.

Pioneering work on the neurobiology of learning has been performed on Aplysia californica. For example, it has been found that synaptic potentiation at sensory-motor neuron synapses require Ca2+ entry through L-type Ca2+ channels and/or Ca2+ mobilization from intracellular stores sensitive to IP3 and ryanodine depending of the potentiation type (7, 36–38). Our previous studies have shown that the soluble ADP-ribosyl cyclase is not only present in the neurons of the Aplysia buccal ganglion, but that its product cADPR, an endogenous modulator of the ryanodine receptors, can enhance the evoked synaptic transmission (39). More recently, we showed that NAADP, the other product of the cyclase, can increase neurotransmitter release at well identified cholinergic synapses (40). To further delineate the Ca2+ signaling pathway mediated by the Aplysia cyclase, we looked at its distribution in the nervous system that has not been characterized.

In this study, we have revealed for the first time that in resting conditions, the soluble Aplysia ADP-ribosyl cyclase is localized in the cytosol of the soma of neurons. Importantly, we have found that depolarization of Aplysia neurons induces the translocation of ADP-ribosyl cyclase from the cytosol into the nucleus. The translocation is dependent on Ca2+ influx through the voltage-dependent L-type Ca2+ channels. We also show that the neuronal nucleus is a Ca2+ store, which could generate specific nucleoplasmic Ca2+ signals in response to three different calcium messengers, cADPR, NAADP, both produced by the ADP-ribosyl cyclase, and IP3. Finally, we propose that the translocation of the Ca2+ signaling enzyme to nucleus following neuronal depolarization may provide a new link between the electrical activity and the biochemical cascades leading to nuclear Ca2+ signals generation.

MATERIALS AND METHODS

Preparation of Isolated Nuclei—Most of the experiments were done on nuclei isolated from abdominal, pedal, and pleural ganglia of adult A. californica and Aplysia punctata. The animals were first perfused with an isotonic MgCl2 solution and the ganglia, after extraction, were pinned in a chamber bathed with artificial seawater (ASW) (NaCl 460 mM; KCl 10 mM; CaCl2 11 mM; MgCl2 25 mM; MgSO4 28 mM; Tris-HCl buffer 10 mM; pH 7.8). The connective tissue packing the neurons was sharply removed with fine forceps. Then the neurons were depolarized by addition of 60 mM KCl (with equimolar substitution of NaCl in the intracellular medium). To measure Ca2+ changes in the intact Aplysia neurons, we used as previously described intracellular injections of Rhod-2 (Kd 570 nM), selected as the best Ca2+-sensitive probe in Aplysia neurons that exhibit autofluorescence at some excitation wavelengths (40). Intracellular injections were performed using air pressure pulses delivered by controlled air valves (Pico Pump, WPI). The Rhod-2 concentration in the injecting pipette was at 1 mM and neurons were injected until they appeared dark red with an estimated dye concentration of 100 μM. Neurons were depolarized by addition of 60 mM KCl (with equimolar substitu-
FIGURE 1. Localization of the Aplysia cyclase in intact nervous ganglia. To visualize the nucleus of the giant neurons, dissected ganglia of Aplysia were stained with a specific nucleic acid marker (SYTO 13 Green or 4',6-diamidino-2-phenylindole (DAPI); A and B, respectively). In resting conditions, Aplysia cyclase (green) was localized in the cytosol of the neuron soma (B). After depolarization of the neurons by addition of KCl, the cyclase translocated into the nucleus (C). D, Ca^{2+} response evoked by a sustained KCl-dependent depolarization of neurons.
Nuclear Translocation of the ADP-ribosyl Cyclase

A

BAPTA-AM + KCl

B

Nifedipin + KCl

C

Bay K-8644

D

Conotoxins + KCl

E

KCl

F/F₀

sec

Control

Nifedipine

BAPTA
tion of NaCl). To visualize DNA in the Aplysia ganglia, we used the cell-permeant SYTO 13 Green from Molecular Probes at a concentration of 5 µM (λex = 488 nm and λem = 509 nm).

Sequence Analysis—The sequence of the Aplysia ADP-ribosyl cyclase was scanned for consensus motifs homologies, using the Prosite Data base on the ExPASy proteomic server.

RESULTS

Localization of the Aplysia Cyclase in Intact Nervous Ganglia—The Aplysia cyclase was first discovered in the ovotestis (28, 42), but its distribution in the nervous system of Aplysia has not been characterized. Typical Aplysia neurons contain a large nucleus surrounded by a thin cytoplasm (Fig. 1A). The localization of the enzyme was revealed by confocal microscopy using a specific antibody raised against the cyclase (41). In resting conditions, the cyclase was almost exclusively localized in the thin cytosol surrounding the nucleus of the soma (Fig. 1B, n = 75/81). To investigate whether the cyclase distribution could change during neuronal activities, the neurons were depolarized for 20 min by the addition of KCl, which evoked a Ca2+ response (Fig. 1D, n = 7). Under depolarizing conditions, nuclear localization of the enzyme was dramatically enhanced (Fig. 1C, n = 35/42). This depolarization-induced Ca2+ elevation and translocation of the cyclase was prevented by prior incubation of the ganglia with the Ca2+ chelator BAPTA-AM (50 µM, 30 min, n = 28/29) (Fig. 2, A, B, and E, n = 4). The next step was to investigate what types of voltage-gated Ca2+ channels could be involved in our observation. Previous studies from our laboratory on Aplysia neurons have shown that during neuronal depolarization the Ca2+ currents are reduced nearly 40–50% in the presence of the L-type Ca2+ channel blockers nifedipine and N-type, P-type voltage-gated Ca2+ channels block nearly 40% of calcium influx during depolarization by conotoxins (43). So, in Aplysia neurons, 80–90% Ca2+ entry occurred through L-type, N-type, and P-type voltage-gated Ca2+ channels.

In our experiments, extracellular addition of the L-type Ca2+ channel inhibitor (nifedipine 10 µM, Sigma, n = 33/33) likewise prevented the translocation and reduced dramatically the Ca2+ responses evoked by KCl (Fig. 2, B and E, n = 4). In addition, activation of the L-type Ca2+ channel by Bay K-8644 at 10 µM evoked nuclear translocation of the cyclase (Fig. 2C, n = 24/53). In contrast, extracellular application of ω-conotoxins (CNVIIA and S VI B, 5 µM), which block the N- and P-type Ca2+ channels, did not affect the depolarization-induced translocation of the cyclase (Fig. 2D, n = 15/20). These data indicate that translocation of the cyclase to the nucleus is a Ca2+-dependent process triggered by Ca2+ entry through the L-type voltage-dependent channels, which, in turn, are activated by membrane depolarization.

The Nuclear Envelope Is a Ca2+ Store Sensitive to Intracellular Ca2+ Releasing Messengers—Cytosolic Ca2+ buffering by the endoplasmic reticulum and the mitochondria (16) could insulate the nucleus (18). Although it is poorly known in neurons, the nucleus could be autonomous by mobilization of Ca2+ sequestered into the nuclear envelope (11). To investigate whether the nuclear Ca2+ stores are responsive to both cADPR (11) and NAADP, the two Ca2+ messengers produced by the Aplysia cyclase, the large nuclei of the Aplysia neurons were isolated. The luminal Ca2+ of the nuclear envelope was measured by loading Mag Fluo-4 (30 µM) into the lumen, using a membrane permeant AM-form of the Ca2+-sensitive dye of low affinity (Fig. 3A). Fluorimetric measurements using confocal microscopy showed that the luminal space could be loaded with Ca2+ following the addition of ATP (5 mM) suggesting that Ca2+-ATPase is involved in nuclear store refilling (Fig. 3B) (n = 3). More importantly, addition of the second messengers IP3 (5–10 µM; n = 8) or cADPR (5–10 mM; n = 8) (Fig. 3, C and D) likewise decreased the Ca2+ concentration in the nuclear envelope, indicating that it is a fully functional Ca2+ store.

NAADP is known to produce a biphasic response, releasing Ca2+ from the sensitive stores at low concentrations while desensitizing its own receptor at high concentrations (44). Various NAADP concentrations, ranging from 10 nM to 100 µM, were thus tested on the isolated nuclei (Fig. 3, E–G). With NAADP in the nanomolar range (10–500 nM), all nuclei examined responded with decreases in Ca2+ contents (n = 3 for each concentration) (Fig. 3, E and F). In stark contrast, at 1 and 10 µM, NAADP failed to evoke a Ca2+ response in 2 of 3 nuclei tested for each concentration and at 100 µM NAADP, 5 of 6 nuclei, showed no response (Fig. 3G). Finally, if the nuclear Ca2+ stores were first depleted by thapsigargin (5 µM), a widely used inhibitor of the sarco(endoplasmic) Ca2+-ATPase, neither IP3 nor NAADP could release any additional Ca2+, indicating that both messengers release Ca2+ from the same thapsigargin-sensitive stores (Fig. 3H, n = 8 and 10, respectively).

To determine whether the Ca2+ release from the nuclear envelope would result in an increase of Ca2+ in the nucleoplasm, a fluorescent Ca2+ probe, Fluo-4 dextran (10 kDa, 20 µM), was loaded into the nucleoplasm (Fig. 4A). Addition of IP3 (10 µM; n = 21), cADPR (5 µM; n = 14), or NAADP (500 nM; n = 37) at the optimal concentrations determined above indeed evoked a modest (supplemental Fig. S1A) but significant increase of the nucleoplasmic Ca2+ concentration (Fig. 4, B–D). In many nuclei, intranuclear Ca2+ fluctuation events called oscillations were observed on the top of a Ca2+ transient response following application of the second messengers (Fig. 4, B–D). The oscillations were characterized by amplitude of about 0.1 ΔF/F and duration of about 10 s and occurred in bursts. The duration of the Ca2+ oscillation period varied among the nuclei and the frequency was estimated by dividing the number of oscillations by the duration of the burst. In 64% of the investigated nuclei, NAADP evoked 0.7 ± 0.06 oscillations/min for 1340 ± 140 s (n = 18/28), whereas IP3 evoked 1.1 ± 0.14 oscillations/min for

FIGURE 2. Pharmacology of the Aplysia cyclase translocation Aplysia neurons. Addition of BAPTA-AM prevented the translocation of the enzyme (A). Nifedipine similarly inhibited the nuclear translocation of the cyclase in response to KCl depolarization (B), whereas activation of L-type Ca2+ channel by Bay K-8644 evoked nuclear translocation of the cyclase (C). Application of ω-conotoxins had no effect on the depolarization-induced translocation (D). E, Ca2+ responses evoked by a sustained KCl depolarization of neurons is markedly reduced in the presence of nifedipine (10 µM) or after pre-treatment of the neurons with BAPTA-AM at 50 µM.
1300 ± 150 s in 55% of the nuclei \((n = 5/9)\). The difference in the mean frequency evoked by IP3 and NAADP was statistically significant \((p = 0.012)\). The mean frequency of cADPR-evoked Ca^{2+} oscillations was 0.9 ± 0.13/min, for 1075 ± 290 s, in 75% of the nuclei \((n = 6/8)\), which was not significantly different from either of the two means listed above.

The nuclear Ca^{2+} oscillations suggest that the release mechanisms involved are capable of self-limiting by feedback. Indeed, none of the three Ca^{2+} messengers were able to totally discharge the nuclear stores. Addition of a Ca^{2+} ionophore (ionomycin, 5 μM) elicited a much larger increase. Also consistent with feedback is the transient nature of the Ca^{2+} increase (supplemental data Fig. S1B). The decline is most likely due to diffusion of Ca^{2+} out of the nuclei through the nuclear pores, following cessation of Ca^{2+} release. As shown in supplemental data Fig. S1A, after the release induced by IP3 had reached the peak and started to decline, addition of Ca^{2+} (10 mM) elicited a rapid and large increase in the nucleoplasmic Ca^{2+} concentration, which could be buffered back by EGTA, indicating that the nuclear pores are permeant to Ca^{2+}, in agreement with previ...
ous work (13, 16, 21, 45, 46). Similar results were obtained after the NAADP- or cADPR-induced release (n = 6).

In contrast to nanomolar concentrations (Fig. 5A), 100 nM NAADP failed to induce an increase of the nucleoplasmic Ca{	extsuperscript{2+}} (n = 9), consistent with the biphasic behavior described above (Fig. 3). However, subsequent addition of cADPR (5 μM) still resulted in a nucleoplasmic Ca{	extsuperscript{2+}} increase (n = 5) (Fig. 5A). Thus, self-desensitization of the NAADP release mechanism did not impair the effect of cADPR on these nuclei, indicating that cADPR and NAADP target different release mechanisms in the nuclear envelope. The specificity of NAADP-evoked Ca{	extsuperscript{2+}} release was verified by testing two inactive analogs of NAADP, nicotinamide adenine dinucleotide phosphate (NADP, 500 nM) and nicotinic acid adenine dinucleotide (NAAD, 500 nM). Both were ineffective; either applied alone or in combination (n = 5) (Fig. 5B).

The Nuclear Ca{	extsuperscript{2+}} Store Supports Ca{	extsuperscript{2+}} Releasing Receptor Interactions—Previous studies showed that NAADP targets acidic Ca{	extsuperscript{2+}} stores, such as lysosomes and endosomes (47, 48). The isolated nuclei we used appeared to be free of these acidic compartments (supplemental data Fig. S1C).

As shown in Fig. 3H, blocking the endoplasmic Ca{	extsuperscript{2+}} pump with thapsigargin (30 min, 5 μM) can deplete the nuclear stores even in the presence of ATP. After store depletion, none of the three messengers, IP{	extsubscript{3}}, cADPR, nor NAADP, were able to evoke any changes of nucleoplasmic Ca{	extsuperscript{2+}} in 16 of 20 nuclei tested (IP{	extsubscript{3}}, n = 6; cADPR n = 4; NAADP, n = 10) (Fig. 5, D–H).

Our previous study has shown that in intact Aplysia neurons the NAADP receptor itself is not sensitive to heparin or ryanodine (40) but in some intact cells, the cytosolic Ca{	extsuperscript{2+}} signals evoked by NAADP require the activation of adjacent IP{	extsubscript{3}} and/or ryanodine receptors through a Ca{	extsuperscript{2+}}-induced Ca{	extsuperscript{2+}} release mechanism (32, 49–54). Heparin (50 μg/ml) was used to block the IP{	extsubscript{3}} receptor, which indeed inhibited or drastically reduced the IP{	extsubscript{3}}-induced Ca{	extsuperscript{2+}} release (Fig. 6D) in 19 nuclei tested. The heparin treatment had no or very little effect on cADPR-elicited Ca{	extsuperscript{2+}} responses (n = 8) (Fig. 6E), but did block NAADP (500 nM) from evoking Ca{	extsuperscript{2+}} release (Fig. 6F) (n = 22/26). This suggests there was strong cooperation between the IP{	extsubscript{3}} and NAADP receptors in the nuclear envelope, amplifying the Ca{	extsuperscript{2+}} signals induced by NAADP.
Nuclear Translocation of the ADP-ribosyl Cyclase

FIGURE 5. Blockade of second messenger-evoked Ca\(^{2+}\) release by thapsigargin. A, self-desensitization of NAADP receptor by 100 \(\mu\)M NAADP did not impair the effect of cADPR (5 \(\mu\)M). A mixture of inactive NAADP analogs, NAAD (500 nM) and NADP (500 nM), failed to increase the Ca\(^{2+}\) concentration in the nucleoplasm (B). Stimulation of the nuclei with IP\(_3\) (10 \(\mu\)M), cADPR (5 \(\mu\)M), or NAADP (500 nM) elicited a nucleoplasmic Ca\(^{2+}\) increase (C, E, and G), whereas messenger stimulation of nuclei pretreated by thapsigargin (5 \(\mu\)M) failed to evoke a nucleoplasmic Ca\(^{2+}\) response (D, F, and H).

Treatment of the Aplysia nuclei with 500 \(\mu\)M ryanodine did not block the Ca\(^{2+}\) release elicited by IP\(_3\) (10 \(\mu\)M) (Fig. 6G), but strongly inhibited both the cADPR (5–10 \(\mu\)M) evoked (Fig. 6H, \(n = 8\)) and NAADP (500 nM) evoked responses (Fig. 6I). In the presence of ryanodine, NAADP failed to induce a Ca\(^{2+}\) release in 22 of 33 nuclei, whereas in the 11 responding nuclei the release was drastically reduced in amplitude (\(n = 11\)). These data indicate that full NAADP-induced Ca\(^{2+}\) responses require functioning ryanodine receptors. Another drug that has been reported to be effective in inhibiting NAADP signaling in intact cells is SKF 96365 (55, 56). The drug (10 \(\mu\)M) had no significant effect on the IP\(_3\)- or cADPR-evoked Ca\(^{2+}\) release (\(n = 4\) for IP\(_3\); \(n = 5\) for cADPR) (Fig. 6, J and K) but blocked the NAADP-evoked response (Fig. 6L) (\(n = 16\)). Our data contrast with what has been described in preparations of nuclei isolated from pancreatic acinar cells, where NAADP was suggested to target the ryanodine receptor (13).

DISCUSSION

NAADP is a Ca\(^{2+}\) releasing molecule that has recently been added to the list of second messengers for Ca\(^{2+}\) mobilization (57). The Ca\(^{2+}\) releasing property of NAADP was first discovered in sea urchin eggs (58) and has since been found in other living organisms including plants and mammals (32, 54, 59). It is known to be involved in regulating a wide range of physiological processes (6, 60–62). Evidence suggests it targets a distinct, but yet uncharacterized, receptor in the thapsigargin-insensitive stores, such as lysosomes or secretory granules (47, 48, 63, 64). Interestingly, it has been recently suggested that the lysosomal Trp-like channel mucolipin I could be the target of NAADP (65). In the same way, the differential effects of SK&F 96365 we observed in our nuclear experiments indicate that the release mechanisms activated by the three Ca\(^{2+}\) messengers have different pharmacology, suggesting separate and distinct receptors are involved (Fig. 7). Our experiments provide evidence for the nuclear envelope as a new Ca\(^{2+}\) store sensitive to NAADP in neurons through the activation of its own receptor. This nuclear NAADP sensitivity could in principle correlate well with the Trp-like channel mucolipin I distribution because it has a putative nuclear localization sequence (66). The density of the NAADP receptor in the nucleus, however, would appear to be low, and its full effect requires the amplification of nearby IP\(_3\) and ryanodine receptors (Figs. 6 and 7). The Ca\(^{2+}\) store distribution can be entirely different in the synapses. Indeed, electrophysiological experiments have shown that neurotransmitter release is enhanced by IP\(_3\), cADPR, or NAADP injections into the intact presynaptic Aplysia neurons. In these injection experiments in the presynaptic neurons the Ca\(^{2+}\) responses evoked by the three messengers are all comparable and independent (40). Thus, the NAADP-induced release is shown to be unaffected by blockage of either the IP\(_3\) or ryanodine receptor, indicating the density of the NAADP receptor would be high enough in the synapses that further amplification by the Ca\(^{2+}\)-induced Ca\(^{2+}\) release is not necessary.

In neurons, nucleoplasmic Ca\(^{2+}\) elevations are particularly important for transcription factor activation. Calcium has been shown to directly bind transcription factors like DREAM (4) or activate the nuclear CaM kinase pathways to regulate gene expression (67). Despite its crucial importance, in neurons, the mechanism involved in the generation of nuclear Ca\(^{2+}\) signals is poorly known. In our experiments, we have shown that the neuronal nucleus is able to generate calcium oscillations in response to second messengers. Nuclear oscillations have been observed in the nucleus of intact starfish oocytes in response to injection of cADPR (68). Similar oscillations have also been
seen in mammalian neurons in response to activation of metabotropic glutamate receptors (69). They are likely to be the result of opening and closing clusters of the release channels, as proposed for the Ca\(^{2+}\) sparks seen in the myocytes. In nuclei from pancreatic acinar cells, no nuclear Ca\(^{2+}\) oscillations were reported for any of the three messengers tested (13). Our study is the first to report that the three well known second messengers evoke nuclear Ca\(^{2+}\) oscillations with distinct frequencies within a single target nucleus. Ca\(^{2+}\) oscillations, especially when occurs in the nucleoplasm, are more efficient in activating transcription factors than a sustained Ca\(^{2+}\) elevation (1). Interestingly, previous studies established that optimal activation of transcription factors like NF-AT is achieved when oscillation frequency is comprised in a window from 0.66/min to 2/min (70, 71), which fits with the second messenger-evoked Ca\(^{2+}\) oscillation frequencies obtained in our study. The CaM kinase II is particularly sensitive to calcium oscillations and has been characterized as a frequency decoder able to transduce these frequencies in different amounts of activity (72). In addition, in our experiments we calculated that the amplitude of the Ca\(^{2+}\) elevation observed in our isolated nuclei is about 200 nM and is in a range reported for substantial activation of the neuronal CaM kinases of 100–400 nM Ca\(^{2+}\)/calmodulin concentrations (72). Finally, the frequency of Ca\(^{2+}\) oscillations is of first importance in the physiology of neurons. For example, it has been found that a 3-fold variation of frequency of the spontaneous Ca\(^{2+}\) spikes in activity in embryonic spinal neuronal regulates the neurotransmitter phenotype expression (73). In this respect, although we have no evidence for it, the various Ca\(^{2+}\) oscillation frequencies observed in our study could provide a mean to discriminate among differential transcription pathways (70, 74).

The presence of three different and functional Ca\(^{2+}\) release mechanisms in a cell provides a high degree of versatility for Ca\(^{2+}\) signaling. These release mechanisms do not need to be distributed uniformly inside the cells. The nuclear translocation of the cyclase in the Aplysia neurons described in this study documents yet a novel way for selective and specific activation of the nuclear Ca\(^{2+}\) stores, which are the main stores in the neurons, which have very little cytoplasm (Fig. 1). In non-neuronal cells, the phospholipase C and protein kinase C, which resulted in nuclear synthesis of IP\(_3\) and diacylglycerol have also been reported to translocate to the nucleus in response to physiological stimuli (12, 20, 24, 75, 76). Here we provide the first evidence showing that the soluble ADP-ribosyl cyclase, the enzyme responsible for the synthesis the other two Ca\(^{2+}\) messengers, cADPR and NAADP, can specifically be induced to translocate into the nucleus. The exact mechanism involved in such a translocation is not yet elucidated. This translocation occurs in a Ca\(^{2+}\)-dependent manner and through
specific L-type voltage-dependent channels activation. We have a primary analysis of the ADP-ribosyl cyclase amino acid sequence, which revealed the presence of putative consensus motifs (supplemental data Fig. S2). Indeed myristoylation and phosphorylation sites for PKC and casein kinase II are present. The myristoylation is a post-translational modification that allows protein attachment to the cytoplasmic face of the intracellular membrane. Very recently, a new concept called "calcium/myristoyl switch" has been reported for targeting protein to different organelles (77–80). This mechanism implies a protein conformational change that exposes the myristoyl group following calcium entry. This new mechanism could be highly relevant to the regulation of the cellular localization of the Aplysia cyclase and opens new perspectives for future investigation.

Two main mechanisms have been suggested for explaining the nuclear Ca\textsuperscript{2+}–dependent transcriptional cascade involving L-type Ca\textsuperscript{2+} channels. One involves Ca\textsuperscript{2+} entry and diffusion to the nucleus and the other involves activation of the calcium-dependent signaling protein at the mouth of the L-type Ca\textsuperscript{2+} channels (80). Our study clearly brings evidence that a synthesizing messenger enzyme could be recruited by Ca\textsuperscript{2+} entering specifically through the voltage-dependent L-type Ca\textsuperscript{2+} channels (8). C, specific nucleoplasmic Ca\textsuperscript{2+} signals can be induced by three different calcium messengers, cADPR, NAADP, both produced by the ADP-ribosyl cyclase, and IP\textsubscript{3}. We show that NAADP acts on its own receptor, which cooperates with the IP\textsubscript{3} and ryanodine receptors to generate nucleoplasmic Ca\textsuperscript{2+} oscillations.

Acknowledgments—We are indebted to M. Cantou from the Station Méditerranéenne de l’Environnement et du Littoral, Sète (France) and O. Richard from the Syndicat Mixte d’Equipement Littoral, Blainville-sur-Mer (France) for collecting Aplysia. We thank Jordi Molgo who provided CN VIIA and S VI B. We thank N. Cheviron for technical assistance and the platform d’imagerie RIO de Gif-sur-Yvette.

FIGURE 7. Nuclear translocation of Aplysia ADP-ribosyl cyclase and specific nuclear Ca\textsuperscript{2+} oscillations generated by the three messengers. In resting conditions, the soluble Aplysia ADP-ribosyl cyclase is localized in the thin cytosol surrounding the nucleus of the soma (A). Depolarization of Aplysia neurons induces the translocation of ADP-ribosyl cyclase from the cytosol into the nucleus. The translocation is dependent on Ca\textsuperscript{2+} influx mainly through the voltage-dependent L-type Ca\textsuperscript{2+} channels (B). C, specific nucleoplasmic Ca\textsuperscript{2+} signals can be induced by three different calcium messengers, cADPR, NAADP, both produced by the ADP-ribosyl cyclase, and IP\textsubscript{3}. We show that NAADP acts on its own receptor, which cooperates with the IP\textsubscript{3} and ryanodine receptors to generate nucleoplasmic Ca\textsuperscript{2+} oscillations.
Nuclear Translocation of the ADP-ribosyl Cyclase

73. Borodinsky, L. N., Root, C. M., Cronin, J. A., Sann, S. B., Gu, X., and Spitzer, N. C. (2004) Nature 429, 523–530
74. Dolmetsch, R. E., Xu, K., and Lewis, R. S. (1998) Nature 392, 933–936
75. Cardenas, C., Muller, M., Jaimovich, E., Perez, F., Buchuk, D., Quest, A. F., and Carrasco, M. A. (2004) J. Biol. Chem. 279, 39122–39131
76. Divecha, N., Banfic, H., and Irvine, R. F. (1993) Cell 74, 405–407
77. O’Callaghan, D. W., and Burgoyne, R. D. (2003) Biochem. Soc. Trans. 31, 963–965
78. O’Callaghan, D. W., Haynes, L. P., and Burgoyne, R. D. (2005) Biochem. J. 391, 231–238
79. O’Callaghan, D. W., Ivings, L., Weiss, J. L., Ashby, M. C., Tepikin, A. V., and Burgoyne, R. D. (2002) J. Biol. Chem. 277, 14227–14237
80. Dolmetsch, R. E., Pajvani, U., Fife, K., Spotts, J. M., and Greenberg, M. E. (2001) Science 294, 333–339