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Ca^{2+} regulates a spectrum of cellular processes including many aspects of neuronal function. Ca^{2+}-sensitive events such as neurite extension and axonal guidance are driven by Ca^{2+} signals that are precisely organized in both time and space. These complex cues result from both Ca^{2+} influx across the plasma membrane and the mobilization of intracellular Ca^{2+} stores. In the present study, using rat cortical neurons, we have examined the effects of the novel intracellular Ca^{2+}-mobilizing messenger nicotinic acid adenine dinucleotide phosphate (NAADP) on neurite length and cytosolic Ca^{2+} levels. We show that NAADP potentiates neurite extension in response to serum and nerve growth factor and stimulates increases in cytosolic Ca^{2+} from bafilomycin-sensitive Ca^{2+} stores. Simultaneous blockade of inositol trisphosphate and ryanodine receptors abolished the effects of NAADP on neurite length and reduced the magnitude of NAADP-mediated Ca^{2+} signals. This is the first report demonstrating functional NAADP receptors in a mammalian neuron. Interplay between NAADP receptors and more established intracellular Ca^{2+} channels may therefore play important signaling roles in the nervous system.

Changes in cytosolic Ca^{2+} concentration regulate a whole host of cellular processes (1). In neurons, Ca^{2+} controls crucial events such as neurotransmitter release and synaptic plasticity (2, 3). Ca^{2+} signals are generated by opening of Ca^{2+} channels located both on the cell surface and the membranes of intracellular Ca^{2+} stores (1). Recent evidence suggests that in addition to inositol trisphosphate (4, 5) and ryanodine (6) receptors, which mediate the release of Ca^{2+} from the (sarco)endoplasmic reticulum, intracellular Ca^{2+} channels sensitive to nicotinic acid adenine dinucleotide phosphate (NAADP)[1] may also be involved in the control of Ca^{2+} dynamics (7–9). In the sea urchin egg, in which the effects of NAADP were first characterized, NAADP targets Ca^{2+} channels with biochemical (10) and pharmacological (11) properties distinct from those of inositol trisphosphate and ryanodine receptors. In addition, these channels appear to be expressed on lysosome-like acidic organelles (the reserve granules) (12). Thus, NAADP-induced Ca^{2+} release is inhibited by agents that dissipate proton gradients but is readily demonstrable in the presence of thapsigargin, an inhibitor of Ca^{2+} pumps on the endoplasmic reticulum (12). NAADP receptors are also unusual in that receptors can, under certain conditions, inactivate before activation (13, 14). Despite the propensity of the release process to inactivate, however, NAADP initiates complex Ca^{2+} signals in a variety of systems (7). This is all the more paradoxical given the inability of NAADP receptors to directly support regenerative Ca^{2+} release through the process of Ca^{2+}-induced Ca^{2+} release (15). Rather, it appears that activation of NAADP receptors provides a trigger release of Ca^{2+} that is then amplified by inositol trisphosphate and ryanodine receptors (7, 14).

Proper development of the central nervous system requires the formation of appropriate synaptic contacts between neurons (16). Neurons are capable of extending axons over considerable distances, a process that involves signaling events within the growth cone in response to a variety of extracellular cues (16). The underlying signal transduction pathways, however, are not well defined, although Ca^{2+} is likely to play key roles (17). Both spontaneous entry of Ca^{2+} across the plasma membrane and Ca^{2+} entry in response to guidance cues such as cell adhesion molecules are crucial for various aspects of neuronal growth (18). Indeed, both the temporal and spatial organization of the ensuing Ca^{2+} signal are likely an important determinant of the growth response. In *Xenopus* spinal neurons, for example, Zheng (19) has demonstrated that imposing localized elevations of Ca^{2+} causes turning of growth cones, with the direction dictated by the average global Ca^{2+} concentration. In addition to the well-characterized role of Ca^{2+} influx, the mobilization of intracellular Ca^{2+} stores through activation of both inositol trisphosphate (20, 21) and ryanodine (22, 23) receptors has also been implicated in the control of neuronal growth. The role of NAADP in process outgrowth, however, has yet to be defined.

Although the Ca^{2+} mobilizing properties of NAADP have been characterized in a variety of cells, corresponding information for neurons is scant, as is knowledge of the downstream consequences of NAADP receptor activation. We therefore investigated possible functional effects of NAADP in mammalian neurons. Our data show for the first time that NAADP-mediated Ca^{2+} changes, through the mobilization of acidic Ca^{2+} stores and

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[1] The abbreviations used are: NAADP, nicotinic acid adenine dinucleotide phosphate; NGF, nerve growth factor.

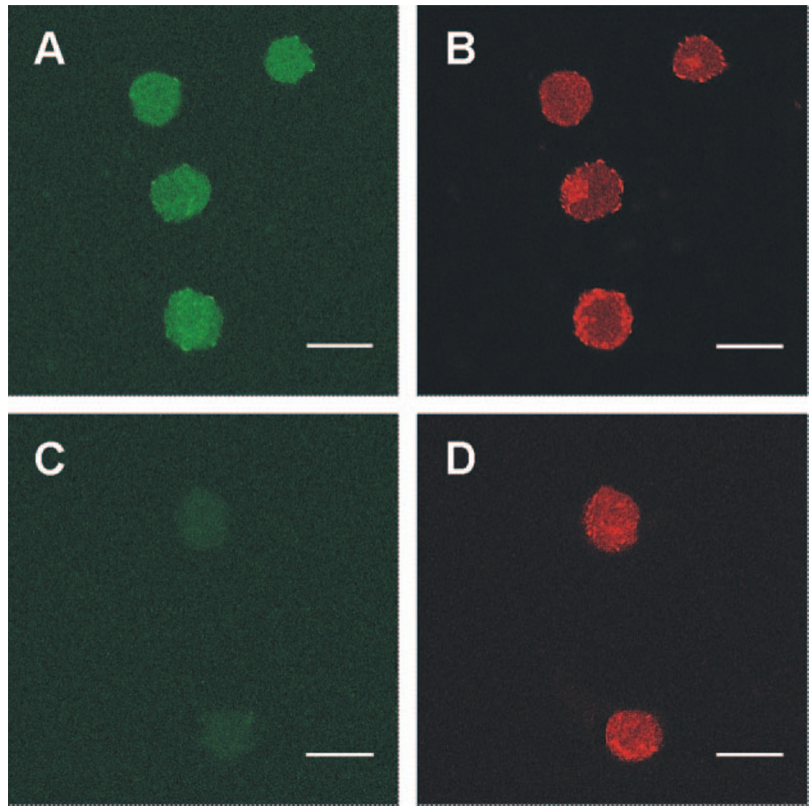

FIG. 1. **Lipsomal delivery of Lucifer yellow into neurons.** Confocal scanning images of Lucifer yellow fluorescence (*A* and *C*, *green*) and neuron-specific enolase immunoreactivity (*B* and *D*, *red*) after perfusion of cultures with liposomes containing 10 μM dye (*A* and *B*) or control liposomes containing buffer only (*C* and *D*). *Scale bar*, 10 μm.

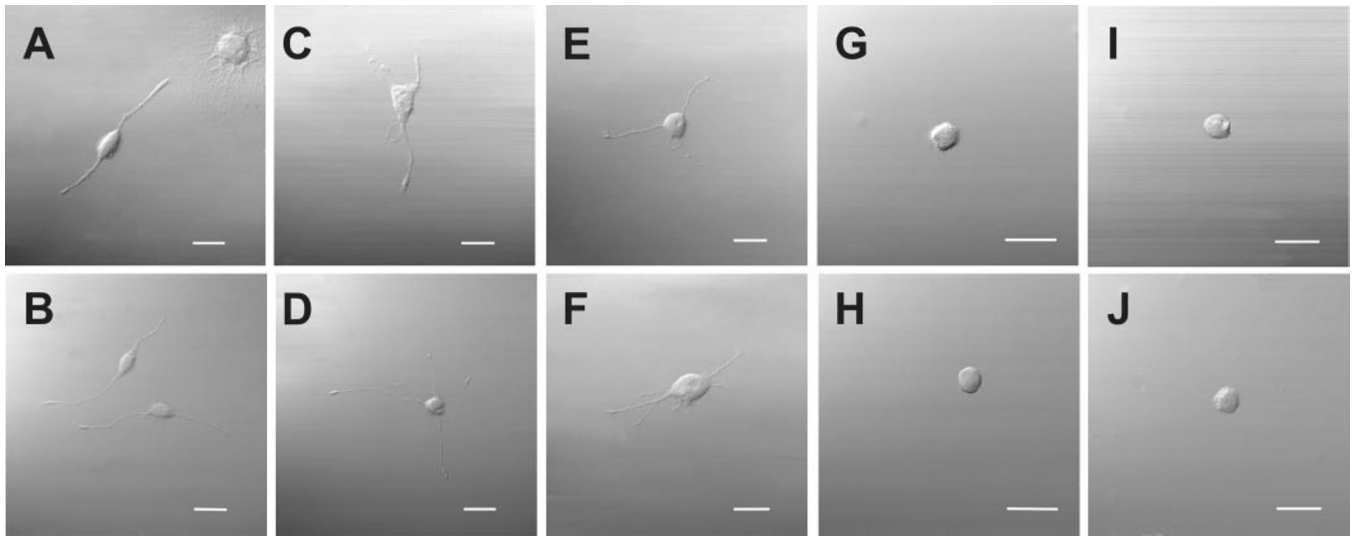

FIG. 2. **Potentiation of neurite outgrowth by NAADP.** *A–J*, representative transmitted light images of neurons cultured in Neurobasal-A™ medium with 10% (v/v) serum (*A* and *B*), B27 supplement + 20 ng/ml NGF (*C* and *D*), B27 supplement + 1 mM dibutyryl cyclic AMP (*E* and *F*), no additions (*G* and *H*), or B27 supplement only (*I* and *J*). Cultures were maintained in the absence (*A*, *C*, *E*, *G*, and *I*) or presence (*B*, *D*, *F*, *H*, and *J*) of NAADP-containing liposomes (100 μM). *Scale bar*, 20 μm.

subsequent amplification by Ca^{2+}-induced Ca^{2+} release, are likely to be important in regulating neurite extension.

EXPERIMENTAL PROCEDURES

Neuronal Cell Culture—Neurons were isolated from the cerebral cortex of newborn rats (4–6 days old) as described previously (24) by enzymatic digestion using 0.5 mg papain/100 mg tissue. Cells were maintained at 37 °C in an atmosphere of 95% O_2 + 5% CO_2 in Neurobasal-A™ medium alone or with the following additions: (i) 10% (v/v) fetal calf serum, (ii) B27 supplement, (iii) B27 supplement + 20 ng/ml NGF (all from Invitrogen), or (iv) B27 supplement + 1 mM N^6,O^2-dibutyryl sodium salt 3′,5′-cyclic AMP (Calbiochem). All culture media contained 20 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Cells were used 2 or 48 h after isolation. In the latter case, glial cell growth was inhibited by addition of the mitotic inhibitor, cytosine β-arabino furanoside (1 μM; Sigma). Cells were treated with liposomes and cell-permeable antagonists in basal medium + B27 supplement for 30 min before transfer to the appropriate medium in the continued presence of the drugs.

Liposome Preparation—NAADP (1–100 μM), Lucifer yellow (10 μM), or heparin (10 mg/ml) was dissolved in 140 mM KCl and incorporated into liposomes prepared using egg phosphatidylcholine (Sigma) and 1,2-dioleoyl-*sn*-glycero-3-ethylphosphocholine (Avanti Polar Lipids) at a 5:1 molar ratio (4 mg lipid/ml aqueous phase) as described previously (25, 26). Unincorporated constituents were removed by dialysis (Sigma dialysis sacs; molecular weight cutoff, 12,400) against Neurobasal-A™ medium (for culture experiments) or Hank's balanced salt solution (for Ca^{2+} measurements) (1:600 (v/v); 150 min; solution change every 30

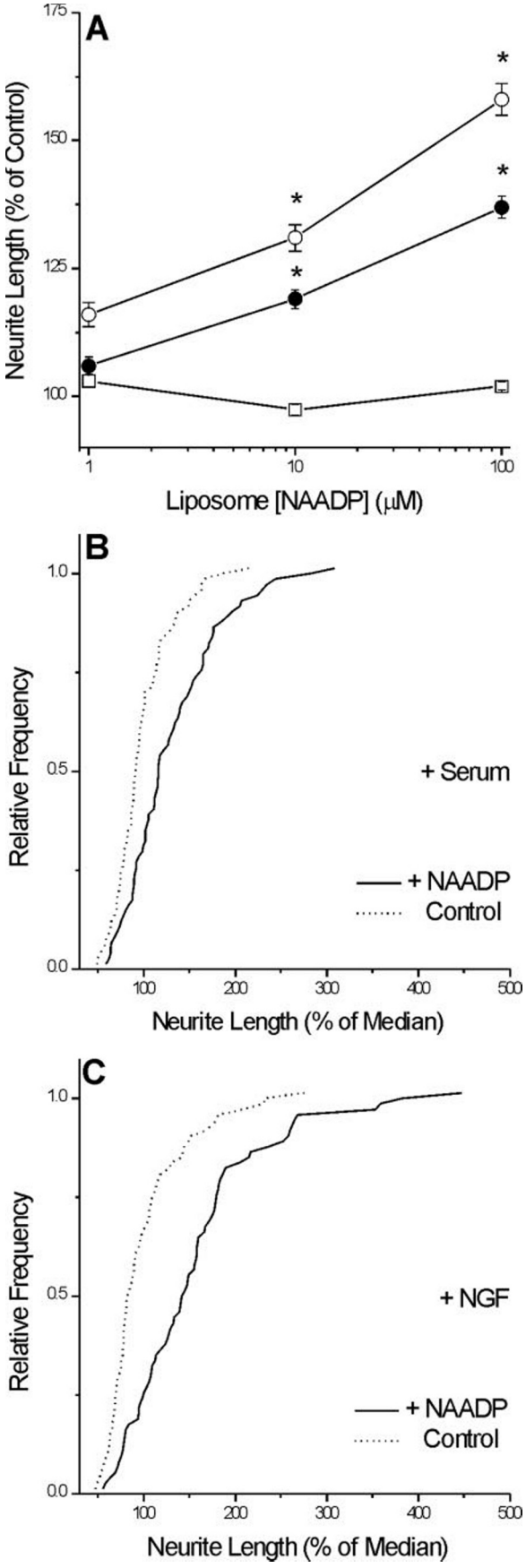

FIG. 3. **Effects of NAADP on neurite length.** *A*, effect of NAADP on neurite outgrowth in cortical neurons cultured for 2 days in medium supplemented with liposomes containing the indicated concentration of NAADP and either serum (●), NGF (○), or dibutyryl cyclic AMP (□). *Asterisks* denote statistical significance ($p < 0.05$) compared with control cultures using a paired *t* test followed by one-way analysis of variance. *B* and *C*, Kolmogorov-Smirnov distribution-free test of neurite length distribution in serum (*B*)- or NGF (*C*)-containing medium in either the absence (*dotted line*) or presence (*solid line*) of NAADP-containing liposomes (100 μM).

min). Liposomes were used at a 1:20 (v/v) final dilution.

Immunocytochemistry—Cells that had been treated with control liposomes or Lucifer yellow-filled liposomes (see above) were fixed in phosphate-buffered saline containing freshly prepared 0.2% (w/v) picric acid and 4% (v/v) paraformaldehyde (20 min). After sequential incubations (in phosphate-buffered saline) with blocking solution (10% (v/v) normal horse serum), primary antibody (anti-mouse neuron-specific enolase IgG; 1:300 dilution; 4 h; Chemicon International), secondary antibody (biotinylated anti-mouse IgG; 1:50 dilution; 1 h; Vector Laboratories), and fluorophore (Avidin Texas Red; 1:50 dilution; 3 h; Vector Laboratories), the coverslips were mounted in Citifluor medium (Ted Pella) for viewing. Lucifer yellow fluorescence and Texas Red fluorescence were captured with a confocal scanning laser microscope (Leica TCS SP2) using excitation/emission wavelengths of 457/520 and 543/620 nm, respectively.

Measurement of Neurite Length—Cells were fixed as described above, and only individual neurons without contacts were used for measurements. The length of the longest neurite (from the cell body to the growth cone tip) was measured using transmitted light images captured with a Leica confocal microscope. For each treatment, a total of 600 neurites (100 neurites per independent culture) were analyzed.

Measurement of Cytosolic Ca^{2+} Concentration—Cytosolic Ca^{2+} concentration measurements were performed as described previously (27). Briefly, freshly dissociated neurons (2 h after isolation) were loaded with the fluorescent Ca^{2+} indicator Fura-2 by incubation of the cells in Hank's balanced salt solution supplemented with 3 μM Fura-2/AM for 45 min and Hank's balanced salt solution alone for an additional 15–60 min (to allow de-esterification of the dye). Coverslips were placed in a custom-designed bath and transferred to the stage of an inverted epifluorescence microscope equipped with a C & L Instruments fluorometer system. Cells were perfused at a flow rate of 2.5 ml/min, and Fura-2 fluorescence (excitation wavelength = 340 and 380 nm, emission wavelength = 520 nm) of single cells was acquired at a frequency of 1 Hz. The ratio of the fluorescence signals obtained (340/380 nm) was converted to Ca^{2+} concentration according to Grynkiewicz *et al.* (28).

RESULTS AND DISCUSSION

We have previously used the liposome technique to effect intracellular delivery of various cell impermeant molecules including NAADP (26, 29). To demonstrate the effectiveness of the methodology in rat cortical neurons, we first characterized delivery of the fluorescent marker Lucifer yellow. Cells were clearly fluorescent when cultures were perfused with liposomes prepared in the presence of the dye (Fig. 1*A*). In contrast, little fluorescence was detected under the same conditions using liposomes filled with buffer alone (Fig. 1*C*). Immunocytochemical analysis with a primary antibody raised to neuron-specific enolase confirmed that imaged cells were neurons (Fig. 1, *B* and *D*).

NAADP has been shown to modulate neurotransmitter release at cholinergic synapses in the buccal ganglion of *Aplysia* (30) and the frog neuromuscular junction (26, 29). To explore possible functional consequences of NAADP-mediated Ca^{2+} increases in rat cortical neurons, we examined the effects of NAADP on neurite extension. When neurons were cultured in the presence of serum, NGF, or cyclic AMP, neurite growth was initiated such that after 2 days in culture, average neurite length was 40 ± 2, 39 ± 2, and 36 ± 2 μm, respectively (n = 600; Fig. 2, *A*, *C*, and *E*). NAADP-containing liposomes (100 μM) dramatically potentiated neurite extension in response to both serum (Fig. 2*B*) and NGF (Fig. 2*D*). The effect of NAADP was concentration-dependent (Fig. 3*A*). Rightward shifts in Kolmogorov-Smirnov plots indicate that NAADP stimulated neurite growth in all neurons (Fig. 3, *B* and *C*). In contrast, NAADP did not affect neurite length in medium supplemented with cell-permeable cyclic AMP (Figs. 2, *E* and *F*, and 3*A*) or

TABLE I

Effect of inositol trisphosphate and ryanodine receptor antagonists on neurite extension

Neuronal cultures were maintained in the presence of serum or NGF with no further additions (control) or the indicated intracellular Ca^{2+} release channel antagonist. Neurite length was measured after culture in the absence (−) or presence (+) of NAADP-containing liposomes (100 μM) or elevated K^+ (6 mM) Antagonist concentrations during liposome preparation were as follows: ryanodine, 100 nM; xestospongin C, 5 μM; and heparin, 10 mg/ml. Stimulation of neuronal growth by NAADP and K^+ (calculated as fold increase) is shown in parentheses.

	Serum			NGF		
	−NAADP	+NAADP	+KCl	−NAADP	+NAADP	+KCl
Control	100%	137 ± 2%[a] (1.4)	128 ± 1.7%[a] (1.3)	100%	158 ± 3%[a] (1.6)	137 ± 2%[a] (1.4)
Ryanodine	86 ± 1%[a]	124 ± 1%[a] (1.4)	105 ± 1.9%[a] (1.2)	91 ± 2%	136 ± 1%[a] (1.5)	122 ± 2%[a] (1.3)
Xestospongin C	86 ± 2%[a]	118 ± 2%[a] (1.4)	108 ± 2% (1.3)	83 ± 1%[a]	113 ± 2%[a] (1.4)	115 ± 2% (1.4)
Heparin	81 ± 1%[a]	118 ± 1%[a] (1.5)	106 ± 2% (1.3)	80 ± 2%[a]	108 ± 2% (1.4)	109 ± 2% (1.4)
Ryanodine + xestspongin C	72 ± 2%[a]	77 ± 2%[a] (1.1)	76 ± 3%[a] (1.1)	68 ± 2%[a]	73 ± 3%[a] (1.1)	74 ± 2%[a] (1.1)
Ryanodine + heparin	68 ± 2%[a]	72 ± 2%[a] (1.1)	68 ± 2%[a] (1.0)	62 ± 2%[a]	64 ± 2%[a] (1.0)	62 ± 2%[a] (1.0)

[a] Statistically significant ($p < 0.05$) compared with control cultures (−NAADP) using a paired *t* test followed by one-way analysis of variance.

initiate neurite extension in control basal medium with or without B27 supplement (Fig. 2, *G–J*). NAADP-induced Ca^{2+} release is therefore not sufficient to induce neurite extension but appears instead to play a specific modulatory role in serum- and NGF-mediated neuronal growth. Liposomal application of either inositol trisphosphate or cyclic ADP-ribose (100 μM) also potentiated neurite extension (data not shown).

It is noteworthy that in other cells such as pancreatic acinar cells, NAADP receptor activation results in the recruitment of inositol trisphosphate and ryanodine receptors through the process of Ca^{2+}-induced Ca^{2+} release (14). Similarly, neurotransmission at the frog neuromuscular junction in response to NAADP and endoplasmic reticulum-based Ca^{2+}-mobilizing messengers displays marked synergism (26). Such orchestration of the release of intracellular Ca^{2+} stores by NAADP could underlie the potentiating effects of NAADP on neurite extension reported here. We therefore examined the effects of inositol trisphosphate and ryanodine receptor antagonists on the stimulation of neurite length by NAADP. Ryanodine, xestospongin C, and heparin caused a modest (<20%) inhibition of neurite length after culture in the presence of serum (Table I). Stimulation of neuronal growth by NAADP, however, was largely unaffected by the antagonists (Table I). In contrast, in the presence of a combination of xestospongin C and ryanodine, which inhibited neurite extension by 28 ± 2%, stimulation by NAADP was almost completely abolished (Table I). Thus, whereas NAADP increased neurite length 1.4-fold in control experiments, in the presence of both inhibitors, stimulation was only 1.1-fold. Essentially similar results were obtained in response to a combination of heparin and ryanodine (Table I). Potentiation of neurite extension by NAADP in the presence of NGF was also much more sensitive to simultaneous block of both inositol trisphosphate and ryanodine receptors than to inhibition of only one class of channels (Table I). These data uncover a requirement for Ca^{2+}-induced Ca^{2+} release in mediating the effects of NAADP on neuronal growth. Moreover, there appears to be functional redundancy in the use of inositol trisphosphate and ryanodine receptors in cortical neurons, as is the case during fertilization of sea urchin eggs (31, 32) and glutamate-mediated hyperpolarization of dopaminergic neurons from rat midbrain (33).

To determine whether Ca^{2+} entry through voltage-sensitive Ca^{2+} channels could affect neuronal growth, we examined the effect of elevated K^+ (6 mM) on neurite length. Stimulation of Ca^{2+} entry potentiated neurite extension 1.3-fold in serum-containing medium and 1.4-fold in the presence of NGF (Table I). Higher concentrations of K^+ (25 mM) were toxic (data not shown). As with NAADP, the effects of depolarization were only modestly affected by inositol trisphosphate or ryanodine receptor antagonists when applied alone (Table I). Additionally, only after simultaneous blockade of the channels were the effects of K^+ on neurite length prevented (Table I). Thus, Ca^{2+}-induced Ca^{2+} release likely amplifies the effects of both NAADP and Ca^{2+} entry through voltage-sensitive Ca^{2+} channels.

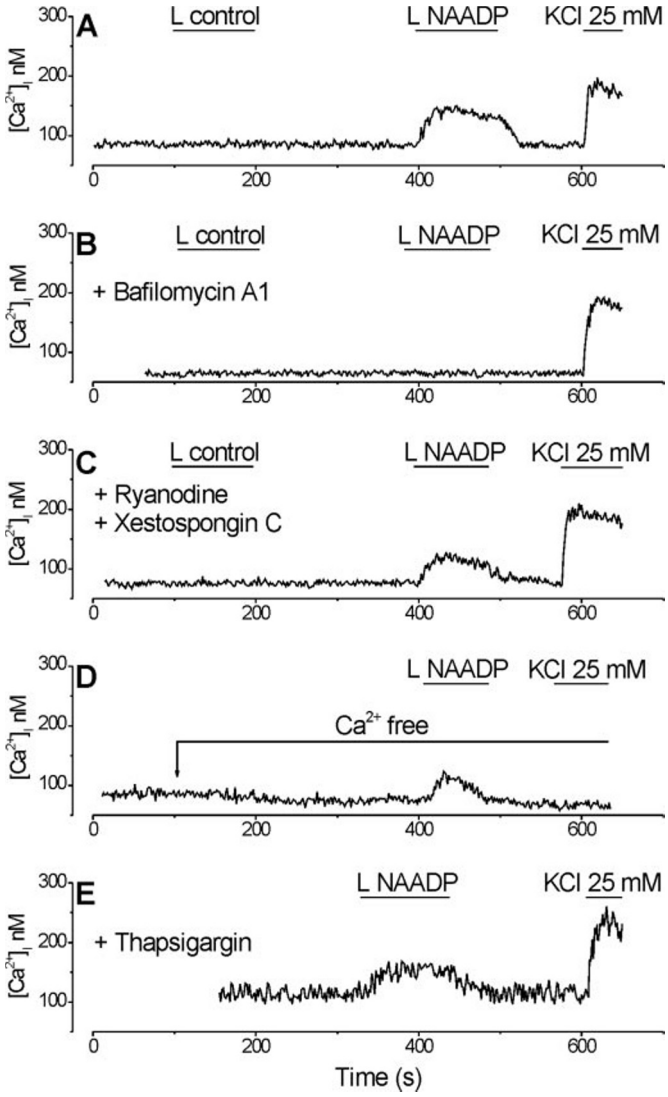

FIG. 4. **NAADP-mediated Ca^{2+} release.** *A*, effect of control liposomes (*L control*), liposomes containing 100 μM NAADP (*L NAADP*), or depolarization with 25 mM K^+ on cytosolic Ca^{2+} concentration of freshly dissociated neurons. *B–E*, similar to the experiment described in *A*, except that before stimulation, neurons were treated with 1 μM bafilomycin A1 (*B*) or 100 nM ryanodine + 5 μM xestospongin C (*C*) for 60 min, Ca^{2+}-free medium (containing 2.5 mM EGTA) for 5 min (*D*), or thapsigargin (1 μM) for 45 min (*E*). Data are from single cells in a typical experiment.

Although NAADP has been demonstrated to stimulate Ca^{2+} release from broken rat brain preparations (34), nothing is known concerning its action in living mammalian neurons. We

therefore examined the effects of NAADP on cytosolic Ca^{2+} concentration in our neuronal cultures. Perfusion of neurons with liposomes filled with buffer only had little effect on cytosolic Ca^{2+} concentration (Fig. 4*A*). Subsequent perfusion with NAADP-filled liposomes (100 μM), however, markedly increased cytolsolic Ca^{2+} by 58 ± 2 nM (n = 6). Importantly, Ca^{2+} responses to NAADP were abolished by the V-type ATPase inhibitor, bafilomycin A1 (Fig. 4*B*; n = 6). Bafilomycin A1, however, did not affect Ca^{2+} increases elicited in response to depolarization of the plasma membrane. Perfusion of cells with elevated K^+ (25 mM) increased cytosolic Ca^{2+} concentration by 96 ± 3 nM (n = 6) and 112 ± 4 nM (n = 6) in the absence and presence of bafilomycin, respectively. These experiments show for the first time that NAADP mediates increases in cytosolic Ca^{2+} in mammalian neurons and that these Ca^{2+} increases likely result from mobilization of acidic Ca^{2+} stores.[2] The latter findings concur with recent reports identifying NAADP-sensitive Ca^{2+} stores as reserve granules in sea urchin eggs (12) and secretory granules in MIN-6 cells (35, 36), both acidic stores of Ca^{2+}. NAADP may also target lysosome-related Ca^{2+} stores in pancreatic acinar cells (36) and smooth muscle cells (37). At the cellular level, expression of NAADP receptors in cortical neurons is consistent with our previous autoradiographical analysis of NAADP binding sites in adult rat brain (38).

NAADP-mediated Ca^{2+} signals were significantly inhibited but not abolished by a combination of xestospongin C and ryanodine (Fig. 4*C*). Ca^{2+} increases in response to NAADP were 58 ± 2 and 39 ± 3 nM in the absence and presence of the inhibitors, respectively. High K^+-induced Ca^{2+} increases (114 ± 5 nM; Fig. 4*C*), however, were similar to those in control experiments (96 ± 3 nM; Fig. 4*A*). Thus, consistent with the effect of the antagonists on the potentiation of neurite growth by NAADP (Table I), Ca^{2+} increases stimulated by NAADP are amplified by inositol trisphosphate and ryanodine receptors. NAADP-mediated Ca^{2+} increases were readily demonstrable in the absence of extracellular Ca^{2+} (Fig. 4*D*) and in the presence of thapsigargin (Fig. 4*E*); peak Ca^{2+} increases were 46 ± 3 and 37 ± 3 nM, respectively (n = 6).

Our demonstration of NAADP-induced Ca^{2+} mobilization in individual neurons, together with previous radiotracer flux studies using brain microsomes (34), and the distinct regional distribution of NAADP binding sites compared with inositol trisphosphate and ryanodine receptors (38) strongly suggest that dedicated Ca^{2+} channels sensitive to NAADP are expressed in the brain. Importantly, we show that their activation modulates a crucial aspect of neuronal growth and that this effect and part of the generated Ca^{2+} signal is dependent upon recruitment of endoplasmic reticulum-based Ca^{2+} release pathways. Cross-talk between intracellular Ca^{2+} channels in mammalian neurons then appears very similar to that in sea urchin eggs (39) and smooth muscle cells (40), in which both the trigger and amplifier components of the NAADP-induced Ca^{2+} signal are readily resolvable. The situation, however, is different from channel "chatter" (7) in pancreatic acinar cells (14). In these cells, NAADP-mediated Ca^{2+} signals are completely blocked by antagonists of inositol trisphosphate or ryanodine receptors, which reflects either a lower density of NAADP receptors (41) or perhaps a more direct interaction of NAADP with the ryanodine receptor (42). It is intriguing that although inositol trisphosphate and ryanodine receptor antagonists have only modest effects on NAADP-induced Ca^{2+} signals in neurons, they are, in combination, able to effectively block the potentiating effects of NAADP on neurite length. These data suggest that the NAADP-stimulated trigger Ca^{2+} signal is not sufficient to mediate the observed functional effects.

Regardless of the exact mode of action of NAADP, the present data support a key role for NAADP in Ca^{2+}-dependent neuronal function. Indeed, NAADP metabolism by brain membranes is stimulated by Ca^{2+}, providing a potential mechanism for the tight control of NAADP-mediated Ca^{2+} signals (43), and CD38, a candidate enzyme for the synthesis of NAADP (44), is expressed throughout early development (45).

Acknowledgments—We thank Steve Bolsover, Chi Li, and Mihai Macovei for useful discussion.

[2] Prolonged incubation of neurons with bafilomycin caused cell death (data not shown), precluding its use to probe the role of acidic Ca^{2+} stores in controlling neurite extension.

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