Cells possess several Ca\(^{2+}\)-mobilizing messengers, which couple stimulation at the cell surface by a multitude of extracellular cues to the regulation of intracellular Ca\(^{2+}\)-sensitive targets. Recent studies suggest that agonists differentially select from this molecular palette to generate their characteristic Ca\(^{2+}\) signals but it is still unclear whether different messengers mediate different functions or whether they act in a redundant fashion. In this study, we compared the effects of nicotinic acid adenine dinucleotide phosphate (NAADP), a novel Ca\(^{2+}\)-mobilizing messenger, with that of the prototypical messenger inositol trisphosphate on cytosolic Ca\(^{2+}\) levels and differentiation status of PC12 cells. We demonstrate that lissosomal delivery of NAADP mediated release of Ca\(^{2+}\) from acidic Ca\(^{2+}\) stores and that this stimulus was sufficient to drive differentiation of the cells to a neuronal-like phenotype. In sharp contrast, cell fate was unaffected by more transient Ca\(^{2+}\) signals generated by inositol trisphosphate-evoked release of endoplasmic reticulum Ca\(^{2+}\) stores. Our data establish for the first time (i) the presence of novel NAADP-sensitive Ca\(^{2+}\) stores in PC12 cells, (ii) a role for NAADP in differentiation, and (iii) that Ca\(^{2+}\)-dependent function can be messenger-specific. Thus, differential recruitment of intracellular Ca\(^{2+}\)-mobilizing messengers and their target Ca\(^{2+}\) stores may represent a robust means of maintaining stimulus fidelity in the control of Ca\(^{2+}\)-dependent cell function.

Changes in cytosolic Ca\(^{2+}\) drive numerous cellular events (1) perhaps the most striking example being the activation of the quiescent egg at fertilization resulting ultimately in the generation of highly differentiated cell types. Given the remarkable multifunctional role of Ca\(^{2+}\), stringent mechanisms must exist within the cell to prevent inappropriate activation of Ca\(^{2+}\)-sensitive targets. Precise organization of Ca\(^{2+}\) signals both temporally and spatially are likely such mechanisms (1). Ca\(^{2+}\) signals are often in the form of oscillations, the frequency of which has profound effects on Ca\(^{2+}\)-dependent output (2–5). Restriction of Ca\(^{2+}\) signals to particular subcellular locations also will ensure the activation of Ca\(^{2+}\) sensors only in that location (6, 7). A full understanding of how these complex Ca\(^{2+}\) signals are generated is then vital to understand how Ca\(^{2+}\) controls cell function.

Cytosolic Ca\(^{2+}\) signals are often generated by agonist-evoked increases in intracellular messengers and the subsequent activation of intracellular Ca\(^{2+}\) channels (1). Inositol trisphosphate is the most ubiquitous of these messengers and is generated in response to a wide range of stimuli that include hormones, growth factors, and neurotransmitters (8). Accumulating evidence indicates that intracellular Ca\(^{2+}\) stores can also be mobilized by NAADP (9–14). Ca\(^{2+}\) release via this NADP metabolite is particularly intriguing because in many cells, NAADP appears to target novel acidic Ca\(^{2+}\) stores (15–19) that are readily distinguishable from the endoplasmic reticulum that houses receptor/calium channels for both inositol trisphosphate and ryanodine (20). Despite this physical segregation, activation of NAADP-sensitive Ca\(^{2+}\) channels is often coupled to activation of endoplasmic reticulum-based Ca\(^{2+}\) channels as evidenced by the sensitivity of NAADP-induced Ca\(^{2+}\) release in intact cells to inhibitors of inositol trisphosphate and/or ryanodine receptors (19, 21–24). NAADP may therefore provide a “trigger” release of Ca\(^{2+}\), which is subsequently propagated by the process of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (21), although direct activation of ryanodine receptors (25–28) and calcium influx by NAADP (29) have been proposed as alternative mechanisms (see Refs. 13 and 30 for discussion).

It has been known for some time that different agonists evoke unique Ca\(^{2+}\) signals or “signatures” (31). The mechanistic basis of this heterogeneity however is not clear. One possibility is that different stimuli may recruit distinct combinations of intracellular messengers. Results of recent studies directly support this hypothesis. In pancreatic acinar cells and smooth muscle cells, desensitization of NAADP receptors or depletion of acidic Ca\(^{2+}\) stores attenuates Ca\(^{2+}\) signals in response to cholecystokinin (17, 21) and endothelin-1 (18), respectively. Importantly, these inhibitory effects are agonist-specific; in acinar cells for example neither acetylcholine- nor bombesin-evoked Ca\(^{2+}\) signals are affected by similar treatments (32, 33). Accordingly, only cholecystokinin stimulates increases in NAADP levels (34). Although specific combinations of intracellular messengers can indeed mimic the effect of extracellular stimuli on cytosolic Ca\(^{2+}\) levels (35), functional correlates of messenger-evoked Ca\(^{2+}\) signals are lacking.

Cell differentiation is a process that occurs not only during embryonic development but also in the adult during regeneration of many tissues. Indeed, the potential of stem cells to develop into different cell types has been the focus of much attention recently (36). Differentiation results because of specific changes in gene expression. Because several transcription factors including CREB (37) and NF-AT (37) are Ca\(^{2+}\)-sensitive, Ca\(^{2+}\) is likely to be involved in the differentiation process. Indeed, buffering of cytosolic Ca\(^{2+}\) with Ca\(^{2+}\) chelators has been shown to prevent differentiation of several cell types including neurons (2), keratinocytes (38), myocytes (39), trophoblasts (40), and NB4 cells (41). Moreover, imposing Ca\(^{2+}\) transients appears sufficient to drive neuro-
transmitter specification in embryonic *Xenopus* spinal neurons (2). Many studies have demonstrated changes in the Ca\(^{2+}\) signaling protein complement upon differentiation (for example see Ref. 42), but whether these changes drive differentiation or are a consequence of altering cell fate is difficult to discern. In particular there is a surprising paucity of information concerning the role of intracellular Ca\(^{2+}\)-mobilizing messengers in differentiation. Although ryanodine receptor-mediated Ca\(^{2+}\) signals have been implicated in the differentiation of skeletal myoblasts (43, 44), cardiac myocytes (39, 45), spermatogenic cells (46), and HL-60 cells (47), the latter via CD-38-mediated production of cADPR, an endogenous modulator of ryanodine receptors (48), direct evidence for inositol trisphosphate receptor involvement is scant. In keratinocytes, phospholipase C-\(\gamma\)1 is necessary for differentiation in response to physiologically relevant elevations in extracellular Ca\(^{2+}\) levels (49). What effect NAADP-mediated Ca\(^{2+}\) signals have on differentiation is not known.

In the present study, using liposomes we have examined the direct effects of NAADP and inositol trisphosphate on modulating differentiation of PC12 cells, a rat pheochromocytoma cell line and a long standing model for neuronal differentiation (50). We show that although both messengers evoke changes in cytosolic Ca\(^{2+}\), these signals are kinetically and mechanistically distinct and that NAADP, but not inositol trisphosphate-mediated Ca\(^{2+}\) increases are sufficient to cause differentiation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—PC12 cells were routinely maintained in RPMI 1640 medium supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 100 units/ml of penicillin, and 100 \(\mu\)g/ml of streptomycin (all from Invitrogen) in an atmosphere of 5% CO\(_2\) at 37 °C. The medium was changed every 72 h.

**Liposome Preparation**—Liposomes were prepared using egg phosphatidylcholine (Sigma) and 1,2-dioleoyl-sn-glycero-3-ethylphosphocholine (Avanti Polar Lipids) at a 9:1 molar ratio (3 mg of lipid/ml of aqueous phase) as described previously (51, 52). The aqueous phase was either buffer (140 mM KCl, pH 6.9) alone or buffer containing Lucifer yellow (10 mM), NAADP (1–100 \(\mu\)M), or IP\(_3\) (1–100 \(\mu\)M). Unincorporated constituents were removed by dialysis (Sigma dialysis sacs, molecular weight cut off of 12,400) against Hanks’ balanced salt solution (for experiments using adherent cells) or RPMI 1640 medium (for culture experiments; 1:600 v/v, 150 min, solution changed every 30 min).

**Liposome Delivery**—Both cells that had been attached to poly-L-lysine-coated coverslips and loosely adherent cells in culture were treated with liposomes (1:15 v/v dilution). For attached cells, the cells were superfused with Hanks’ balanced salt solution supplemented with liposomes at a flow rate of 0.4 ml/min. For cells in culture, the cells were first serum-starved for 12 h, and then cell suspensions were incubated with liposomes in serum-free medium for 2 h with gentle agitation.

**Measurement of Lucifer Yellow Fluorescence**—Adherent cells were superfused for 2 min with Lucifer yellow-containing liposomes. Unincorporated liposomes were removed by manual rinsing of the coverslips with Hanks’ balanced salt solution, and the cells were fixed with 4% paraformaldehyde. Confocal fluorescence images (emission wavelength >520 nm) were captured following excitation at 425 nm.

**Measurement of NAADP Levels**—NAADP levels were determined based on the method described previously (53). Suspensions of PC12 cells (5–10 \(\times\) 10\(^6\)) that had been treated with liposomes were washed by brief centrifugation, and cell pellets were resuspended in two volumes of ice-cold 10% w/v trichloroacetic acid. After a 5-min incubation, the samples were centrifuged at 12,100 \(\times\) g for 1 min, and the supernatants were recovered and neutralized by back extraction with water-saturated ether (5 \(\times\) 1 ml) followed by addition of 100 mM HEPES (pH 7.2). The samples and known concentrations of NAADP were incubated with sea urchin egg homogenates (0.25% v/v) prepared as described previously (54) in binding buffer consisting of 20 mM HEPES (pH 7.2), 250 mM potassium gluconate, 250 mM N-methyl-D-glucamine, and 1 mM MgCl\(_2\). Following a 1–2 h incubation, enzymatically prepared \(^{32}\)PNAADP (55) was added (0.5 nM) to saturate unoccupied NAADP receptors, and the incubations were filtered through glass fiber filters using a cell harvester to separate bound and free ligand. The radioactivity of the filters was determined by Cerenkov counting. The sensitivity of this assay was markedly enhanced by the addition of unlabeled NAADP before the radiolabel (53). This was necessary to detect NAADP levels in unstimulated cells.

**Measurement of Cytosolic Ca\(^{2+}\) Concentration**—Cytosolic Ca\(^{2+}\) concentration measurements were performed as described previously (19). Attached cells were incubated with 4 \(\mu\)M Fura-2 AM (Molecular Probes) in Hanks’ balanced salt solution at room temperature for 45 min in the dark, washed three times with dye-free buffer, and then incubated for a further 45 min to allow for complete de-esterification of the dye. The coverslips were subsequently mounted in a custom-designed bath on the stage of a S300 Axiovert Nikon inverted microscope equipped with a C & L Instruments fluorometer system. Cells were routinely superfused with Hanks’ balanced salt solution at a flow rate of 2.5 ml/min except during liposome addition (see above). Fura-2 fluorescence (emission = 510 nm) following alternate excitation at 340 nm and 380 nm was acquired at a frequency of 20 Hz.

**Organelle Localization**—For the localization of acidic compartments and the endoplasmic reticulum in the same cells, cells were incubated with a combination of Lysotracker Green (1 \(\mu\)M) and ER-Tracker Blue-White DPX (100 nM) for 15 min. Confocal fluorescence images of the dyes were captured following excitation at 488 nm (emission = 505–530 nm) and 364 nm (emission >385 nm), respectively, using a 63× objective. Acidic compartments were also localized using Lysotracker Red (1 \(\mu\)M, excitation = 543 nm, emission >620 nm) following a 1-h pretreatment with vehicle Me\(_2\)SO (0.1% v/v), glycyrl-1-phenylalanine-\(\beta\)-naphthylamide (GPN) (200 \(\mu\)M), or bafilomycin-A1 (1 \(\mu\)M). In these experiments, the magnification was increased to 100× and z stack images were captured. All dyes were from Molecular Probes.

**Differentiation**—Cells suspensions were treated with liposomes (as described above) or nerve growth factor (100 ng/ml, 2 h) and then plated on to poly-L-lysine-coated coverslips in complete medium and cultured for 7 days. In some experiments, cells were incubated with BAPTA-AM (10 \(\mu\)M, 45 min) followed by three washes to remove the Ca\(^{2+}\) chelator, prior to liposome addition. Cells were fixed in freshly prepared 0.2% picric acid, 4% paraformaldehyde for 20 min. The coverslips were then blocked with 10% normal horse serum for 2 h and incubated sequentially with mouse monoclonal anti-tyrosine hydroxylase antibody (1:750; Chemicon International, Temecula, CA) for 4 h, biotinylated anti-mouse IgG (1:50, Vector Laboratories, Burlingame, CA) for 1 h, and fluorescein Avidin D (Vector Laboratories) for 3 h. All dilutions were done in phosphate-buffered saline, and coverslips were rinsed several times between incubations. The coverslips were mounted in Citifluor (Ted Pella, Redding, CA) sealed, and confocal fluorescence images were captured using excitation and emission wavelengths of 488 and 520 nm, respectively. Cells were considered differentiated if neurite length exceeded that of the cell body.
RESULTS AND DISCUSSION

We have previously used liposomes to effect intracellular delivery of NAADP and other cell impermeant molecules into neuronal preparations (19, 52). To demonstrate the effectiveness of the methodology in PC12 cells, we characterized delivery of the fluorescent marker Lucifer yellow (Fig. 1, A and B). Confocal imaging of cells that been attached to coverslips and superfused with Lucifer yellow-containing liposomes for 2 min revealed a uniform distribution of the dye throughout the cell (Fig. 1B, left). Little fluorescence was detected upon perfusion of cells with control liposomes prepared in the presence of buffer only (Fig. 1B, middle). These data clearly demonstrate that cytosolic delivery of liposomal constituents is achieved rapidly as reported (56). Extracellular application of the dye resulted in much weaker punctate fluorescence confined to the cell periphery (Fig. 1B, right) most likely reflecting endocytosis.

To further quantify liposomal delivery, cell suspensions were incubated with liposomes encapsulating NAADP (100 μM), acid extracts were prepared, and NAADP content was measured using a radioreceptor assay (Fig. 1C). As shown in Fig. 1C, the concentration of NAADP in extracts from cells treated with NAADP-containing liposomes was markedly higher than in extracts from cells treated with control liposomes containing buffer alone. These data demonstrate that cellular levels of NAADP can be readily elevated in populations of PC12 cells in a non-invasive manner.

Having established experimental conditions for the successful delivery of molecules into PC12 cells, we first characterized the effect of NAADP (100 μM) and inositol trisphosphate (100 μM) on cytosolic Ca²⁺ concentration (Fig. 2). Both messengers evoked robust cytosolic Ca²⁺ increases in attached cells loaded with the fluorescent Ca²⁺ indicator.
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Thus, these vesicles are strong candidates for NAADP-sensitive Ca\textsuperscript{2+}ically, Ca\textsuperscript{2+} increases in response to NAADP and inositol trisphosphate were similar, the kinetics of the evoked changes were quite different (Fig. 2A). Specifically, Ca\textsuperscript{2+} increases in response to NAADP were slower to peak and more sustained than those mediated by inositol trisphosphate. We quantified the time taken for the cytosolic Ca\textsuperscript{2+} concentration to peak and then return to 50% of its peak value following stimulation with each messenger. Intriguingly the latter “recovery index” for NAADP was nearly an order of magnitude greater than that for inositol trisphosphate (Fig. 2B). Clearly then, NAADP and inositol trisphosphate mediate kinetically different Ca\textsuperscript{2+} increases.

We next examined the pharmacology of messenger-evoked Ca\textsuperscript{2+} signals. In these experiments, cells were transiently superfused with liposomes containing 100 µM NAADP (Fig. 3A) or 100 µM inositol trisphosphate (Fig. 3B). Under these conditions, the Ca\textsuperscript{2+} signals in response to the messengers receded upon washout of the liposomes most likely reflecting rapid metabolism upon termination of delivery. Importantly, control liposomes did not evoke any changes in cytosolic Ca\textsuperscript{2+} levels (Fig. 3, A and B).

Prior incubation with thapsigargin, a sarcoplasmic reticulum Ca\textsuperscript{2+} ATPase inhibitor, which depletes endoplasmic reticulum Ca\textsuperscript{2+} stores, reduced but did not abolish NAADP-evoked Ca\textsuperscript{2+} increases (Fig. 3A). Peak Ca\textsuperscript{2+} responses in response to NAADP after thapsigargin treatment were ~50% of control responses (Fig. 3C). To probe the possible involvement of lysosomes in NAADP-mediated Ca\textsuperscript{2+} changes (15, 17, 18), we compared the effects of NAADP following osmotic lysis of these organelles with GPN, a cathepsin C substrate. GPN pretreatment abolished the effects of NAADP (Fig. 3, A and C). Essentially identical results were obtained with the V-type ATPase inhibitor bafilomycin A1 which prevents organelle acidification (Fig. 3, A and C). In contrast to the effects of NAADP, inositol trisphosphate-evoked Ca\textsuperscript{2+} mobilization was completely blocked by thapsigargin and unaffected by pretreatment with GPN and bafilomycin A1 (Fig. 3, B and D). Results with the latter confirm the specificity of the inhibitors employed. Stimulation of cells with a lower concentration of either NAADP or inositol trisphosphate (1 µM) failed to significantly affect cytosolic calcium concentration (Fig. 3, C and D).

We compared the distribution of acidic compartments and the endoplasmic reticulum in live PC12 cells. As shown in Fig. 4A, the acidic compartments tended to form perinuclear clusters, whereas the endoplasmic reticulum was more diffuse. Individual acidic vesicles could be resolved at higher magnification (Fig. 4B). When cells were pretreated with GPN or bafilomycin-A1, vesicular staining was abolished (Fig. 4B). Thus, these vesicles are strong candidates for NAADP-sensitive Ca\textsuperscript{2+} stores.
including neurotrophins such as nerve growth factor. To explore the role of intracellular Ca\(^{2+}\) stores in differentiation, we examined the effects of NAADP and inositol trisphosphate on the morphology of PC12 cells. In these experiments, cell suspensions were incubated for 2 h in the presence of control liposomes or liposomes containing either NAADP or inositol trisphosphate, and then cells were cultured (in the absence of liposomes) for 7 days. The effects of NAADP on cell morphology were dramatic. Remarkably, as shown in Fig. 5, NAADP differentiated a significant proportion of cells in the absence of trophic support. The observed phenotype was highly reminiscent of that following nerve growth factor treatment (Fig. 5A). In stark contrast, liposomes containing inositol trisphosphate had little effect on cell morphology (Fig. 5) despite the demonstrated ability of this messenger to evoke Ca\(^{2+}\) changes (Fig. 3, B and D). As expected, control liposomes did not induce differentiation (Fig. 5). A lower concentration of either NAADP or inositol trisphosphate (1 \(\mu\)M) were also without effect (Fig. 5B).

To determine whether the effects of NAADP on cell morphology were Ca\(^{2+}\)-dependent, we examined the effect of NAADP-containing liposomes in cells that had been loaded with the Ca\(^{2+}\) chelator BAPTA. Our loading conditions were optimized such that BAPTA completely prevented Ca\(^{2+}\)-signals in response to both NAADP and inositol trisphosphate (data not shown). In BAPTA-treated cultures, NAADP did not affect cell fate (Fig. 5B). Importantly, this treatment did not compromise cell viability. Trypan blue staining indicated that 93 \(\%\) of the cells were viable. Thus the Ca\(^{2+}\)-dependent outcome in this particular scenario appears exquisitely sensitive to the Ca\(^{2+}\)-mobilizing messenger.

Although NAADP evokes changes in cytosolic Ca\(^{2+}\) concentration in many cells, little is known concerning its role in neurons and less still concerning downstream functional consequences. Here we have shown for the first time that mobilization of acidic lysosomal-like Ca\(^{2+}\) stores by NAADP is sufficient to mediate differentiation of a neuronal cell model. Combined with the ability of NAADP to modulate neurotransmission (52, 57, 58) and potentiate neurite outgrowth (19), NAADP may
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well play important signaling roles in the nervous system (59). Indeed, receptors for NAADP are widespread in the mammalian brain (55), and brain homogenates readily metabolize NAADP in a Ca\(^{2+}\)-dependent fashion (60). The functional effects of NAADP-mediated Ca\(^{2+}\) signals on differentiation reported here are unprecedented and clearly highly specific because Ca\(^{2+}\) increases by inositol trisphosphate were unable to modify cell fate. The lack of effect of inositol trisphosphate on differentiation is consistent with a previous report using PC12 cells (61). We propose that only sustained Ca\(^{2+}\) elevation is consistent with a previous report using PC12 cells (61). We modify cell fate. The lack of effect of inositol trisphosphate on differentiation is consistent with a previous report using PC12 cells (61). We propose that only sustained Ca\(^{2+}\) elevation.

To conclude, we provide a clear example of different Ca\(^{2+}\) mobilizing messengers mediating quite different cellular responses likely through the generation of temporally distinct Ca\(^{2+}\) signals. Ca\(^{2+}\)-dependent function may thus be tailored by selective recruitment of intracellular Ca\(^{2+}\)-mobilizing messengers.

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