Cell-Permeant Small-Molecule Modulators of NAADP-Mediated Ca^{2+} Release

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Summary

Nicotinic acid adenine dinucleotide phosphate (NAADP, 1) is the most potent intracellular Ca^{2+} mobilizing agent in important mammalian cells and tissues, yet the identity of the NAADP receptor is elusive. Significantly, the coenzyme NADP is completely inactive in this respect. Current studies are restricted by the paucity of any chemical probes beyond NAADP itself, and importantly, none is cell permeant. We report simple nicotinic acid-derived pyridinium analogs as low molecular weight compounds that (1) inhibit Ca^{2+} release via the NAADP receptor (IC$_{50}$ ~15μM – 1 mM), (2) compete with NAADP binding, (3) cross the cell membrane of sea urchin eggs to inhibit NAADP-evoked Ca^{2+} release, and (4) selectively ablate NAADP-dependent Ca^{2+} oscillations induced by the external gastric peptide hormone agonist cholecystokinin (CCK) in murine pancreatic acinar cells.

Introduction

Ca^{2+} signals govern many global aspects of the cell, while their shape in time and space is orchestrated by mixing between separate stores [1]. Each of these is addressed by specific small molecules called second messengers that are produced within cells in response to external stimuli [2, 3]. Cyclic adenosine dinucleotide phosphate ribose (cADPR) and inositol 1,4,5-triphosphate (IP$_3$) and their respective targets, the ryanodine (RYR) and IP$_3$ receptors (IP$_3$R), are well known examples. Studies involving microinjection of NAADP, or photoactivation of its caged derivative [4], have shown that NAADP plays a key role in cholecystokinin- [5], but not

bombesin- [6] or cholinergic- [7], mediated Ca^{2+} signaling in pancreatic acinar cells. In pancreatic β cells, glucose-, but not acetylcholine-, mediated signals are affected, and here endogenous levels of NAADP have been shown to increase in response to the glucose stimulus, confirming its role as a second messenger [2, 8, 9]. NAADP has also been implicated in the regulation of Ca^{2+} signaling in other cell types, including T cells [10], heart myocytes [11], and neurons [12–14], yet little is known about the biological machinery with which this endogenous compound interacts.

Interestingly, the concentration-response relationship for NAADP is bell-shaped in intact mammalian cells; Ca^{2+} release is observed at nanomolar concentrations of NAADP, but a thousand-fold increase in its concentration causes no release and completely inactivates the store [5, 9, 10]. This autoinhibitory effect has been central to the characterization of this Ca^{2+} release mechanism so far.

The location of the NAADP store has to date been refined to an acidic, lysosome-related organelle discrete from the IP$_3$R and RYRs that are located at the endoplasmic reticulum [15, 16]. These findings are contentious, however; for example, one alternative model proposes that NAADP directly interacts with RYRs [17]. The absence of selective, cell-permeant chemical tools that interact at the NAADP receptor has so far restricted investigations. Various L-type Ca^{2+} channel blockers (diltiazem and dihydropyridines) and potassium channel blockers (tetrahexylammonium [THA]) selectively inhibit NAADP-induced release but, crucially, do not compete with [^{32}P]NAADP for binding at the receptor [18]. Triazine dyes, in particular reactive red 120 (RR120), were reported to reversibly bind to the NAADP receptor (IC$_{50}$ 1.4 μM) and mobilize Ca^{2+} at high concentrations (100 μM) [19]. These compounds are not cell permeant, however, and lack selectivity because they also interact with IP$_3$ receptors. Work on full-scale NAADP analogs confirmed that display of a negative charge at the pyridine 3 position was essential for activity; for example, NADP and its 3-hydroxymethylpyridinium and 4-carboxypyridinium variants are all inactive, whereas the 3-sulfonic acid derivative induced Ca^{2+} release, albeit with reduced potency [20]. Later experiments confirmed the importance of this part of the molecule for both binding affinity and Ca^{2+} release, leading the authors to conclude that rational design of potential antagonists should initially focus on mimicking other parts of the second messenger [21]. This range of analogs is, however, largely constrained to those available from base-exchange facilitated by ADP-ribosyl cyclases/NAD glycohydrolases (E.C.3.2.2.5) [22, 23]. We recently reported a flexible route to NAADP via total synthesis of NADP that will offer a wider scope for structural modulation of the whole messenger (for detailed examination of the chemical biology of NAADP), but the general utility of such tools is likely to be limited by the cost and practicability of synthesis [24]. Studies to investigate rational design of small-molecule analogs were therefore initiated.

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Results and Discussion

We sought simple truncated analogs that retain the 3-carboxy group and the charged pyridinum scaffold to exploit the tight binding pocket envisaged to be responsible for the remarkable selectivity observed at the receptor. Alkylation of nicotinic acid [25] provided rapid access to a range of trial compounds for biological evaluation (Figure 1). Broken egg preparations of the sea urchin *Lytechinus pictus* are an ideal model for compound evaluation, showing Ca^{2+} release from separately addressable Ca^{2+} release mechanisms and stores. The egg homogenate comprises functional vesicles that fully sequester Ca^{2+} in ATP-supplemented buffer and then release Ca^{2+} from discrete stores upon stimulus with second messengers [26]. Concentration-dependent Ca^{2+} release can therefore be measured using an appropriate reporter dye, such as Fluo-3, and a simple cuvette-based fluorimetry assay.

NAADP-induced Ca^{2+} release in sea urchin egg homogenate is strongly concentration dependent; 10–250 nM concentrations of the dinucleotide typically cause a rapid increase in fluorescence followed by subsequent decay back to baseline as free Ca^{2+} is returned to the stores. A unique and useful feature of the sea urchin system is that treatment with subthreshold concentrations of NAADP (0.2 nM) prevents Ca^{2+} release upon subsequent challenge with normally releasing concentrations of NAADP [27].

Alkyl pyridinium compounds were therefore evaluated, initially at 1 mM, for their ability to interact with each aspect of the NAADP-sensitive Ca^{2+} release mechanism, including release and inactivation. Most compounds tested were derived by alkylation of nicotinic acid using simple hydrophobic reagents such as benzyl bromide or allyl bromide and proved to be inactive under the test conditions. Notably, two of the compounds appeared to modulate NAADP-induced Ca^{2+} release.
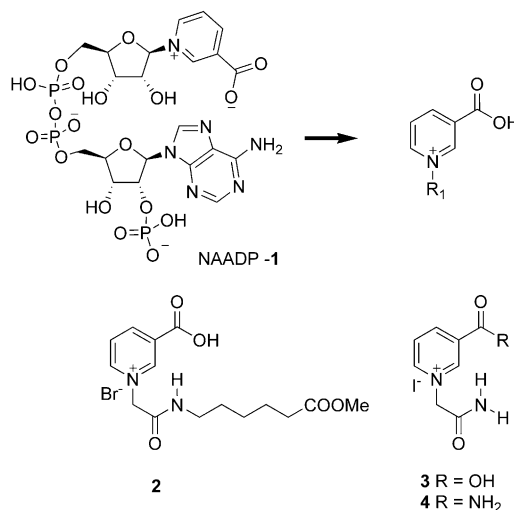

Figure 1. The Structure Of NAADP and Pyridinium Analogs **2** and **3**

Compound **2**, obtained by reaction between nicotinic acid and 6-(2-bromo-acetylamino)-hexanoic acid methyl ester diminished the extent of NAADP-induced Ca^{2+} release without affecting subsequent challenge with IP_3, or cADPR (Figure 2B). Evaluation across a range of concentrations revealed that this compound is not particularly potent, however, with an approximate IC_{50} of around 500 μM (Figure 2C). That this pyridinium analog is weakly competitive at the NAADP binding site was confirmed when the ratio of bound and free NAADP was measured after microfiltration of homogenate first treated with **2** in the presence of 0.2 nM [^{32}P]NAADP. Under these conditions high micromolar concentrations of the pyridinium compounds were able to significantly reduce the specific binding of [^{32}P]NAADP (0.2 nM) (Figure 2D).
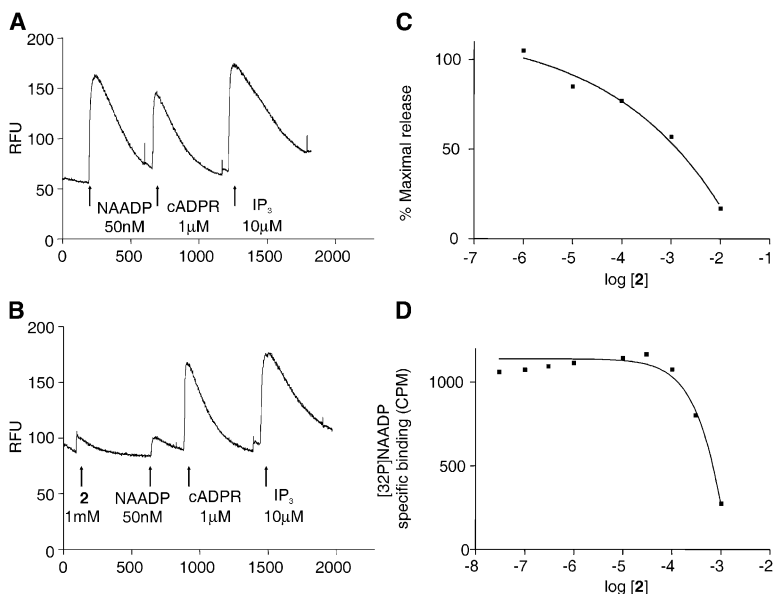

Figure 2. The Effect Of Pyridinium **2** on NAADP-Induced Ca^{2+} Release from Sea Urchin Egg Homogenate

(A) NAADP, cADPR, and IP_3 all induce Ca^{2+} release from sea urchin egg homogenates. Samples were diluted to 2.5% in GluIM in the presence of regenerating system and kept at 17°C with agitation for 3 hr to facilitate Ca^{2+} uptake into stores. Ca^{2+} release was determined by an increase of Fluo-3 fluorescence at 526 nm. Data are expressed as released [Ca^{2+}] as determined by fluorescence in arbitrary units. n = >3. RFU = relative fluorescence units. The effects of maximal concentrations of each of these agents are shown.

(B) Pretreatment of sea urchin egg homogenate with the pyridinium compound **2** (1 mM) selectively inhibits NAADP (250 nM)-induced calcium release but does not significantly affect release evoked by either IP_3 (10 μM) or cADPR (1 μM).

(C) Concentration-dependence of inhibition of NAADP-evoked Ca^{2+} release by pyridinium **2**.

(D) Effect of various concentrations of pyridinium **2** on [^{32}P]NAADP binding to egg membranes.
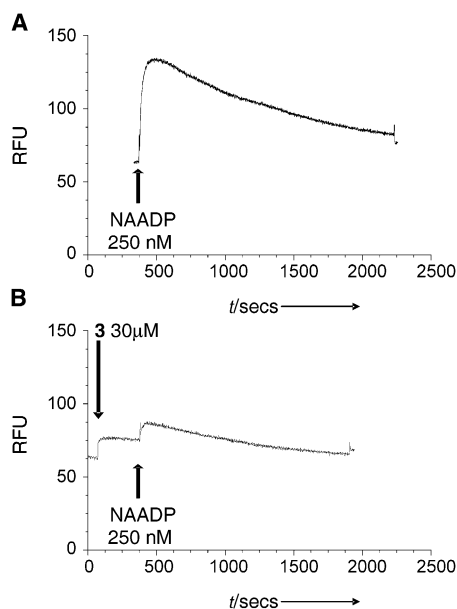
Figure 3. Treatment of Sea Urchin Egg Homogenate with Pyridinium 3 Substantially Reduces NAADP-Induced Ca^{2+} Release

(A) Normal effect of NAADP (250 nM) on Ca^{2+} release from egg homogenates.

(B) Pyridinium compound 3 (30 μM) alone causes a small stable increase in fluorescence. Subsequent addition of NAADP (250 nM) induces a much reduced Ca^{2+} release compared to the control (A).

A more simple acetamide-derived compound, 3, proved to be a much more potent modulator of NAADP-induced Ca^{2+} release. A significant reduction in the effect of 250 nM NAADP on Ca^{2+} release from broken sea urchin egg preparations was observed in the presence 30 μM pyridinium 3 (Figure 3B). Evaluation across a range of concentrations suggested an approximate IC$_{50}$ of around 15 μM (Figure 4A). Notably, neither unmodified nicotinic acid, nicotinic acid riboside, nor the nicotinamide-derived compound 4 caused this effect (data not shown). At a 30 μM concentration of 3, a small amount of Ca^{2+} release was observed at approximately 15% of that induced by 250 nM NAADP. Plotting the maximal Ca^{2+} release of the pyridinium compound 3 alone confirmed this result and indicated that higher concentrations of the compound (>100 μM) appear to cause moderate Ca^{2+} release from this store, suggesting that the compound is a partial agonist. This effect was reduced by pretreatment of egg homogenates with a desensitizing concentration of NAADP (3 nM; data not shown) [27]. Furthermore, the compound does not affect Ca^{2+} release from other stores controlled either by cADPR or InsP$_3$ (Figure 4B).

Total specific binding of [^{32}P]NAADP (B$_{max}$) was reduced from 69% ± 3% to 46% ± 3% (n = 3) in the presence of 10 μM acetamide-derived pyridinium 3, but was not affected at lower concentrations of the small-molecule analog (1 and 100 nM) (Figure 4C). The compound was weakly competitive with [^{32}P]NAADP binding to egg membranes with an IC$_{50}$ of around 90 μM (Figure 4D).

Single-cell studies on intact sea urchin eggs using confocal microscopy, the reporter dye Ca^{2+} green dextran, and photoactivation of caged NAADP showed that, despite their polar character, the compounds were indeed cell permeant and clearly selective for the NAADP receptor within these cells. Brief UV laser flash photolysis (351 and 364 nm) induced the characteristic Ca^{2+} transient in untreated eggs (Figure 5A), but not in those first incubated with pyridinium 3 (10 mM) in extracellular sea water buffer (Figure 5B). In contrast, other
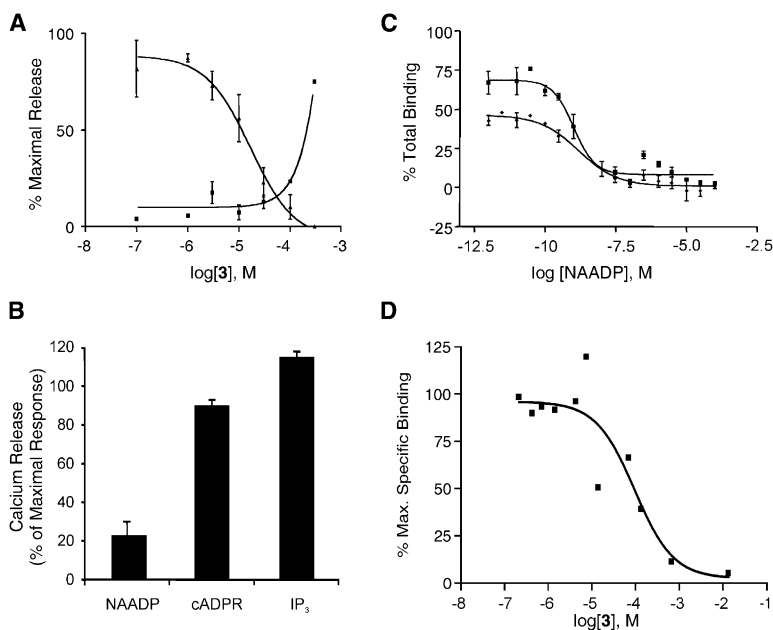

Figure 4. Effects of Varied Concentrations of Nicotinic Acid Derivative 3 on NAADP-Mediated Ca^{2+} Mobilization and Binding

(A) Compound 3 inhibits NAADP-mediated Ca^{2+} release in sea urchin egg homogenate with an apparent IC$_{50}$ of around 15 μM. Data are expressed as percentage of Ca^{2+} release induced by 250 nM NAADP alone, and bars represent the mean ± standard error of the mean of three independent experiments. Filled triangles indicate the percentage release induced by NAADP in the presence of pyridinium 3. Filled squares indicate the effect of 3 alone. At concentrations greater than 100 μM, the compound induces a Ca^{2+} release itself.

(B) Pyridinium 3 is selective because it inhibits Ca^{2+} release induced by NAADP, but has little effect on Ca^{2+} release in sea urchin egg homogenate induced by maximal concentrations of cADPR and IP$_3$. Data are expressed as percentage of Ca^{2+} release induced by NAADP, cADPR, or IP$_3$ in the absence of 3, and bars represent the mean ± standard error of the mean of three independent experiments.

(C) 10 μM pyridinium 3 reduces the B$_{max}$ from 69% ± 3% to 46% ± 3%. Bars represent the mean ± standard error of the mean of three independent experiments. Data are expressed as percentage of total binding. Filled squares indicate specific binding of [^{32}P]NAADP in the presence of increasing concentrations of NAADP; filled diamonds indicate binding in the presence of 10 μM derivative 3.

(D) [^{32}P]NAADP (0.2 nM) specific binding to egg membranes is reduced by 3 in a concentration-dependent manner with an approximate IC$_{50}$ of 90 μM.
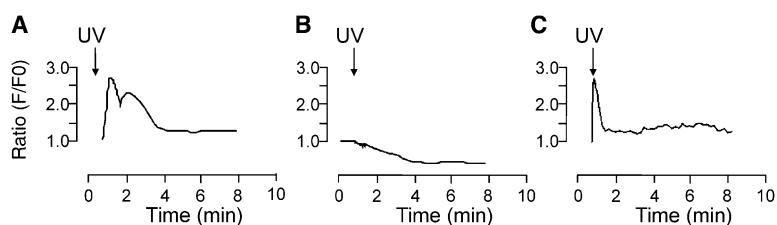
Figure 5. External Application of Pyridinium 3 Abolishes Ca^{2+} Release in Response to Photolysis of Caged NAADP in Intact Sea Urchin Eggs

Ca^{2+} is measured by the ratio of fluorescence observed for reporter dye, Ca^{2+} green dextran, before and after photolysis plotted against time (minutes).

(A) The normal response of an intact sea urchin egg to photolytic activation of intracellular caged NAADP.

(B) Extracellular compound 3 (10 mM) in sea water buffer reduced this response.

(C) Nicotinamide applied at the same external concentration to sea water buffer has no effect on the initial NAADP response. The specificity of extracellular application of the pyridinium 3 compound can thus be assessed by comparing it to the concentrations of other cell-permeant molecules. We used 10 mM, which is 300-fold higher than that required for half-maximal inhibition in the sea urchin egg homogenate. This is comparable to similar experiments involving cell-permeant cAMP and cGMP (Bromo or diacetyl forms) that are typically added at extracellular concentrations between 0.1 and 1 mM [31], 100–10,000 times higher concentration than that required to elicit a response in homogenized preparations [32].

analogs, including nicotinamide, did not abolish NAADP-evoked Ca^{2+} release (Figure 5C). The notable absence of second-phase Ca^{2+} release in the nictonamide-treated cells may reflect the established role of cADPR in mediating the prolongation of the calcium signal in sea urchin eggs, as also observed at fertilization [28, 29].

These promising results encouraged examination of the compound in mammalian cells. Pancreatic acinar cells have been widely used for research into agonist-specific Ca^{2+} signaling. Control of fluid secretion, exocytosis, and trophic effects are controlled by Ca^{2+} signals of subtly different shape depending on whether they are induced by gastrointestinal peptides, such as cholecystokinin (CCK) and bombesin, or the neurotransmitter acetylcholine. Addition of CCK induces characteristic Ca^{2+} oscillations that cannot be recreated by combination of InsP$_3$ or cADPR [3] and that, in contrast to bombesin and acetylcholine, are linked to NAADP production [30]. Administration of low concentrations of NAADP to these cells effectively reproduces the same signals as CCK, while high concentrations selectively inhibit these signals as a result of the distinctive autoinactivation properties described earlier [5].

Normally, CCK-induced Ca^{2+} oscillations persist for several minutes for as long as pancreatic acinar cells are exposed to the peptide [5, 30]. Most significantly, addition of the acetamide derivative 3 to the extracellular milieu significantly attenuated CCK-induced signals, provoking a decrease in the frequency and amplitude of the oscillations, which quickly retreated to baseline (Figure 6A). In the complementary experiment, CCK failed to stimulate more than weak, irregular signals in cells that had first been treated with the same small-molecule modulator (Figure 6B). In contrast, the effects of extracellular pyridinium 3 on acetylcholine-evoked Ca^{2+} responses were much less pronounced, consistent with an absence of a role for NAADP in mediating the calcium-mobilizing effects of this agonist [7] (Figure 6C).

These data highlight simple pyridinium compounds as the first cell-permeant small molecules that modulate NAADP-evoked Ca^{2+} mobilization in both mammalian pancreatic acinar cells and in intact sea urchin eggs. These compounds are designed to exploit the apparently very strong discrimination between NADP and NAADP observed in all known NAADP-responsive cells, which presumably arises from a tight, specific carboxy-

anion binding pocket local to the as yet uncharacterized receptor. Alternative models, such as binding at an allosteric regulation site, cannot be ruled out and will require detailed characterization of the NAADP receptor. The simple synthetic route toward these prototypical compounds should ensure their general utility to the community. Meanwhile, their low molecular weight also encourages further investigation and optimization of the chemical space around this structure. More potent compounds that target the nicotinic acid binding pocket of the NAADP receptor will explore the idea that these compounds will be agonists [21]; our
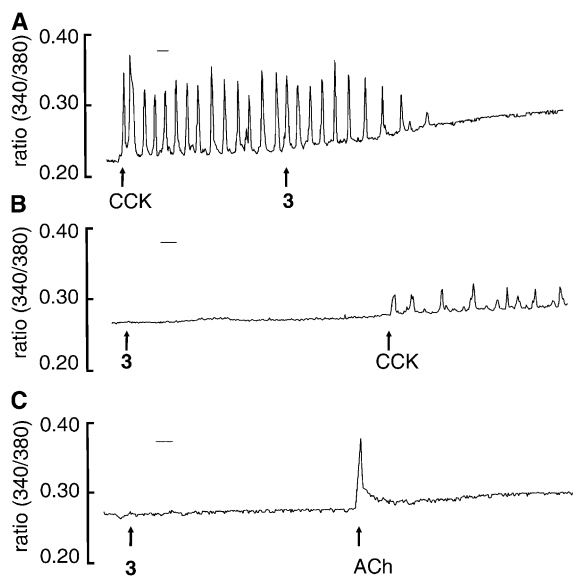

Figure 6. CCK-Induced Ca^{2+} Oscillations in Murine Pancreatic Acinar Cells Are Reduced or Inhibited by External Application of 3

Ratio of fluorescence emitted at 510 nm, upon alternate excitation at 340/380 nm, by fura2-AM (a Ca^{2+}-dependent fluorophore) plotted against time. Scale bars represent 100 s.

(A) External addition of 3 (1 mM) leads to oscillations of reduced frequency and magnitude that decay to zero.

(B) Cells first incubated with 3 (1 mM) and then treated with CCK (5 pM) show very weak Ca^{2+} oscillations with greatly reduced frequency.

(C) In contrast the effects on CCK-mediated Ca^{2+} spikes, the response to acetylcholine (50 nM) after extracellular application of 3 (1 mM) persists with little change.

earlier observation that acetamide-derived compound **3** may be a partial agonist might support this idea. These preliminary results outline an interesting new direction for exploration of the chemistry of the NAADP receptor, although an expansion of the repertoire of molecules under evaluation will be required to develop the insight necessary to improve their potency. More detailed studies of the structure-activity relationships for these and related structures are underway. Elaborated scaffolds with fluorescent or affinity functionality that may facilitate assay, facilitate localization, or indeed assist in the purification of the biological target for NAADP are also in preparation. Additionally, the combination of modulator and target may provide the basis for the development of potential novel therapeutic strategies, and we are currently extending functional studies to other significant cell types.

Significance

We report small-molecule pyridinium compounds designed to mimic the nicotinic acid portion of NAADP. In the absence of sequence or structural data, we exploited the significant selectivity for NAADP over NADP that might arise from a tight binding subsite at the putative receptor. Reaction between nicotinic acid and alkyl halides provided a small array of pyridinium compounds that were evaluated for their effect on Ca²⁺ release in fresh sea urchin egg homogenate. Two analogs emerged from these studies that diminished Ca²⁺ release induced by NAADP but not by the other second messengers cADPR and IP₃, the most potent of which (IC₅₀ ~15 μM) was derived from reaction with bromoacetamide. At >10 μM concentration, this pyridinium ligand was competitive at the NAADP binding site and reduced the total specific binding of [³²P]NAADP (B_{max}) from 69% ± 3% to 46% ± 3% (n = 3). Penetration of the cell membrane was demonstrated in intact sea urchin eggs, where exogenous application of the compound abolished transient Ca²⁺ release induced by photolysis of caged NAADP. In murine pancreatic acinar cells, the frequency and amplitude of characteristic Ca²⁺ oscillations induced by the gastrointestinal agonist cholecystokinin (CCK) were decreased to baseline upon external application of the pyridinium analog, while cells first treated with the same compound showed only weak Ca²⁺ oscillations with irregular frequency in response to CCK. Together, these results define the first cell-permeant modulator of NAADP-induced Ca²⁺ release and are highly significant because current investigations are restricted to close relatives of NAADP that are mainly cell impermeant and require additional techniques to bypass the cell membrane. We anticipate that the relatively simple low molecular weight scaffold reported herein can be easily developed as more effective molecular probes of the intracellular chemistry and biology of NAADP and potentially even as new therapeutics.

Experimental Procedures

Chemistry: General
NMR spectra were recorded using JEOL JMN GX-270 or Bruker AV-400 spectrometer for ¹H (270 or 400 MHz) and ¹³C (75 or 100 MHz).

Chemical shifts (δ) are given in ppm relative to residual solvent peaks, and coupling constants (J values) are in Hertz. Mass spectra data were collected on a Micromass LCT mass spectrometer system using electrospray ionization (ESI).

General Procedure
All reagents and solvents were from commercial suppliers and used as supplied. A solution of nicotinic acid (100 mg, 0.81 mmol) and the appropriate alkyl halide (0.81 mmol) was heated in DMF at 50ºC for 10 hr, and then concentrated, suspended in CH₃OH, and precipitated by addition of ethyl acetate.

1-Carbamoylmethyl-3-Carboxy-Pyridinium Iodide 3
Reaction with 2-iodoacetamide (150 mg, 0.812 mmol) provided the compound as a yellow amorphous solid (175 mg, 70%) mp: 223ºC–225ºC; ¹H-NMR (270 MHz, D₂O): δ 9.24 (s, 1H), 9.0 (d, J 8.2, 1H), 8.89 (d, J 4.8, 1H), 8.17 (m, 1H) and 5.56 (2H, s); ¹³C-NMR (75 MHz, D₂O): δ 169.5, 167.3, 149.3, 148.6, 148.1, 135.6, 129.5, 63.2. IR (KBr) 3379, 1701, 1665 cm⁻¹. HRMS (m/z): [M]⁻ calcd for C₈H₇N₂O₃ 179.0457; found, 179.0491 (100%), [2MH]⁻ calcd for C₁₆H₁₅N₄O₆ 359.09912; found, 359.1024 (90%) [25].

3-Carboxy-1-[(5-Methoxycarbonyl-Pentylcarbamoyl)-Methyl]-Pyridinium Bromide 2
Reaction with 6-(2-bromo-acetylamino)-hexanoic acid methyl ester (550 mg, 2.18 mmol) and nicotinic acid (269 mg, 2.18 mmol) yielded a beige amorphous solid (250 mg, 30%) ¹H-NMR (400 MHz, D₂O): 9.29 (s, 1H), 9.05 (d, J 7.9, 1H), 8.93 (d, J 6.2, 1H), 8.22–8.19 (dd, J 7.9, 6.2,1H), 5.22 (s, 2H), 3.66 (s, 3H), 3.29 (t, J 6.8, 2H), 2.40–2.35 (m 2H), 1.66–1.49 (m, 4H) and 1.38–1.29 (m, 2H). δ_C (100 MHz, D₂O) 177.5, 165.3, 165.2, 147.8, 146.9, 146.6, 134.5, 128.0, 61.9, 52.1, 39.7, 33.5, 27.7, 25.4 and 23.8. m/z [FAB⁺] 309.1 (M⁺, 100%) HRMS (m/z): [M]⁺ calcd for C₁₅H₂₁N₂O₅ 309.1450; found, 309.1456.

Biological Evaluation
Sea Urchin Egg Homogenate Ca²⁺ Release Assay
Sea urchin egg homogenate was prepared and diluted to 2.5% in GluIM consisting of 250 mM potassium gluconate, 250 mM N-methylglucamine, 20 mM HEPES (pH 7.2), 1 mM MgCl₂, 1.0 mM ATP, 10 mM phosphocreatine, 10 units/ml creatine phosphokinase, 1 mM sodium azide, and 3 μM fluo-3 in the presence of regenerating system and kept at 17ºC with agitation for 3 hr to facilitate Ca²⁺ uptake into stores. Ca²⁺ release was determined by measuring the increase in Fluo-3 fluorescence at 526 nm.
[³²P]NAADP Binding to 0.5% Sea Urchin Egg Homogenate
[³²P]NAADP was obtained as described [33]. Dilute homogenate (as above) was preincubated with acetamide derivative **3** for 10 min, and then 0.2 nM [³²P]NAADP was added and the mixture was incubated at room temperature for a further 15 min. Samples were filtered through Whatman GF/B filters to separate bound and free [³²P]NAADP ligand [34].
Intact Sea Urchin Eggs
Sea urchin eggs were microinjected with a solution containing the Ca²⁺ reporter dye, Ca²⁺ green dextran, and caged NAADP [4] and incubated in sea water containing the test pyridinium compound **3** (10 mM). Fluorescence intensity was imaged using a Leica confocal microscope using an excitation wavelength of 488 nm.

Sea urchin eggs of *Lytechinus pictus* were obtained by intracoelomic injection of 0.5 M KCl shed into artificial sea water (435 mM NaCl, 40 mM MgCl₂, 15 mM MgSO₄, 11 mM CaCl₂, 10 mM KCl, 2.5 mM NaHCO₃, 1 mM EDTA), dejellied by passing through a 90 mm nylon mesh, and then washed twice by centrifugation. Eggs were transferred to polylysine-coated glass coverslips for microinjection and microscopy. Oregon green 488 BAPTA (1,2-bis(2-aminophenoxy)-ethane-N,N,N′,N′-tetraacetic acid dextran; Molecular Probes) was pressure-microinjected (Picospritzer; World Precision Instruments). The Ca²⁺-sensitive dye was imaged by laser-scanning confocal microscopy (Leica model TCS NT) using the 488 nm line of an argon ion laser for excitation, and the emission was long pass-filtered (515 nm) and detected with a photomultiplier tube. Caged NAADP (²⁹P-(1-(2-nitrophenyl)ethyl) NAADP; Molecular Probes) was purified further by high-performance liquid chromatography to remove small amounts of contaminating free NAADP. Caged NAADP was photolyzed with ultraviolet light (351 and 364 nm lines) from an argon ion laser

(Enterprise model 651; Coherent) that was directed into the scanning head by a quartz fiber optic cable. The spatial location of photolysis was controlled via a shutter that was placed in the light path of the ultraviolet laser. This resulted in a band of UV across the image with the position and width of the band being controllable. The confocal images were processed with the software NIH Image to create a self ratio by dividing the intensity (F) of each image on a pixel-by-pixel basis by the intensity of an image acquired before stimulation (F_0). Time courses of F/F_0 are plotted against time.

Murine Pancreatic Acinar Cells

All experiments were conducted at room temperature. Pancreatic acinar cells were isolated from mice, dispersed by collagen treatment, and then seeded onto polylysine-coated number 1 glass coverslips and loaded by incubating cells with 1–5 μM fura-2 acetoxymethylester (Molecular Probes; Leiden, Holland) for 60 min. The cells were subsequently washed, maintained in buffer (140 mM NaCl, 4.7 mM KCl, 1.1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 10 mM glucose [pH 7.2]) and used immediately. Compound **3** (1 mM) was added before or after Cholecystokinin (CCK; 5 pM) or acetylcholine (ACh; 50 nM) was added, the cells were excited alternately with 340 and 380 nm light (emission 510 nm), and the ratio of the intensities of emitted light at the two excitation wavelengths were recorded using a 12 bit CCD camera (MicroMax; Princeton Instruments, NJ) and plotted against time.

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