Activation of a Calcium Entry Pathway by Sodium Pyrithione in the Bag Cell Neurons of *Aplysia*

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ABSTRACT: **The ability of sodium pyrithione (NaP), an agent that produces delayed neuropathy in some species, to alter neuronal physiology was accessed using ratiometric imaging of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in fura PE-filled cultured *Aplysia* bag cell neurons. Bath-application of NaP evoked a $[Ca^{2+}]_i$ elevation in both somata and neurites with an EC_{50} of ≈300 n*M* and a Hill coefficient of ≈1. The response required the presence of external Ca^{2+}, had an onset of 3–5 min, and generally reached a maximum within 30 min. 2-Methyl-sulfonylpyridine, a metabolite and close structural analog of NaP, did not elevate $[Ca^{2+}]_i$. Under whole-cell current-clamp recording, NaP produced a ≈14 mV depolarization of resting membrane potential that was dependent on external Ca^{2+}. These data suggested that NaP stimulates Ca^{2+} entry across the plasma membrane. To minimize the possibility that a change in cytosolic pH was the basis for NaP-induced Ca^{2+} entry, bag cell neuron intracellular pH was estimated with the dye 2′,7′-bis(carboxyethyl-5(6)-carboxy-fluorescein acetoxy methylester. Exposure of the neurons to NaP did not alter intracellular pH. The slow onset and sustained nature of the NaP response suggested that a cation exchange mechanism coupled either directly or indirectly to Ca^{2+} entry could underlie the phenomenon. However, neither ouabain, a Na^+/K^+ ATPase inhibitor, nor removal of extracellular Na^+, which eliminates Na^+/Ca^{2+} exchanger activity, altered the NaP-induced $[Ca^{2+}]_i$ elevation. Finally, the possibility that NaP gates a Ca^{2+}-permeable ion channel in the plasma membrane was examined. NaP did not appear to activate two major forms of bag cell neuron Ca^{2+}-permeable ion channels, as Ca^{2+} entry was unaffected by inhibition of voltage-gated Ca^{2+} channels using nifedipine or by inhibition of a voltage-dependent, nonselective cation channel using a high concentration of tetrodotoxin. In contrast, two potential store-operated Ca^{2+} entry current inhibitors, SKF-96365 and Ni^{2+}, attenuated NaP-induced Ca^{2+} entry. We conclude that NaP activates a slow, persistent Ca^{2+} influx in *Aplysia* bag cell neurons.** © 2004 Wiley Periodicals, Inc. J Neurobiol 60: 411–423, 2004

Keywords: **calcium channel; fura-2; neuropathy; store-operated channels**

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INTRODUCTION

Pyrithione (*N*-hydroxypyridine-2-thione), usually administered in the form of its sodium, zinc, or copper salt, is a broad-spectrum fungistatic and antimicrobial agent. Pyrithione has been found to produce reversible neurotoxicity in rodents. This effect is characterized by the failure of transmission at the neuromuscular junction (Ross and Lawhorn, 1990) and by biochemical and histopathological changes in peripheral motor nerves. This is followed by subsequent histopathological changes in the associated skeletal muscles that are innervated by these nerves (Snyder et al., 1979). Although rodent models have been a primary focus on the neurotoxic actions of pyrithiones, it is evident that other species are also sensitive (Lansdown, 1991; Delahunt et al., 1962). Aquatic animals are also particularly sensitive to the actions of pyrithione (Goka, 1999). A variety of effects have been reported for the actions of sodium pyrithione on cultured cell lines (Moller et al., 2002; Santa Maria et al., 1996). Neither the molecular targets of these actions nor the cellular mechanisms that produce the neurotoxic effects of pyrithiones are, however, yet known.

The bag cell neurons of the marine snail, *Aplysia californica*, have been used to investigate prolonged changes in neuronal excitability, intracellular Ca^{2+} regulation, and Ca^{2+} influx, and the role of each of these processes in neuropeptide secretion (Kupfermann and Kandel, 1970; Conn and Kaczmarek, 1989; Knox et al., 1992; Wayne and Wong, 1994; Jonas et al., 1997; Magoski et al., 2000). In the present study, we have tested the actions of sodium pyrithione (NaP) on intracellular Ca^{2+} levels and Ca^{2+} influx in these neurons using ratiometric dye imaging. Our data show that NaP gates a Ca^{2+} entry pathway in bag cell neurons, and that the pharmacology of this pathway resembles that of store-operated Ca^{2+} channels in certain nonexcitable cells.

MATERIALS AND METHODS

Isolation of Bag Cell Neurons, Salines, and Pharmacological Agents

Adult *A. californica* (200–250 g) were anesthetized by injection of isotonic $MgCl_2$ (50% of body weight), and the abdominal ganglia, along with the pleural-abdominal connectives, were excised. To make primary cultures of bag cell neurons, ganglia were incubated for 18 h at room temperature in a solution of dispase (13.3 mg/mL; Boehringer Mannheim) dissolved in normal artificial sea water [nASW; containing in m*M*: 11 $CaCl_2$, 55 $MgCl_2$, 460 NaCl, 10.4 KCl, 15 *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid (HEPES), and 5.6 glucose; 100 U/mL penicillin, and 0.1 mg/mL streptomycin; pH 7.8 with NaOH]. Bag cell clusters were then dissected from their surrounding connective tissue and the neurons were dispersed onto glass coverslips (VWR #1 48366045) coated with poly-D-lysine (1 mg/mL, molecular weight 70,000–150,000; Sigma P0899) attached to drilled-out 35 mm Petri dishes containing nASW. Cultures were maintained for 1–3 days in a 14°C incubator.

Most experiments were carried out in nASW. When an absence of extracellular Ca^{2+} was required, the $CaCl_2$ was omitted and 0.5 m*M* ethylene glycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added to make Ca^{2+}-free ASW. In some cases, the $CaCl_2$ was omitted but the EGTA was not added, making nominally Ca^{2+}-free ASW. To make Na^+-free ASW, the NaCl was replaced with tetra ethyl ammonium (TEA)-Cl or TEA-Br. Salts for salines were obtained from American Bioanalytical, J.T. Baker, Mallinckrodt, or Sigma.

All drugs were dissolved and stored as concentrated stocks, with a final concentration being achieved by either adding a small volume (1–20 μL) of stock directly to the bath or making a larger volume of bath solution with the drug at final concentration and exchanging the entire bath. Care was taken to pipette any small volume of concentrated stock as far away from the neurons as possible. The source of pharmacological agents was as follows: NaP and 2-methyl-sulfonylpyridine were synthesized by Arch Chemicals; SKF 96365 (1-(β-[3(4-methoxyphenyl)propoxy]-4-methoxy-phenethyl))-imidazole hydrochloride (567310) was from Calbiochem; $NiCl_2$ (N54) was from Fisher; 4-amino pyridine (A0152), cromolyn (C0399), econazole (E4632), $GdCl_3$ (G7532), $LaCl_3$ (L4131), nifedipine (N7634), ouabain (O3125), tetrodotoxin (T5651) were from Sigma-Aldrich. With the following three exceptions all drug stocks were made in distilled water: econazole was first dissolved in 1:1 chloroform:methanol and then added to the saline; nifedipine and SKF 96365 were first dissolved in dimethyl sulfoxide (DMSO) and then added to the saline. The final concentration of DMSO was 0.01 μ*M*, which in control experiments had no effect on $[Ca^{2+}]_i$.

Intracellular Ca^{2+} Imaging in Bag Cell Neurons

Bag cell neuron somata were microinjected with 10 m*M* fura PE3 K^+ salt (TefLabs, 0110; Vondran et al., 1995) by pressure ejection from intracellular microelectrodes (electrical resistance 30–50 MΩ when back filled with 3 *M* KCl). A range of 10–15 (900 ms) pulses was required to fill the neurons with an optimum amount of dye, estimated to be 50–100 μ*M*, following which the neurons were allowed to rest for 30 min. Neurons were then placed on the recording chamber of a Nikon Diaphot inverted microscope equipped with a 40X objective [Nikon Plan Fluor Numerical Aperture (NA) = 1.3] or a 10X objective [Nikon Fluor (NA) = 0.5]. The illumination system was a 75 W Xenon arc lamp, coupled to the microscope via a fiber optic cable, and a

computer-controlled grating/monochromator based excitation system (Photon Technology Inc). Fluorescent images were acquired with a Hamamatsu C2400 iCCD camera. The culture densities were such that one to eight bag cell neurons could be monitored in a single field when using the 10X objective. Emission images at 510 nm fluorescence from a single field were sequentially acquired at 340 and 380 nm excitation wavelengths, with an acquisition time (including frame averaging when necessary) for a single full-frame (256 × 520 pixels) of 1–4 s. Ca^{2+} concentration sampling was performed at 30 or 60 s intervals. An IBM compatible personal computer was used to sample $[Ca^{2+}]_i$ in real time and save the value to hard disk. The camera gain voltage was set based on the initial fluorescence intensity of the cells at the beginning of each experiment and was maintained constant thereafter. Between acquisition episodes, the excitation illumination was blocked by automatic shutter control. The ratio R of the fluorescence intensities (converted to pixel values) from the 340 and 380 nm excitation wavelength evoked images was used to calculate the free $[Ca^{2+}]_i$ from the relationship, $[Ca^{2+}]_i = Kd \cdot Q(R - R_{min})/(R_{max} - R)$ (Grynkiewicz et al., 1985). R_{min}, R_{max}, and Q were determined in intact bag cell neurons by applying 1–10 μM digitonin (Molecular Probes D-8449) in Ca^{2+}-free buffer (0.5 mM EGTA) followed by perfusion with media containing a saturating amount of Ca^{2+} (11 mM). The constant Q was determined from the ratio of 380 nm evoked fura PE3 fluorescence in Ca^{2+}-free and 11 mM Ca^{2+} ASW. Values for R_{min}, R_{max}, and Q ranged from 0.11 to 0.33, 5.1 to 7.5, and $8.7 \cdot 10^{-6}$ to $10.2 \cdot 10^{-6}$, respectively, while the Kd was 204 nM. Corrections for background fluorescence and camera dark current were carried as described previously (Knox et al., 1996) and incorporated into the acquisition program.

Intracellular Ca^{2+} Imaging in HEK 293 Cells

Some experiments were performed on a line of human embryonic kidney cells (HEK 293). These cells stably expressed a recombinant form of the trpC4 channel and were a gift from Dr. Lou H. Philipson (Departments of Medicine and Pharmacology, University of Chicago). Briefly, HEK cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (Gibco) supplemented with 10% fetal bovine serum and antibiotic/antimycotic (Gibco) in a 5% CO_2 air environment at 37°C. Cells were grown on the same glass coverslips coated with poly-D-lysine and attached to drilled-out 35 mm Petri dishes as were used for the bag cell neurons. For imaging experiments, cells were loaded for 1 h at 37°C with 20 μM fura PE3-AM (TefLabs 0108), which was prepared by combining one part fura PE3-AM with one part 20% (w/v) pluronic acid and then diluted in culture medium. The excess extracellular dye was then washed away by twice rinsing with saline (see below). Cells were left to equilibrate for ≈30 min in saline prior to imaging. The saline used during imaging consisted of, in mM: 1 $CaCl_2$, 140 NaCl, 3 KCl, 29 glucose, and 10 HEPES; pH 7.2 with NaOH. HEK 293 cell $[Ca^{2+}]_i$ was determined using the 40X objective and in the same manner as for bag cell neuron $[Ca^{2+}]_i$.

Intracellular H^+ Imaging in Bag Cell Neurons

As with $[Ca^{2+}]_i$, pH was estimated optically. Bag cell neurons were dye loaded in ASW (22°C) for 20 min with 2′,7′-cis(carboxyethyl)-5,6-carboxyfluorescein acetoxymethy ester (BCECF-AM; TefLabs) at a concentration of 10 μM, and allowed to equilibrate for 30 min prior to experiments. The neurons were then transferred to the recording microscope and alternately excited by 495 and 440 nm light (the isosbestic wavelength for BCECF) at 30 s intervals. Fluorescence emission above the 535 nm was barrier filtered (Omega Optical, Brattleborough, VT), measured, ratioed (490/440 nm), and saved to hard disk.

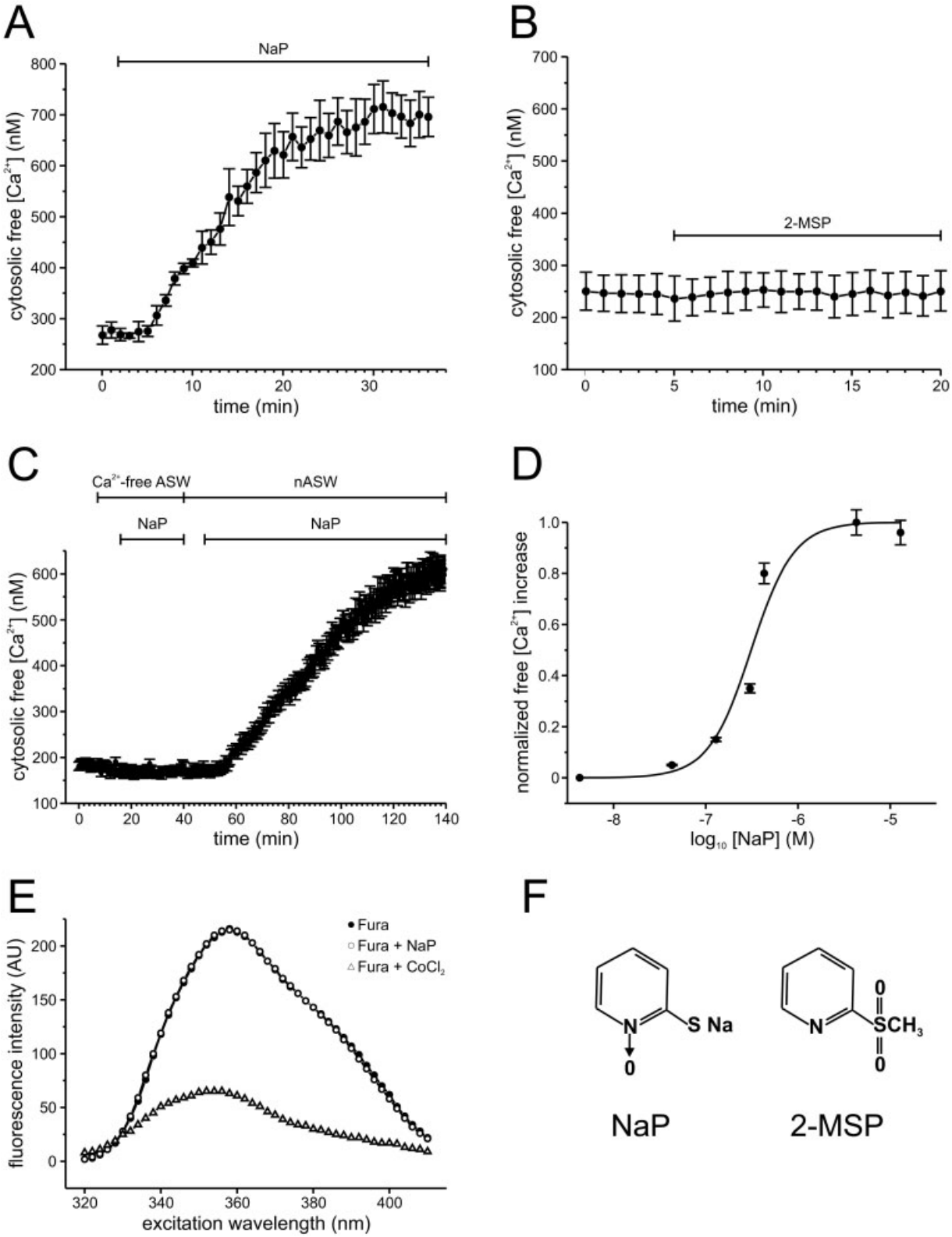
Imaging Data Presentation

Imaging data files (PTI.csv files) were subsequently imported off-line into the spreadsheet program Origin (version 4.1, MicroCal) for further analysis. Data were expressed over time as the mean $[Ca^{2+}]_i$ or BCECF 440/485 ratio fluorescence ± the standard error of the mean for multiple neurons.

Electrophysiology in Bag Cell Neurons

Current-clamp recordings were made from bag cell neurons using an EPC-7 amplifier (List-Electronics) and the tight-seal, whole-cell method. Microelectrodes were pulled from 1.5 mm internal diameter, borosilicate glass capillaries and had a resistance of 0.9–1.2 MΩ when filled with intracellular saline [IS; containing in mM: 500 K-aspartate, 70 KCl, 0.595 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 11 glucose, 0.77 EGTA, 10 glutathione, 5 ATP (grade 2, disodium salt; Sigma), and 0.1 GTP (type 3, disodium salt; Sigma); pH 7.3 with KOH; estimated free $[Ca^{2+}]$ = 300 nM]. Voltage signals were acquired using pCLAMP (version 6.02; Axon Instruments) data acquisition and analysis software. Signals were filtered at 3 kHz and sampled at 1.5 Hz.

Cell-attached patch clamp recordings were made using the same apparatus and materials as per the whole-cell current-clamp recordings, the difference being that the pipette was filled with nASW. Seal resistance was monitored using the Ohm's law based seal test function of pCLAMP. Capacitance, a measure of the extent of electrical continuity between the pipette and the interior of the neuron, was read directly from the amplifier capacitance compensation controls.


A
NaP
cytosolic free [Ca2+] (nM)
time (min)
B
2-MSP
cytosolic free [Ca2+] (nM)
time (min)
C
Ca2+-free ASW
nASW
NaP
NaP
cytosolic free [Ca2+] (nM)
time (min)
D
normalized free [Ca2+] increase
log10 [NaP] (M)
E
Fura
Fura + NaP
Fura + CoCl2
fluorescence intensity (AU)
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F
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Figure 1

RESULTS

NaP Stimulates a Slow Onset, Prolonged Elevation of $[Ca^{2+}]_i$

In normal external Ca^{2+} (11 m*M*) containing ASW, bath application of 10 μM NaP [Fig. 1(F)] induced a slow elevation of $[Ca^{2+}]_i$ in isolated bag cell neurons [Fig. 1(A)]. The onset of the response was normally within 3–5 min, and reached peak levels between 20–40 min. Exposure of bag cell neurons to similar amounts of 2-methyl-sulfonylpyridine, a metabolite with similar structural features to NaP [Fig. 1(F)], did not alter $[Ca^{2+}]_i$ [Fig. 1(B)]. Furthermore, in Ca^{2+}-free ASW (no added Ca^{2+} plus 0.5 m*M* EGTA) there was no change in cytosolic Ca^{2+} levels [Fig. 1(C)]. Attempts to reverse the effect of NaP by washing in nASW were for the most part unsuccessful, although in some cases a small, partial reversal was observed, suggesting that once NaP activates this particular Ca^{2+} entry pathway it remains open. The NaP evoked rise in $[Ca^{2+}]_i$ was dose-dependent, with an EC_{50} value of ≈300 n*M* and a Hill coefficient of ≈1.0 [Fig. 1(D)].

Because NaP is water soluble, and is ionized at pH 7.8, it is unlikely to be membrane permeant under our experimental conditions. Nevertheless, to test the possibility that NaP enters the neuronal cytosol and interferes with the spectral properties of fura PE3, thereby producing an apparent Ca^{2+} rise, the absorption spectra of fura PE3 was monitored in the absence and presence of NaP. As shown in Figure 1(E), 10 μM NaP had no effect on the absorption spectra of fura PE3, thereby eliminating the possibility of such an artifact; furthermore, as a positive control, we demonstrated potent quenching of fura PE3 fluorescence by Co^{2+}.

It is known that NaP can exhibit Zn^{2+} ionophore activity in rat cortical neurons when extracellular Ca^{2+} is replaced by 1 m*M* Zn^{2+} (Sensi et al., 1997). This type of experiment has implicated changes in intracellular free Zn^{2+} to be causal in certain types of brain injury (Choi and Koh, 1998). Although none of the salines used in our experiments contained added Zn^{2+}, we considered the possibility that our solutions had contaminating levels of Zn^{2+} sufficient to be transferred intracellularly by NaP ionophore activity and alter fura PE3 fluorescence. First, to address the possibility that the presence of EGTA in the Ca^{2+}-free ASW could have resulted in the chelation of trace amounts of Zn^{2+}, we tested the effects of NaP in nominally Ca^{2+}-free ASW (no added Ca^{2+} *and* no EGTA). Using this saline, 10 μM NaP failed to produce a robust change in fura fluorescence [Fig. 2(A)], supporting the hypothesis that NaP selectively stimulates Ca^{2+} entry under our experimental conditions. Second, if the actions of NaP were attributable to Zn^{2+} ionophore activity, one would predict that any cell type loaded with fura should respond to NaP with an increase in fluorescence ratio, assuming that all of our salines have contaminating Zn^{2+} from either our double distilled water or laboratory salts. We found, however, that fura PE3-AM loaded HEK 293 cells expressing recombinant trpC4 channels do not undergo an elevation in fluorescence ratio following application of 10 μM NaP [Fig. 2(B)]. Finally, the response of bag cell neurons to NaP could not be prevented by addition of 50 μM *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), a very high affinity Zn^{2+} chelator, to the external medium (n = 15, data not shown). Collectively, these experiments indicate that NaP selectively stimulates Ca^{2+} entry into bag cell neurons.

NaP Elicits a Ca^{2+}-Dependent Depolarization

Whole-cell current-clamp recording from cultured bag cell neurons showed that 10 μM NaP elicited a 14

Figure 1 Dose-dependent NaP-induced $[Ca^{2+}]_i$ elevation in fura PE3 loaded bag cell neurons. (A) Bath-application of 10 μM NaP elicits a relatively slow onset, sustained ≈450 n*M* elevation of $[Ca^{2+}]_i$ (n = 8). (B) In a different set of neurons, application of 10 μM 2-methyl-sulfonylpyridine (2-MSP) fails to produce a change in $[Ca^{2+}]_i$ (n = 4; similar results were seen in seven other neurons). (C) The NaP-induced elevation of $[Ca^{2+}]_i$ is dependent on extracellular Ca^{2+} and not due to the release of intracellular Ca^{2+}. When 10 μM NaP is introduced in Ca^{2+}-free ASW, no change in $[Ca^{2+}]_i$ occurs; however, following bath exchange to nASW (which contains 11 m*M* Ca^{2+}) NaP application results in a Ca^{2+} elevation (n = 10). (D) Cumulative dose-response curve from 12 neurons for the NaP-induced rise in cytosolic free $[Ca^{2+}]_i$ showed an EC_{50} = ≈300 n*M* and a Hill coefficient = ≈1. (E) *In vitro* excitation spectra of 10 m*M* fura PE3 alone (closed circles), and in the presence of 4.3 m*M* NaP (open circles) and 10 m*M* $CoCl_2$ (open triangles), indicating that NaP does not interfere with the spectral properties of the Ca^{2+} indicator dye. AU, arbitrary units. (F) The chemical structures of sodium pyrithione (NaP) and 2-methyl-sulfonylpyridine (2-MSP).

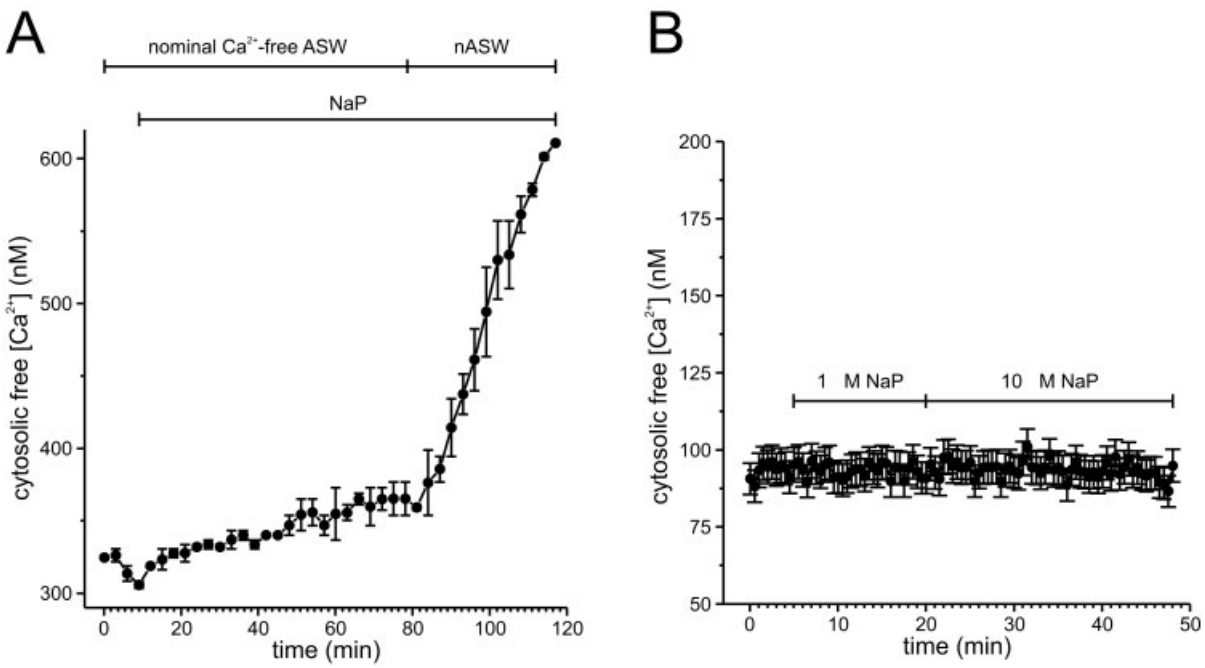

Figure 2 NaP selectively elicits Ca^{2+} influx into bag cell neurons. (A) When 10 μM NaP is bath-applied in nominally Ca^{2+}-free ASW (no added $CaCl_2$ and no EGTA), the $[Ca^{2+}]_i$ shows only a very a small increase—most likely due to residual Ca^{2+} in the bath. Upon exchange to nASW (11 mM Ca^{2+}) while maintaining the presence of NaP, the $[Ca^{2+}]_i$ elevates markedly ($n = 4$). (B) This is in contrast to fura PE3-AM loaded HEK 293 cells expressing recombinant trpC4 channels, where bath-application of 10 μM NaP in a Ca^{2+}-containing saline does not produce a change in $[Ca^{2+}]_i$ ($n = 15$).

$\pm$ 2.0 mV depolarization ($n = 6$) in the presence of extracellular Ca^{2+}, but only $\approx$1 mV depolarization in Ca^{2+}-free ASW [Fig. 3(A,B)]. The amplitude and onset of the NaP evoked depolarization are similar to that evoked by thapsigargin, which depolarizes bag cell neurons by stimulating, through intracellular Ca^{2+} release, a Ca^{2+}-activated, voltage-independent, nonselective, monovalent cation conductance (I_{Tg}) in bag cell neurons (Knox et al., 1996).

We also performed cell-attached recordings with nASW and 10 mM NaP in the pipette to test the possibility that NaP could increase Ca^{2+} permeability by acting in a manner analogous to antifungal drugs such as nystatin. For these recordings, we used a 1,000-fold higher concentration of NaP in the pipette solution as compared to what was typically bath-applied in other experiments. Measurements from five neurons showed that 10 mM NaP in the pipette did not alter the seal resistance or capacitance from the onset of seal formation (2.84 $\pm$ 0.39 MΩ and 5.99 $\pm$ 0.11 pF) through 30 min of cell-attached recording (2.70 $\pm$ 0.40 M° and 5.97 $\pm$ 0.10 pF; $p > 0.05$ for both sets of data, Student's t test). This is contrary to the effects of nystatin, which we have used routinely to make perforated-patch recordings from a number of cell types.

NaP-Induced Ca^{2+} Elevation Is Unaffected by Inhibition of Plasma Membrane Ca^{2+} Transport

The slow onset and maintenance of the NaP-induced Ca^{2+} entry suggested the possible involvement of a Ca^{2+} transport mechanism. For example, if NaP were to stimulate the plasma membrane Na^+/Ca^{2+} exchanger to run in reverse, which does occur in neurons under certain conditions (for example, elevated internal Na^+; Yu and Choi, 1997), one would predict a slow rise in $[Ca^{2+}]_i$. To test this, we eliminated the Na^+/Ca^{2+} pump by complete substitution of external Na^+ with TEA-Cl. We have shown previously that the bag cell neuron Na^+/Ca^{2+} exchanger is most active under conditions of high Ca^{2+} load, such as during store depletion, and that removal of external Na^+ inhibits Na^+/Ca^{2+} exchanger activity (Knox et al., 1996). As shown in Figure 4(A), the NaP-induced Ca^{2+} elevation was not altered under these conditions. Similarly, inhibiting the Na^+/K^+ ATPase with

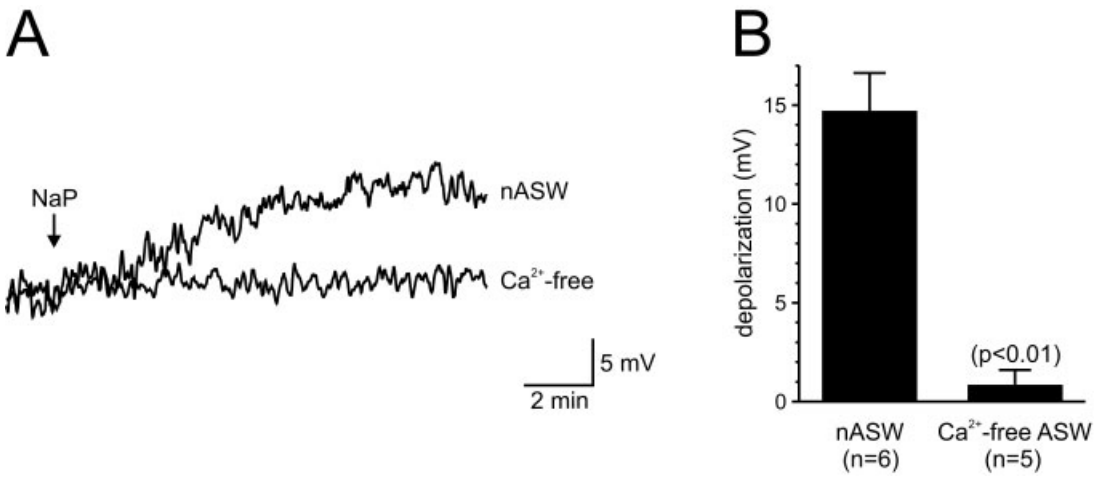

Figure 3 NaP depolarizes bag cell neurons. (A) Whole-cell current-clamp measurement of membrane potential in two isolated neurons, one in nASW (dark trace) and the other in Ca^{2+}-free ASW (light trace). The onset of the depolarization to 10 μM bath-applied NaP in nASW is comparable to the NaP-induced Ca^{2+} elevation measured in fura PE3 loaded neurons. When NaP is applied in the absence of extracellular Ca^{2+}, no depolarization is elicited. (B) The mean NaP-evoked depolarization was $\approx$15 mV for Ca^{2+} containing nASW ($n = 6$), and was significantly different (Student's t test) from the $\approx$1 mV in Ca^{2+}-free ASW ($n = 5$). The average resting membrane potential of these bag cell neurons, prior to NaP application, was -52.7 ± 6.0 mV in nASW and -51.0 ± 3.3 mV in Ca^{2+}-free ASW (data not shown).

1 mM ouabain had no effect on the ability of 10 μM NaP to raise $[Ca^{2+}]_i$, thereby eliminating the Na^+/K^+ pump, and Na^+ homeostasis as a possible pathway of action for NaP [Fig. 4(B)].

NaP Does Not Alter Intracellular pH

The fact that the biocidal activity of NaP is related to its ability to collapse pH gradients across microbial membranes (Dinning et al., 1998), as well as the knowledge that intracellular pH and Ca^{2+} homeostasis are mechanistically linked (Wiegmann et al., 1993), led us to measure the intracellular pH of NaP-treated neurons using the fluorescent pH indicator dye BCECF. As indicated in Figure 5, 10 μM NaP did not alter the 440/490 nm fluorescence emission ratio of BCECF, whereas our positive control, 4-amino pyridine (4-AP; a strong base) rapidly alkalinized the cytosol, indicated by the drop in the BCECF fluorescence ratio. Note that the cytoplasmic pH recovers to baseline in the continued presence of 4-AP and NaP, indicating that the normal proton exchange processes that regulate intracellular pH are not compromised by extracellular NaP. In addition, we observed the normal characteristic rebound acidification of the cytosol following washout of 4-AP.

NaP-Induced Ca^{2+} Elevation Is Unaffected by Voltage-Gated Ca^{2+} and Cation Channel Blockers but Is Inhibited by Store-Operated Ca^{2+} Channel Blockers

Possible modes of NaP-stimulated Ca^{2+} entry include voltage-operated Ca^{2+} channels and nonselective cation channels that are Ca^{2+} permeable. Pretreatment with the dihydropyridine nifedipine (50 μM), which blocks voltage-gated Ca^{2+} channels in bag cell neurons (Strong et al., 1987; Nerbonne and Gurney, 1987), failed to attenuate the effect of 10 μM NaP, suggesting that voltage-dependent Ca^{2+} channels are not the target of NaP [Fig. 6(A)]. Likewise, 100 μM tetrodotoxin (TTX), which blocks a Ca^{2+}-permeable, voltage-dependent cation channel in bag cell neurons (Wilson et al., 1996; Magoski et al., 2000), did not alter the NaP-induced Ca^{2+} elevation [Fig. 6(B)]. This would preclude the involvement of a nonselective, Ca^{2+}-permeable conductance in mediating the response to NaP.

A slow, sustained rise in $[Ca^{2+}]_i$, similar to that produced by NaP in bag cell neurons, occurs as a result of ligand-gated/store-operated capacitative Ca^{2+} entry in many nonexcitable cells (Putney, 1986, 1990). In those cases, Ca^{2+} entry is stimulated by release or depletion of intracellular Ca^{2+} stores and

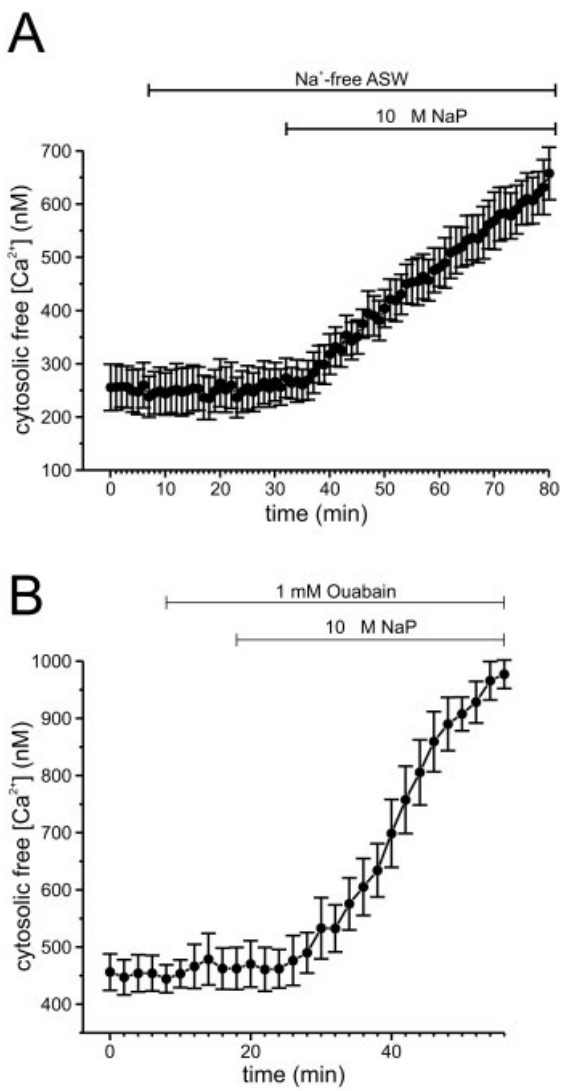

Figure 4 NaP-induced $[Ca^{2+}]_i$ elevation is unaffected by inhibition of plasma membrane Ca^{2+} and Na^+/K^+ pumps. (A) Replacement of extracellular NaCl with TEA-Cl has no effect on the ability of 10 μM NaP to raise $[Ca^{2+}]_i$ ($n = 10$). The same results were also obtained using the Br^- salt of TEA (data not shown), ruling out the involvement of a Cl^- conductance. We have previously established that while removal of external Na^+ eliminates $Na^+\backslash Ca^{2+}$ exchanger activity, the basal activity of the exchanger is fairly low, unless a large load of intracellular Ca^{2+} is present (Knox et al., 1996). This would explain the absence of a $[Ca^{2+}]_i$ elevation following the initial removal of external Na^+. (B) Similarly, inhibition of the $Na^+\backslash K^+$-ATPase pump with 1 mM ouabain is without effect on the response to 10 μM NaP ($n = 5$).

may be blocked by organic compounds, such as the chromone, cromolyn (Cox et al., 1998), and the imidazoles, econazole (Daly et al., 1995) and SKF-96365 (Fasolato et al., 1990; Merritt et al., 1990; Blayney et al., 1991; Cabello and Schilling, 1993; Doi et al., 2000), as well as by the metals, Gd^{3+}, La^{3+}, and Ni^{2+} (Hoth and Penner, 1992; Ross and Cahalan, 1995; Zhang and McCloskey, 1995). Pretreatment with cromolyn (200 μM, $n = 3$; 400 μM, $n = 1$), econazole (30 μM, $n = 2$), Gd^{3+} (50 μM, $n = 1$; 100 μM, $n = 1$), or La^{3+} (10 μM, $n = 2$; 100 μM, $n = 1$) did not prevent the $[Ca^{2+}]_i$ elevation induced by 10 μM NaP; however, both Ni^{2+} and SKF-96365 were effective at attenuating the response. Figure 7(A) shows that pretreatment of cultured bag cell neurons with 20 μM SKF-96365 effectively eliminates the ability of 10 μM NaP to elevate $[Ca^{2+}]_i$. Similarly, when 100 μM Ni^{2+} is introduced and 10 μM NaP is applied, the normally marked and steady increase in $[Ca^{2+}]_i$ is both diminished and irregular [Fig. 7(B)]. In addition, subsequent application of Ni^{2+} after $[Ca^{2+}]_i$ had been elevated by NaP produced a prompt reduction of $[Ca^{2+}]_i$ ($n = 2$, data not shown).

DISCUSSION

Using Ca^{2+} imaging of fura PE3 loaded cells, we have demonstrated that NaP gates a form of Ca^{2+} entry in the bag cell neurons. The lack of NaP-induced Ca^{2+} elevation in Ca^{2+}-free ASW rules out a

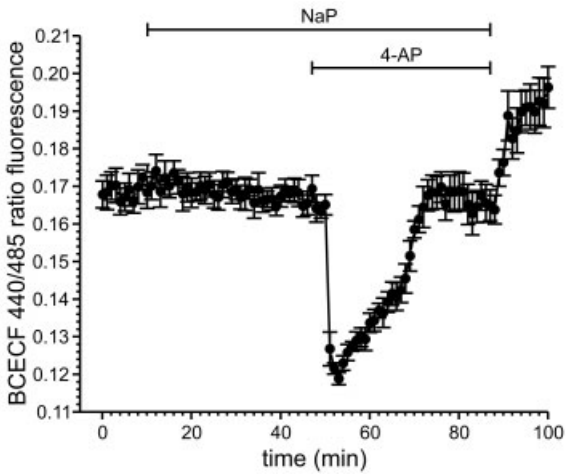

Figure 5 NaP does not alter cytosolic pH. NaP-induced Ca^{2+} elevation does not change the intracellular H^+ concentration, whereas, as expected, application of the strong base, 2 mM 4-aminopyridine (4-AP), rapidly alkalinizes the neuronal cytosol ($n = 12$). Notice that in the continued presence of NaP and 4-AP, the pH returns towards control level, suggesting that NaP does not compromise the activity of endogenous H^+ regulatory processes. Additionally we observe a characteristic rebound acidification of the cytosol following base washout.

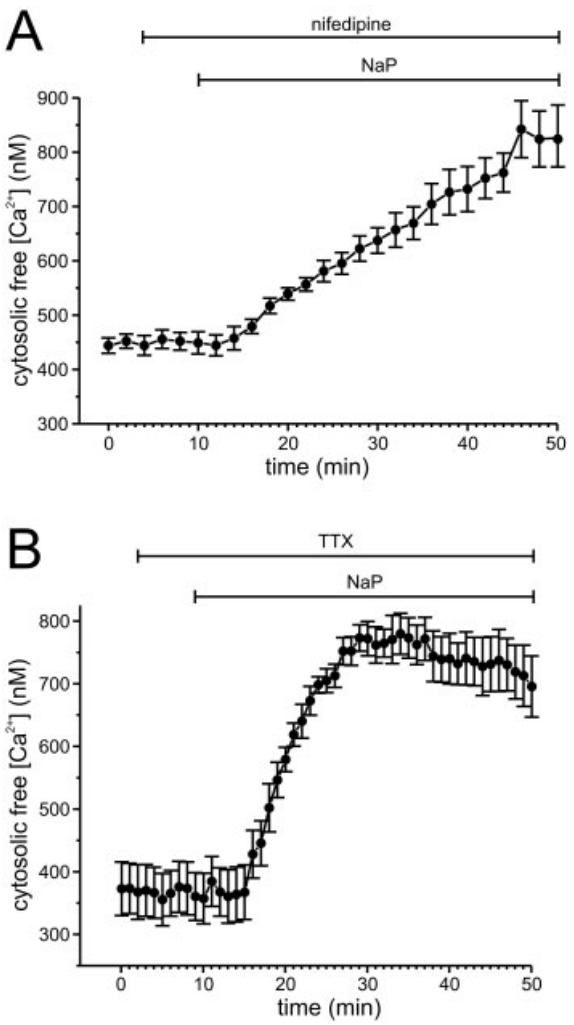

Figure 6 NaP-induced $[Ca^{2+}]_i$ elevation persists in the presence of voltage-gated Ca^{2+} and cation channel blockers. Treatment of isolated neurons with 50 μM nifedipine [(A), $n = 7$] or 100 μM tetrodotoxin [TTX; (B), $n = 8$] does not inhibit NaP-induced Ca^{2+} elevation. Nifedipine selectively abolishes the basal, voltage-dependent Ca^{2+} current in bag cell neurons (Strong et al., 1987; Nerbonne and Gurney, 1987), while the high concentration of TTX is sufficient to block a voltage-dependent, nonselective cation channel also found in these neurons (Wilson et al., 1996; Magoski et al., 2000). Collectively these results rule out an involvement for direct or indirect voltage-gated Ca^{2+} entry in NaP-induced Ca^{2+} elevation.

mechanism involving release of Ca^{2+} from internal stores. Although the molecular basis of this form of calcium entry, and its biological significance for the physiological properties of these neurons, are not yet known, there are several plausible candidates. Both SKF-96356 (Fasolato et al., 1990; Merritt et al., 1990; Blayney et al., 1991; Cabello and Schilling, 1993; Doi et al., 2000) and Ni^{2+} (Hoth and Penner, 1993) have been established as blockers of store-operated currents. Thus, the pharmacology of the NaP-induced Ca^{2+} rise and its time course are similar to store-operated Ca^{2+} entry in several non-neuronal cell

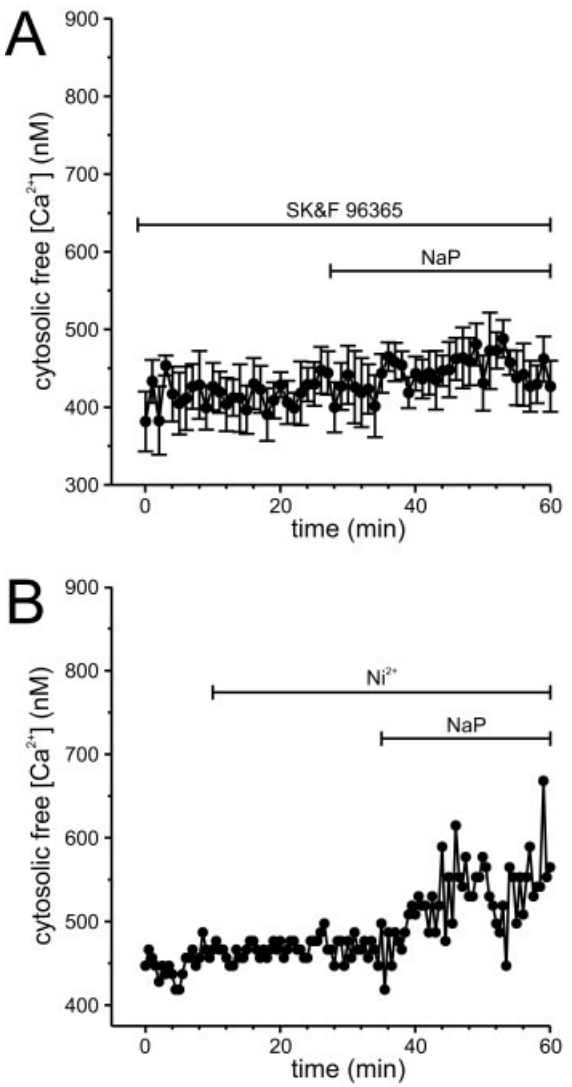

Figure 7 The store-operated Ca^{2+} channel blockers, SKF-96365 and Ni^{2+}, inhibit NaP-induced $[Ca^{2+}]_i$ elevation. (A) In an experiment monitoring four bag cell neurons, the response to 10 μM NaP in nASW is essentially abolished when the cells are pretreated with 20 μM SKF-96365, an imidazole known to block store-operated Ca^{2+} entry in nonexcitable cells. Similar results were seen in three other experiments involving 19 additional bag cell neurons. (B) In a single experiment, application of 100 μM Ni^{2+}, a nonspecific blocker of store-operated Ca^{2+} channels, does not alter $[Ca^{2+}]_i$, but suppresses the response to 10 μM NaP. In the presence of Ni^{2+}, the normally robust and steady NaP response is diminished to a running average of only $\approx$100 nM and overall is rather nonuniform. Similar results were obtained in four experiments.

types, including endothelial cells, mast cells, smooth muscle cells, and T-lymphocytes (Lewis and Cahalan, 1990, 1995; Merritt et al., 1990; Putney, 1990; Hoth and Penner, 1992; Cabello and Schilling, 1993; Doi et al., 2000). It is possible, therefore, that the NaP activates a conductance pathway with properties similar to Ca^{2+} release-activated Ca^{2+}currents/Ca^{2+} depletion-activated Ca^{2+} currents that underlie capacitative Ca^{2+} entry in such cells (Putney, 1990; Luckhoff and Clapham, 1994). The ineffectiveness of other store-operated Ca^{2+} channel blockers, particularly La^{3+} and Gd^{3+}, on the NaP-induced $[Ca^{2+}]_i$ elevation could reflect a difference in pharmacology between molluskan and mammalian channels. Moreover, there exist multiple types of store-operated currents, including the ultra-low conductance/high selectivity CRAC-like channels (Lewis and Cahalan, 1990; Hoth and Penner, 1992; Luckhoff and Clapham, 1994) and lower selectivity store-operated cation channels (Curtis and Scholfield, 2001; Trepakova et al., 2001; Albert and Large, 2002). Although store-operated Ca^{2+} entry via CRAC-like channels has not been described in neurons, some excitable cells, such as PC12 and chromaffin cells, possess currents resembling the higher conductance/ lower Ca^{2+}-selective store-operated currents of nonexcitable cells (Fasolato et al., 1990; Fomina and Nowycky, 1999). Parenthetically, we have also found that both La^{3+} and Gd^{3+} are ineffective at blocking true, depletion-activated Ca^{2+} entry elicited by thapsigargin or cyclopiazonic acid treatment (manuscript in preparation).

The observation that NaP causes a sustained rise in $[Ca^{2+}]_i$ is an interesting one. Given that the response showed very modest reversibility, it is possible that NaP binds and permanently locks a Ca^{2+} channel in a permissive state, in a manner analogous to a ligand-gated channel. Although its pharmacology suggests a store-operated current, it was not necessary to deplete stores of intracellular Ca^{2+} to observe activation by NaP. Thus, it is possible that the binding of NaP produces direct activation of a conductance that normally requires store depletion. The mechanism by which SKF-96365 inhibits the action of NaP is most likely to represent pore block, rather than competition with NaP for a binding site on the channel, because the low level of reversibility suggests that NaP is bound tightly to its target.

The alternative possibility is that NaP acts on a novel calcium entry pathway that is quite distinct from store-operated channels. For example, in some cells, calcium entry pathways that share some of the pharmacological properties of store-operated channels, but are not affected by store-depletion, have been found to be activated by second messengers such as arachidonic acid (Broad et al., 1999). The specificity of pharmacological agents that block store-operated Ca^{2+} channels is not absolute, and it is possible that SKF-96365 or Ni^{2+}, and NaP itself, act on an unrelated target. For example, the NaP-sensitive Ca^{2+} influx pathway could represent an uncharacterized member of the transient receptor potential (trp) family of channels (Petersen et al., 1995; Philipp et al., 1996; Zitt et al., 1996; Boulay et al., 1997; Montell, 1997; Ma et al., 2000), many of whom have not yet been assigned specific biological functions, but which are widely expressed in both excitable and nonexcitable cells. In addition, the sensitivity of the NaP-activated Ca^{2+} influx pathway to Ni^{2+} could indicate a T-type Ca^{2+} channel as the source of Ca^{2+} entry. We feel, however, that this is unlikely, given that neither our laboratories, nor other laboratories that work on bag cell neurons, have ever observed T-type Ca^{2+} currents in these cells; furthermore, there are, to our knowledge, no reports of T-type Ca^{2+} currents being blocked by SKF-96365.

In addition to demonstrating that NaP may be a useful pharmacological tool for activating slow neuronal Ca^{2+} entry, our data raise the question of the physiological significance of this mode of Ca^{2+} entry in neuronal function. In T-lymphocytes for example, store-operated Ca^{2+} channels not only regulate the replenishment of intracellular Ca^{2+} stores required for second messenger signaling pathways involving phosphoinositide turnover or Ca^{2+}-induced Ca^{2+}-release (Tsien and Tsien, 1990; Friel and Tsien, 1992), but they also control cell proliferation and selected gene transcription. The NaP-regulated Ca^{2+} entry pathway could contribute to Ca^{2+} homeostasis and/or receptor-mediated Ca^{2+} mobilization responses, especially under conditions when Ca^{2+} store replenishment by voltage-gated Ca^{2+} entry is absent. This may be the case in the rat cerebellum, where metabotropic glutamate receptor signaling via the IP_3 pathway occurs in the absence of postsynaptic conductance changes (Svoboda and Mainen, 1999). Maintenance of this type of synaptic signaling necessitates a mechanism for replenishing the neuritic Ca^{2+} pool acted on by IP_3. A regulated resting Ca^{2+} conductance would be an obvious candidate under these conditions.

The physiological role of the bag cell neurons is to initiate egg-laying behavior in *Aplysia* through the neurohemal secretion of a peptide cocktail into the animal's circulation (Kupfermann and Kandel, 1970). Peptide secretion is achieved through the afterdischarge, a marked and prolonged change in excitability that occurs following brief synaptic stimulation to the bag cell neurons in the intact abdominal ganglion (Kupfermann and Kandel, 1970; Conn and Kacz-

marek, 1989). The afterdischarge is associated with depolarization, bursts of action potentials, a release of Ca^{2+} from intracellular stores, and ultimately secretion (Conn and Kaczmarek, 1987; Wayne and Wong, 1994; Fisher et al., 1994). Similar phenomena are also observed during pharmacologically induced afterdischarge-like behavior in cultured bag cell neurons (Magoski et al., 2000). Using the vibrating Ca^{2+}-sensitive microelectrode technique, we previously demonstrated that thapsigargin-mediated depletion of the endoplasmic reticular Ca^{2+} store stimulates Ca^{2+} influx across the bag cell neuron plasma membrane (Knox et al., 1996). The Ca^{2+} entry pathway activated by NaP and blocked by SKF-96365 and Ni^{2+} may be the same as the pathway initiated by thapsigargin. Such a conductance could, along with voltage-gated Ca^{2+} entry, play a role in replenishing intracellular Ca^{2+} stores following Ca^{2+} release during the bag cell neuron afterdischarge.

Peptide secretion from the bag cell neurons can also be elicited in the absence of action potential firing by the application of insulin to the intact cluster (Jonas et al., 1997). This form of secretion appears to be the result of insulin triggering the release of intracellular Ca^{2+} from a novel store that is prevalent both in the soma and the neurite tips (the site of release), but does not correspond to the endoplasmic reticulum or the mitochondria. Because insulin does not initiate an afterdischarge, or even depolarize the bag cell neurons in any significant manner (Jonas et al., 1996), replenishment of the insulin-activated Ca^{2+} store would require some form of voltage-independent, store-operated Ca^{2+} entry pathway. Conceivably, the NaP-sensitive conductance could act as a source for Ca^{2+} store repletion under such circumstances. Interestingly, Fomina and Nowicky (1999) reported that activation of a store-operated Ca^{2+}-permeable channel in chromaffin cells was sufficient to stimulate exocytosis at negative membrane potentials. Like chromaffin cells, bag cell neurons are neuroendocrine in nature, thus, the potential exists for a NaP-sensitive Ca^{2+} entry pathway to play a direct role in neuropeptide secretion.

How does NaP bring about depolarization of bag cell neurons? A likely possibility is through activation of a Ca^{2+}-sensitive cation channel available for opening at the resting membrane potential. We know that Ca^{2+} released in response to treatment with the store depleting agent, thapsigargin, depolarizes bag cell neurons through activation of I_{Tg}, a voltage-independent, nonselective cation current (Knox et al., 1996). I_{Tg} is distinct from the Ca^{2+} entry pathway activated by store depletion, particularly in the fact that it appears to be selective only for monovalent cations, and while we cannot rule out the possibility that store depletion *per se* elicits I_{Tg}, the evidence suggests that this cation channel is activated by Ca^{2+} itself (Knox et al., 1996; Whim and Kaczmarek, 1998). It is therefore likely that NaP-evoked Ca^{2+} entry also triggers activation of this nonselective cation current and depolarizes the neurons.

Over the relatively short time periods of our experiments, the NaP-induced elevation of $[Ca^{2+}]_i$ in *Aplysia* bag cell neurons did not appear to alter the health of these cells. However, similar $[Ca^{2+}]_i$ elevations in other, more sensitive cell types could be deleterious (Tymianski, 1996; Sattler and Tymianski, 2000). Thus, the neurotoxic effects of NaP in rodents (Snyder et al., 1979; Ross and Lawhorn, 1990) could be explained in part by its action on $[Ca^{2+}]_i$. Consistent with this hypothesis, we have found application of NaP to rat motor neurons *in vitro* not only causes an elevation of $[Ca^{2+}]_i$, but also results in the death of a significant number of these cultured neurons (manuscript in preparation). Thus, it may be that the normal high external Ca^{2+} environment of the bag cell neurons renders them significantly less susceptible to Ca^{2+}-induced toxicity.

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