Inositol Trisphosphate Releases Intracellularly Stored Calcium and Modulates Ion Channels in Molluscan Neurons

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Stimulation of the bag cell neurons of *Aplysia* triggers a long-lasting afterdischarge in these cells. *In vivo*, such a discharge causes the onset of a sequence of reproductive behaviors. We have found that treatments that trigger discharges *in vitro* stimulate the hydrolysis of phosphoinositides in the bag cell neurons, as measured by increased incorporation of ³H-inositol into fractions containing membrane lipids and water-soluble inositol phosphates.

The electrophysiological effects of inositol trisphosphate, one of the products of phosphoinositide turnover that has been shown to mobilize intracellular calcium in non-neuronal cells, were investigated using isolated bag cell neurons in cell culture. Microinjection of inositol trisphosphate into cultured bag cell neurons caused a transient hyperpolarization of the membrane (~35 sec), together with an increase in conductance. This effect of inositol trisphosphate was abolished by 50 mM tetraethylammonium ions. Inositol trisphosphate also reduced the amplitude of action potentials. Injection of calcium ions directly into bag cell neurons mimicked these responses seen after inositol trisphosphate injection.

Using the cell-attached patch-clamp technique in conjunction with inositol trisphosphate microinjection, we observed that inositol trisphosphate evoked increases in the activity of a channel carrying outward current at the resting potential and more positive potentials. The estimated slope conductance of the channel modulated by inositol trisphosphate was ~40 pS, and its reversal potential was close to that predicted for potassium ions. The increased opening of this channel in response to inositol trisphosphate injection appeared to result from a transient shift of its voltage-dependence to more negative potentials. In a few cases, inositol trisphosphate injection also elicited an increase in the activity of a channel passing inward current at rest.

Direct measurements of changes in intracellular calcium in response to inositol trisphosphate were made using digital imaging of isolated neurons loaded with the fluorescent calcium indicator fura-2. These revealed that injection of inositol trisphosphate significantly elevated intracellular calcium levels, and that this inositol trisphosphate-induced rise in cytosolic calcium was not affected by removal of extracel-

lular calcium. In contrast to the effects of trains of action potentials in calcium-containing media, which produced increases in calcium primarily in neurites, the inositol trisphosphate-induced elevation of calcium appeared more localized to the somata of these neurons.

Our results provide evidence that phosphoinositide-derived second messengers play a role in stimulus-induced discharge in the bag cell neurons and that one of these, inositol trisphosphate, directly mobilizes intracellularly stored calcium ions in these neurons.

The bag cell neurons of *Aplysia* comprise a well-defined system for studying the long-term modulation of neuronal excitability by second-messenger systems. These neurons are found in 2 discrete clusters in the abdominal ganglion and receive input from the head ganglia via the pleuroabdominal nerves. Although these neurons have relatively negative resting potentials and do not generate any spontaneous electrical activity, brief electrical stimulation of the pleuroabdominal connective or exposure to neuroactive peptides from the atrial gland triggers a prolonged afterdischarge of synchronous action potentials (Kupfermann and Kandel, 1970; Heller et al., 1980). Immediately following stimulation, the bag cell neurons hyperpolarize transiently (~15–45 sec) before entering the extended phase of spontaneous firing, which lasts approximately 30 min. During the discharge, the calcium component of the action potentials is enhanced, and the cells secrete several neuroactive peptides that initiate a sequence of reproductive behaviors in the animal (Dudek and Blankenship, 1977; Dudek et al., 1979; Kaczmarek et al., 1982). After the end of the discharge, the cells enter a period of relative inhibition for about 20 hr, during which time further stimulation fails to trigger long-lasting discharges (Kaczmarek and Kauer, 1983).

One of the second-messenger systems responsible for the shift in excitability of the bag cell neurons following brief stimulation is the adenylate cyclase system (Kaczmarek et al., 1978; Jennings et al., 1982; Kauer and Kaczmarek, 1985). Another second-messenger system, linked to the calcium/phospholipid-dependent enzyme protein kinase C, may also play a role in the generation of the afterdischarge. Activation of protein kinase C by phorbol esters or synthetic diacylglycerols, or direct microinjection of the kinase into isolated bag cell neurons, has been shown to enhance action potentials by increasing the calcium current (DeRiemer et al., 1985b). This effect of protein kinase C results from the unmasking of a previously covert species of voltage-dependent calcium channel (Strong et al., 1987). Inhibitors of protein kinase C have been shown to depress the en-

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hancement of calcium action potentials during a discharge in the bag cell neurons (P. J. Conn, J. A. Strong, and L. K. Kaczmarek, unpublished observations).

Stimulation of protein kinase C activity is believed to occur in response to physiological stimuli that activate phosphoinositide hydrolysis. Hydrolysis of the membrane lipid phosphatidylinositol 4,5,-bisphosphate to the products diacylglycerol and inositol trisphosphate (IP₃) constitutes a transmembrane communication system thought to control a variety of cellular functions (Berridge, 1984). Diacylglycerol formed as a result of stimulation can activate protein kinase C at the plasma membrane (Nishizuka, 1984). IP₃, on the other hand, is released into the cytoplasm, where it is believed to act primarily as a stimulus for the release of intracellularly stored calcium (Berridge, 1983; Streb et al., 1983; Berridge and Irvine, 1984). Studies carried out on various excitable cell types, including *Aplysia* neurons, have described certain electrophysiological effects of IP₃ that may result from the release of intracellular calcium (Brown et al., 1984; Fein et al., 1984; Higashida et al., 1986; Sawada et al., 1987).

In this study, we have investigated the regulation of inositol polyphosphate production in the bag cell neurons in response to stimuli that trigger afterdischarge, and we have examined the effects of IP₃ on the electrical properties of the bag cells. Moreover, using fura-2 imaging techniques (Connor, 1986; Tsien and Poenie, 1986), we have demonstrated directly that IP₃ causes release of intracellularly stored calcium ions in the somata of these neurons.

Materials and Methods

Phosphoinositide metabolism. *Aplysia californica* (~500 gm) were obtained from Alacritty Marine Biological Services and were maintained in artificial seawater (ASW) at 14°C. The abdominal ganglion was dissected out with the pleuroabdominal connective nerves, and the bag cell clusters and their associated connective nerves were cut away from the rest of the ganglion. For some experiments, the atrial gland was also dissected from the reproductive tract and homogenized in ASW (460 mM NaCl, 10.4 mM KCl, 11 mM CaCl₂, 55 mM MgCl₂, 10 mM Tris-HCl, pH 7.8) with protease inhibitors (250 µg/ml chicken egg white trypsin inhibitor; 250 µg/ml soybean trypsin inhibitor; 25 mg/100 ml bacitracin). Both clusters of bag cell neurons were incubated for 2 hr in 200 µl ASW containing 1 mCi/ml of ³H-inositol (15 Ci/mM). The clusters were then rinsed 5 times in ASW and placed in ASW containing 30 mM LiCl for 20 min. Lithium chloride has been shown to amplify the accumulation of inositol phosphates in stimulated tissue (Berridge et al., 1982). Experimental and control clusters were then separated and placed in petri dishes containing fresh ASW/30 mM LiCl. At this point, one of 3 different treatments was applied. The experimental cluster was either exposed to atrial gland extract for 30 min, to forskolin (50 µM) and theophylline (1 mM) for 15 min, or was stimulated electrically for 1 min. Treatment with forskolin and theophylline elevates cAMP levels in the bag cells by 200% (Kauer and Kaczmarek, 1985). Control clusters were exposed to vehicle or left unstimulated. Electrical stimulation was carried out by placing a suction electrode at the distal end of the pleuroabdominal connective nerve. Stimulus trains were given for 1 min at a frequency of 6 Hz and an intensity sufficient to trigger action potentials in the bag cell neurons (2–10 V). Clusters were homogenized 5 min after stimulation. Experimental and control clusters were each homogenized in 500 µl of distilled water. Chloroform/methanol (1200 µl), 1:1 was then added to separate the aqueous and lipid phases. Aliquots of the lipid fraction containing phospholipids that had incorporated ³H-inositol were counted directly, and the aqueous fraction was subjected to one of 2 procedures to quantitate water-soluble inositol phosphates.

In most experiments, the aqueous phase was mixed with 500 µl of Dowex-1 (formate form) anion-exchange resin, centrifuged briefly (1600 gm, 30 sec), and the supernatant was counted as a measure of free ³H-inositol. These counts, which, to a large extent, represent as-yet unmetabolized ³H-inositol that had been taken up by the cells, were used

to estimate the relative sizes of ³H-inositol pools available for stimulus-induced hydrolysis in different clusters and to normalize the counts in the inositol phosphate fractions. The resin was then washed 4 times with 4 ml of 5 mM cold inositol. Finally, 0.5 ml of 0.1 M formic acid/1.0 M ammonium formate was added to displace all the inositol phosphates, and aliquots of the supernatant were counted.

In some experiments the aqueous fraction was passed through a 1 ml Dowex-1 anion-exchange column, as described by Berridge et al. (1983), to separate different inositol phosphates. After the aqueous phase was applied to the column, the column was washed with 10 ml of distilled water to remove the free ³H-inositol. The counts in these fractions were used to normalize the counts in the inositol phosphate peaks. Four fractions, containing glycerophosphate, inositol-1-phosphate, inositol bisphosphate, and inositol trisphosphate, respectively, were eluted sequentially with 8 ml 5 mM sodium borate/60 mM sodium formate (glycerophosphate), 8 ml 0.1 M formic acid/0.2 M ammonium formate (IP₁), 15 ml 0.1 M formic acid/0.4 M ammonium formate (IP₂), and then 8 ml 0.1 M formic acid/1.0 M ammonium formate (IP₃).

Cell culture. The method used to prepare isolated bag cells in primary culture was similar to that already described (Kaczmarek et al., 1979). The abdominal ganglion was removed and incubated overnight in a neutral protease solution (25 mg/ml) for 17 hr at room temperature. The bag cell cluster was then isolated from surrounding tissue, and cells were dispersed with a Pasteur pipette into culture dishes containing modified Eagle's medium (MEM) or Leibovitz medium L15, containing seawater salts. For acute experiments in calcium-free saline, these media were replaced by inorganic saline containing 2 mM EGTA, in which calcium was replaced by magnesium (Connor and Hockberger, 1984).

Electrophysiology and microinjection. Intracellular recordings were made using conventional techniques. Microelectrodes used for injections were filled with 1,4,5-IP₃ (0.8 mM IP₃, 0.6 M KCl) or calcium (10.0 or 50.0 mM CaCl₂, 4.0 M K-acetate), and these solutions were injected into single bag cell neurons by application of pressure to the inside of the electrode (50–250 msec pulses of 20–80 psi) (Kaczmarek et al., 1980). Voltage recordings and current injections were made using either a second, independent KCl-containing electrode in the cell, or, in some cases, using the pressure-injection microelectrode itself. The cells used in these experiments had resting potentials between –35 and –60 mV.

Single-channel recordings were made using the cell-attached or inside-out patch-clamp technique (Hamill et al., 1981). Pipettes contained ASW. For cell-attached recording, a fire-polished micropipette (0.5–3 MΩ) was first sealed to the cell membrane and the cell was then penetrated with a microelectrode for pressure injection. After being filtered at 100 Hz, single-channel recordings were stored in digitized form and on chart records. In experiments on inside-out, cell-free patches, cell-attached pipettes were first withdrawn from cells so as to expose the inner surface of the membrane patch to MEM. The bathing solution was then rapidly exchanged for a solution containing 1.2 mM MgCl₂, 570 mM KCl, 10 mM HEPES, 11 mM glucose, and 0.77 mM EGTA. Calcium concentrations of 10^{–9}, 10^{–8}, 10^{–7}, and 10^{–6} M at the cytoplasmic face of the membrane were obtained by adding 0.0, 0.07 mM, 0.385 mM, and 0.77 mM CaCl₂ to this solution.

Calcium imaging. Bag cell neurons growing on #1 glass coverslips coated with polylysine were loaded with fura-2 by pressure injection through a microelectrode. Final intracellular fura-2 concentrations were estimated by comparing the fluorescence (475–525 nm) of loaded cells, using 360 nm excitation, with that of indicator at known concentrations in glass capillaries whose diameters were close to those of isolated cells. Working indicator concentrations of 30–60 µM were employed. Analytical measurements on single neurons were made with a cooled, charge-coupled device (CCD)-based imaging system described in detail elsewhere (Connor, 1986; Connor et al., 1987a, b). A 25× glycerine objective was used in the epifluorescence measurements rather than the 40× one employed in previous work, owing to the large size of the neurons. Fura-2 fluorescence increases maximally with the binding of calcium at 340 nm excitation and decreases with calcium binding at 380 nm excitation (Grynkiewicz et al., 1985; Tsien et al., 1985; Williams et al., 1985). The ratio of fluorescence in digitized images taken at 340 and 380 nm excitation reflects calcium concentrations.

After loading cells with the indicator dye, the fura-2-containing microelectrodes were withdrawn and the cells were repenetrated with microelectrodes containing either IP₃ in KCl or KCl (0.6 M) alone. In a few cases, IP₃ was injected iontophoretically, but better results were obtained with pressure injection. Increases in the calcium concentration

Figure 1. Increases in incorporation of ³H-inositol into inositol phosphate-containing aqueous fractions and into inositol-containing lipid fractions following stimulation of bag cell clusters. Pairs of bag cell clusters were incubated in ³H-inositol for 2 hr and then in LiCl for 20 min. Following stimulation by electrical pulses to the pleuroabdominal connective nerve, exposure to atrial gland extract, or exposure to 50 μM forskolin and 1 mM theophylline, aqueous and lipid fractions were prepared as described in Materials and Methods. Electrical stimulation, *n* = 7; atrial gland extract, *n* = 3; forskolin/theophylline, *n* = 7.

were deemed significant if there was a noticeable intracellular gradient of calcium following injection of IP₃, and if the 340:380 nm ratio of fluorescence increased by at least 10%. Images were stored and analyzed with a DEC LSI 11-73-based computer and photographed directly from a video monitor. Indicator fluorescence ratios have been converted to calcium concentrations using the relation,

$$[Ca^{2+}] = K_d \left(\frac{R - R_{min}}{R_{max} - R} \right) (F_0/F_s),$$

where R_{min} (0.45) and R_{max} (11.5) are minimum and maximum ratios observed in the apparatus for limiting values of calcium; F_0/F_s is the fluorescence ratio for 380 nm excitation at limiting values of calcium. The indicator dissociation constant (K_d) of 760 nM was employed (Gryniewicz et al., 1985), which, to some extent, takes into account the higher ionic strength encountered when working with marine animals. This K_d was used in calculating calcium levels. The computed calcium levels are coded in false-color, blue to red, signifying low to high levels.

Results

Phosphoinositide metabolism

Afterdischarges may be triggered in the bag cell neurons in 3 different ways: (1) brief electrical stimulation of an afferent input, (2) application of atrial gland peptides (Heller et al., 1980), and (3) elevation of intracellular cAMP levels (Kaczmarek et al., 1978; Kauer and Kaczmarek, 1985). We found that all 3 of these treatments stimulated the metabolism of phosphoinositides in intact bag cell clusters, as indicated by increased incorporation of ³H-inositol into water-soluble inositol phosphates and into inositol-containing lipids (Fig. 1). Electrical stimulation of an afferent nerve to the bag cells, and elevation of cAMP levels in the cells by forskolin and theophylline, led to similar increases in the production of both inositol phosphates and inositol-containing lipids, whereas atrial gland extract produced a slightly larger response. When fractions containing inositol

polyphosphates were further fractionated to separate mono-, bis-, and trisphosphates (with higher phosphates), radioactivity was detected in all 3 fractions.

Electrophysiological effects of inositol trisphosphate injections

The electrical effects of inositol phosphates were investigated using isolated bag cell neurons in cell culture. Figure 2 illustrates a representative response of the membrane potential of an isolated neuron to a single injection of IP₃ (0.8 mM in electrode). In this experiment, IP₃ injection was followed within a few seconds by a hyperpolarization lasting approximately 60 sec and reaching a peak amplitude of 15 mV. Overall, 66 injections of IP₃ were carried out in 14 cells at their resting potential (~−45 to −60 mV), and the amplitude of observed hyperpolarizations ranged between 2 and 16 mV, with a mean response of 7 ± 0.4 mV ($\pm$ SEM). The duration of the hyperpolarizations ranged between 14 and 75 sec in different cells, with a mean of 35 ± 1.5 sec. The cells did not desensitize to IP₃. As many as 18 injections were carried out in one cell, and a similar response was elicited by each injection. The hyperpolarization elicited by IP₃ could be eliminated by the potassium channel blocker tetraethylammonium ions (TEA; 50–100 mM) in the extracellular medium (*n* = 4; Fig. 2). The response to IP₃ could not be mimicked by injections of myo-inositol, inositol-1-phosphate, inositol-1,3,4,5 tetrakisphosphate, ATP, or KCl.

Figure 2*b* shows that the IP₃-induced hyperpolarization occurred with an increase in membrane conductance. At the peak of the hyperpolarization, the input resistance of this cell, measured by the amplitude of voltage deflections elicited by constant hyperpolarizing current pulses, was decreased by 20%. Input resistance was monitored during 25 IP₃ injections in 12 cells, and the mean decrease was $26 \pm 3.5\%$. The effect of IP₃ on input resistance was not due to the shift in membrane potential,

Figure 2. Voltage recordings of isolated bag cell neurons in culture injected with IP_3 . *a*, Hyperpolarizing voltage response of a bag cell neuron to intracellular pressure injection of IP_3 (arrow). *b*, IP_3 injection in the same cell produces an increase in membrane conductance, measured by a reduction in the voltage response of the cell to constant hyperpolarizing current pulses. *c*, *d*, Response to IP_3 injection in a different cell before (*c*) and after (*d*) exposure to 50 mM TEA.

because passive hyperpolarization of cells by up to 16 mV with current injection did not significantly lower input resistance.

Figure 3 shows that injection of IP_3 also attenuated the size of action potentials evoked by depolarizing current pulses. Figure 3*a* illustrates action potentials elicited before IP_3 injection. Figure 3*b* was recorded 10 sec after injection, and the action potentials are clearly diminished in height. Recovery, shown in Figure 3*c*, was evident within 2 min. In some cases, as in Figure 3, recovery was associated with a rebound enhancement of action potential height, but this was not observed in all experiments. As with the effect of IP_3 on input resistance, passive hyperpolarization of the cell membrane could not mimic the reduction in the amplitude of action potentials induced by IP_3 .

Figure 3. Effect of IP_3 injection on the amplitude of action potentials evoked by depolarizing current pulses (lowest trace). *a*, Action potentials elicited by depolarizing current pulses before injection of IP_3 . *b*, Action potentials are greatly attenuated following IP_3 injection. *c*, Recovery is evident within 5 min after injection.

Control injections of myo-inositol, inositol-1-phosphate, ATP, or KCl did not significantly influence input resistance or the amplitude of action potentials.

The characteristics of the response of isolated bag cell neurons

Figure 4. Voltage recordings from an isolated bag cell neuron injected with calcium chloride. *a*, Intracellular pressure injection of calcium chloride transiently hyperpolarizes the cell. *b*, Injection of calcium chloride produces an increase in membrane conductance, measured as a decrease in the voltage response to a train of constant hyperpolarizing current pulses.

Figure 5. Effect of IP₃ injection on single-channel activity recorded in cell-attached patches on isolated bag cell neurons. *A*, In a patch maintained 50 mV positive to rest, IP₃ elicits the repeated opening of an outward channel presumed to be a calcium-activated potassium channel. *B*, Pooled data from 3 experiments illustrating the time course of the opening of single channels passing outward current following IP₃ injection.

to IP₃ injection, in particular the amplitude and duration of the IP₃-induced hyperpolarization, closely match those of the transient hyperpolarization that is observed immediately following stimulation that triggers a subsequent afterdischarge in intact clusters of bag cell neurons within the abdominal ganglion. Moreover, the transient hyperpolarization in the intact system is eliminated by 50–100 mM TEA, and is associated with a depression of action potential amplitude (Kaczmarek et al., 1982, and unpublished observations), as is the IP₃-induced hyperpolarization. Nevertheless, because selective inhibitors of IP₃ formation are lacking, the relationship between the IP₃ response of isolated neurons and the stimulus-induced hyperpolarization in intact clusters cannot be tested definitively.

Intracellular injection of calcium ions

The electrical effects of IP₃ injection in isolated bag cell neurons could be mimicked by direct injection of calcium ions. Figure 4 illustrates an example in which calcium injection produced a hyperpolarization that lasted about 40 sec. Figure 4*b* shows that the input resistance of the cell decreased following calcium injection and recovered with the same time course as the membrane potential. Nineteen injections of calcium chloride in 8 cells elicited a mean hyperpolarization of 18 ± 2.8 mV. The mean duration of the response was 37 ± 11.3 sec, and the input

resistance measured after 4 injections in 2 cells was reduced by 49% at the peak of the hyperpolarization. Calcium injection also attenuated the size of action potentials by 47% (3 injections in 2 cells). The response to calcium was usually more rapid in onset than that to IP₃ injection.

Single-channel recordings

To investigate the single-channel events underlying the electrical effects of IP₃ and calcium injections, cells were penetrated with an IP₃ or calcium-containing microelectrode after a patch electrode was sealed to the membrane for cell-attached recording. Figure 5*A* shows a continuous current recording from a membrane patch held 50 mV positive to rest before and after injection of IP₃. Few openings were observed during the 16 sec prior to IP₃ injection. However, after the injection, the activity of a channel carrying outward current increased for a period of 34 sec and then subsided. Figure 5*B* represents data pooled from 3 experiments, showing that the time course of the increase in such single-channel openings elicited by IP₃ injection matched the time course of IP₃ effects observed in the current-clamp experiments. In total, 31 injections in 7 cells elicited dramatic increases in outward channel activity at rest and at potentials up to +100 mV positive to rest. The latency from the time of injection to the increase in observed channel openings varied

Figure 6. IP_3 induces an alteration in the voltage-dependence of channel activity recorded in cell-attached patches. Traces are representative examples of the probability of opening of the IP_3 -modulated channel, before and after IP_3 injection, at several different voltages. The values for the holding potentials of the patch are shown next to the individual traces and are given relative to the resting potential, such that the top traces are at the resting potential. After IP_3 injection, the probability of the channel's opening is increased at all voltages examined.

from 2 sec to approximately 1 min, but was most often less than 15 sec. The increase in the probability of channel opening usually lasted between 20 and 80 sec, but in 2 cases the response was prolonged for 3–4 min. Figure 6 demonstrates that the channel opened by IP_3 injection is also gated by voltage, and that the IP_3 -induced increase in channel activity results from

an alteration in the voltage dependence of channel opening. Figure 6 shows recordings from a cell-attached patch before and after IP_3 injection at 7 voltages. Prior to IP_3 injection, prolonged channel openings occurred to a significant extent only at positive potentials (60–100 mV positive to rest). IP_3 injection was followed by an increase in the probability of opening at all holding

Figure 7. Current-voltage relation of the channel modulated by IP_3 . Data shown were obtained following 2 separate injections of IP_3 in the same cell. The estimated slope conductance of the channel is ~ 40 pS and the extrapolated reversal potential is close to E_K . No corrections were made for membrane potential changes due to IP_3 injection.

Figure 8. Effect of IP_3 injection on single-channel activity recorded from a cell-attached patch held at the resting potential. In this experiment, IP_3 injection was followed by a burst of openings of a channel passing inward current.

potentials, with prolonged ($> \sim 20$ msec) openings now occurring at the resting potential. The open channel current-voltage relation for this IP_3 -induced channel displayed some outward rectification, as illustrated in Figure 7. The slope conductance over the voltage range 0 to +70 mV from rest was ~ 40 pS, and the extrapolated reversal potential (37 mV negative to rest) was close to that predicted for potassium ions. Similar results were obtained in 3 experiments in which the IP_3 response was examined at multiple holding potentials.

A response similar to that following IP_3 injection was also observed on injection of calcium ions. Twelve injections of calcium chloride in 5 cells increased the activity of channels passing outward current, recorded at patch potentials between rest and 86 mV positive to rest. The latency of the response to calcium injections ranged between 2 and 25 sec, and the duration of the response varied between 3 and 200 sec.

In addition to increasing the activity of the channel described above, IP_3 could also evoke openings of a channel passing inward current. Four injections of IP_3 in 3 cells elicited sharp increases in the activity of such a channel, whose openings in the absence of IP_3 were observed only rarely. One example of this is shown in Figure 8. A channel carrying 1 pA of inward current at the resting potential opened numerous times during a period of 35 sec postinjection, whereas before injection and after this 35 sec burst the channel opened very rarely. Because this response was seen so infrequently, it was not investigated in more detail.

Recordings made from cell-free inside-out patches revealed at least one calcium-activated potassium channel, with prop-

erties that match those of the IP_3 -modulated channel observed in cell-attached patches. When the inner surface of the membrane was bathed in solutions containing 10^{-9} , 10^{-8} , 10^{-7} , and 10^{-6} M calcium chloride, we observed channels whose probability of opening increased with calcium concentration. With 570 mM K^+ ions at the cytoplasmic face of the patches, the slope conductance of calcium-activated potassium channels we observed most often (5 out of 7 patches) averaged 60 pS over the voltage range -100 to 0 mV. We examined the effect of exposing the internal surface of the membrane to IP_3 , and found that it did not induce increased channel activity in cell-free patches containing calcium-activated potassium channels ($n = 3$) (not shown).

Calcium imaging

The electrophysiological effects of IP_3 are consistent with the hypothesis that IP_3 induces an elevation of intracellular calcium concentration in bag cell neurons. To test this hypothesis directly, we used isolated bag cell neurons loaded with the calcium indicator fura-2 (Grynkiewicz et al., 1985) to examine the effects of electrical stimulation and of IP_3 injection on intracellular calcium levels. The responses to IP_3 injection were tested in 11 cells, using both iontophoretic and pressure-injection techniques. IP_3 was injected intracellularly into bag cells using a single 1 sec pulse or a series of repeated pulses at approximately 0.5 Hz (Figs. 9, 10). Twenty-four out of 25 injections of IP_3 elicited significant increases in intracellular calcium in these neurons. The magnitude of these increases in the calcium signal varied between 10 and 400% (ratio of fluorescence images taken at 340 and 380 nm excitation), and depended on the number of injections, the electrode size, and the use of pressure or iontophoretic injection. Not surprisingly, the largest responses were obtained with longer pressure injection through large microelectrodes. Pressure injection of KCl alone had no effect on intracellular calcium concentrations.

To investigate whether the IP_3 -induced elevation of cytosolic calcium occurred through release from intracellular stores or entry across the plasma membrane, we injected IP_3 into 3 cells bathed in calcium-free medium. Figure 9 illustrates that, in the absence of extracellular calcium, a single, 1 sec injection of IP_3 , comparable to that used in the electrophysiological experiments, evoked a significant increase in the cytosolic calcium signal. Before injection (Fig. 9*a*), calcium levels were around 300 nM. Immediately following the injection of IP_3 , a gradient of intracellular calcium was recorded, with the greatest increase occurring near the electrode tip (793 nM). Intermediate calcium levels of ~ 415 –536 nM were found elsewhere in the soma, and only a very slight increase was detected in the processes furthest from

Figure 9. Effects of IP_3 injection on the intracellular $[\text{Ca}^{2+}]$ of a fura-2-loaded bag cell neuron in calcium-free medium. On the left is a Nomarski image of the cell. The boxes on the cell indicate 3 locations in which the fluorescence ratio was measured. Ratios have been converted to approximate calcium concentrations. Locations will be referred to as 1, 2, and 3, beginning with the box closest to the microelectrode at the left side of the cell and going clockwise. *a*, The increase in intracellular calcium evoked by a single injection of IP_3 , comparable to that used in the electrophysiological experiments (Figs. 2, 3, 5, 6). In the resting (control) image, calcium levels are (1) 356 nM, (2) 273 nM, and (3) 296 nM. Immediately following the 1 sec injection of IP_3 , calcium levels rise to (1) 793 nM, (2) 536 nM, and (3) 415 nM. Fifteen seconds after the injection, they have risen further in the soma: (1) 861 nM, (2) 793 nM, and (3) 727 nM. Eighty seconds after the injection, calcium levels revert to control values: (1) 356 nM, (2) 296 nM, and (3) 356 nM. *b*, The effect of multiple injections of IP_3 in calcium-free medium. Values in the control are (1) 171 nM, (2) 132 nM, and (3) 132 nM. Immediately following 15 sec of repetitive IP_3 injections, they rise to (1) 1.30 μM , (2) 1.46 μM , and (3) 930 nM. Progressive recovery is evident 45 sec after the injections: (1) 930 nM, (2) 793 nM, and (3) 598 nM; and, 2 min after the injections, (1) 356 nM, (2) 296 nM, and (3) 270 nM. The top of the color scale represents a fluorescence ratio of 2.5 (1.30 μM) in *a* and 2.8 (1.54 μM) in *b*.

a.

b.

the injection site. Fifteen seconds after the injection, the calcium signal was further elevated and was relatively uniform in all regions of the soma (727–861 nM). Recovery to control calcium levels (around 330 nM) occurred within 80 sec after IP₃ injection.

Figure 9*b* demonstrates that injecting a greater quantity of IP₃ produced a larger increase in intracellular calcium. The calcium concentration in the soma rose to over 1 μ M, as compared to 132–171 nM before injection. Moreover, even after multiple injections of IP₃, the response was still preferentially localized to the soma, although smaller increases were recorded in the processes. Eighty percent recovery of calcium levels was observed 2 min after the end of the injections. Pressure injection of KCl had no effect on the calcium signal.

The spatial distribution of calcium elevation following IP₃ injection could be clearly differentiated from that seen after depolarizing current injection in cells bathed in calcium-containing media, especially when neurite outgrowth had occurred. Acutely dissociated bag cell neurons are spherical, or spherical with short stumps of neurite attached, but by the second day in culture have usually sprouted considerable neuritic outgrowth (Kaczmarek et al., 1979). Immediately following electrical stimulation in such day 2 cells, a large increase in the calcium signal could be recorded in the neurites with little or no effect visible in the cell body. This behavior was in sharp contrast with that observed before neurites had sprouted (0–4 hr after dissociation). During that period, large calcium transients could be evoked in the soma by the same number of action potentials (not shown). Figure 10*a* shows the changes in calcium following a train of 15 action potentials in an isolated neuron in culture. Immediately following stimulation, a marked increase in the calcium signal could be recorded in the neurites, with a significantly smaller increase occurring in the soma.

Figure 10*b* compares the effect of electrical stimulation with that of IP₃ injection in the same cell. The Nomarski image of the cell shows numerous fine processes extending from the soma. The control fluorescence ratio image of the cell after penetration with an IP₃-containing microelectrode shows the resting level of calcium to be uniformly low in soma (~260 nM) and processes. After electrical stimulation of 25 action potentials (4/sec), a dramatic rise in the calcium concentration occurred almost exclusively in the processes (~1 μ M). The final image in the series illustrates the effect of 7 repetitive IP₃ injections (20 psi, 1 sec, 0.5 Hz). The elevation of calcium was localized within the soma, reaching approximately 530 nM, and in contrast to the effect of depolarizing current injection, the processes were relatively unaffected. This was a consistent feature of all 11 IP₃-injected cells, in which calcium increases within the soma were accompanied only by much smaller changes or no change in the neurites.

In addition to differences between the intracellular distributions of responses to IP₃ injection and to depolarization, there were also differences in the time course of the 2 responses. The increase in calcium concentration following IP₃ injection was rapid in onset and continued to rise for many seconds after the

end of injection before declining to preinjection levels (see Fig. 9*a*). In comparison, the response to one or more depolarizing current pulses began to decline immediately after stimulation. Elevated calcium levels following electrical stimulation usually recovered within 20–40 sec, whereas the response following IP₃ injection lasted for 1–3 min.

Discussion

Our studies have provided a direct demonstration that IP₃ acts to elevate intracellular calcium ions within neuronal somata, and that this effect is largely independent of extracellular calcium. Previous work on IP₃ actions in intact, excitable cells has provided indirect evidence for such an elevation using electrophysiological techniques (Brown et al., 1984; Fein et al., 1984; Higashida et al., 1986; Sawada et al., 1987). Therefore, with respect to this important control mechanism, neurons closely resemble nonexcitable cells in which this pathway has been investigated in detail (Berridge, 1983; Streb et al., 1983; Burgess et al., 1984, 1985; Gershengorn et al., 1984; Prentki et al., 1984).

Our data also suggest that IP₃ formation plays a role in the onset of the afterdischarge in the bag cell neurons. Previous work with these cells (Kaczmarek et al., 1978) has shown that stimuli that trigger discharges result in an elevation of cAMP levels, and that such elevations produce a variety of effects on potassium conductances, which promote the ability of these cells to afterdischarge (Kaczmarek and Strumwasser, 1981, 1984; Strong, 1984; Strong and Kaczmarek, 1986). We have now found that stimuli capable of triggering the bag cell neurons to discharge also induce hydrolysis of phosphoinositides. Not only are electrical stimulation of the connective nerve and exposure to atrial gland extract able to stimulate phosphoinositide metabolism in the bag cell neurons, but exposure of the cells to forskolin and theophylline, a treatment that directly elevates cAMP levels in these cells, also increases phosphoinositide turnover. The mechanism by which forskolin/theophylline stimulates phosphoinositide metabolism in the bag cell neurons is not yet known. It could be that an elevation of cAMP directly influences enzymes involved in phosphoinositide turnover. Alternatively, since these agents can trigger discharges, phosphoinositide metabolism could be stimulated indirectly by substances released from the bag cell neurons during a discharge.

The mobilization of intracellular calcium by IP₃ could influence a variety of cellular processes at the onset of the afterdischarge in the bag cell neurons. In addition to promoting the secretion of neuroactive peptides from these neurons, an elevation of calcium would be expected to promote the activity of calcium-dependent enzymes, such as the calcium/calmodulin- and calcium/phospholipid-dependent protein kinases (DeRiemer et al., 1984, 1985a). The activation of the latter protein kinase enhances voltage-dependent calcium current (DeRiemer et al., 1985b; Strong et al., 1987) and contributes to the subsequent transformation of the electrical properties of the bag cell neurons. The pattern of a transient cellular response to IP₃ followed by a sustained response to protein kinase C activation

Figure 10. Effects of electrical stimulation and IP₃ injection on the intracellular calcium concentration of isolated bag cell neurons loaded with fura-2. *a*, Digitized images of the ratio of fluorescence at 340 and 380 nm excitation, reflecting the intracellular distribution of Ca²⁺ ions. *Center*, The calcium signal following a train of 15 action potentials. *b*, *Left*, the transmitted light (Nomarski) image of a different cell. The next 3 panels show the fluorescence ratio images before stimulation (*control*), following 25 action potentials, and immediately after 7 injections of IP₃ at 0.5 Hz. Cells shown had been cultured the previous day. The vertical color scale at the right represents the fluorescence ratio values, with the top of the scale (pink) equal to a maximum ratio of 2.5.

a.

control

action potentials

recovery

b.

control

action potentials

IP₃ injection

has been suggested for the agonist-induced stimulation of a variety of non-neuronal cell types. For example, synergistic actions of calcium and protein kinase C appear to underlie the physiological response to cellular stimulation of pituitary cells (Delbecke et al., 1984), adrenal glomerulosa cells (Kojima et al., 1984), and pancreatic beta cells (Zawalich et al., 1983). Our study suggests that a similar synergism between IP₃ and protein kinase C may contribute to the prolonged electrical and neurosecretory activity that results from brief synaptic stimulation of these cells, leading to the initiation of a series of reproductive behaviors in the animal.

The electrophysiological effects of IP₃ injection are consistent with those expected to result from an IP₃-induced release of intracellular calcium. A major component of the potassium current in bag cell neurons has been shown to be calcium dependent (Kaczmarek and Strumwasser, 1984; Strong and Kaczmarek, 1986). The activation of a calcium-dependent potassium conductance would be expected to produce a hyperpolarization and a decrease in input resistance, as was seen in response to IP₃ injection. The decrease in input resistance could contribute to the attenuation of action potentials. Moreover, an elevation of intracellular calcium could also produce calcium-dependent inactivation of calcium current (Eckert and Tillotson, 1981), further attenuating action potentials, which, in bag cell neurons, have a large calcium component (Kaczmarek and Strumwasser, 1984; DeRiemer et al., 1985b). In single-channel recordings, the major response to IP₃ injection was the opening of a channel whose properties resemble those of calcium-activated potassium channels described in other preparations. In particular, the shift in voltage-dependence of the probability of channel opening to more negative potentials that we observed in response to IP₃ has been demonstrated for calcium-activated potassium channels from a variety of sources in response to an elevation of calcium concentration at the cytoplasmic face of the channel (Lux et al., 1981; Moczydlowski and Latorre, 1983; Ewald and Levitan, 1987; Miller, 1987). Further supporting the hypothesis that the effects of IP₃ are mediated indirectly through the release of intracellular calcium, we found that injection of calcium ions mimicked the responses seen after IP₃ injection. In addition, we found no direct effect of IP₃ on the opening of outward channels in cell-free patches from bag cell neurons.

In a few experiments, IP₃ also increased the activity of a channel carrying inward current at the resting potential. One possible explanation is that the bag cell membrane contains a calcium-activated, nonspecific cation channel (Yellen, 1982). Alternatively, IP₃ may act on the membrane itself to open a cation-permeable channel, perhaps an IP₃-sensitive calcium channel, as has been described for T-lymphocytes (Kuno and Gardner, 1987).

Fluorescence images of cells preloaded with the calcium indicator fura-2 confirmed that IP₃ injection produces a sharp rise in cytosolic calcium in the somata of these neurons. This response to IP₃ was not affected by removal of extracellular calcium. Thus, we conclude that IP₃ elevates cytosolic calcium through release from intracellular stores. It is possible that calcium-induced calcium release also contributes to the progressive elevation of calcium following IP₃ injection (Fabiato, 1982). The elevated levels of calcium evoked by IP₃ injection were localized primarily to the somata, although smaller changes in calcium concentration could be observed in the neurites. This pattern is in sharp contrast to that seen with electrical stimulation of bag cell neurons in calcium-containing medium. There are several

possible reasons why IP₃ elevates calcium levels in the neurites to a much smaller extent than in the soma. It is unlikely that IP₃ fails to reach the neurites because of simple diffusional barriers, as we have observed that a variety of fluorescent dyes rapidly fill both the somata and neurites following intracellular injection. It is possible, however, that neurites contain a significantly lower density of calcium storage sites, or that hydrolysis of IP₃ limits its entry into the neurites.

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