Effect of Intracellular Injection of Inositol Trisphosphate on Cytosolic Calcium and Membrane Currents in *Aplysia* Neurons

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Pacemaker cells of *Aplysia californica* display a regular bursting that results from a complex interplay of Ca^{2+} -mediated conductances and a continuous influx and extrusion of Ca^{2+} . The effect of the second messenger 1,4,5-inositol trisphosphate (InsP_3) on intracellular free Ca^{2+} concentration (Ca_i) regulation and electrical properties was investigated in identified neurons of the abdominal ganglion (R15, L2–L4, L6). Double-barreled Ca-selective microelectrodes were used to pressure inject InsP_3 and measure Ca_i at the same point. Brief injection of InsP_3 resulted in an average increase of Ca_i of $9.2 \pm 10.0 \mu\text{M}$ ($\pm\text{SE}$; $n = 14$) that decayed in about 1 min. The InsP_3 -induced elevation of Ca_i increased in a dose-dependent manner and saturated when large amounts of InsP_3 were injected. The InsP_3 -induced Ca_i increase was the result of mobilization from intracellular stores; Ca_i could be repeatedly mobilized by InsP_3 in cells superfused with 0 Ca artificial seawater for more than 60 min. Following multiple injections of InsP_3 , there was no evidence of immediate inhibition or facilitation. The spatial nature of the InsP_3 -induced Ca_i increase was investigated by moving the double-barreled Ca-selective microelectrode tip in a stepwise manner relative to the membrane surface. The largest InsP_3 -induced Ca_i increases were measured in an area 0–80 μm from the membrane surface; some cells had their largest InsP_3 -induced Ca_i increase some 120–160 μm away from the membrane. Injection of InsP_3 in a bursting neuron induced an immediate train of action potentials followed by membrane hyperpolarization and a decrease in the burst frequency. Injection of InsP_3 in voltage-clamped cells induced a biphasic response: a rapid inward current followed by a more prolonged outward current; the temporal overlap of the currents was depth dependent. Injection of InsP_3 or Ca^{2+} from a double-barreled injecting electrode induced currents that were different in waveform and time course, indicating that part of the conductance change induced by InsP_3 is direct and not mediated by the mobilized Ca^{2+} . In BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid]-loaded cells, the InsP_3 -induced inward current was mostly unaffected while the Ca-induced outward current was

largely attenuated. The results suggest that InsP_3 mobilizes Ca^{2+} from discrete intracellular compartments and induces distinct changes in membrane currents that seem to be independent of the Ca_i increase.

The bursting pattern displayed by molluscan pacemaker cells appears to result from a complex interplay of Ca^{2+} -mediated conductances and a continuous accumulation and extrusion of Ca^{2+} (Gorman et al., 1981; Adams, 1985; Kramer and Zucker, 1985a,b). The Ca^{2+} that accumulates with membrane activity was shown to be implicated in the gating mechanisms of two membrane conductance systems. One is a membrane channel system selectively permeable to K^+ whose conductance depends on the level of intracellular free Ca^{2+} concentration (Ca_i) (Meech, 1974; Gorman and Hermann, 1979; see, however, Adams and Levitan, 1985; Kramer and Zucker, 1985a,b). The other is the Ca conductance system, whose inactivation appears to be mediated by accumulated Ca^{2+} (Tillotson, 1979).

In view of the sensitivity of these membrane conductances to intracellular Ca^{2+} , second messengers like 1,4,5-inositol trisphosphate (InsP_3) may have important effects on membrane currents and Ca_i regulation in nerve cells. There is also evidence that the bursting activity of pacemaker neurons can be modulated by several neurotransmitters and neuropeptides (Levitan et al., 1979; Smith, 1980); some of these neurotransmitters were found to exert their action on membrane channels through variations in cAMP, or independently of it (Levitan, 1985). It is unknown whether any of these neurotransmitters mobilize Ca_i . It is also unclear what would be the advantages of mobilized Ca_i in addition to the well-characterized Ca^{2+} influx/Ca conductance system. One possibility would be for the Ca signal resulting from the opening of voltage-activated Ca^{2+} channels to be amplified by mobilization of Ca_i through Ca-dependent formation of InsP_3 (Eberhard and Holz, 1988; Harootunian et al., 1991). An added advantage would be to segregate entering and mobilized Ca^{2+} because they may have different target molecules.

In the present study, I have investigated the effect of InsP_3 on Ca_i regulation and electrical properties of *Aplysia* neurons from the abdominal ganglion. Using double-barreled Ca-selective microelectrodes, I found that brief intracellular injections of InsP_3 mobilize Ca_i from intracellular stores. Moreover, I tried to dissociate its Ca^{2+} mobilization role from its direct action on the membrane conductance by injecting InsP_3 and Ca^{2+} side by side at the same point. I found that InsP_3 induces changes in membrane currents that are distinct from the ones induced by Ca^{2+} injections.

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Some of the results have been reported previously in abstract form (Levy, 1987, 1988, 1989).

Materials and Methods

Preparation. Specimens of *Aplysia californica* (200–300 gm) were obtained from Alacritty Marine Biological Services (Redondo Beach, CA) and kept in artificial seawater (Instant Ocean, Mentor, OH) at 14–16°C. Experiments were performed on identified neurons (R15, L2–L4, L6) of the abdominal ganglion (Frazier et al., 1967), known to have substantial Ca currents. Some injections were made in R2 cells. The methods for dissecting and recording have appeared previously (Gorman et al., 1984). Briefly, the abdominal ganglion was dissected out of the animal, treated with protease (Pronase, Calbiochem, San Diego, CA; 5–7 mg in 2 ml of seawater for 20 min at room temperature), and then pinned to the bottom of a 1 ml recording chamber and desheathed. Ganglia were continuously superfused with normal artificial seawater (NSW) having the following composition (in mM: NaCl, 500; KCl, 10; CaCl₂, 10; MgCl₂, 50; and Tris.HCl, 10, pH 7.8). In 0 Ca artificial seawater (0Ca-SW) solutions, Mg²⁺ was substituted for Ca²⁺ and 2 mM EGTA [ethyleneglycol-bis-(β -aminoethylether)-*N,N'*-tetra-acetic acid] were added. Experiments were carried out at about 18°C.

Injections. I intracellularly injected the following solutions: 10 μ M to 1 mM D-myo-inositol 1,4,5-trisphosphate (Sigma, St. Louis, MO); 100 μ M Ins(2,4,5)P₃ (Calbiochem, San Diego, CA); 100 μ M Ins(1,3,4,5)P₄ (Calbiochem); 1 mM myo-inositol (Sigma); 10 mM CaCl₂; 100 mM BAPTA [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid; Molecular Probes, Eugene, OR]. Except as noted, all substances were dissolved in a carrier solution containing 200 mM K-acetate and 20 mg/ml of a red dye (Reactive Red, Sigma). To prevent contamination of the injection barrel by Ca²⁺, the following precautions were taken: (1) the K-acetate solution was passed through a chelating ion-exchange resin (Chelex-100, Bio-Rad, Richmond, CA); (2) the injection barrel stayed a very short time in the bathing solution (NSW has 10 mM Ca²⁺) prior to impalement; (3) in some experiments insertion of the injection electrode was made in 0Ca-SW. The red dye served as a visual marker to monitor successful injections. Pressure injection with this dye was easily seen both in the bath and inside the cell. I found that I could reliably inject solutions intracellularly if I could first visualize ejection of the red solution in the bath prior to inserting the electrode. I carefully checked that pressure injections did result in ejection of a red dot inside the cell. Control injections in the bath were always made after withdrawal of the injection electrodes from the cell.

Most intracellular injections were made from double-barreled microelectrodes made from θ -glass capillaries (style 1A, R & D Glass Co., Spencerville, MD). One barrel was typically used to pressure inject the substance of choice while the adjacent barrel, selective to Ca²⁺, was used to measure the resulting Ca_i change at the same location (see below and Fig. 1A). Alternatively, the adjacent barrel was used to inject a different substance so side-by-side comparison of effects on membrane currents could be made. Rapid injections were achieved by applying brief pressure pulses using a programmable pulse generator (Ionoptix Instruments, Milton, MA) and an electropneumatic valve (Clippard, Cincinnati, OH). The output of the pulse generator was connected to the chart recorder and was used to monitor the timing of the pressure pulse (stimulus monitor). To estimate the amount injected, I routinely made injections into an oil droplet or in air and measured the diameter of the droplet (Corson and Fein, 1983). A rather large injection (70 psi, 2 sec) ejected about 40 pl, which amounts to 0.3–0.9% of the cell volume for cells 200–300 μ m in diameter. Intracellular injections ranged from 32 psi/0.1 sec to 70 psi/2 sec. Injections were made at different depths within the cell by moving the injection electrode by steps of 5–15 μ m using a Huxley-type micromanipulator (Custom Medical Research Equipment, Glendora, NJ).

Ca_i measurement. Measurements of Ca_i were made using Ca-sensitive microelectrodes of the membrane type in which a column of sensor selective to Ca²⁺ is contained in the open tip of a glass micropipette. The sensor mixture (Fluka 21048, Ronkonkoma, NY), based on the ligand ETH 1001 (Oehme et al., 1976), was made syrupy by adding polyvinyl chloride (13–16%) and tetrahydrofuran (Aldrich, Milwaukee, WI). Double-barreled Ca-selective microelectrodes were used. The aim was to make point injections with one barrel and measure the resulting change in Ca_i with the adjacent Ca²⁺-selective barrel (Fig. 1A). One advantage is that I can make spatial measurements by moving the electrode tip within the cell (Gorman et al., 1984). The fabrication of

Figure 1. A, Schematic diagram of the experimental arrangement for directly measuring InsP₃-induced Ca increases. The nerve cell is impaled with voltage (electrode 1) and current passing (i) electrodes. The double-barreled Ca electrode is inserted and advanced or withdrawn in 5–15 μ m increments. At each increment, InsP₃ is pressure injected through the nonactive barrel (electrode 3) and measured at the same point with the adjacent Ca-sensitive barrel (electrode 2). Intracellularly, V_m is subtracted from the voltage measured by the Ca electrode to yield the differential Ca signal ($E_{Ca} - V_m$). In turn, the Ca signal is converted to Ca²⁺ concentrations using a calibration curve. B, Typical InsP₃-induced Ca signal increase and control injection of carrier solution. The top trace is the differential Ca signal; the lower trace is the stimulus monitor (command voltage to the electropneumatic valve). Left panel, the double-barreled Ca electrode contained 10 μ M InsP₃ and 20 mM K-acetate. A 2-sec-long pressure injection through the InsP₃ barrel (55 psi) resulted in a negative-going signal followed by a large Ca signal measured by the Ca electrode. The corresponding Ca_i increase was about 5 μ M. The Ca_i increase returned to baseline within 50 sec ($t_{1/2}$ = 5 sec). Right panel, the same cell was then impaled with a different double-barreled Ca electrode containing only the carrier solution. In response to a similar 2-sec-long pressure injection (40 psi), the Ca signal consisted mostly of a negative-going potential.

double-barreled Ca-selective microelectrodes is essentially as described by Munoz et al. (1983). After silanization using TMSDMA (*N,N*-Dimethyltrimethylsilylamine; Fluka 41720) at 250°C, the tip of the double-barreled microelectrode is beveled to 2–3 μ m. The active barrel is back-filled with a Ca-buffered solution (about 0.5 μ M free Ca²⁺, 300 mM KCl) and filled with sensor by dipping the tip and using suction. The columns of sensor are usually 30–100 μ m long. The resistance of the Ca-sensitive barrel is usually 50–500 G Ω .

The response time of the Ca microelectrodes was routinely measured using a fast solution change system described elsewhere (Levy and Fein, 1985; Levy and Tillotson, 1987). Following a step change in Ca²⁺ concentration from 0.1 μ M to 1 μ M, Ca electrodes typically reach 50% and 90% of their final potential in about 100 msec and 1.5 sec, respectively. The time to peak as well as the magnitude of the InsP₃-induced Ca signals measured in this study is most probably affected by the response time of the Ca electrodes. The falling phase of several seconds is, however, unlikely to be affected. For an extensive discussion of the dynamic response of Ca electrodes, see Levy and Tillotson (1987).

The calibration of the Ca-sensitive barrel and the composition of the calibrating solutions are described in detail elsewhere (Levy and Fein,

Figure 2. Dependence of InsP_3 -induced Ca_i increase on InsP_3 concentration. *A*, Effect of pressure pulse duration on the amplitude of the InsP_3 -induced Ca_i increase in an R15 cell (same experimental arrangement as in Fig. 1*A*). *Top trace*, differential Ca signal; *bottom trace*, stimulus monitor. The pulse duration (sec) is shown under each trace. The measured Ca signal increases monotonically with increasing pulse durations. The time intervals following each injection were, respectively, 68 sec after the 0.2 injection, 104 sec after the 0.4, 112 sec after the 0.6, and 160 sec after the 0.8 injection. *B*, plot of the peak ΔCa_i versus pressure pulse durations taken from *A* to show that the increase in Ca_i saturates at longer pulse durations. Pressure pulse was kept at 50 psi throughout; the injection barrel contained 1 mM InsP_3 ; the electrode tip was 90 μm from the cytoplasmic membrane.

1985; Levy and Tillotson, 1988). Although the ion-sensitive electrodes measure activities, the values are expressed as the intracellular free ion concentrations. The activity coefficient should remain constant on the assumption that the extracellular medium has approximately the same ionic strength as the intracellular one and the calibrating solutions.

Electrical arrangement. Figure 1*A* shows a schematic diagram of the recording system. The Ca microelectrode potential (E_{Ca}) was recorded by a custom-built high-input impedance electrometer (Pelagic Instruments, Falmouth, MA) based on the AD515L operational amplifier (Analog Devices, Norwood, MA). A separate intracellular voltage electrode was used to measure the cell's membrane potential (V_m). The potential of the V_m electrode was subtracted from that of the Ca -sensitive barrel to give the Ca signal, $E_{\text{Ca}} - V_m$, which is a measure of Ca_i . In most experiments, a current-passing electrode was also inserted into the cell in order to voltage clamp the nerve cells. Both the V_m and the current electrode were filled with 3 M KCl and had resistances of 2–5 M Ω . A bath electrode was connected to an I - V converter having a 5 M Ω feedback resistor and was used to measure the total membrane current (I_m). Unless otherwise noted, all cells were voltage clamped at -45 mV. V_m , I_m , the stimulus monitor, and the Ca signal were recorded continuously on a multichannel recorder (Gould, Cleveland, OH). The Ca signal recorded and shown on all figures was filtered by a 5 Hz low-pass filter. In some experiments, the Ca signal, V_m , I_m , and stimulus monitor were also recorded (16 bit resolution) using a PCM recording adaptor (Unitrade DAS 601, Dagan, Minneapolis, MN) for subsequent playback and analysis.

Injection artifacts. Figure 1*B* shows an InsP_3 -induced Ca_i increase in an L2 cell. In response to injected InsP_3 , the Ca signal consisted of a fast negative-going potential followed by a positive-going potential with a time to peak of about 1 sec (left panel). The Ca signal increase corresponded to a Ca_i increase of about 5 μM . The Ca_i increase recovered within 50 sec with a half-time of about 5 sec. Pressure injection of the carrier solution alone, in the same cell, resulted in a Ca signal consisting mostly of the negative-going potential; this indicated that the negative-going potential was an injection artifact (right panel). Extensive control injections were made in solutions of different ionic content and Ca buffering capacities. Control injections of InsP_3 , usually made before insertion into cells, always resulted in an injection artifact in all solutions tested. In some cases, the injection artifact consisted of a fast negative-going followed by a fast positive-going signal; this type of injection artifact differed from a normal InsP_3 -induced Ca_i increase in two respects. Its entire time course only lasted the length of the pressure pulse and its amplitude or time course did not change when control injections were made in solutions of different Ca buffering capacities. By contrast, the InsP_3 -induced Ca signal changes seen intracellularly outlasted the pressure pulse by about 1 min (Figs. 1–4). Another evidence that the injection artifact cannot explain the normal InsP_3 -induced Ca_i increase can be seen in Figure 3: the Ca signal amplitude, following a standard InsP_3 injection, depends strongly on the spatial location of the double-barreled Ca -selective microelectrode.

Results

The InsP_3 -induced increase in Ca_i

The range for the peak Ca_i increase in 14 cells varied from 0.34 to 35.5 μM . Three cells (all from the same ganglion) had no measurable InsP_3 -induced Ca_i increase. The peak Ca_i increase refers here to the one generally measured at the site of highest InsP_3 -induced Ca_i increase (see Spatial variations of the InsP_3 -induced Ca_i increase, below).

The InsP_3 -induced elevation of Ca_i increased in a dose-dependent manner. Figure 2*A* shows a series of Ca_i increases induced by brief injections of InsP_3 of increasing durations in an R15 cell. The peak Ca_i increased monotonically with increasing pulse durations, and eventually saturated as large amounts of InsP_3 were injected. Figure 2*B* shows a plot of the peak Ca_i increase versus InsP_3 injection times; the linear portion could be fitted by a straight line that had a slope of 0.26 μM per 100 msec injection for a pressure of 50 psi.

The InsP_3 -induced increase in Ca_i occurred through release from intracellular stores. There was no noticeable change in amplitude or time course of the Ca signal when the bathing solution was switched to 0Ca-SW. Ca_i could be repeatedly mobilized by InsP_3 in cells superfused in 0Ca-SW for more than 60 min.

Spatial variations of the InsP_3 -induced Ca^{2+} release

It is known that the InsP_3 released by hydrolysis of PIP_2 (phosphatidylinositol 4,5-bisphosphate) mobilizes Ca^{2+} only from some parts of the cell (Berridge and Irvine, 1989). Recent evidence has shown that InsP_3 -sensitive Ca stores are organized as discrete compartments (Berridge, 1989) that may be functionally independent (Parker and Yao, 1992). To investigate a spatial distribution of the InsP_3 -sensitive stores, I made intracellular injections of InsP_3 at different distances from the plasma membrane. I show in Figure 3 that the Ca_i increase seems to be spatially restricted to some areas of the cell. Figure 3*A* shows an example where the greatest Ca signal, in response to identical InsP_3 injections, occurred closer to the plasma membrane. The size of the Ca signal did not depend on the sequence in which InsP_3 injections were given. Figure 3*B* shows the spatial profile

of the Ca signal over a longer distance; plotted is the peak Ca_i recorded by the Ca electrode versus the depth of penetration. The peak Ca signal increases steeply as the electrode tip is positioned closer to the membrane. Representative Ca signals at four different depths are shown in the inset. The small Ca signals measured deep in the cell are not due to deterioration of the cell or clogging of the injection electrodes because injections started first deeper in the cell and proceeded toward the membrane.

A word of caution concerning the *absolute* depths reported in Figure 3. The zero depth was defined as the position of the electrode tip prior to its removal from the cell by a 5–15 μm step. This so-called zero could be approximate because of the problem of dipping of the membrane when the microelectrode is pushed in and out of the cell. Furthermore, *Aplysia* neurons have numerous invaginations of the surface membrane (Coggeshall, 1967). However, the *relative* distance between any two sites (e.g., between 30 and 60 μm) should be fairly accurate.

The spatial variations of the Ca signal varied from cell to cell. The InsP_3 -sensitive region was not always closest to the membrane; Figure 3C shows an example of a cell where the largest InsP_3 -induced Ca_i increase was located some 75 μm from the inner membrane. By making reversible movements of 15 μm , I could repeatedly measure InsP_3 -induced Ca_i increases that were larger *away* from the membrane than closer to it. Figure 3D shows the spatial profile for the same cell. It is evident in this R15 cell that the Ca_i increase, induced by identical InsP_3 injection, gets smaller as the double-barreled Ca-selective microelectrode is approaching the membrane.

If we take 250–300 μm as the average diameter of the neurons where the present measurements were made, then the histogram shown on Figure 3E reveals that the largest InsP_3 -induced Ca_i increases measured are contained in a region 0–80 μm from the inner membrane. This may represent the area where the density of discrete compartments is larger.

Summation and saturation of the InsP_3 -induced Ca_i increase

It has been reported that the action of InsP_3 on the Ca^{2+} pool is highly cooperative (Meyer et al., 1988; Berridge and Irvine, 1989) and that paired injections of InsP_3 produce facilitation (Parker and Miledi, 1989). On the other hand, in *Limulus* photoreceptors, the Ca_i increase inhibits the ability of InsP_3 to release additional Ca from internal stores (Payne et al., 1988, 1990; Levy and Payne, 1991). To test for the mechanism of modulation in *Aplysia* neurons, I did multiple injections of InsP_3 separated by varying lengths of time. I found no evidence of either facilitation or negative feedback.

Figure 4A shows that identical pressure injections of InsP_3 evoke identical increases in Ca_i after Ca_i has returned to baseline before the next injection. In the absence of any negative feedback, the slowness of the recovery of Ca_i following injections of InsP_3 (Figs. 1–3) might suggest that multiple injections, if spaced close enough, could in fact induce summation of the Ca signal. Figure 4B shows indeed that successive Ca signals summate when evoked by successive InsP_3 injections. The level of Ca_i during a train of injections usually reached a plateau after the fourth or fifth injection, no matter how long the train was.

The level of Ca_i reached was usually maximal in a given cell when the injection of InsP_3 was continuous. Figure 4C shows superimposed Ca signals induced by injections of increasing amounts of InsP_3 in the same cell as Figure 4B. The injections (100 μM InsP_3 , 70 psi) lasted 26 and 80 sec, respectively; the

Figure 3. Spatial variation of the InsP_3 -induced Ca_i increase: examples of greatest Ca^{2+} release near the membrane (*A*, *B*) or away from it (*C*, *D*). In both *A* and *C*, arrowheads indicate injection of InsP_3 ; the numbers on the left indicate approximate depths in relation to zero position. *A*: Top trace, Ca signal in response to a brief injection. Moving the electrode in by 60 μm resulted in a much smaller Ca signal (middle trace). Movement of the electrode back to its original position restored the original Ca signal (bottom trace). *B*: Plot of InsP_3 -induced Ca_i increase versus the depth of the injecting electrode. The double-barreled electrode was first inserted deeply into a neuron; it was then withdrawn in steps of 15 μm , with its tip approaching the membrane surface. Following each change in depth, InsP_3 was pressure injected and Ca_i measured. Representative records at four different depths are shown in the inset. The measured amplitude of the Ca electrode potential, induced by a standard InsP_3 injection (10 μM -filled electrode, 30 psi, 400 msec), increases steeply as the electrode tip is positioned closer to the membrane surface. See text for zero depth definition. *C*: Top trace, Ca signal in response to a brief injection. Advancing the electrode by 15 μm resulted in a larger Ca signal (middle trace). Withdrawing of the electrode by 15 μm restored the original Ca signal (bottom trace). *D*: Plot of InsP_3 -induced Ca_i increase versus the depth of the injecting electrode (same protocol as *B*). This time, the measured amplitude of the Ca electrode potential, induced by a standard InsP_3 injection (100 μM -filled electrode, 40 psi, 20 msec), decreases as the electrode tip is positioned closer to the membrane surface. *E*: The histogram shows the distribution of the largest InsP_3 -induced Ca_i increase in several nerve cells.

rise time and decay to baseline of both Ca signals were nearly identical; the effect of the larger injection was simply to lengthen the duration of the Ca signal increase. The Ca signal reached a plateau, meaning that a steady state seemed to be reached between the rate of Ca^{2+} release and the rate of buffering.

Figure 4. Summation and saturation of the InsP_3 -induced Ca_i increase. **A**, Lack of facilitation and feedback inhibition as evidenced by identical Ca_i increases induced by identical, successive InsP_3 injections. The Ca_i increase was $1.4 \mu\text{M}$ at the peak of both transients. The time of the injections (50 psi, 400 msec, $1 \text{ mM } \text{InsP}_3$) is shown in the lower trace. The small potential changes during the Ca_i recovery are electrical artifacts due to inadequate spatial control of the membrane potential. R15 cell; depth, $90 \mu\text{m}$. **B**, Ca_i changes induced by multiple, identical pressure injections of InsP_3 ($100 \mu\text{M}$ solution). The lower trace shows the timing of the pulses (500 msec each, 70 psi). Injections 1–3 were presented after Ca_i reached a plateau and before it started recovering; under these conditions the Ca_i signals summate and the total peak Ca_i increases. Hardly any additional Ca_i increase is seen after injection 4. The negative-going potentials are injection artifacts (see Fig. 1B). **C**, Saturation of the Ca_i increase by pressure injection of increasing amounts of InsP_3 ($100 \mu\text{M}$ solution). The Ca_i signals resulting from injections of different durations (as marked by bars below the traces) were superimposed. The injections lasted 26 and 80 sec (70 psi); the rise time and decay to baseline of both Ca_i signals were nearly identical; the effect of the larger injection was simply to lengthen the duration of the Ca_i signal increase. Both **B** and **C**: cell R2, depth $30 \mu\text{m}$. The maximum increase in this cell was reached during the continuous InsP_3 injection and not during successive, multiple brief injections.

I checked whether interfering with the inositol phosphate metabolism by using lithium would modify the InsP_3 -induced Ca_i increase. Lithium has been shown to inhibit the breakdown of InsP_1 by inhibiting InsP_1 phosphatase. When I superfused nerve cells with Li^+ (45 mM, substituted for Na^+), I saw no difference in the amplitude or waveform of the InsP_3 -induced Ca_i increase (two experiments).

Injection of other inositol polyphosphates

I checked the specificity of the Ca -mobilizing effect of $\text{Ins}(1,4,5)\text{P}_3$ by injecting related compounds. It was reported that $\text{Ins}(2,4,5)\text{P}_3$ is an efficient Ca mobilizer with a potency that is less than $\text{Ins}(1,4,5)\text{P}_3$ (Irvine and Moor, 1986). What makes $\text{Ins}(2,4,5)\text{P}_3$ an interesting compound is that, unlike $\text{Ins}(1,4,5)\text{P}_3$, it cannot

be phosphorylated to $\text{Ins}(1,3,4,5)\text{P}_4$, which supposedly promotes Ca^{2+} entry (Berridge and Irvine, 1989). I found that both $\text{Ins}(2,4,5)\text{P}_3$ and $\text{Ins}(1,3,4,5)\text{P}_4$ were able to mobilize Ca^{2+} , but in general the amounts injected had to be increased in order to see increases in Ca_i comparable to those mobilized by $\text{Ins}(1,4,5)\text{P}_3$. I also found that both inositol polyphosphates were able to mobilize Ca^{2+} in 0Ca-SW .

Effect of InsP_3 injections on bursting activity

The pacemaker cycle of *Aplysia* bursting neurons has two membrane conductance systems both dependent on changes in Ca_i (Gorman et al., 1981; Adams, 1985; Kramer and Zucker, 1985a,b). Moreover, the bursting can be modulated by several neurotransmitters (Levitan et al., 1979; Smith, 1980). It is possible that some agonists modulate membrane conductances by releasing InsP_3 . To test the hypothesis that changes in membrane properties could be directly modulated by InsP_3 (or indirectly through Ca^{2+} mobilization), I injected InsP_3 and measured changes in membrane potential or total membrane current. Figure 5 shows that injection of InsP_3 had a marked effect on the bursting frequency and the membrane potential of an R15 pacemaker neuron. Injection of $1 \text{ mM } \text{InsP}_3$ induced an immediate train of action potentials (arrowheads 1–3) followed by membrane hyperpolarization. After the cell recovered from the hyperpolarization, the burst frequency had noticeably decreased. Both the InsP_3 -induced immediate discharge of action potentials and the hyperpolarization depended on the amount of InsP_3 injected. Large injections (arrowhead 3) increased the duration of the interburst hyperpolarization without increasing further its amplitude. Additional injections during the prolonged hyperpolarization caused the cell to fire action potentials (not shown).

Effect of InsP_3 injection on total membrane currents

To gain insight into the mechanism of action of InsP_3 on the bursting activity, I injected InsP_3 in voltage-clamped cells and monitored the total membrane current. Conventional, single-barreled micropipettes filled with InsP_3 were used for these experiments. Figure 6A shows that injection of InsP_3 in an R15 cell resulted in a biphasic response: a relatively rapid inward current followed by a more prolonged outward current. I also found that the relative magnitude of the inward and outward current changed with depth within the cell. Figure 6B shows a plot of peak inward and outward current versus the depth of the injecting electrode; the peak inward current increased linearly as the injection electrode approached the inner membrane. The outward current remained small until the injecting electrode approached the membrane. Scholz et al. (1988) described a similar biphasic response caused by InsP_3 injections in *Aplysia*. Sawada et al. (1987) found only outward currents in *Aplysia kurodai* neurons. The biphasic response shown in Figure 6A resembles the response caused by Ca^{2+} injections in *Helix* neurons (Hofmeier and Lux, 1981) and could be the Ca^{2+} -dependent, nonspecific cation current responsible for maintaining repetitive spiking during the burst of *Helix* (Swandulla and Lux, 1985) and *Aplysia* (Kramer and Zucker, 1985a,b) neurons.

For control injections, I used double-barreled injecting microelectrodes, where one barrel was filled with *myo*-inositol and the other barrel with InsP_3 . Figure 6C shows that control injections of $1 \text{ mM } \text{myo}$ -inositol at depths of $50 \mu\text{m}$ (left panel) and $30 \mu\text{m}$ (right panel) did not produce any appreciable change in total membrane current. Injections of InsP_3 at the same site

Figure 5. Effect of InsP_3 injections on bursting frequency and membrane potential of an R15 *Aplysia* neuron. The cell was impaled with a voltage electrode and a single micropipette filled with 1 mM InsP_3 . The 0 potential is shown by a dotted line. Each injection (30 psi) is indicated by an arrowhead. Arrowheads 1, 2, and 3 correspond to 200 msec, 400 msec, and 600 msec long injections, respectively. Each injection increases the frequency of the action potentials (because of the slow time scale, these are seen as a thick vertical line following the injection). The increased frequency is followed by a hyperpolarization whose duration seems to depend on the amount of InsP_3 injected.

induced large changes in membrane current. It is important to do the control injections at the same site since the membrane current changes are highly depth dependent.

Differential effects of Ca^{2+} and InsP_3 injections on membrane currents

To find out whether the effect of InsP_3 on membrane currents is primary or mediated by the Ca^{2+} released by InsP_3 , I intracellularly injected Ca^{2+} and InsP_3 at the same site. I used double-barreled injecting microelectrodes filled with 10 mM Ca^{2+} and 1 mM InsP_3 . Figure 7 shows the effects of such injections on membrane currents in two types of neurons, R15 and L3. The depth of the injecting electrode is given in the Figure 7 caption. Membrane currents induced by Ca^{2+} or InsP_3 injections differed in waveform and time course. Ca injections typically induced outward currents, while InsP_3 injections induced inward currents. Similar results were found in a total of 12 cells from 12 different ganglia. To test directly the hypothesis that InsP_3 activates membrane currents that seem to be independent of the

Ca_i increase, InsP_3 and Ca were injected side by side in the presence of BAPTA. BAPTA was chosen over EGTA, because it is a much faster Ca buffer; for example, certain Ca -mediated effects were found to be resistant to EGTA, but not to BAPTA (Adler et al., 1991; Frank and Fein, 1991). Figure 8 shows that in the presence of BAPTA, the Ca -induced outward current was substantially reduced while the InsP_3 -induced inward current was largely unaffected. The InsP_3 -induced current changes were resistant to BAPTA in three more cells. In three other cells, BAPTA effectively chelated intracellular Ca^{2+} , since the Ca -dependent slow tail currents, induced by depolarization, were greatly reduced. These results suggest that the current changes induced by InsP_3 injections may be primary and are not explained solely by a Ca_i increase.

Simultaneous measurement of membrane current and Ca_i induced by InsP_3 injections

The fact that the membrane conductance changes are different for Ca^{2+} and InsP_3 injections does not exclude that the mem-

Figure 6. Effect of intracellular InsP_3 injection on membrane current of an R15 cell. The cell was voltage clamped at -45 mV and the total membrane current measured as illustrated in Figure 1A, except that the double-barreled microelectrode was replaced by a single-barreled injection electrode. **A**, Pressure injection of InsP_3 (275 μM solution, 50 psi, 200 msec) results in a biphasic response that consists of a rapidly developing inward current followed by a more prolonged outward current. **B**, Plot of the peak inward and outward currents induced by single injections of InsP_3 versus the depth of the injecting electrode; the peak inward current increased linearly as the injection electrode approached the inner membrane, while the outward current did not start increasing until the injecting electrode approached the inner membrane. **C**, Injection of 1 mM *myo*-inositol at depths of 50 μm (left) and 30 μm (right) did not result in any noticeable increase in membrane current, whereas injection of 1 mM InsP_3 at the same sites had a marked effect. Both compounds were injected using a double-barreled injecting electrode.

Figure 7. Effect of injecting Ca^{2+} and InsP_3 at the same site on membrane currents. *Inset*, Experimental arrangement showing the double-barreled injecting electrode that allows delivery of either Ca^{2+} (10 mM solution) or InsP_3 (1 mM) solution at the same point. Cells: *A*, *C*, R15; *B*, L3. Depth of the injecting electrodes: *A*, 60 μm ; *B*, 40 μm ; *C*, 25 μm .

brane current and the Ca^{2+} mobilized by InsP_3 may be related. One way to find out is to follow changes in membrane current and Ca_i simultaneously in response to InsP_3 injections. Figure 9*A* shows such changes in an R15 cell. In the inset of Figure 9*A*, the recorded Ca_i signal (voltage) was converted to a linear ion concentration scale to facilitate comparison with the inverted and normalized membrane current trace. The inset shows that although the current and the Ca_i trace start rising simul-

taneously, the InsP_3 -induced membrane current recovers faster than the Ca_i signal. The same is true for the Cl^- current and the Ca_i signal changes induced by InsP_3 in oocytes (Parker and Ivorra, 1990a). The inset of Figure 9*A* also shows that the peak Ca_i increase is delayed in relation to the peak membrane current, which is probably the result of the relative slowness of the Ca -selective microelectrode; the true Ca_i peak is probably larger and faster.

Another way to assess whether the Ca_i signal and the membrane current are related is by looking at their relative spatial variation following InsP_3 injections. Figure 9*B* shows a plot of peak Ca_i increase and peak inward current versus the depth of the InsP_3 -injecting double-barreled Ca electrode. Both peaks changed relatively by the same amount as the injecting electrode approached the surface membrane. So, although side-by-side Ca^{2+} and InsP_3 injections induce different current waveforms, it looks as though the InsP_3 -induced membrane current changes may be spatially related to the mobilized Ca^{2+} . Further experiments will be necessary to separate the effects of Ca^{2+} and InsP_3 on membrane currents in molluscan neurons.

Discussion

The results suggest that intracellular injections of InsP_3 in identified nerve cells of *Aplysia californica* mobilize Ca^{2+} from intracellular stores and also induce distinct changes in membrane currents that seem to be independent of the Ca_i increase.

In previous studies, the effect of InsP_3 in nerve cells of *Aplysia kurodai* (Sawada et al., 1987) and *Aplysia californica* (Sholz et al., 1988) was indirectly shown to mobilize Ca^{2+} , by measuring membrane currents. Fink et al. (1988) have shown, using digital imaging of fura-2 fluorescence, that InsP_3 increases Ca_i in *Aplysia* bag cells. In this study, I directly measured InsP_3 -induced Ca_i increases using double-barreled Ca -selective microelectrodes. I found that the InsP_3 -induced Ca_i increase was graded with the amount of InsP_3 injected. A similar dose-response

Figure 8. The InsP_3 -induced membrane current change subsists in BAPTA-loaded cells. Side-by-side injection of InsP_3 and Ca^{2+} before (*upper trace*) and after pressure injection of BAPTA (15 injections, 60 psi, 200 msec each). The increased Ca buffering capacity was effective in reducing the Ca -induced outward current, but not the InsP_3 -induced inward current (same experimental arrangement as the inset of Fig. 7, except that the V_m electrode was filled with 100 mM BAPTA).

Figure 9. Temporal relations of membrane current and Ca_i measured simultaneously. *A*, Comparison of the time course of membrane current (middle trace) and measured Ca_i signal (top trace) induced by intracellular injection of $InsP_3$ in an R15 cell. Note how the membrane current recovers a few seconds following the injection whereas the Ca_i signal increase is still relatively high; it took about 80 sec for the Ca_i signal to reach the baseline (not shown). In the inset, the current trace was inverted and normalized to the Ca_i signal trace to show the shift in peak amplitude in relation to the Ca_i signal; the Ca_i signal was converted from voltage (which varies with the log of the Ca^{2+} concentration) to a linear Ca^{2+} concentration scale. Both traces in the inset were sampled at 0.5 kHz and digitized. The double-barreled Ca -selective microelectrode was about 5 μm from the membrane surface. Injection: 50 psi, 400 msec, 1 mM $InsP_3$ solution. *B*, Spatial variations of the Ca_i increase and membrane current induced by $InsP_3$ injection. Plotted are the peak Ca_i increase and membrane current versus the depth of the double-barreled microelectrode; the peak current was normalized to the peak of the largest Ca_i increase (5 μm point). Same protocol as Figure 3*B*. R15 cell, 50 psi, 400 msec injections, 1 mM $InsP_3$ solution.

relationship was found in other preparations using fura-2 fluorescence (Meyer et al., 1988) or ^{45}Ca (Gawler et al., 1990; Finch et al., 1991). Our results also agree with those of Sawada et al. (1987) for the $InsP_3$ -activated outward current, and those of Parker and Ivorra (1990b) for chloride currents and Ca_i increases.

The intracellular Ca store has supposedly a limited capacity to release Ca^{2+} and thus to sustain a Ca_i increase, other mechanisms must exist, notably Ca influx (Michell, 1975; Putney, 1986; Berridge and Irvine, 1989; Takemura and Putney, 1989). Matthews et al. (1989) found evidence for $InsP_3$ -induced Ca influx in mast cells. In this study, I was able to induce Ca^{2+} increases by $InsP_3$ injections in 0Ca-SW repeatedly for up to 60 min. This may mean that *Aplysia* nerve cells have unusually large stores of Ca^{2+} or that the released Ca^{2+} is returned to the $InsP_3$ -sensitive store efficiently.

A complex aspect of the $InsP_3$ signaling system is the way the $InsP_3$ -induced Ca_i increase is modulated. In many cell types, Ca^{2+} ions inhibit the action of $InsP_3$ to induce further mobilization of Ca^{2+} from intracellular stores (Payne et al., 1988, 1990; Berridge, 1989; Parker and Ivorra, 1990a). This feedback inhibition is preceded in some preparations by facilitation, whereby a prior elevation of Ca^{2+} potentiates the effect of $InsP_3$ (Sawada et al., 1987; Parker and Ivorra, 1990a; Finch et al., 1991). The feedback inhibition was recently shown to be mediated by the lingering elevation of Ca_i that follows mobilization of Ca^{2+} by $InsP_3$ (Levy and Payne, 1991; Payne, 1991). The results of Figure 4*B* show that the initial elevation of Ca_i does not inhibit subsequent increases of Ca_i by $InsP_3$. Desensitization occurred only after several injections have elevated Ca_i further. One possibility is that different preparations have different thresholds of elevated Ca_i to produce desensitization.

The mobilization of Ca^{2+} by $InsP_3$ has been shown in many systems to be spatially restricted to some areas of the cells (Levy, 1987; Payne and Fein, 1987; Parker and Ivorra, 1990b). Berridge (1989) found that the $InsP_3$ -induced Cl^- current desensitization in *Xenopus* oocytes was restricted to the point of injection and proposed that the $InsP_3$ -sensitive pool is composed of

discrete compartments, a concept supported by recent evidence (Parker and Yao, 1992). The results presented on Figure 3 show strong evidence that the $InsP_3$ -induced Ca_i increase is nonuniform in *Aplysia* nerve cells. Regarding our own experimental design, which required moving the double-barreled Ca microelectrodes and injecting at different points, could the apparent spatial localization be the result of some artifact like reversible electrode clogging or limited dissipation of injected $InsP_3$? Electrode clogging cannot explain differences that can be measured in both directions of penetration depending on where the site of highest $InsP_3$ -induced Ca_i increase is located. Also I can reversibly induce different magnitudes of $InsP_3$ -induced Ca_i increase simply by moving the injection electrode back and forth (Fig. 3*A,C*). In my own hands, I have always seen injection electrodes getting clogged irreversibly. Regarding possible limitations to the diffusion of $InsP_3$, it could be argued that as the injection electrode approaches the plasma membrane, the diffusion of the injected $InsP_3$ is more restricted due to the presence of the membrane, and this could explain a larger mobilized Ca^{2+} concentration at that point. The results of Figure 3, *C* and *E*, argue against restricted diffusion as the cause of spatial differences in $InsP_3$ -induced Ca_i increase that I measure, since the largest $InsP_3$ -induced Ca_i increase was found in some cells to be some 80–120 μm away from the membrane. It is of interest to mention that the Ca buffering capacity in *Aplysia* nerve cells is largest near the plasma membrane (Tillotson and Gorman, 1980), so it is possible that the largest $InsP_3$ -induced Ca_i increase seen near the plasma membrane would have been larger if not for the increased Ca buffering capacity. I conclude that the spatial difference in ability for a given injection of $InsP_3$ to mobilize Ca^{2+} reflects a real difference in localization of the internal stores of Ca .

If we support the concept of separate local compartments (Berridge, 1989; Parker and Yao, 1992), the differences in Ca signals that I measure could be due to the proximity of the injection electrode to a more or less dense population of compartments. Further support to the concept of discrete compartments may come from an interesting result from the inset of

Figure 3B: regardless of the size of the Ca signal, the time to peak of the InsP_3 -induced Ca_i increase seems to be the same. This may mean that the injected InsP_3 is reaching Ca stores at each depth, only the difference in the number of compartments makes it that larger responses are obtained when more sites are simultaneously activated by InsP_3 . A similar time to peak of the Ca signal at depths of 30 and 90 μm (Fig. 3A) is unlikely to be due to some distortion by the dynamic response of the Ca electrode. If the small Ca signal at 90 μm was the result of Ca^{2+} diffusing from a site near the plasma membrane, its time to peak would have been much larger, as demonstrated before in similar conditions (Levy and Fein, 1985). The significance of this highly localized distribution of InsP_3 -sensitive Ca stores is unclear. One possibility is that Ca^{2+} is mobilized by InsP_3 where it is most effective. For example, in *Limulus* photoreceptors, phototransduction is rapid and efficient because of the close proximity of the visual pigment rhodopsin, the sites of release of InsP_3 , the InsP_3 -sensitive Ca stores, and the Ca -sensitive receptors (Payne and Fein, 1987). A consideration in the case of *Aplysia* nerve cells, where Ca influx is substantial, is that it may be important to segregate entering and mobilized Ca^{2+} ions because they may have different target molecules. Alternatively, Ca influx could be more or less potentiated through mobilized Ca , by reaching a different number of discrete compartments.

An interesting aspect of molluscan pacemaker potentials is that they can be altered by neurohormones like vasopressin or egg-laying hormone (Levitan et al., 1979; Smith, 1980). These neurohormones could act through G-protein activation of phospholipase C. In accordance with previous findings (Scholz et al., 1988), my data suggest that InsP_3 could play a role in modulating the bursting activity of *Aplysia* pacemaker neurons. InsP_3 injection into bursting R15 neurons causes an immediate train of action potentials followed by membrane hyperpolarization and later by a decrease in burst frequency (Fig. 5). It is not clear whether the initial membrane hyperpolarization is due to a direct effect of InsP_3 or to an increased entry of Ca^{2+} following the train of action potentials. Thomas and Gorman (1977) have shown that increased Ca^{2+} entry (by passing current during a burst) does result in a larger hyperpolarization, but under this condition it is not followed by a decrease in interburst frequency. One has to keep in mind that a neurotransmitter would release both InsP_3 and diacylglycerol. The effect of the latter on bursting is not known. Strong et al. (1987) have shown that activation of protein kinase C in *Aplysia* bag cells leads to enhancement of Ca currents. In preliminary experiments, we found that *Aplysia* neurons from the abdominal ganglion (devoid of bag cells) contain all the endogenous inositol phosphates and lipids (S. Levy and E. Szuts, unpublished observations).

The presence of the Ca -mobilizing effect of InsP_3 in a cell system does not necessarily preclude its role in directly modulating membrane currents. Whether InsP_3 directly activates ion channels in the plasma membrane remains controversial (Irvine, 1987; Matthews et al., 1989). My results of Figures 7 and 8 show evidence that the membrane current changes induced by InsP_3 injection in *Aplysia* nerve cells may be primary and are not due solely to a Ca_i increase. I cannot rule out that InsP_3 may be converted to InsP_4 or some other messenger that itself would induce changes in membrane currents (but see Snyder et al., 1988). Some differences in the time course of the responses to both compounds are expected since InsP_3 and Ca^{2+} do not probably have the same diffusion rate; InsP_3 could diffuse less freely to the membrane, but Ca^{2+} would be strongly buffered.

One difference could be that the area of the membrane reached by InsP_3 (if it is not rapidly degraded) is probably larger than the one reached by a point injection of Ca^{2+} . It seems more likely, however, that InsP_3 (or one of its products), in addition to mobilizing Ca^{2+} from intracellular stores, directly activates a conductance change in the membrane. A strong evidence is that the InsP_3 -induced inward current is resistant to BAPTA, whereas the Ca -induced outward current is not (Fig. 8). The fact that BAPTA is almost without effect on the InsP_3 -induced membrane current excludes the possibility that it is the InsP_3 -induced Ca_i increase that activates a membrane current change. Scholz et al. (1988) also found that Ca^{2+} injections do not mimic InsP_3 injections in *Aplysia* neurons, but their injections were made from separate micropipettes, and hence not necessarily at the same site. In oocytes, Parker and Ivorra (1990a) found that injections of InsP_3 or Ca^{2+} induce a chloride conductance; however, Ca^{2+} injection induced a larger Ca_i increase than InsP_3 , but a smaller membrane current change.

There are several reports of similar effects of Ca^{2+} and InsP_3 injections on membrane currents (Sawada et al., 1987; Fink et al., 1988). I have seen no evidence of an effect of preinjecting one compound on the response to the second compound, even at short time intervals; however, I have not done a thorough time interval study. As shown on Figure 7, our typical procedure was to wait for the current induced by the first injection to reach baseline before making the second injection. It is therefore unlikely that the differences in current waveform between InsP_3 - and Ca^{2+} -induced current changes are due to the effect of prior injection of InsP_3 on Ca^{2+} -induced current changes or vice versa.

In conclusion, I was able to dissociate the Ca^{2+} mobilizing role of InsP_3 from its direct action on the plasma membrane conductance. Furthermore, my results call for caution in using InsP_3 -induced ion current changes as an assay of intracellular free Ca^{2+} concentration changes (DeLisle et al., 1990; Komori and Bolton, 1991). It is clear as shown in this study and others that changes in membrane currents are not a faithful indicator of changes in Ca_i .

References

- Adams WB (1985) Slow depolarizing and hyperpolarizing currents which mediate bursting in *Aplysia* neurone R15. *J Physiol (Lond)* 360:51–68.
- Adams WB, Levitan IB (1985) Voltage and ion dependences of the slow currents which mediate bursting in *Aplysia* neurone R15. *J Physiol (Lond)* 360:69–93.
- Adler EM, Augustine GJ, Duffy SN, Charlton MP (1991) Alien intracellular calcium chelators attenuate neurotransmitter release at the squid giant synapse. *J Neurosci* 11:1496–1507.
- Berridge MJ (1989) Inositol 1,4,5-trisphosphate-induced Ca mobilization is localized in *Xenopus* oocytes. *Proc R Soc Lond [Biol]* 238:235–243.
- Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. *Nature* 341:197–205.
- Coggeshall RE (1967) A light and electron microscope study of the abdominal ganglion of *Aplysia californica*. *J Neurophysiol* 30:1263–1287.
- Corson DW, Fein A (1983) Quantitative pressure injection of picoliter volumes into *Limulus* photoreceptors. *Biophys J* 44:299–304.
- DeLisle S, Krause K-H, Denning G, Potter BVL, Welsh MJ (1990) Effect of inositol trisphosphate and Ca^{2+} on oscillating elevations of Ca_i in *Xenopus* oocytes. *J Biol Chem* 265:11726–11730.
- Eberhard DA, Holz RW (1988) Intracellular Ca activates phospholipase C. *Trends Neurosci* 11:518–520.
- Finch EA, Turner TJ, Goldin SM (1991) Calcium as coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* 252:443–446.
- Fink LA, Connor JA, Kaczmarek LK (1988) Inositol trisphosphate

- releases intracellularly stored calcium and modulates ion channels in molluscan neurons. *J Neurosci* 8:2544–2555.
- Frank TM, Fein A (1991) The role of the inositol phosphate cascade in visual excitation of invertebrate microvillar photoreceptors. *J Gen Physiol* 97:697–723.
- Frazier WT, Kandel ER, Kupferman I, Waziri R, Coggeshall RE (1967) Morphological and functional properties of identified cells in the abdominal ganglion of *Aplysia californica*. *J Neurophysiol* 30:1288–1351.
- Gawler DJ, Potter BVL, Nahorski SR (1990) Inositol 1,3,4,5-tetrakisphosphate-induced release of intracellular Ca^{2+} in SH-SY5Y neuroblastoma cells. *Biochem J* 272:519–524.
- Gorman ALF, Hermann A (1979) Internal effects of divalent cations on potassium permeability in molluscan neurons. *J Physiol (Lond)* 296:395–410.
- Gorman ALF, Hermann A, Thomas MV (1981) Intracellular calcium and the control of neuronal pacemaker activity. *Fed Proc* 40:2233–2239.
- Gorman ALF, Levy S, Nasi E, Tillotson D (1984) Intracellular calcium measured with calcium-sensitive microelectrodes and arsenazo III in voltage-clamped *Aplysia* neurones. *J Physiol (Lond)* 353:127–142.
- Harootunian AT, Kao JPY, Paranjape S, Tsien RY (1991) Generation of Ca oscillations in fibroblasts by positive feedback between Ca and IP_3 . *Science* 251:75–78.
- Hofmeier G, Lux HD (1981) The time courses of intracellular free calcium and related electrical effects after injection of CaCl_2 into neurons of the snail, *Helix pomatia*. *Pfluegers Arch* 391:242–251.
- Irvine RF (1987) Inositol phosphates and calcium entry. *Nature* 328:386.
- Irvine RF, Moor RM (1986) Micro-injection of IP_4 activates sea urchin eggs by a mechanism dependent on external Ca^{2+} . *Biochem J* 240:917–920.
- Komori S, Bolton TB (1991) Calcium release induced by inositol 1,4,5-trisphosphate in single rabbit intestinal smooth muscle cells. *J Physiol (Lond)* 433:495–517.
- Kramer RH, Zucker RS (1985a) Calcium-dependent inward current in *Aplysia* bursting pacemaker neurons. *J Physiol (Lond)* 362:107–130.
- Kramer RH, Zucker RS (1985b) Ca-induced inactivation of Ca current causes the interburst hyperpolarization of *Aplysia* bursting neurones. *J Physiol (Lond)* 362:131–160.
- Levitan IB (1985) Phosphorylation of ion channels. *J Membr Biol* 87:177–190.
- Levitan IB, Harmar AJ, Adams WB (1979) Synaptic and hormonal regulation of a neuronal oscillator: a search for molecular mechanisms. *J Exp Biol* 81:131–151.
- Levy S (1987) Intracellular injection of IP_3 releases Ca from intracellular stores in *Aplysia* bursting neurons. *Biophys J* 51:424a.
- Levy S (1988) Effects of intracellular injection of inositol derivatives on Ca_i , bursting activity and membrane currents of molluscan neurons. *J Gen Physiol* 92:2a.
- Levy S (1989) Differential effects of IP_3 and Ca^{2+} injections on membrane currents of *Aplysia* neurons. *Biophys J* 55:436a.
- Levy S, Fein A (1985) Relationship between light sensitivity and intracellular free Ca concentration in *Limulus* ventral photoreceptors: a quantitative study using Ca-selective microelectrodes. *J Gen Physiol* 85:805–841.
- Levy S, Payne R (1991) Inhibition of the IP_3 -induced Ca release in *Limulus* ventral photoreceptors requires the continuous presence of elevated resting Ca_i . *Biophys J* 59:531a.
- Levy S, Tillotson D (1987) Ability of the Ca^{2+} selective electrode to measure fast and local Ca^{2+} transients in nerve cells. *Can J Physiol Pharmacol* 65:904–914.
- Levy S, Tillotson D (1988) Effect of Na and Ca gradients on intracellular free Ca in voltage clamped *Aplysia* neurons. *Brain Res* 474:333–342.
- Matthews G, Neher E, Penner R (1989) Cl^- conductance activated by external agonists and internal messengers in rat peritoneal mast cells. *J Physiol (Lond)* 418:131–144.
- Meech RW (1974) The sensitivity of *Helix aspersa* neurones to injected calcium ions. *J Physiol (Lond)* 237:259–277.
- Meyer T, Holowka D, Stryer L (1988) Highly cooperative opening of Ca channels by IP_3 . *Science* 240:653–656.
- Michell RH (1975) Inositol phospholipids and cell surface receptor function. *Biochem Biophys Acta* 415:81–147.
- Munoz J-L, Deyhimi F, Coles JA (1983) Silanization of glass in the making of ion-selective microelectrodes. *J Neurosci Methods* 8:231–247.
- Oehme M, Kessler M, Simon W (1976) Neutral carrier Ca^{2+} microelectrode. *Chimia* 30:204–206.
- Parker I, Ivorra I (1990a) Inhibition by Ca^{2+} of inositol trisphosphate-mediated Ca^{2+} liberation: a possible mechanism for oscillatory release of Ca^{2+} . *Proc Natl Acad Sci USA* 87:260–264.
- Parker I, Ivorra I (1990b) Localized all-or-none calcium liberation by inositol triphosphate. *Science* 250:977–979.
- Parker I, Miledi R (1989) Nonlinearity and facilitation in phosphoinositide signalling studied by the use of caged inositol trisphosphate in *Xenopus* oocytes. *J Neurosci* 9:4068–4077.
- Parker I, Yao Y (1992) Regenerative release of calcium from functionally discrete subcellular stores by inositol trisphosphate. *Proc R Soc Lond [Biol]* 246:269–274.
- Payne R (1991) Injection of 1 mM EGTA reverses desensitization of inositol trisphosphate-induced calcium release in *Limulus* photoreceptors. *Biophys J* 59:540a.
- Payne R, Fein A (1987) Inositol 1,4,5 trisphosphate releases calcium from specialized sites within *Limulus* photoreceptors. *J Cell Biol* 104:933–937.
- Payne R, Walz B, Levy S, Fein A (1988) The localization of Ca release by inositol trisphosphate in *Limulus* photoreceptors and its control by negative feedback. *Philos Trans R Soc Lond [Biol]* 320:359–379.
- Payne R, Flores TM, Fein A (1990) Feedback inhibition by calcium limits the release of calcium by inositol trisphosphate in *Limulus* ventral photoreceptors. *Neuron* 4:547–555.
- Putney JW Jr (1986) A model for receptor-regulated Ca entry. *Cell Calcium* 7:1–12.
- Sawada M, Ichinose M, Maeno T (1987) Ionic mechanism of the outward current induced by intracellular injection of inositol trisphosphate into *Aplysia* neurons. *J Neurosci* 7:1470–1483.
- Scholz KP, Cleary LJ, Byrne JH (1988) Inositol 1,4,5 trisphosphate alters bursting pacemaker activity in *Aplysia* neurons: voltage-clamp analysis of effects on calcium currents. *J Neurophysiol* 60:86–104.
- Smith TG Jr (1980) Ionic conductances in bursting pacemaker cells and their hormonal modulation. *Cold Spring Harbor Rep Neurosci* 1:135–143.
- Snyder P, Krause K, Welsh M (1988) Inositol trisphosphate isomers, but not inositol 1,3,4,5-tetrakisphosphate, induce calcium influx in *Xenopus laevis* oocytes. *J Biol Chem* 263:11048–11051.
- Strong JA, Fox AP, Tsien RW, Kaczmarek LK (1987) Stimulation of protein kinase C recruits covert calcium channels in *Aplysia* bag cell neurons. *Nature* 325:714–717.
- Swandulla D, Lux HD (1985) Activation of a nonspecific cation conductance by intracellular Ca elevation in bursting pacemaker neurons of *Helix pomatia*. *J Neurophysiol* 54:1430–1443.
- Takemura H, Putney JW Jr (1989) Capacitative Ca entry in parotid acinar cells. *Biochem J* 258:409–412.
- Thomas MV, Gorman ALF (1977) Internal calcium changes in a bursting pacemaker neuron measured with arsenazo III. *Science* 196:531–533.
- Tillotson D (1979) Inactivation of Ca conductance dependent on entry of Ca^{2+} ions in molluscan neurons. *Proc Natl Acad Sci USA* 76:1497–1500.
- Tillotson D, Gorman ALF (1980) Non-uniform Ca^{2+} buffer distribution in a nerve cell body. *Nature* 286:816–817.