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Ionic Mechanism of the Outward Current Induced by Intracellular Injection of Inositol Trisphosphate into *Aplysia* Neurons

Masashi Sawada, Mitsuyuki Ichinose, and Takashi Maeno

Department of Physiology, Shimane Medical University, Izumo, Japan

Inositol 1,4,5-trisphosphate ($InsP_3$) has been proposed to be the intracellular second messenger in the mobilization of Ca^{2+} from intracellular stores in a variety of cell types. The ionic mechanism of the effect of intracellularly injected $InsP_3$ on the membrane of identified neurons (R9–R12) of *Aplysia kurodai* was investigated with conventional voltage-clamp, pressure-injection, and ion-substitution techniques. Brief pressure injection of $InsP_3$ into a neuron voltage-clamped at −40 mV reproducibly induced an outward current (10–60 sec in duration, 20–60 nA in amplitude) associated with a conductance increase. The current was increased by depolarization and decreased by hyperpolarization up to −80 mV, where it disappeared. Extracellular application of tetraethylammonium (TEA; 5 mM) blocked the $InsP_3$-induced outward current, and the current was not affected by the presence of bath-applied 4-aminopyridine (4-AP; 5 mM). The $InsP_3$-induced outward current recorded at a holding potential of −40 mV increased in amplitude in low-K^+ solutions and decreased in amplitude in high-K^+ solutions. Alteration of $[Cl^-]_o$, as well as perfusion with Ca^{2+} free plus 2 mM EGTA solution, did not affect the outward current. The $InsP_3$-induced outward current was found to disappear when the neuron was injected with the Ca^{2+} chelator EGTA. The outward current evoked by repeated $InsP_3$ injection at low doses exhibited summation and facilitation and, at high doses, was shown to desensitize. The calmodulin inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7; 20–50 μM), inhibited both the $InsP_3$-induced and the Ca^{2+}-activated outward currents. An intracellular pressure injection of Ca^{2+} ions into the same identified neuron was shown to produce an outward current associated with a K^+ conductance increase similar to the $InsP_3$-induced current, and the current was blocked by bath-applied TEA (5 mM). These results suggest that brief pressure injection of $InsP_3$ into certain identified neurons of *Aplysia* induces a 4-AP-resistant, TEA-sensitive K^+ current activated by increased intracellular free Ca^{2+} concentration, and this increase might be the result of the mobilization of Ca^{2+} from intracellular stores by $InsP_3$.

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Correspondence should be addressed to Dr. Masashi Sawada at the above address.

It has been proposed that hydrolysis of phosphatidylinositol-4,5-bisphosphate ($PI\text{-}P_2$) is involved in the control of a variety of physiological processes (Michell, 1975; Berridge, 1984). Recent studies have implicated inositol 1,4,5-trisphosphate ($InsP_3$) as an intracellular messenger in the cascade mediating hormone-, neurotransmitter-, and light-induced physiological responses (Berridge, 1983; Streb et al., 1983; Fein et al., 1984; Joseph et al., 1984; Oron et al., 1985; Waloga and Anderson, 1985; Hashimoto et al., 1986).

Berridge and his collaborators (1983) have demonstrated that the first measurable reaction following cell membrane receptor activation is a rapid hydrolysis of $PI\text{-}P_2$, and that the product of this reaction, $InsP_3$, could cause a release of nonmitochondrial Ca^{2+}. These findings have been verified in other systems (Agranoff et al., 1983; Poggioli et al., 1983; Putney et al., 1983). It has recently been demonstrated that *Limulus* ventral photoreceptors possess the pathways for the synthesis of $PI\text{-}P_2$ and for its subsequent light-induced hydrolysis to produce $InsP_3$ (Brown et al., 1984). In addition, the intracellular injection of $InsP_3$ into the *Limulus* photoreceptor (Brown et al., 1984; Fein et al., 1984) and into salamander rod (Waloga and Anderson, 1985) has been shown to mimic several aspects of the light-induced response. Furthermore, Oron and his collaborators (1985) have demonstrated that the intracellular injection of $InsP_3$ into the *Xenopus* oocyte mimics a muscarinic depolarizing chloride current. These results are consistent with the possibility that $InsP_3$ might play a role as an intracellular messenger mediating an increase in the conductance of the neuronal membrane activated by neurotransmitters. In molluscan neurons in which Ca^{2+}-activated K^+ conductance has been studied extensively, it has been shown that a small increase in intracellular Ca^{2+} can produce a sizable increase in K^+ conductance (Hermann and Gorman, 1979; Hermann and Hartung, 1982).

In the present study, we investigated the ionic mechanism of an $InsP_3$-induced outward current using micropressure injection of $InsP_3$ into identified neurons (R9–R12) of *Aplysia kurodai* and compared the current with a Ca^{2+}-activated K^+ current recorded from the same neuron. Our results indicate that brief micropressure injection of $InsP_3$ into these identified neurons of *Aplysia* induces an outward current associated with an increase in K^+ conductance and showing the same ionic and pharmacological properties as currents evoked by Ca^{2+} injection into the same neuron.

Materials and Methods

Identified R9–R12 (nomenclature of Frazier et al., 1967) neurons in the abdominal ganglion of *Aplysia* and approximately 80 animals, weighing from 100 to 300 gm, were used in this study. Isolated, desheathed abdominal ganglia were pinned, ventral surface upward, to a Sylgard

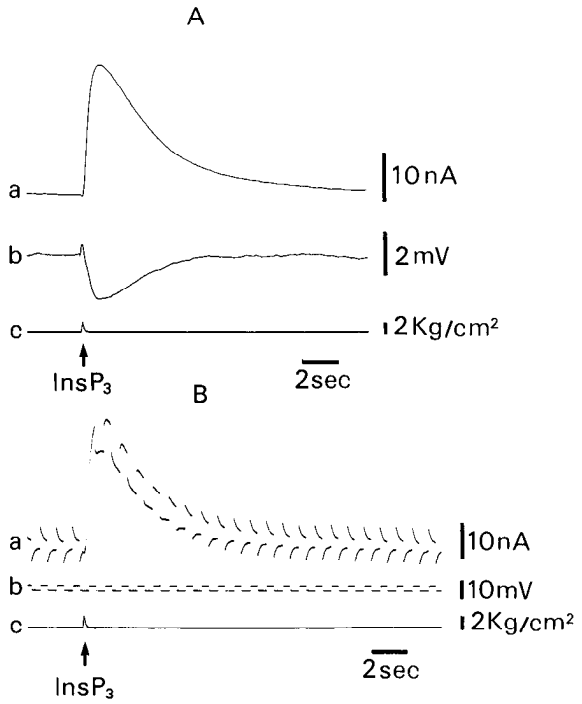

Figure 1. Micropressure injection of $InsP_3$ into identified neuron R12. $InsP_3$ was prepared from human erythrocyte ghosts and injected by a single constant pressure pulse (60 msec in duration, 2 kg/cm² in intensity; calculated final concentration of $InsP_3$, 0.4 μM). $InsP_3$-induced outward current (*A,a*), $InsP_3$-induced hyperpolarizing response (*A,b*), and $InsP_3$-induced outward current associated with an increase in the membrane conductance (*B*). *A, a*: Neuron R12 in voltage-clamp at a holding potential of −40 mV; *b*: neuron R12 in current-clamp with membrane potential of −40 mV; *c*: pressure monitor for intracellular injection of $InsP_3$. *B, a*: Membrane current; *b*: holding potential (−40 mV); *c*: pressure monitor. $InsP_3$ was injected by a constant pressure pulse (70 msec duration; 2 kg/cm² intensity). Constant hyperpolarizing command pulses (2 mV) with a duration of 500 msec were injected every 1 sec and the current required for these pulses was recorded for measurement of membrane conductance (*B*). Note that the $InsP_3$-induced outward current was typically associated with an apparent increase in the membrane conductance.

(Dow-Corning) floor in a 0.3 ml perfusion chamber containing artificial *Aplysia* Ringer's: 587 mM Na^+, 12 mM K^+, 671 mM Cl^-, 14 mM Ca^{2+}, and 52 mM Mg^{2+} (a slight modification of the *Aplysia* Ringer's originally made by Sato et al., 1968). The pH was adjusted to 7.8 with Tris-(hydroxymethyl)-aminomethane and HCl. The rate of perfusion was 4 ml/min. The K^+-deficient solutions were produced by mixing appropriate amounts of K^+-free *Aplysia* Ringer's with normal *Aplysia* Ringer's. When the Ringer's solution required a low Cl^- ion concentration, the Cl^- ion was replaced by an acetate ion. Zero Ca^{2+}–2 mM EGTA seawater was made by substituting 14 mM $MgCl_2$ for 14 mM $CaCl_2$ and adding 2 mM EGTA to artificial *Aplysia* Ringer's.

An identified neuron was impaled with a double- or triple-barreled microelectrode. One barrel, filled with 4 M K acetate, was connected to a preamplifier in order to record the membrane potential; the other barrel, filled with either $InsP_3$ (0.8 mM) or $CaCl_2$ (10 mM), was used for pressure injection. A second microelectrode, filled with 4 M K acetate, was inserted into the neuron for voltage-clamp. The neuron was voltage-clamped conventionally with a 2-microelectrode system (using the voltage-clamp amplifiers CEZ-1100 and MEZ-7101; Nihon Kohden) and held at resting membrane potential. An Ag–AgCl electrode with an agar–seawater bridge was immersed in the bath and served as the indifferent electrode and virtual ground. The membrane potential and the current were monitored by a dual-beam oscilloscope (VC-9A; Nihon Kohden) and a 4-channel pen recorder (Recti Horiz-8K; Sanei) with selected signal filtering. In order to evaluate the change in membrane conductance, the resting membrane was hyperpolarized every 1 sec by a voltage pulse of 2 mV with a duration of 500 msec, and the current required for this constant hyperpolarizing pulse was recorded before and during the responses to $InsP_3$ or Ca^{2+}, injected intracellularly into the identified neuron.

Several substances were injected intracellularly, with pressure pulses of 0.8–2.0 kg/cm², for 10–900 msec (using a PPM-2 pneumatic pump; (Medical System Corp.). Either double-barreled or triple-barreled electrodes were used for intracellular injection of $InsP_3$ (0.8 mM), *myo*-inositol (1 mM), and $CaCl_2$ (10 mM). The second barrel was always filled with 4 M K acetate and used to record membrane potential. Electrodes for pressure injection were filled with 20 mg/ml Procion red and one of the above substances dissolved in distilled water (pH = 7.4). Procion red was used to allow visual verification that solutions were being injected into the neurons. Control injections of either Procion red or *myo*-inositol had no obvious adverse effects on membrane current and conductance of the neurons (Fig. 2, *A, C*).

In several experiments, the volume of solution pressure-injected was quantified using a modification of the method of McCaman et al. (1977). Calibration of a particular microelectrode was accomplished by measurement of the diameter of the spherical droplet formed under varying

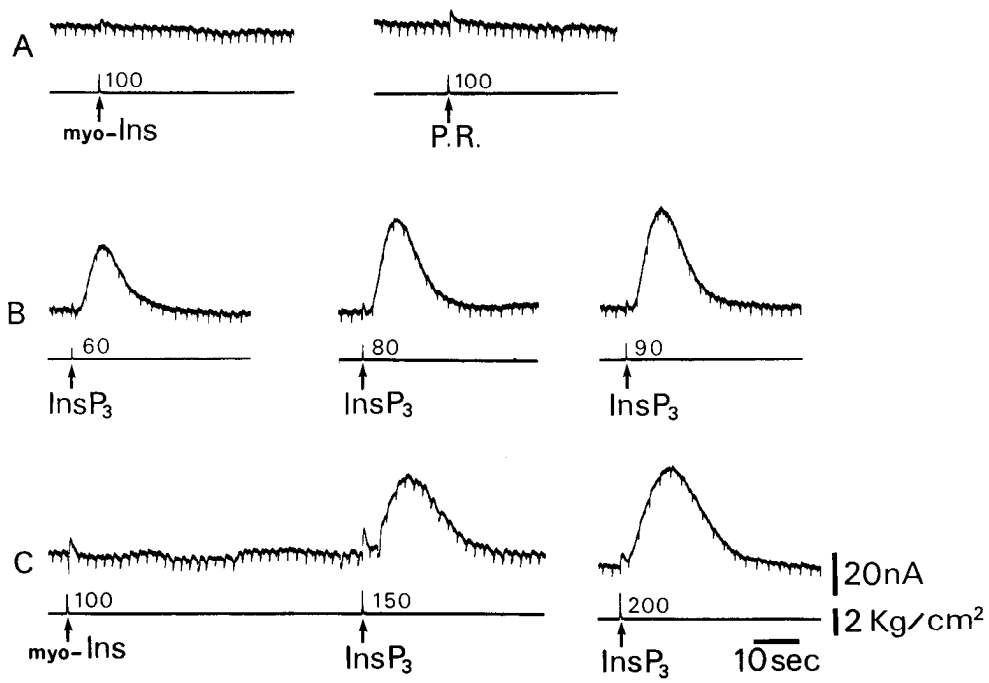

Figure 2. Intracellular injection of *myo*-inositol (*myo-ins, A,C*), Procion red (*P.R., A*), and $InsP_3$ (*B,C*) into neuron R12 from a triple-barreled microelectrode. *myo*-Inositol was injected by a constant pressure pulse (100 msec, 2 kg/cm²) using 1 barrel filled with 1 mM *myo*-inositol. Procion red was injected by a constant pressure pulse (100 msec, 2 kg/cm²) using the second barrel filled with 20 mg/ml Procion red solution. $InsP_3$ was injected by pressure pulses (60–200 msec, 2 kg/cm²) using the third barrel filled with 0.8 mM $InsP_3$ (Amersham). Holding potential of neuron R12 was −35 mV. *Numbers* represent the duration of the pressure pulse (msec) in this and subsequent figures. Note that neither *myo*-inositol nor Procion red induced any changes in membrane current, but $InsP_3$ injected into the same neuron R12 induced the outward current in a dose-dependent manner.

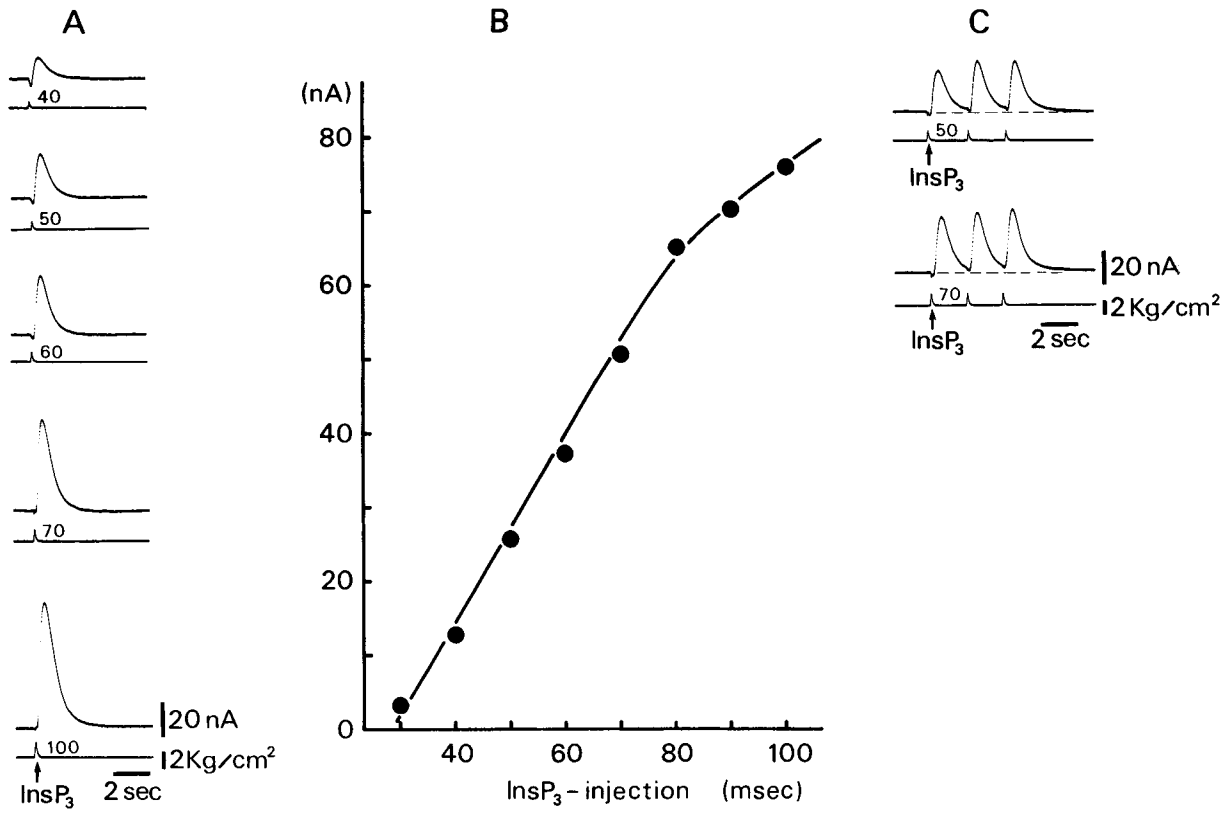

Figure 3. $InsP_3$ outward currents induced by $InsP_3$ injections of different durations at a constant pressure intensity (2 kg/cm²) into neuron R12. One of a triple-barreled microelectrode was filled with 0.8 mM $InsP_3$. Pressure-injected $InsP_3$ in this and subsequent experiments (except in Fig. 5*A*) was obtained from Amersham. *A*, Membrane current responses to pressure-injected $InsP_3$ of different durations. *B*, Relationship between the duration of pressure for the $InsP_3$ injection and the peak of the $InsP_3$-induced outward current. *C*, Effect of repetitive injection of $InsP_3$ into neuron R12. $InsP_3$ was injected by constant pressure pulses (50 msec, 2 kg/cm², *top*; 70 msec, 2 kg/cm², *bottom*) every 2 sec into neuron R12. Holding potential, −35 mV.

pressure- and pulse-duration combinations. Droplets were formed in the neuron or were ejected into oil (Toray AH 200-100) and the diameter was measured with an ocular micrometer (Hara et al., 1985). Injection quantities were in the range of 0.01–0.3% of the cell volume and were delivered at a pressure-pulse duration of 10–900 msec under constant pressure (2 kg/cm²). A pressure pulse of 250 msec duration usually produced an injection on the order of 0.05% of the cell volume. This was normally sufficient to induce responses.

In an initial series of experiments, $InsP_3$ was prepared by incubating human erythrocyte ghosts with $CaCl_2$, followed by a Dowex formate column separation, and then desalted by elution from a Dowex chloride column with 1 M LiCl, and the LiCl removed with ethanol (Downes et al., 1982). In most experiments, D-*myo*-inositol 1,4,5-trisphosphate ($InsP_3$ isolated from bovine brain) (Amersham) was pressure-injected into the identified neurons. Mircropressure injection of $InsP_3$, obtained either from human erythrocyte ghosts or from bovine brain, was shown to induce a similar outward current in the identified neurons.

Drugs applied extracellularly were added to the artificial *Aplysia* Ringer's. The following drugs were used: *myo*-inositol (Sigma); $InsP_3$ (potassium salt; Amersham); 4-aminopyridine (4-AP; Nakarai); tetraethylammonium chloride (TEA; Sigma); EGTA (Sigma); *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7; Sigma); ACh (Nakarai); and Procion red (Inolex).

All experiments were performed at room temperature (19–22°C).

Results

Identification of neuron

The identified neurons (R9–R12) are usually in the ventral right rostral quarter of the abdominal ganglion of *Aplysia kurodai*. These neurons are homologous to R9–R12 of *Aplysia californica* (Frazier et al., 1967) in appearance (white), location, and physiological properties (regular firing and hyperpolarizing response to ACh) (Koester and Kandel, 1977). The majority of the data presented here were obtained using identified neuron R12.

$InsP_3$-induced outward current

Micropressure-injected $InsP_3$ caused a marked hyperpolarization in the nonclamped neuron R12. Clamping the neuron at its resting potential level (−40 mV) and reinjecting $InsP_3$ with the same dose resulted in the development of a transmembrane outward current with the same time course as the membrane hyperpolarization in the nonclamped condition (Fig. 1*A*). Although both the time course and the delay from the beginning of the injection to the onset of the $InsP_3$-induced outward current recorded at −40 to −45 mV were found to be neuron- and ganglion-dependent, they remained the same for a given neuron in a given ganglion. The change in input conductance produced by $InsP_3$ injection was directly measured by applying constant-voltage hyperpolarizing pulses while clamping the neuron at resting membrane potential (Fig. 1*B*). The $InsP_3$-induced outward current reached a maximum amplitude (20–60 nA) within 1 sec and lasted at least 10–60 sec. It was typically associated with an apparent increase in membrane conductance, as illustrated in Figure 1*B* and subsequent figures. At the peak of the $InsP_3$-induced outward current there was a 400% increase in input-membrane conductance (Fig. 1*B*). The outward current response to injected $InsP_3$ was observed in over 86 of the identified neurons studied (54 R12, 18 R11, 6 R10, and 8 R9). With a single injection (pressure pulse duration, 60 msec; pressure

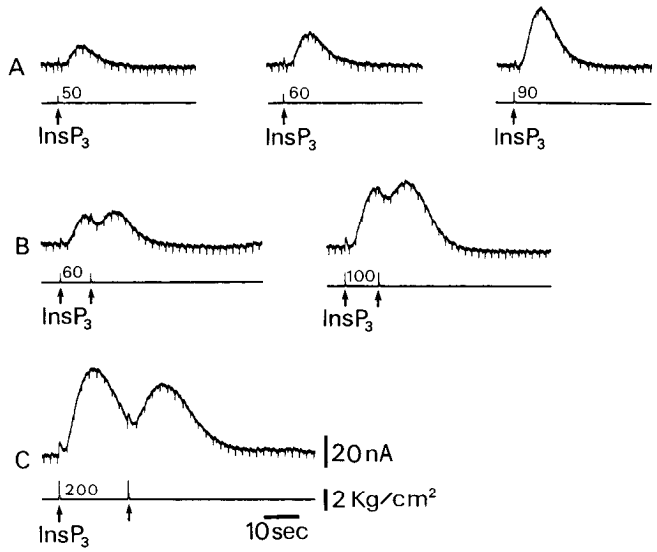

Figure 4. Intracellular injection of $InsP_3$ at different pulse durations into neuron R12. Holding potential, −35 mV. *A*, $InsP_3$ was injected by 3 different pressure pulses (50, 60, and 90 msec; 2 kg/cm²). *B*, $InsP_3$ was injected by a short pressure pulse (60 msec, 2 kg/cm²) (*left*) and by two 100 msec pulses at short intervals (*right*). *C*, $InsP_3$ was injected by two 200 msec pressure pulses (2 kg/cm²). Note that, in *B*, repeated injection of $InsP_3$ at low amounts caused a summation, but in *C* caused a desensitization at higher amounts.

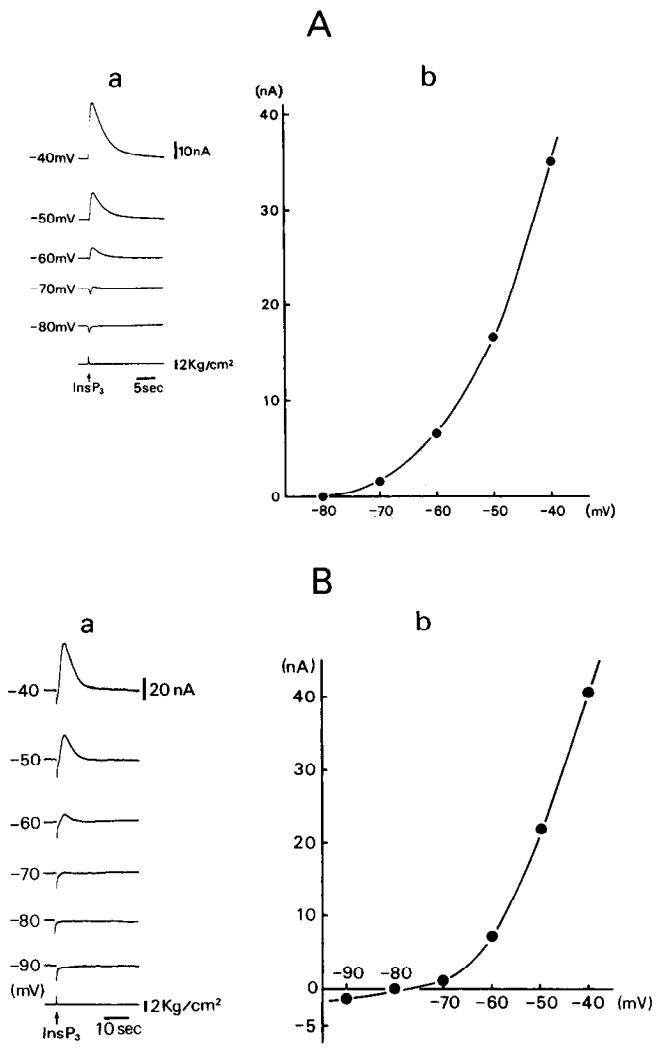

Figure 5. $InsP_3$-induced outward currents (*A,a*) at different holding potentials and voltage sensitivity of these currents (*A,b*) recorded from the same neuron used in Figure 1 (R12). Pressure-injected $InsP_3$ in this experiment was prepared from human erythrocyte ghosts. $InsP_3$ was injected by a constant pressure pulse (100 msec, 2 kg/cm²). Note that the $InsP_3$-induced outward current decreased at holding potentials more hyperpolarized than −40 mV and disappeared near the predicted equilibrium potential for K^+ ions (−80 mV). $InsP_3$-induced outward currents (*B,a*) at different holding potentials, and voltage sensitivity of these currents (*B,b*) recorded from neuron R12 in a different preparation. Pressure-injected $InsP_3$ in this experiment was obtained from Amersham. $InsP_3$ was injected by a single constant pressure pulse (100 msec, 2 kg/cm²). Note that the $InsP_3$-induced outward current reversed at hyperpolarized holding potentials more negative than −80 mV, and the reversal potential of the $InsP_3$-induced outward current was −78.5 mV.

intensity, 2 kg/cm²), the calculated concentration of $InsP_3$ in neuron R12 (300 μm in diameter; cell volume, 14.2 nl) was 0.4 μM when the injection electrode was filled with 0.8 mM $InsP_3$ (see Methods in Hara et al., 1985).

In order to check the specificity of the inhibitory effect of $InsP_3$, a variety of control solutions was also pressure-injected into the neurons and found to be without a noticeable effect on the membrane current. As a rule, injection of either *myo*-inositol or Procion red, using the same injection pressure pulse as with the $InsP_3$ injection (the electrode used was filled with 1 mM *myo*-inositol or 20 mg/ml Procion red solution), did not induce any observable current response (Fig. 2*A,C*).

Both the amplitude and delay (the time measured between the pressure artifact and the onset of response) of the $InsP_3$-induced outward current obtained by injection of $InsP_3$ at a medium-sized dose were dependent on the position of the injection electrode. A larger amplitude and shorter delay of the $InsP_3$-induced outward current were recorded when the injection electrode was positioned nearer the cell membrane than the central soma. The $InsP_3$-induced outward current at a holding potential of −35 mV increased in a dose-dependent manner, i.e., the current amplitude increased with an increase in the duration of the pressure pulse (Fig. 3, *A, B*). The peak of the outward current responses rose linearly with the duration of the pulse for $InsP_3$ injection. The straight line fitted to the experimental points has a slope of 57 nA per 50 msec-injected $InsP_3$ load, using a constant pressure intensity of 2 kg/cm², but does not pass through the origin, which indicates that a certain minimum amount of $InsP_3$ has to be injected to activate the outward current. With increasing duration of the injection pressure pulse, the delay from the beginning of injection to the onset of response is shortened, the rise of the outward current faster, and the time for the outward current to peak is prolonged.

Repetitive injection of $InsP_3$ pulses of brief duration at short intervals (2 sec) leads to an increased amplitude of the $InsP_3$-induced outward current. With an interval of 2 sec, the second $InsP_3$-induced outward current was increased, although the 2 responses did not overlap (Fig. 3*C*, top). The descriptive term facilitation may be used for this phenomenon. When the $InsP_3$ injections followed more closely in time (less than 2 sec), the $InsP_3$-induced outward current overlapped and summation also occurred (Fig. 3*C*, bottom; Fig. 4*B*). On the other hand, repetitive injections at short intervals of higher amounts of $InsP_3$ into neuron R12 led to a decreased amplitude of the second $InsP_3$-induced outward current, indicating that desensitization had occurred (Fig. 4*C*).

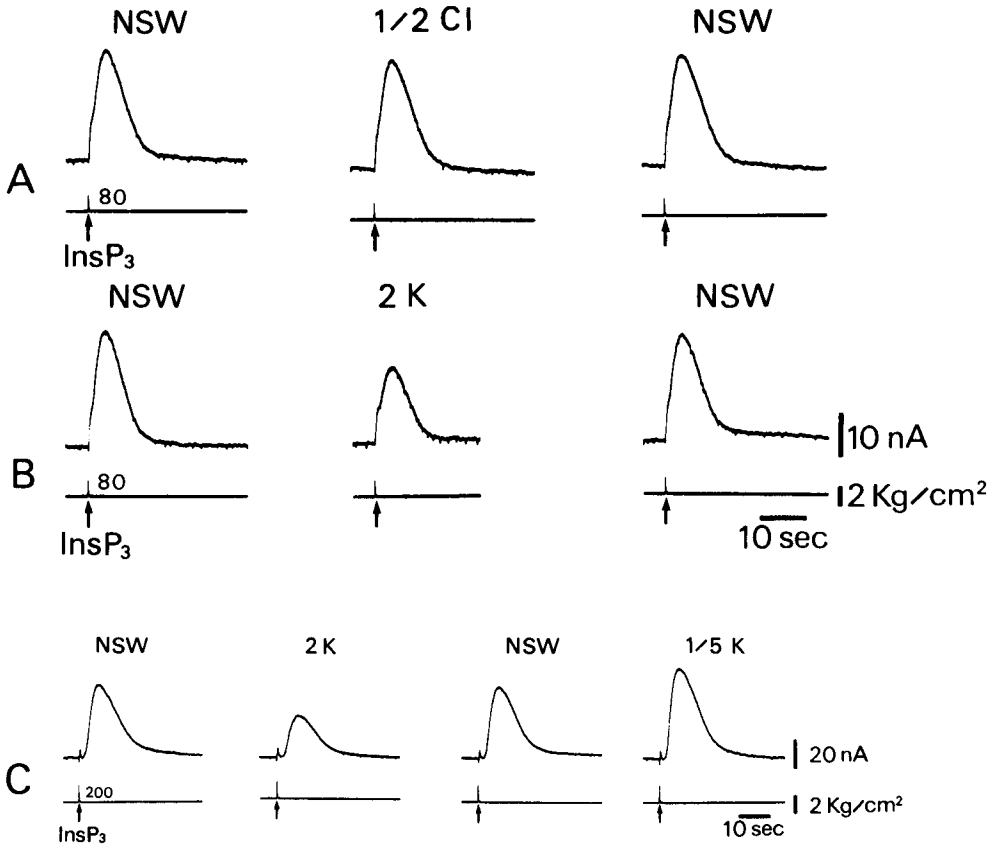

Figure 6. Lack of effect of Cl^-_0-deficient seawater (*A*) and effects of high K^+_0 seawater (*B,C*) and low-K^+_0 seawater (*C*) on the $InsP_3$-induced outward current recorded from neuron R12. Holding potentials were −45 mV in *A* and *B*, −35 mV in *C*. $InsP_3$ was injected by a single constant pressure pulse (80 msec, 2 kg/cm² in *A* and *B*; 200 msec, 2 kg/cm² in *C*). Normal seawater *NSW* represents normal *Aplysia* Ringer's in this and subsequent figures. *A, Left*: control; *center*: nine minutes after R12 was exposed to Cl^-_0-deficient (0.5× normal Cl^-) seawater; *right*: nine minutes after washout. *B, Left*: control; *center*: five minutes after R12 was exposed to high-K^+_0 (2× normal K^+) seawater; *right*: nine minutes after washout. *C, Left*: control; *center* (*left side*): six minutes after R12 was exposed to high-K^+_0 (2× normal K^+) seawater; *center* (*right side*): nine minutes after washout; *right*: six minutes after R12 was exposed to K^+_0-deficient (0.2× normal K^+) seawater.

Ionic mechanism of the $InsP_3$-induced outward current

Effect of holding potential. We have explored the voltage sensitivity of the $InsP_3$-induced outward current by clamping the same R12 neuron used in Figure 1 to various holding potentials, ranging from −40 to −80 mV, and measuring the amplitude of these currents. Figure 5*A,b* shows a plot of the $InsP_3$-induced outward current versus membrane holding potential. The relationship between the $InsP_3$-induced outward current amplitude and holding potential is not linear. The $InsP_3$-induced outward current decreased at holding potentials more hyperpolarized than −40 mV and disappeared near the predicted equilibrium potential for K^+ ions of the *Aplysia* neuron (−80 mV). In neuron R12 from another preparation, the $InsP_3$-induced outward current reversed at hyperpolarized holding potentials more negative than −80 mV (Fig. 5*B,a*). In this case, the reversal potential of the $InsP_3$-induced current was −78.5 mV (Fig. 5*B,b*). This is reasonably close to the K^+ equilibrium potential of −80 mV estimated for the molluscan neuron (*Aplysia*) with K^+-sensitive electrodes (Kunze and Brown, 1971).

To determine which ions contribute to the $InsP_3$-induced outward current, the external ionic compositions were altered and several specific blockers of ionic channels were applied.

Low Cl^-_0 effect. The effect of reducing the extracellular chloride concentration (Cl^-_0) on the $InsP_3$-induced outward current elicited by a single constant pressure injection of $InsP_3$ is illustrated in Figure 6*A*. Reducing Cl^-_0 from 671 to 335 mM (replaced with acetate) caused little change in the $InsP_3$-induced outward current recorded at a holding potential of −45 mV.

Effect of altered K^+_0. That the $InsP_3$-induced outward current reversed at ca. −80 mV suggests that the current was caused by an increase in K^+ conductance. The effects of increasing and decreasing the extracellular potassium concentration (K^+_0) on the $InsP_3$-induced outward current, elicited by a single constant pressure injection of $InsP_3$ into neuron R12, are illustrated in Figure 6, *B, C*. If the $InsP_3$-induced outward current were due simply to an increase in K^+ conductance, one would expect that the current recorded at a holding potential of −45 mV would be increased during exposure to a K^+_0-deficient solution and decreased in a high-K^+_0 solution. Increasing K^+_0 from 12 to 24 mM caused a marked decrease in the $InsP_3$-induced outward current (58.2 ± 7.5% of control, mean ± SD; $n = 3$). Conversely, decreasing K^+_0 from 12 to 2.4 mM caused an increase in the $InsP_3$-induced current (124.3 ± 11.2%, mean ± SD; $n = 3$). These results suggest that the $InsP_3$-induced outward current recorded from neuron R12 is due to an increase in K^+ conductance but not in Cl^- conductance.

Effects of TEA and 4-AP. In *Helix* neurons (Meech and Standen, 1975) and in *Aplysia* neurons (Hermann and Gorman, 1979; Hermann and Hartung, 1982), extracellularly applied TEA blocks both Ca^{2+}-activated and voltage-activated outward currents. Figure 7*A* shows an example of the $InsP_3$-induced outward current before and after external application of TEA. In 5 mM TEA, the $InsP_3$-induced outward current was reduced to about 21% of the control. This blocking effect always completely disappeared 12 min after the cessation of TEA perfusion. On the other hand, external application of 5 mM 4-aminopyridine (4-AP) had no effect on the $InsP_3$-induced outward current recorded from the same R12 neuron (Fig. 7*B*), whereas at similar concentration it blocks a voltage-dependent fast K^+ current in molluscan neurons (Thompson, 1982).

Effects of external Ca^{2+} depletion and intracellular injection

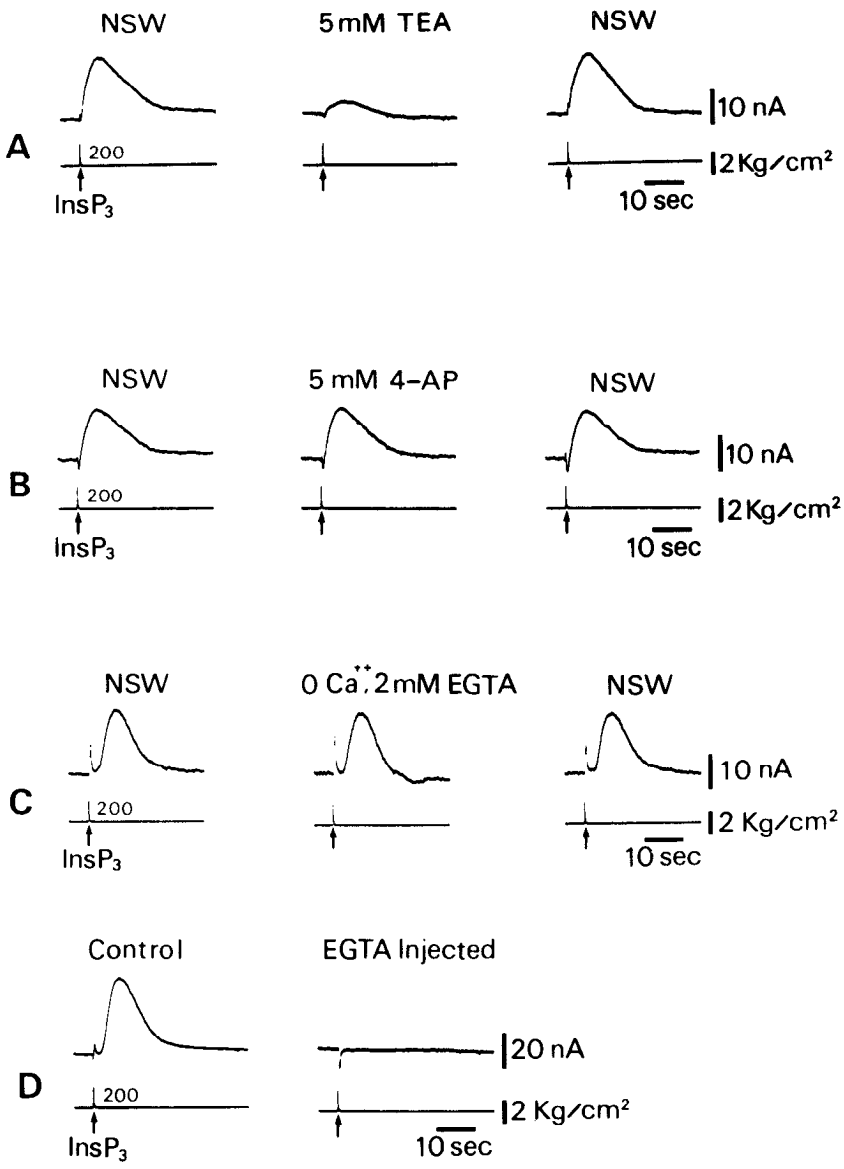

Figure 7. Effects of 5 mM TEA (*A*), 5 mM 4-AP (*B*), 0 Ca^{2+}–2 mM EGTA seawater (*C*), and intracellular injection of EGTA (*D*) on the $InsP_3$-induced outward current recorded from neuron R12. Holding potential was −45 mV. $InsP_3$ was injected by a single constant pressure pulse (200 msec, 2 kg/cm²). *A, Left*: control; *center*: nine minutes after R12 was exposed to 5 mM TEA; *right*: twelve minutes after washout. *B, Left*: control; *center*: nine minutes after R12 was exposed to 5 mM 4-AP; *right*: twelve minutes after washout. *C, Left*: control; *center*: twelve minutes after R12 was exposed to 0 Ca^{2+}–2 mM EGTA seawater; *right*: nineteen minutes after washout. *D*, $InsP_3$-induced outward current before (*left*) and after (*right*) intracellular injection of EGTA. EGTA was pressure-injected into neuron R12 with seven pulses (each pulse was of 200 msec duration and 1 kg/cm² intensity), using 1 barrel of the triple-barreled microelectrode filled with 100 mM EGTA plus 400 mM KCl. The calculated final intracellular concentration of EGTA was about 500 μM. Note that the $InsP_3$-induced outward current was greatly reduced by 5 mM TEA and completely abolished by intracellular injection of EGTA.

of EGTA. To determine whether the $InsP_3$-induced outward current was dependent on the entry of Ca^{2+} ions from the extracellular space, the effect of external Ca^{2+} depletion on the current was studied. A 0 Ca^{2+}–2 mM EGTA solution was made by substituting Mg^{2+} for Ca^{2+} and adding 2 mM EGTA to artificial *Aplysia* Ringer's. The $InsP_3$-induced outward current, recorded at a holding potential of −45 mV, persisted in 0 Ca^{2+}–2 mM EGTA *Aplysia* Ringer's (Fig. 7*C*; $n = 4$). To further evaluate the contribution of Ca^{2+}-activated conductance changes, 4 R12 neurons were pressure-injected with EGTA to chelate intracellular calcium ions. When EGTA was pressure-injected into the neuron, the $InsP_3$-induced outward current was completely abolished and the effect of internal EGTA could not be recovered by washings that lasted up to 1 hr (Fig. 7*D*). These results suggest that micropressure injection of $InsP_3$ into identified neurons of *Aplysia* can induce an outward K^+ current activated by increased intracellular free Ca^{2+} concentration, and that the $InsP_3$-induced outward current is likely to be a Ca^{2+}-activated K^+-conductance increase; however, Ca^{2+} entry from the extracellular space is not required.

Intracellular injection of Ca^{2+}

Ca^{2+}-activated K^+ current can be induced by the influx of Ca^{2+} through voltage-dependent ion channels in the cell membrane or by direct injection of Ca^{2+} into the neuron (Meech, 1978). By means of iontophoretic injection of Ca^{2+} into *Aplysia* neurons under voltage-clamp conditions it has been shown that K^+ ions are the primary charge carriers giving rise to an outward current (Gorman and Hermann, 1979; Hermann and Hartung, 1982). On the other hand, it has been reported that, using pressure injection, Ca^{2+} ions in *Helix* neurons activate an inward current followed by a prolonged outward current (Hofmeier and Lux, 1981).

In order to compare the $InsP_3$-induced outward current with the Ca^{2+}-activated K^+ current, both $InsP_3$ and Ca^{2+} ions were micropressure-injected into the same identified neuron. Intracellular injection of Ca^{2+} always caused a dose-dependent transient outward current associated with a conductance increase in neuron R12, which was voltage-clamped at a holding potential of −40 mV (Fig. 8*A*). The peak of the outward current responses

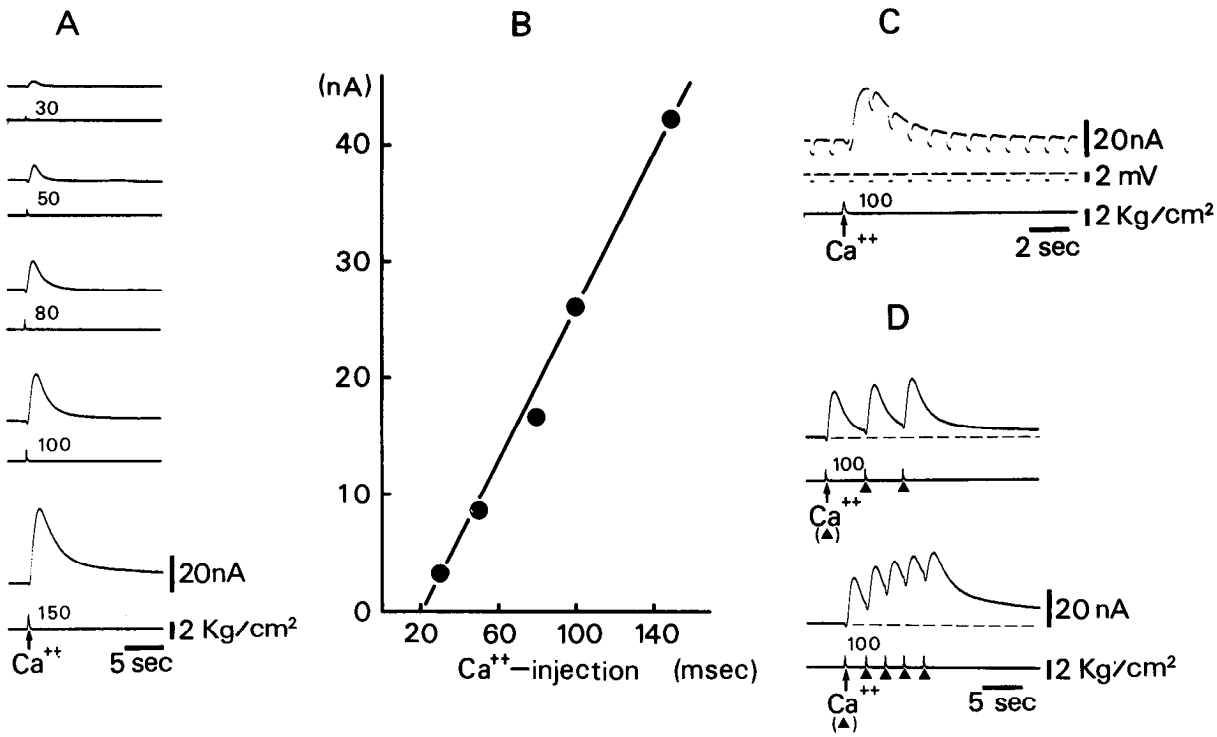

Figure 8. Ca^{2+}-activated outward currents induced by $CaCl_2$ injections of different durations at a constant pressure intensity (2 kg/cm²) into neuron R12. Holding potential, −40 mV. *A*, Membrane current responses to pressure-injected $CaCl_2$ at different pulse durations. *B*, Plot of data in *A*; relationship between the duration of pressure for the $CaCl_2$ injection and the peak of the Ca^{2+}-activated outward current. *C*, Ca^{2+}-activated outward current associated with an increase in membrane conductance. Same nomenclature as in Figure 1*B*. $CaCl_2$ was injected by a single constant pressure pulse (100 msec, 2 kg/cm²). *D*, Effect of repetitive injection of $CaCl_2$ into neuron R12. $CaCl_2$ was injected by constant pulse (100 msec, 2 kg/cm²) every 5 sec (*top*) or every 2.5 sec (*bottom*) into the same neuron. Note that the peak outward current activated by $CaCl_2$ injection rose linearly with the duration of the pressure pulse for the $CaCl_2$ injection and that repeated injection of $CaCl_2$ caused a summation.

rose linearly with the duration of the Ca^{2+} injection. The straight line fitted to the experimental points (Fig. 8*B*) had a slope of 33 nA per 100 msec-injected Ca^{2+} load, using a constant pressure intensity of 2 kg/cm². The line did not pass through the origin, which indicates that a certain minimum number of Ca^{2+} ions must be injected in order to activate the outward current. With a single injection (pressure pulse duration, 100 msec; pressure intensity, 2 kg/cm²), the calculated concentration of Ca^{2+} in neuron R12 (300 μm diameter; cell volume, 14.2 nl) was 5 μM when the injection electrode was filled with 10 mM $CaCl_2$ (see Methods in Hara et al., 1985). With increasing duration of the injection pressure pulse, the rise of the outward current was faster and the duration of the outward current prolonged.

Repetitive injections of Ca^{2+} at relatively short intervals lead to overlap and/or summation (Fig. 8*D*). Both the amplitude and the delay from the beginning of injection to the onset of the Ca^{2+}-activated outward current were dependent on the position of the injection electrode (similar to the findings for $InsP_3$ injections). Larger amplitude and shorter delay of the Ca^{2+}-activated currents were recorded when the injection electrode was positioned nearer the cell membrane than the center of the soma.

Ionic mechanism of the Ca^{2+}-activated outward current

Effect of holding potential. The Ca^{2+}-activated outward current was typically associated with an apparent increase in the membrane conductance, as illustrated in Figures 8*C* and 9*A*. At the peak of the Ca^{2+}-activated outward current, membrane input conductance increased from 170% (Fig. 8*C*) to 550% of the control (Fig. 9*A*). Figure 9*C* shows a plot of the Ca^{2+}-activated outward current recorded from neuron R12 versus membrane holding potential. The relationship between the Ca^{2+}-activated outward current amplitude and holding potential is not linear. The outward current decreased at holding potentials more hyperpolarized than −45 mV and disappeared near the predicted equilibrium potential for K^+ ions (−80 mV). These data confirm the finding of others (Hermann and Hartung, 1982; Kehoe, 1985) that the increase in K^+ conductance induced by intracellular injection of Ca^{2+} ions into certain molluscan neurons is highly voltage-dependent, disappearing at hyperpolarizing potentials.

Effects of TEA, 4-AP, and high K^+_o. Figure 10*A* presents an example of the Ca^{2+}-activated outward current before and after external application of TEA. In 5 mM TEA, the Ca^{2+}-activated outward current was reduced to 23.8% of the control. This blocking effect always disappeared completely 12 min after the cessation of TEA perfusion. On the other hand, external application of 5 mM 4-AP had no effect on the Ca^{2+}-activated outward current recorded from the same neuron R12 (Fig. 10*B*). Increasing K^+_o from 12 to 24 mM caused a marked decrease in the Ca^{2+}-activated outward current recorded from R12 (62.3 ± 8.7% of the control, mean ± SD; $n = 3$) (Fig. 10*C*). In another experiment, in which both Ca^{2+} and $InsP_3$ were successfully injected into neuron R12, bath-applied TEA, at a concentration of 2 mM for 6–9 min, reduced the Ca^{2+}-activated outward current to 61.2% of the control and the $InsP_3$-induced outward current to 44.4% of the control (Fig. 11*B*). We have not performed a sufficient number of experiments to determine whether the difference between the reduction of the Ca^{2+} and $InsP_3$ re-

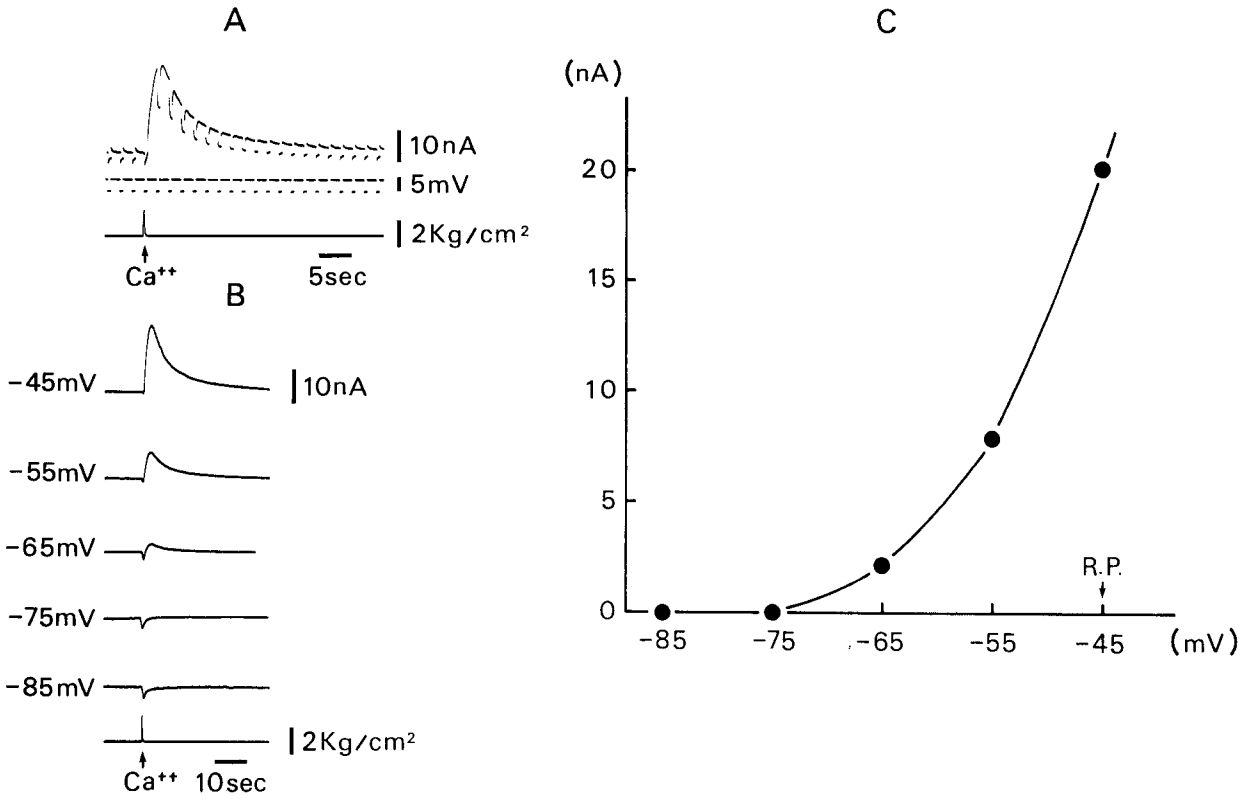

Figure 9. Ca^{2+}-activated outward current associated with an increase in membrane conductance (*A*), Ca^{2+}-activated outward currents at different holding potentials (*B*), and a plot of the voltage sensitivity of these currents recorded from neuron R12 (*C*) $CaCl_2$ was injected by a single constant pressure pulse (200 msec, 2 kg/cm²). Holding potential was −45 mV in *A*. Note that the Ca^{2+}-activated outward current decreased at hyperpolarized holding potentials more negative than −45 mV, disappeared near the predicted equilibrium potential for K^+ ions (−80 mV), and was associated with an apparent increase in membrane conductance.

sponses is significant. However, in separate experiments, the percentages of reduction were always quite similar (see above), which suggests that $InsP_3$ and Ca^{2+} modulate the same membrane channel. On the other hand, both Ca^{2+}-activated and $InsP_3$-induced outward currents recorded from the same neuron R12 were not influenced by bath application of 4-AP at a concentration of 5 mM for 6–9 min (Fig. 11*D*). This suggests that brief micropressure injection of $InsP_3$ into neuron R12 of *Aplysia* can induce an outward current associated with an increase in K^+ conductance, and that the current is identical to the Ca^{2+}-activated K^+ outward current produced by Ca^{2+} injection into the same neuron, judging from the ionic mechanism and pharmacological properties.

Comparison between the $InsP_3$-induced outward current and the Ca^{2+}-activated outward current

To evaluate further the properties of the $InsP_3$-induced outward current and of the Ca^{2+}-activated outward current, $InsP_3$ and $CaCl_2$ were micropressure-injected into the same identified neuron R12 using 2 different barrels of a triple-barreled microelectrode. First, $InsP_3$ was injected into the neuron using a single constant pressure pulse with a comparatively short duration; $InsP_3$-induced outward current was recorded as a "control" response. After sufficient time (30 sec) had passed after the first injection of $InsP_3$, $CaCl_2$ was injected into the same neuron using the other barrel. The amplitude of the Ca^{2+}-activated outward current was made identical to that of the $InsP_3$-induced outward current by adjusting the duration of the pressure pulse for the Ca^{2+} injection (Fig. 12*A,a,b*). Second, the interval between the first injection of $InsP_3$ and subsequent injection of Ca^{2+} was varied (Fig. 12*A,c–g*). Pressure-injected $InsP_3$, followed by Ca^{2+} injection at short intervals (less than 3 sec), caused an apparent increase in the outward Ca^{2+}-induced current as compared to that induced by individual Ca^{2+} injections (Fig. 12*A,c–f*). This increase could be due to summation or, alternatively, prior $InsP_3$ might potentiate a subsequent response to Ca^{2+} injection. Figure 12, *B, C* supports the hypothesis that there may be some potentiation. For example, pressure-injected Ca^{2+} followed by $InsP_3$ injection at short intervals (less than 2.5 sec) did not cause any increase in the outward current induced by $InsP_3$ (Fig. 12*B,c,d*). Furthermore, in another R12 neuron the effects were clearly distinguishable from simple summation (Fig. 12*C*). In this example, Ca^{2+} injection after preinjection of $InsP_3$ at short intervals (2.5 sec) produced a response that was larger than that produced by Ca^{2+} injection alone. These results indicate that the Ca^{2+}-activated outward current induced by injection of $CaCl_2$ is potentiated after preinjection of $InsP_3$ at short intervals. This potentiation might result from the addition of injected Ca^{2+} to the intracellular free Ca^{2+} released from stores by $InsP_3$.

Effects of W-7 on the $InsP_3$-induced and Ca^{2+}-activated outward currents

Calmodulin is the common Ca^{2+}-binding protein that mediates ubiquitous cellular responses to increases in intracellular Ca^{2+} (Kakiuchi et al., 1970; Cheung, 1981; Weiss et al., 1982). In the bag cell neurons of *Aplysia,* calmodulin inhibitors such as trifluoperazine (TFP) and *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide (W-7) have been shown to inhibit calcium/

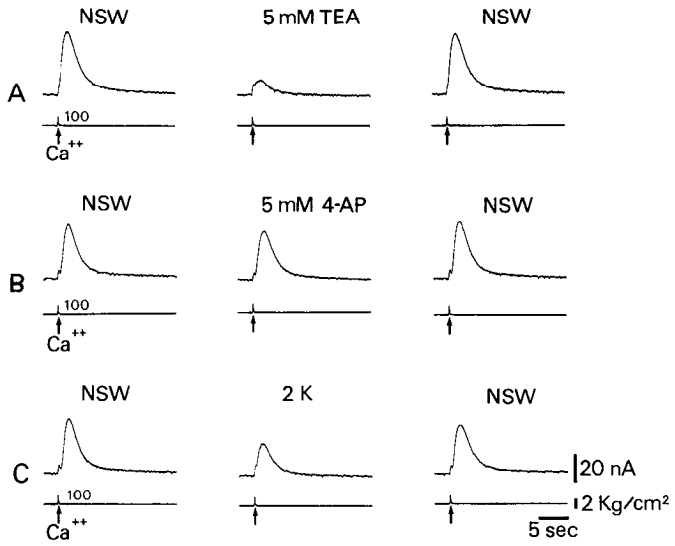

Figure 10. Effects of 5 mM TEA (*A*), 5 mM 4-AP (*B*), and high-K^+_o seawater (*C*) on the Ca^{2+}-activated outward current recorded from neuron R12. Holding potential was −40 mV. $CaCl_2$ was injected by a single constant pressure pulse (100 msec, 2 kg/cm²). *A, Left*: control; *center*: nine minutes after R12 was exposed to 5 mM TEA; *right*: twelve minutes after washout. *B, Left*: control; *center*: nine minutes after R12 was exposed to 5 mM 4-AP; *right*: twelve minutes after washout. *C, Left*: control; *center*: six minutes after R12 was exposed to high K^+_o (2× normal K^+) seawater; *right*: nine minutes after washout. Note that the Ca^{2+}-activated outward current was greatly reduced by 5 mM TEA.

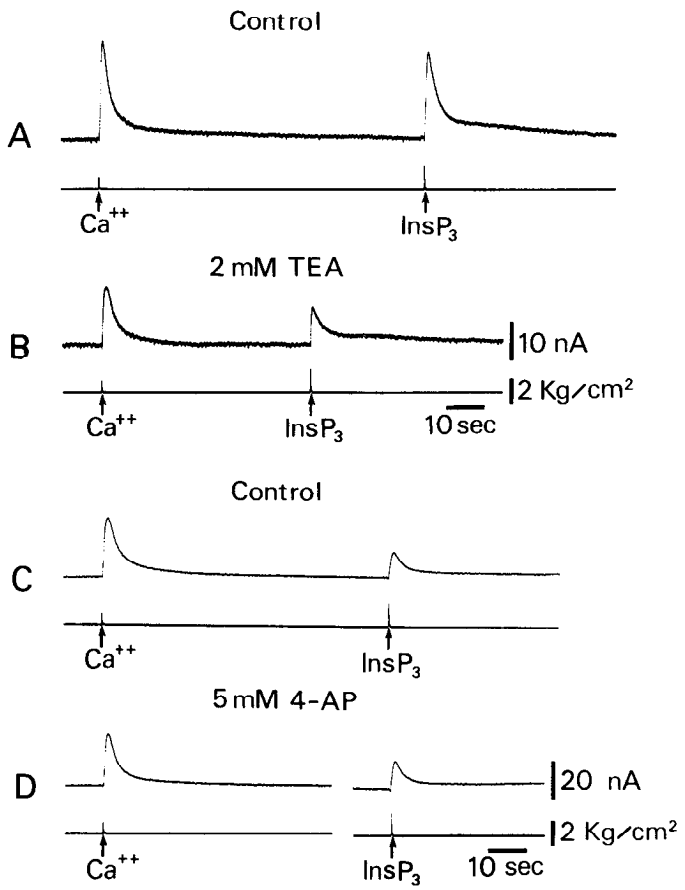

Figure 11. Effects of 2 mM TEA on the Ca^{2+}-activated and the $InsP_3$-induced outward current (*B*), and effects of 5 mM 4-AP on the Ca^{2+}-activated and the $InsP_3$-induced outward current (*D*) recorded from the same neuron R12. Holding potential was −35 mV. $CaCl_2$ was injected by a single constant pressure pulse (70 msec, 2 kg/cm²) using 1 barrel of the triple-barreled microelectrode. $InsP_3$ was injected by a constant pressure pulse (170 msec, 2 kg/cm²) using a second barrel of the microelectrode. *A, C*, Control outward current responses to $CaCl_2$ and $InsP_3$ injection. *B*, Six to nine minutes after R12 was exposed to 2 mM TEA. *D*, Six to nine minutes after R12 was exposed to 5 mM 4-AP. Note that both the Ca^{2+}-activated outward current and the $InsP_3$-induced outward current were inhibited by bath-applied TEA.

calmodulin-dependent enzymes as well as the calcium–phospholipid-dependent protein kinase *in vitro* (DeRiemer et al., 1983, 1984). Therefore, we studied the effects of W-7 on both the $InsP_3$-induced and Ca^{2+}-activated outward current recorded from the same R12 neuron.

Extracellular application of W-7 (20–50 μM) reduced both the $InsP_3$-induced and the Ca^{2+}-activated outward currents (Fig. 13). The effect of W-7 on both these currents was only partially reversible, even after prolonged (>1 hr) washings. In another neuron R12 from a different preparation, bath-applied W-7, at a concentration of 10 μM for 6 min, reduced the $InsP_3$-induced and Ca^{2+}-activated outward currents to 29.1 and 46.1% of their control values, respectively (not illustrated). Figure 14 shows the time course of the inhibitory effects of W-7 on both the $InsP_3$-induced outward current (*A*) and the Ca^{2+}-activated outward current (*B*), recorded from the same neuron R12. Bath-applied W-7 at a concentration of 50 μM for 13 min reduced the $InsP_3$-induced outward current to 17.3% of the control and the Ca^{2+}-activated outward current to 4.6% of the control. The maximal inhibitory effects of W-7 on these outward currents were obtained several minutes after washout had begun, and the effects were again only partially reversible, even after prolonged (>1 hr) washings.

Discussion

The present study demonstrates that brief micropressure injection of $InsP_3$ into identified neurons (R9–R12) of *Aplysia* induces an outward current associated with an increase in K^+ conductance, and the current shows the same ionic and pharmacological properties as those evoked by Ca^{2+} injection into the same neuron. Furthermore, the $InsP_3$-induced outward current is activated by the increased Ca^{2+} concentration resulting from mobilization of Ca^{2+} from intracellular stores, but not from Ca^{2+} in the extracellular space. Both the $InsP_3$-induced outward current and the Ca^{2+}-activated outward current in the neuron may involve a common mechanism mediated by the calcium/calmodulin-dependent enzyme or the calcium–phospholipid-dependent protein kinase for the opening of a specific K^+ channel.

The hypothesis that the $InsP_3$-induced outward current is due to an increase in K^+ conductance activated by an increased Ca^{2+} concentration via mobilization of Ca^{2+} from intracellular storage sites is supported by the following observations: (1) The $InsP_3$-induced outward current is sensitive to changes in the extracellular K^+ concentration, but not to the extracellular Cl^- concentration. (2) The $InsP_3$-induced outward current has a reversal potential near the potassium equilibrium potential (−80 mV). (3) Extracellular 2 mM EGTA in 0 Ca^{2+} does not affect the $InsP_3$-induced outward current; however, intracellularly injected EGTA completely blocks the current. (4) The $InsP_3$-induced outward current is identical to a Ca^{2+}-activated K^+ outward current induced by injection of $CaCl_2$ that was recorded from the same neuron, according to ionic dependency and phar-

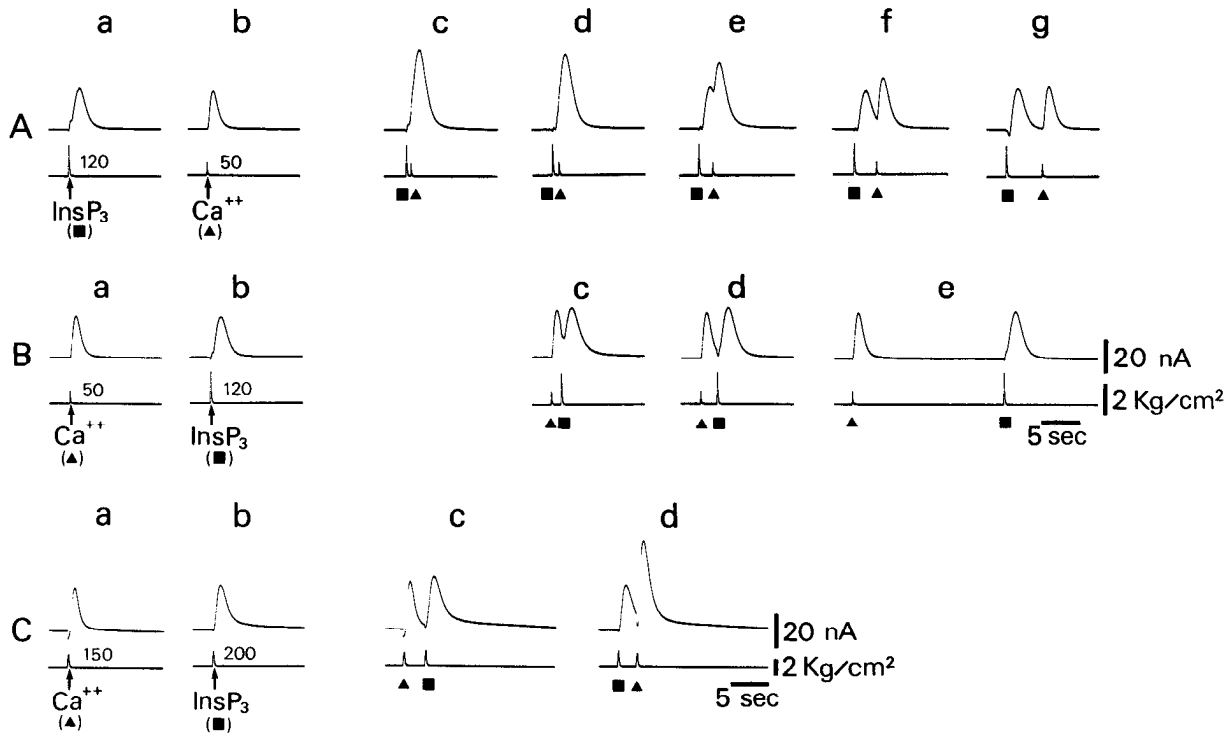

Figure 12. Comparison of the $InsP_3$-induced outward current with the Ca^{2+}-activated outward current recorded from the same neuron R12 (*A, B*) and from another neuron R12 from a different preparation (*C*). $InsP_3$ was injected using 1 barrel of the triple-barreled microelectrode and $CaCl_2$ was injected using the other barrel of the microelectrode. $InsP_3$ was injected by a single constant pressure pulse (120 msec, 2 kg/cm² in *A* and *B*; 200 msec, 2 kg/cm² in *C*). $CaCl_2$ was injected by a single constant pressure pulse (50 msec, 2 kg/cm² in *A* and *B*; 150 msec, 2 kg/cm² in *C*). Holding potential was −45 mV in *A* and *B*, −40 mV in *C*. *A,a, B,b, C,b,* Control outward current responses to $InsP_3$ injection; *A,b, B, a, C,a,* Outward current responses to $CaCl_2$ injection made to be of the same amplitude as that of the $InsP_3$-induced response by adjusting the pressure pulse duration (see text). *A,c–g,* Outward current responses to paired injection of $InsP_3$ (*closed squares*) followed by $CaCl_2$ (*closed triangles*) at different intervals. *B,c–e,* Outward current responses to paired injections of $CaCl_2$ (*closed triangles*) followed by $InsP_3$ (*closed squares*) at different intervals. *C,c,* Outward current responses to a paired injection of $CaCl_2$ (*closed triangle*) followed by $InsP_3$ (*closed square*) at short intervals (2.5 sec). *C,d,* Outward current response to a paired injection of $InsP_3$ (*closed square*) followed by $CaCl_2$ (*closed triangle*) at short intervals (2.5 sec). Note that pressure-injected $InsP_3$ followed by $CaCl_2$ injection at short intervals caused a large increase in the outward current.

macological properties (Hermann and Gorman, 1979; Hermann and Hartung, 1982).

cAMP has been shown to be the likely mediator of many transmitter-induced diminutions in K^+ conductance (Castellucci et al., 1980; Deterre et al., 1982; Siegelbaum et al., 1982). In the report of Kaczmarek and Strumwasser (1984), cAMP appears to diminish a Ca^{2+}-activated K^+ conductance as well as the rapidly inactivating, voltage-gated A-current (Strong, 1984) in the bag cells of *Aplysia*. On the other hand, serotonin, dopamine, and stimulation of the branchial nerve all cause the R15 neuron of *Aplysia* to hyperpolarize and stop bursting (Drummond et al., 1980). A series of biochemical, pharmacological, and electrophysiological experiments have satisfied all the criteria (Greengard, 1978) necessary to implicate cAMP as an intracellular second messenger for this response (Drummond et al., 1980). Furthermore, in the *Aplysia* ILD (inhibition of long duration) neurons, forskolin, a potent activator of adenylate cyclase (Seamon and Daly, 1982) mimics a dopamine-induced outward current and cAMP may play a role as second messenger mediating the dopamine-induced K^+ conductance increase (Sawada et al., 1980).

In addition to cAMP, another series of intracellular second messengers has recently been shown to function as transducing agents, relaying information from the external cell surface to intracellular enzymes for the purpose of modifying cell function and neuronal transmission. There have been 2 recent advances in our knowledge of intracellular signaling mechanisms. One of these was the discovery of a Ca^{2+}- and phospholipid-dependent protein kinase, or protein kinase C (Nishizuka, 1984), which has a greatly increased affinity for Ca^{2+} and enhanced activity in the presence of diacylglycerol. The other was the demonstration that $InsP_3$ causes Ca^{2+} release from a vesicular, nonmitochondrial pool (Streb et al., 1983; Burgess et al., 1984; Joseph et al., 1984) and has the attributes of a Ca^{2+}-mobilizing second messenger.

Hydrolysis of PI-P_2 by phospholipase C produces diacylglycerol and $InsP_3$, and these products play an essential role in cell activity as second messengers. In particular, $InsP_3$ is known to release Ca^{2+} from storage sites in a variety of cell types (Berridge, 1984; Berridge and Irvine, 1984).

Recently Oron and his collaborators (1985) demonstrated that the direct intracellular injection of $InsP_3$ into a *Xenopus* oocyte mimicked a muscarinic depolarizing chloride current. In addition, the intracellular pressure injection of $InsP_3$ into *Limulus* photoreceptors (Brown et al., 1984; Fein et al., 1984) and salamander rods (Waloga and Anderson, 1985) has been shown to mimic several aspects of the light-induced response. Furthermore, the effects of $InsP_3$, on the electrical properties of the peptidergic bag cell neurons of *Aplysia* have been investigated by Fink and her coworkers (1985), and they reported that in-

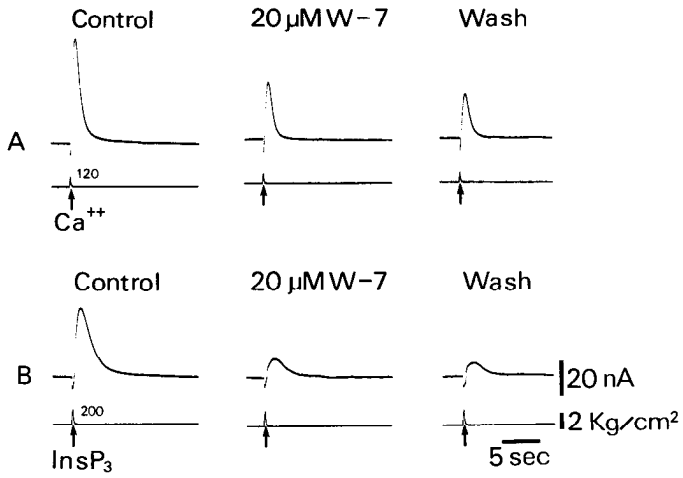

Figure 13. Inhibitory effects of 20 μM W-7 on the Ca^{2+}-activated outward current (*A*) and the $InsP_3$-induced outward current (*B*), recorded from the same neuron R12. Holding potential was −40 mV. $CaCl_2$ was injected by a single constant pressure pulse (120 msec, 2 kg/cm²) using 1 barrel of the triple-barreled microelectrode. $InsP_3$ was injected by a single constant pressure pulse (200 msec, 2 kg/cm²) using another barrel of the microelectrode. *A* and *B, Left*: control outward current responses to $CaCl_2$ and $InsP_3$ injection; *center*: six to nine minutes after R12 was exposed to W-7; *right*: forty to forty-five minutes afer washout. Note that both the Ca^{2+}-activated and $InsP_3$-induced outward current were inhibited in the presence of W-7 at low concentrations and that these inhibitory effects were only partially reversible, even after prolonged (>1 hr) washings.

tracellularly injected $InsP_3$ hyperpolarized the bag cell membrane and caused a reduction in the amplitude of action potentials evoked by depolarizing current.

The results presented in this study strongly suggest that Ca^{2+} is the intracellular mediator of the $InsP_3$-induced outward current in identified neurons of *Aplysia kurodai*. This suggestion is supported by the following facts: (1) intracellularly injected Ca^{2+} induces a dose-dependent K^+ conductance increase similar to that produced by $InsP_3$; (2) intracellular Ca^{2+} depletion by means of EGTA injection eventually inhibits the $InsP_3$-induced outward current; (3) the calmodulin inhibitor W-7 (Hidaka et al., 1981) inhibits the K^+ outward current evoked by both $InsP_3$ and by $CaCl_2$ injection. The efficacy of EGTA in buffering the internal Ca^{2+} was verified by demonstrating that the EGTA completely eliminated the Ca^{2+}-dependent K^+ current activated by depolarization of neurons in the pleural ganglion of *Aplysia* (Kehoe and Marty, 1980; Kehoe, 1985).

Because the $InsP_3$-induced outward current is persistent in 0 Ca^{2+}–2 mM EGTA seawater, it was concluded that most of the Ca^{2+} ions required for the $InsP_3$-induced K^+ outward current are mobilized from intracellular storage sites. It has been reported that the release of intracellular Ca^{2+} does not require influx of extracellular Ca^{2+} for the activation of smooth muscle by transmitter (Bond et al., 1984) and that Ca^{2+} release is not influenced by inhibitors of mitochondrial oxidative phosphorylation (Somlyo et al., 1985). Furthermore, in *Xenopus* oocytes, Dascal and his coworkers (1985) have demonstrated that neither external Ca^{2+} depletion nor the Ca channel blockers nifedipine and Mn^{2+} were able to fully block the Cl^- current evoked by ACh, and they concluded that most of the Ca^{2+} ions required for this current were mobilized from intracellular stores. Recent electron-probe analytic studies have directly demonstrated that the sarcoplasmic reticulum (SR) is the source of intracellular Ca^{2+} released by norepinephrine in the rabbit main pulmonary artery (Kowarski et al., 1985). It has recently been reported as well that $InsP_3$ releases Ca^{2+} from the sarcoplasmic reticulum of vascular smooth muscle (Suematsu et al., 1984) and the cardiac sarcoplasmic reticulum of canine ventricular muscle (Hirata et al., 1984).

It is well established that Ca^{2+} is important in regulating K^+ conductance increases in molluscan neurons (Meech 1978; Hermann and Hartung, 1982; Deitmer and Eckert, 1985) and the Cl^- conductance increase in *Xenopus* oocytes (Dascal et al., 1985).

When, in the same identified R12 neuron of *Aplysia*, the $InsP_3$-induced outward currents are compared with the Ca^{2+}-activated outward current, bath-applied TEA at 2 mM reduced these currents to about 40–60% of their control values, but neither the $InsP_3$-induced nor the Ca^{2+}-activated outward current was influenced by bath-applied 4-AP at 5 mM (Fig. 11, *B, D*). These data suggest that a parallel can be drawn between the $InsP_3$-induced outward current and the Ca^{2+}-activated outward current, although there are some components that are resistant to TEA in both the $InsP_3$-induced and Ca^{2+}-activated outward currents. A TEA-resistant, Ca^{2+}-dependent K^+ conductance has been observed previously in response to a depolarizing pulse in voltage-clamped neurons in a variety of preparations, e.g., bullfrog sympathetic ganglion neurons (Pennefather et al., 1985), neuroblastoma cells (Moolenaar and Spector, 1979), and molluscan neurons (Aldrich et al., 1979; Smith and Zuckler, 1980; Barish and Thompson, 1983; Deitmer and Eckert, 1985; Kehoe, 1985). In certain molluscan neurons, intracellularly injected Ca^{2+} ions induce a long-duration, K^+-dependent response (Meech, 1974; Hofmeier and Lux, 1981), but the sensitivity of the conductance to TEA and the possible mediation of intracellular $InsP_3$ were not evaluated.

As was shown in Figures 13 and 14, the calmodulin inhibitor W-7 almost completely inhibits both the $InsP_3$-induced outward current and the Ca^{2+}-activated outward current. These effects do not appear to be secondary to interference with changes of the resting membrane conductance by bath-applied W-7 because the resting holding current and membrane conductance of identified neurons were not affected in the presence of W-7. The inhibitory effects of W-7 on these currents were only partially reversible, even after prolonged washings (Fig. 14). In another system, *Xenopus* oocytes, Dascal and his collaborators (1985) have shown that extracellular application of TFP, the widely used calmodulin inhibitor (Weiss et al., 1982), inhibited both the Cl^- current evoked by ACh and the Cl^- current induced by intracellular injection of Ca^{2+}. The inhibitory effect of TFP on these currents was also only partially reversible, even after prolonged washings.

Calmodulin inhibitors, such as TFP and W-7, have been shown to inhibit calcium/calmodulin-dependent enzymes as well as the calcium–phospholipid-dependent protein kinase *in vitro* in the bag cell neurons of *Aplysia* (DeRiemer et al., 1984). The mechanism by which $InsP_3$ causes the opening of K^+ channels is not known. Since W-7 can inhibit the $InsP_3$-induced outward current as well as the Ca^{2+}-activated outward current, both currents, recorded from identified neurons of *Aplysia*, may involve a common mechanism mediated by the calcium/calmodulin-dependent enzyme or the calcium–phospholipid-dependent protein kinase for the final opening of a specific K^+ channel. In other systems, it has been reported that the Ca^{2+}–calmodulin complex can regulate phosphorylation of cellular proteins and

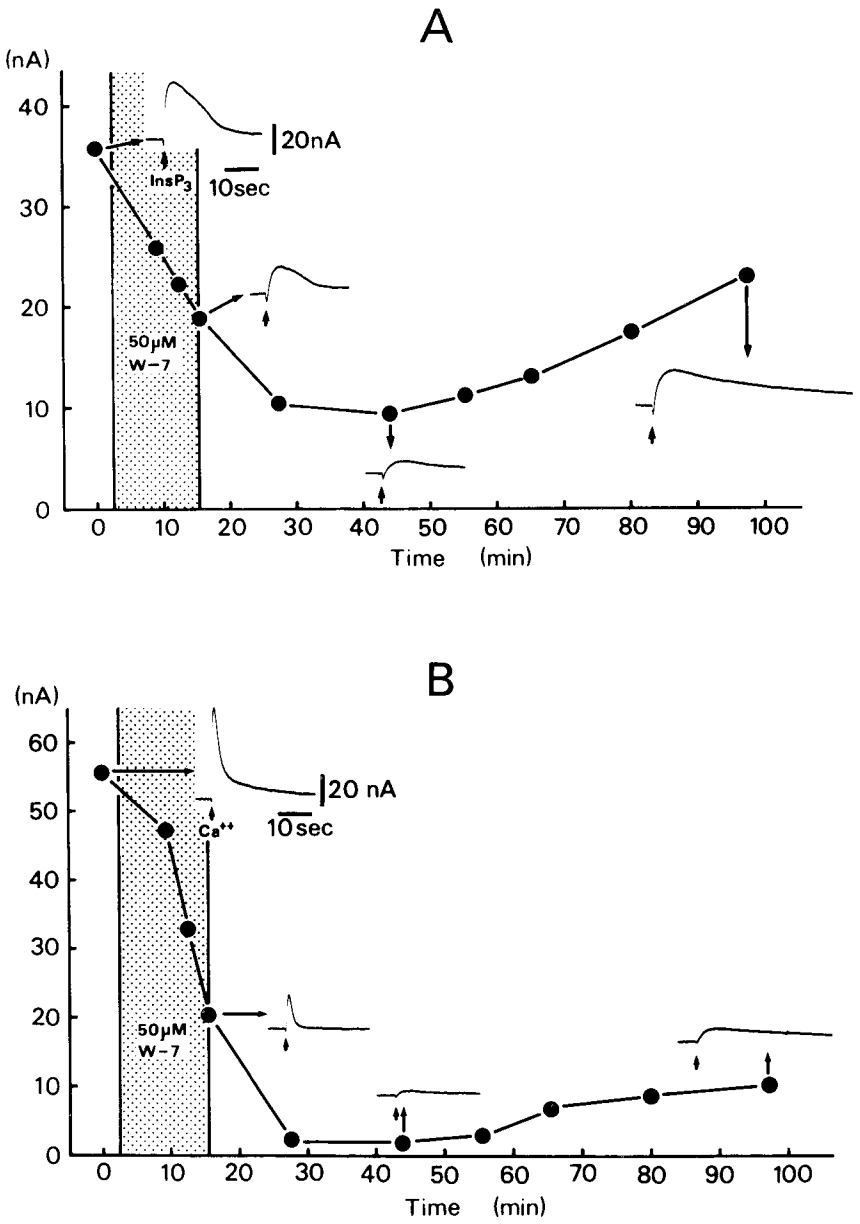

Figure 14. Time course of the inhibitory effects of 50 μM W-7 on the $InsP_3$-induced outward current (*A*) and the Ca^{2+}-activated outward current (*B*) recorded from the same neuron R12. Holding potential was −40 mV. W-7 was bath-applied for 13 min (*stippled areas*). $InsP_3$ was injected by a single constant pressure pulse (60 msec, 2 kg/cm² in *A*) and $CaCl_2$ by a single pressure pulse (30 msec, 2 kg/cm² in *B*). Note that the inhibitory effects of W-7 on these currents were, again, only partially reversible, even after prolonged (>1 hr) washings.

activate the Ca^{2+}-dependent cyclic nucleotide phosphodiesterase (Means and Dedman, 1980; Cheung, 1981; Stoclet, 1981). On the other hand, De Peyer and his collaborators (1982) have shown that addition of the catalytic subunit of cAMP-dependent protein kinase to internally perfused *Helix* neurons increases a Ca^{2+}-dependent net outward current, and have proposed that cAMP-dependent protein phosphylation regulates the Ca^{2+}-activated K^+ conductance in these neurons. It seems likely that changes in $InsP_3$ levels may coordinate a variety of biochemical changes induced by other second messengers, such as cAMP and diacylglycerol. Further studies are required to examine whether the time course and amount of intracellular $InsP_3$ production induced by the neurotransmitter are correlated with an increase in intracellular free Ca^{2+} concentration in neurons using intracellular Ca^{2+} as an intermediate second-messenger transducer.

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