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The Contributions of Protein Kinase A and Protein Kinase C to the Actions of 5-HT on the L-Type Ca^{2+} Current of the Sensory Neurons in *Aplysia*

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In the sensory neurons of *Aplysia*, 5-HT acts through cAMP to reduce current flow through two classes of K^+ channels, the S-K^+ channel and a transient K^+ channel (I_{KV}). In addition, 5-HT increases a voltage-dependent, nifedipine-sensitive Ca^{2+} current. In this article we show that, while the effect on the S-K^+ channel is mediated exclusively by cAMP, the effect on the Ca^{2+} current can be mimicked by phorbol 12,13-dibutyrate (PDBu) as well as by intracellular injection of cAMP. We then use specific blockers of protein kinase C (PKC) and the cAMP-dependent protein kinase A (PKA) to examine the roles of PKC and PKA in mediating the effect of 5-HT on the nifedipine-sensitive Ca^{2+} current.

We find that H-7, a kinase inhibitor that appears to inhibit PKC more effectively than PKA in intact *Aplysia* neurons, reverses the increase in the Ca^{2+} current produced by PDBu. Moreover, H-7 partially blocks the effect of 5-HT on the Ca^{2+} current without affecting the decrease in the S-K^+ current. A more specific PKC inhibitor (the 19–31 pseudosubstrate of PKC) also partially blocks the increase in the Ca^{2+} current produced by 5-HT, suggesting that this increase is mediated by PKC. Rp-cAMPS, a specific blocker of PKA, did not block the increase in the Ca^{2+} current produced by 5-HT, suggesting that the effect of 5-HT on this current may be mediated to only a small extent by PKA. The effect of 5-HT on the S-K^+ current and the Ca^{2+} current can also be separated on basis of the time course of their appearance. The fact that the decrease in the S-K^+ current precedes the increase in Ca^{2+} current suggests that there may be a temporal difference in the activation of the two kinase systems.

[Key words: protein kinase C (PKC), protein kinase A (PKA), Aplysia sensory neurons, phorbol esters, calcium current, second messengers, current modulation]

Serotonin (5-HT) facilitates the synaptic connection between sensory and motor neurons of the gill-withdrawal reflex of *Aplysia californica*. This facilitation is accompanied by a decrease in a background current, the S-K^+ current (Klein et al., 1982; Siegelbaum et al., 1982) as well as a transient K^+ current, I_{KV} (Baxter and Byrne, 1990; Goldsmith and Abrams, 1992; Hochner and Kandel, 1992). The effect on the S-K^+ current and on I_{KV} can be simulated by increasing the intracellular level of cAMP or of the cAMP-dependent protein kinase A (PKA). In addition to these two K^+ currents, Edmonds et al. (1987) found that 5-HT also modulates a slowly inactivating component of the Ca^{2+} current. Interestingly, modulation of this slowly inactivating current does not contribute directly to the facilitation of transmitter release (see Edmonds et al., 1990).

Since in addition to increasing intracellular cAMP (Bernier et al., 1982) 5-HT also causes translocation of an isoform of protein kinase C (PKC; Braha et al., 1990; Chirardi et al., 1990; Sacktor and Schwartz, 1990), we examined the possible role of cAMP-dependent phosphorylation via PKA and of Ca^{2+}-phospholipid–dependent phosphorylation via PKC on the serotonergic modulation of this Ca^{2+} current. For comparison, we also examined another representative current, the S-K^+ current. We find that the S-K^+ current is modulated only by the cAMP-dependent PKA (for parallel data on I_{KV}, see Hochner and Kandel, 1992, and Goldsmith and Abrams, 1992). It is not affected by the activation of PKC, nor is it blocked by H-7. By contrast, 5-HT modulation of the slowly inactivating Ca^{2+} current can be simulated by both cAMP and phorbol esters. However, the modulation of this current by 5-HT is not blocked by inhibiting PKA but is partially blocked by inhibiting PKC. Taken together, the data suggest that whereas the serotonergic modulation of the S-K^+ current is mediated by PKA, the modulation of the Ca^{2+} current seems to involve primarily PKC.

Materials and Methods

Cell preparation. Experiments were done on primary cell cultures prepared by the method described by Schacher and Proshansky (1983) and Rayport and Schacher (1986). Pleural and abdominal ganglia were dissected from adult *Aplysia* (100–150 gm) anesthetized with 50–75 ml of isotonic $MgCl_2$. The ganglia were incubated for 2–2.5 hr at 34°C in 1% protease type IX (Sigma) in L15 (modified for *Aplysia*; Flow Laboratories). After rinsing, the ganglia were transferred to a Sylgard dish containing 1% fetal calf serum in L15, or 50% L15 and 50% filtered *Aplysia* hemolymph. The ganglia were pinned and desheathed, and in-

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dividual cells were removed with a micropipette. The cells were transferred to polylysine-coated dishes containing 50% L15 and 50% hemolymph. In some voltage-clamp experiments, cells were plated on uncoated dishes and grown in modified L15 alone. These cultures grew fewer processes, which was advantageous for voltage-clamp experiments.

Materials were purchased from Sigma, unless otherwise stated. Serotonin creatinine sulfate (5-HT), was dissolved in distilled water to form a stock solution of 10 mM, and diluted in the bath solution before application. It was usually applied to the bath to give a final concentration of 10 μM. The inactive α-isomer and the active β-isomer of phorbol dibutyrate (PDBu; LC Services Corp.) were dissolved in dimethyl sulfoxide (DMSO) to form a stock solution of 10–100 mM and kept at −20°C. Before each experiment they were diluted with the recording medium and applied directly to the bath to give the specified concentration. The membrane-permeable cAMP analog 8-(4-chlorophenylthio)-cAMP (CPTcAMP) was dissolved in distilled water at 5 mM and applied to the bath to give a final concentration of 100 μM in conjunction with 100 μM isobutylmethylxanthine (IBMX) in 0.1% DMSO. Another membrane-permeable analog of cAMP, 8-benzylthio-cAMP (BTcAMP), was dissolved in distilled water at 10 mM and applied to the bath to give final concentration of 100–200 μM. 1-(5-Isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7) purchased from Seikagaku America, Inc., was dissolved in distilled water and aliquots were lyophilized and kept at 4°C. Before each experiment it was dissolved in the recording medium and applied at final concentrations of 100, 200, or 400 μM. The same aliquots were used for the biochemical assays.

Electrophysiology. Action potentials, membrane potential, and membrane resistance of sensory neurons were studied by conventional intracellular techniques, using glass microelectrodes containing 3 M KCl (resistance of 10–15 MΩ) and an Axoclamp 2A amplifier in the current-clamp configuration ("bridge mode"). The experiments were carried out in artificial seawater (ASW), which contained (in mM) 460 NaCl, 11 $CaCl_2$, 10 KCl, 55 $MgCl_2$, and 10 HEPES, at pH 7.6–7.8. Voltage-clamp experiments were done in the discontinuous single-electrode voltage-clamp mode (Axoclamp 2A), using the whole-cell recording configuration (Hamill et al., 1981). Pipette resistance was 1–2 MΩ, and sampling frequency ranged between 5 and 10 kHz. In some experiments the cells were voltage clamped using a List EPC-7 (Medical Systems Corp.). A few voltage-clamp experiments were done using the conventional two-electrode voltage clamp with electrodes containing 3 M KCl or 2 M CsCl. These experiments gave results qualitatively similar to those done in the whole-cell recording configuration. One exception is that in some of the two-electrode voltage clamps the amplitude of the measured Ca^{2+} was smaller and inactivated faster, probably due to some "washout" of the Ca^{2+} current, which was prevented in the whole-cell configuration by introduction of Ca^{2+} chelating agents. In the voltage-clamp experiments, 20 mV hyperpolarizing steps were applied to monitor the leak current. In the records shown, the leak was subtracted when indicated. The voltage-clamp experiments were carried out in ASW or in high tetraethylammonium (TEA) medium containing (in mM) 450 or 460 TEA-chloride (Kodak), 11 or 20 $CaCl_2$, 55 $MgCl_2$, 10 KCl, 10 HEPES, and 0.1 3,4-diaminopyridine (3,4-DAP). In earlier experiments, 60 μg/ml tetrodotoxin (Calbiochem) was added to block current through sodium channels, but in later ones, as all sodium was replaced by TEA, we found it unnecessary. The intracellular (pipette) solution used to fill the patch electrodes contained (in mM) 450 KCl or K-Asp, 5.1 $MgCl_2$, 100 HEPES, 10 BAPTA or 2 EGTA, 5 Na_2ATP, 0.1 Na_2GTP, 10 reduced glutathione, and 100 glucose. Cells were held at −50 mV and stepped to +10 mV every 10–20 sec. In ASW, with this holding potential, high frequency of stimulation, and depolarization steps, the I_{KV} current is mostly inactivated and is unlikely to contribute to the measured current, but other K^+ currents still obscure the Ca^{2+} current. In the high TEA medium (460 mM TEA and 0.1 mM 3,4-DAP) most K^+ channels other than the S-K^+ are blocked (Klein et al., 1982; Shuster and Siegelbaum, 1987; Baxter and Byrne, 1989), and Ca^{2+} currents can be observed. Since the high extracellular TEA did not block all of the K^+ current, in experiments where compete isolation of the Ca^{2+} current was desired, the K^+ salts of the intracellular (pipette) solution were replaced by Cs-Asp or CsCl. Confirmation of adequate blockade of all K^+ currents under these conditions came from the observation that the outward tail currents observed with K^+-containing pipette solution were eliminated (Edmonds et al., 1990).

In experiments using the PKA inhibitor Rp-cAMPS (Biolog), Ca^{2+} currents were examined with patch pipettes using the whole-cell clamp configuration (Hamill et al., 1981). The pipette solution contained (in mM) 450 CsCl, 2 $MgCl_2$, 100 HEPES, 5 Na_2ATP, 1 Na_2GTP, 10 reduced glutathionine, 100 glucose, and 10 EGTA, pH 7.3; extracellular solution contained 450 TEA-Br (Kodak), 10 KCl, 11 $CaCl_2$, 55 $MgCl_2$, and 10 HEPES, pH 7.5. In experiments where the voltage response to 5-HT was examined, KCl was used instead of CsCl in the pipette solution and the extracellular medium had the relative ionic composition of normal *Aplysia* saline, with all the concentrations reduced by 5%. In both types of experiments, two cells less than 200 μM apart were monitored at the same time; the pipette recording from one cell contained intracellular solution with 500 μM Rp-cAMPS, while the other contained the same solution without Rp-cAMPS. Responses of the two cells to the same application of 5-HT were compared.

A synthetic peptide inhibitor of PKC (PKCI), a 13 amino acid (19–31) pseudosubstrate (House and Kemp, 1987), was kindly provided by Bruce Kemp (Melbourne, Australia). Stock solutions (10 mM) of the inhibitor in distilled water were aliquoted and kept at −70°C. The peptide inhibitor was pressure injected into the sensory cells from micropipettes containing 0.5 M K-acetate with 200–500 μM PKCI and 0.2% fast green. The PKCI was injected into the cell by applying repeated 20 msec pulses of 10 psi. Injection was monitored by visual detection of fast green in the cell. Experiments were discarded in which the pressure injection caused a large increase in leakage (monitored by 20 mV hyperpolarizing pulses).

In some experiments where a fast exchange of solutions was required we applied the various drugs using a multibarrel microperfusion system similar to the one used in Boll and Lux (1985).

Data analysis. Data were recorded on magnetic tape from which records were transferred to a Gould pen recorder and to an IBM clone computer. The data were analyzed either directly from the pen records or from the tape by using the SPIKE program (Hilal Associates). Isolation of current modulated by a drug was achieved by digital subtraction of the relevant membrane currents (using the SPIKE program). Current amplitudes were measured at the peak of the current (~10 msec after the beginning of the pulse) or at the end of the pulse (100–150 msec after the beginning of the pulse). The effects of treatment were calculated by the percentage change from control [(treatment − control)/control × 100]. Statistical comparisons were performed by using the paired-sample two-tailed *t* test.

Biochemical assays of the effect of H-7 on PKC and PKA in Aplysia. PKC activity was assayed as described by Sacktor and Schwartz (1990): the neural components of the pleural-pedal ganglia of adult *Aplysia* were removed in 50% ASW and 50% isotonic $MgCl_2$. They were transferred to a glass–glass microhomogenizer containing 300 μl of homogenizing buffer at 4°C. The homogenizing buffer contained Tris-HCl, pH 7.5, 50 mM; EGTA, 1 mM; $MgCl_2$, 10 mM; 2-mercaptoethanol, 5 mM; phenylmethylsulfonyl fluoride, 0.1 mM; aprotinin, 50 Kallikrein units/ml; benzamidine HCl, 5 mM; and leupeptin, 0.1 mM (all from Sigma). The homogenate was centrifugated at 1000 × *g* for 30 sec at 4°C to remove poorly homogenized material. The supernatant was centrifuged again at 100,000 × *g* for 30 min; the supernatant (cytosol) was then diluted 1:5 with the homogenizing buffer containing Triton X-100 (0.05% final detergent concentration) and used for assaying PKC activity by histone phosphorylation. At this dilution, which contained less than 0.3 μg protein per 10μl sample (determined by Bradford microassay, Bio-Rad, Richmond, CA), the effect of endogenous inhibitors on histone phosphorylation was negligible. The reaction mixture contained Tris-HCl, pH 7.5, 50 mM; $MgCl_2$, 10 mM; PKA inhibitor type II (Sigma), 0.1 mg/ml; histone HI (III-S), 0.6 mg/ml; phosphatidylserine (Avanti Polar Lipids, Birmingham, AL), 150 μg/ml; 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), 200 nM; and 10 μl of the supernatant. The reaction was started with ^{32}P-ATP (10 μl) 50 μM (New England Nuclear, Boston, MA; 1 μCi/tube). After 30 min at 20°C, the reaction was terminated by spotting 40 μl onto Whatman phosphocellulose paper, and then washed six times (5 min each) with 500 ml of 0.425% phosphoric acid and counted by scintillation. Protein kinase C was measured in duplicate as the difference between the experimental (with phosphatidylserine and TPA) and the control (without the activators).

PKA assay. Protein kinase A was assayed in a final volume of 50 μl by phosphorylation of the synthetic peptide Kemptide. The reaction mixture contained 100 μM Kemptide, 50 mM MOPS-Na, pH 6.8, 15 mM $MgCl_2$, 0.1 mg/ml bovine serum albumin (BSA), 0.1% Nonidet P40; 0.25 mM IBMX, 1 mM D-dithiothreitol, and the neuronal supernatant. The experimental tubes contained 10 μM cAMP, while the control was with no cAMP. The reaction was carried out at 20°C and initiated by the addition of ^{32}P-ATP at various concentrations. It was

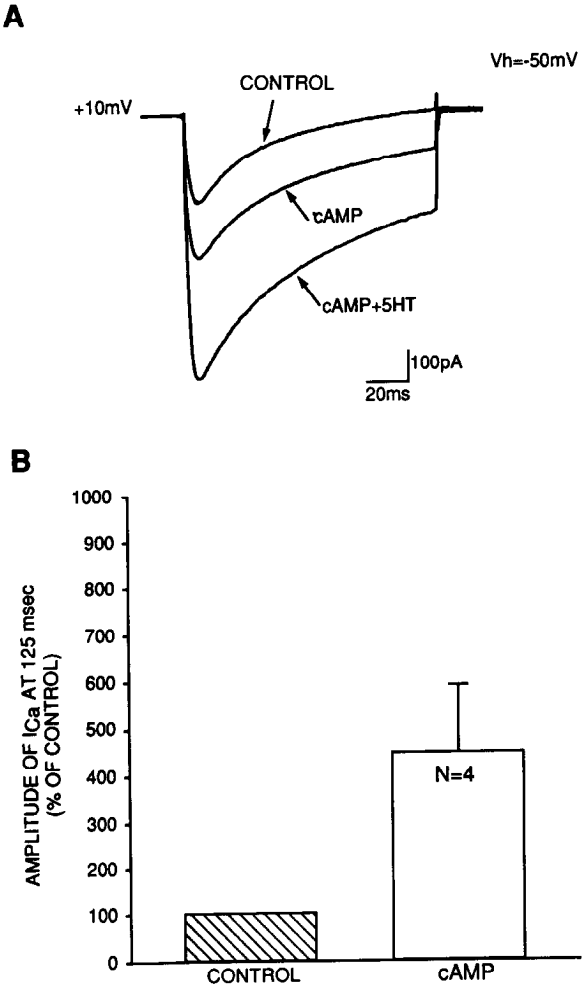

Figure 1. cAMP injections enhance the slowly inactivating Ca^{2+} current. *A*, A sensory neuron was impaled with a microelectrode containing intracellular recording solution plus 200 mM cAMP. After obtaining a stable recording with a Cs^+-containing pipette solution, 5-HT (10 μM) was added. Experiments were performed in the presence of 100μM IBMX. Leak was subtracted from the membrane currents. *B*, Group data show the average percentage increase in the sustained current (at 125 msec) obtained with cAMP injection ($n = 4$).

terminated after 5 min and 10 min, as described for the PKC assay. The activity of PKA was measured as the difference in the cpm readings of the experimental (with cAMP) and the control (without cAMP), thus subtracting phosphorylation by other kinases.

K_i values for H-7 were determined by assaying the activity (cpm) of the kinases at different concentrations of H-7 and a constant concentration of the substrate (ATP). Plots of 1/cpm as a function of [H-7] (Dixon plot) were constructed, where the intercept with the horizontal axis is equal to $-K_i(1 + [S]/K_m)$. Alternatively, the IC_{50} was determined from plots of the activity as a function of [H-7], from which the K_i value was isolated using the equation, $IC_{50} = K_i(1 + [S]/K_m)$, with $[S]$ being the ATP concentration (10μM or 1000μM). The K_m for ATP was determined by assaying the activity of the kinases at different ATP concentrations, and was found to be 15.5 μM for PKA assay and 16.5 μM for PKC assay.

Results

cAMP enhances the Ca^{2+} current in sensory neurons

Previous experiments using a two-electrode voltage clamp, and loading the cells with Cs^+ in the presence of the antibiotic nystatin, failed to reveal any direct modulation of the Ca^{2+} current by 5-HT (Klein and Kandel, 1980), probably as a result of a "washout" of the Ca^{2+} current or of components required for its modulation. Edmonds et al. (1987, 1990) have now reexamined this question using the whole-cell recording technique, with a patch pipette solution containing Cs^+ and Ca^{2+} chelating agents, that yields improved resolution of the Ca^{2+} current and prevents the "washout." With this method, they found two distinct components of Ca^{2+} current in *Aplysia* sensory neurons, a slowly inactivating current that is blocked by the dihydropyridine antagonist nifedipine, and a rapidly inactivating component that is reduced by the presynaptic inhibitory peptide FMRFamide (Edmonds et al., 1990). 5-HT selectively enhanced the slowly inactivating, dihydropyridine-sensitive current, although modulation of this slowly inactivating current did not contribute directly to the facilitation of transmitter release (see Edmonds et al., 1990).

Table 1. The dose-response relationship between the cAMP concentration in the pipette and the effect on the Ca^{2+} current measured at the peak and at the end of the pulse

cAMP concentration in the pipette	Change in peak current (nA) ± SEM	Change at the end of the pulse (nA) ± SEM
1 mM ($n = 4$)	+0.25 ± 0.18	−0.09 ± 0.08
2.5 mM ($n = 5$)	−0.04 ± 0.12	−0.22 ± 0.10
5 mM ($n = 11$)	−0.91 ± 0.21	−0.87 ± 0.16
50 mM ($n = 8$)	−0.96 ± 0.26	−0.64 ± 0.31

5-HT activates both cAMP and PKC second-messenger pathways. To explore the second messengers involved in the modulation of the Ca^{2+} current, we first examined the role of cAMP. After achieving a stable Ca^{2+} current recording with a patch pipette containing Cs^+, we injected cAMP intracellularly in the presence of 100 μM IBMX, by impaling the sensory neuron with an electrode containing the whole-cell pipette solution plus 200 mM cAMP. cAMP injection resulted in a large and rapid (10–20 sec to maximum effect) increase in the Ca^{2+} current to 446 ± 145% of initial value ($n = 4$; see Fig. 1). In contrast, 5′-AMP injection had no significant effect ($n = 4$). This current resembles the slowly inactivating nifedipine-sensitive Ca^{2+} current described by Edmonds et al. (1987, 1990) and shown to be modulated by 5-HT. Although cAMP simulates the action of 5-HT, in three experiments in which 5-HT was applied subsequent to the cAMP injection, 5-HT still produced a large enhancement of the slowly inactivating current as shown in Figure 1*A*. The failure of cAMP to occlude the response to 5-HT may be due to the fact that the injected dose of cAMP had not been saturating.

To address this possibility, we studied the dose–response relationship between the cAMP concentration in the cell and the effect on the Ca^{2+} current. Whole-cell recordings were performed with patch pipette solutions containing 1 mM, 2.5 mM, 5 mM, and 50 mM cAMP. The increase in Ca^{2+} current saturated with 5 mM cAMP in the pipette (see Table 1), while application of 5-HT still produced a larger increase in the Ca^{2+} current. These results suggest that 5-HT also utilizes another, cAMP-independent, second-messenger system to enhance the Ca^{2+} current. The findings of Braha et al. (1990), Sacktor and Schwartz (1990), Sossin and Schwartz (1992), and Ghirardi et al., (1992) suggest that 5-HT recruits PKC in the sensory neurons. We thus ex-

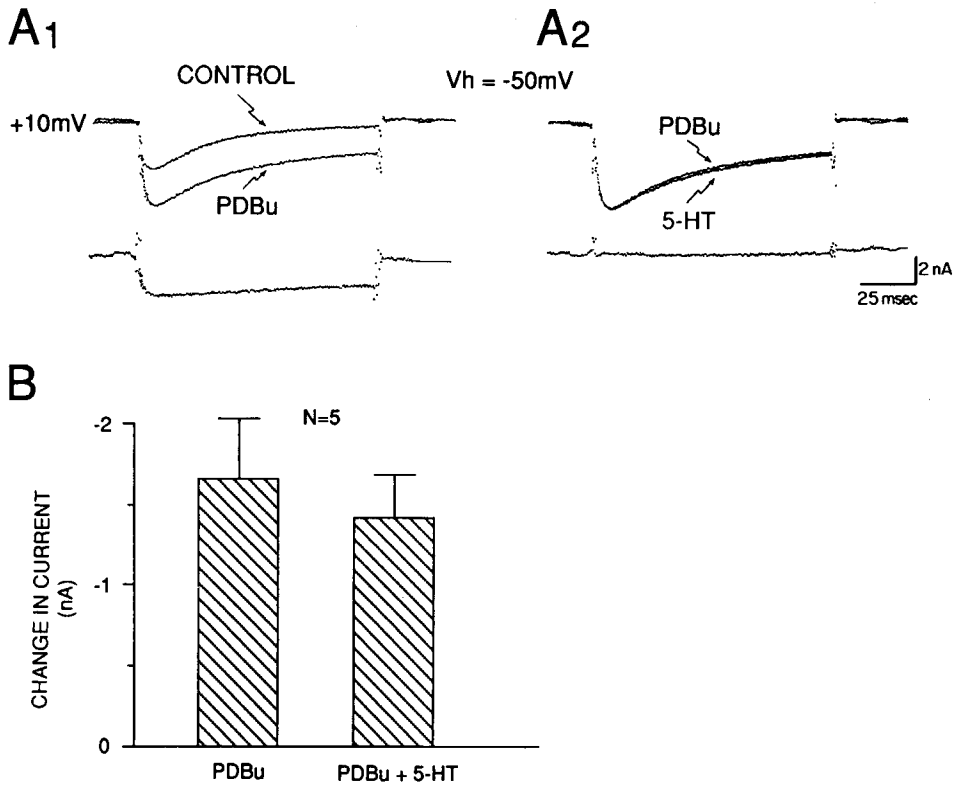

Figure 2. *A*, Application of PDBu enhances the slowly inactivating Ca^{2+} current. *1:Upper traces*, Superposition of leak subtracted membrane currents before (*CONTROL*) and 10 min after application of 20 nM PDBu. *Bottom trace*, The difference current. *2*, Application of PDBu occludes the effect of 5-HT on the slowly inactivating Ca^{2+} current. *Upper traces*, Superposition of the membrane currents in the presence of 20 nM PDBu and after application of 10 μM 5-HT (same cell as in *1*). Application of 5-HT (in the presence of PDBu) did not cause a further increase in the Ca^{2+} current. *Bottom trace*, The difference current. The small change by 5-HT is probably due to closure of residual S-K^+ channels. *B*, Group data showing the difference in peak inward current from control produced by 20 nM PDBu and by subsequent application of 10 μM 5-HT.

amined in detail the effect of PKC activation on the slowly inactivating Ca^{2+} current.

Phorbol esters also modulate the slowly inactivating Ca^{2+} current

To activate PKC, we used phorbol esters. Figure 2 shows that 20 nM PDBu enhances the macroscopic Ca^{2+} current. The difference current (Fig. 2*A1*, bottom trace) reveals that the modulated current is kinetically similar to the slowly inactivating Ca^{2+} current described in the sensory cells by Edmonds et al. (1990). The current enhanced by PDBu is blocked by 100 μM Cd^{2+}, and its voltage dependence is typical of voltage-dependent Ca^{2+} currents in the sensory neurons. The current is bell-shaped with a maximum between +10 mV and +20 mV (data not shown).

To address whether the phorbol ester–sensitive current is identical to the one modulated by 5-HT, we examined the interaction between 5-HT and phorbol ester. If the currents are distinct and independent, then 5-HT should show an increase in Ca^{2+} current after pretreatment with phorbol ester. As shown in Figure 2*A2*, when the Ca^{2+} current is enhanced by phorbol ester the response to 5-HT is occluded, suggesting that phorbol ester and 5-HT are modulating the same current. In fact, in three of five such experiments, 5-HT caused no change in the current, while in two experiments 5-HT actually decreased the peak current by 19% and 28% (see group data, Fig. 2*B*).

As is the case with the current modulated by 5-HT, the current induced by PDBu is also blocked by nifedipine (Fig. 3). In four cells, 5 μM nifedipine reduced Ca^{2+} current by an average of 0.27 nA (0.075 SEM; Fig. 3*B*; $p < 0.01$, two-tailed t test). After washout of nifedipine, 20 nM PDBu produced an average increase in the current of 0.95 nA (0.085 SEM; $p < 0.005$, two-tailed t test). Reapplication of 5 μM nifedipine in the presence of PDBu reduced the Ca^{2+} current by an average of 0.80 nA (0.135 SEM; Fig. 3*A2*; $p < 0.01$, two-tailed t test).

PDBu does not affect the S-K^+ channel, spike broadening, or afterhyperpolarization in sensory cells

Whereas the cAMP-dependent PKA modulates the S-K^+ channel (Klein and Kandel, 1980; Klein et al., 1982; Siegelbaum et al., 1982; Shuster et al., 1985), phorbol ester does not. Thus, 10 μM 5-HT causes a conductance decrease associated with a characteristic depolarization and a reduction in the afterhyperpolarization following a spike. Application of 100 nM PDBu causes neither a decrease in membrane conductance, depolarization, nor a reduction in the afterhyperpolarization (Fig. 4*A*).

In addition, concentrations of PDBu (10 nM) that produce maximal facilitation of transmitter release (Braha et al., 1990) have a negligible effect on the duration of the action potential (less than 2%), compared to the 22.7% increase produced by 5-HT (4.16 SEM, $p < 0.005$, $n = 5$) (Fig. 4*B*). PDBu also did not reduce accommodation of action potential firing in response to prolonged depolarizing pulses (Fig. 4*C*). The depolarization, decrease in membrane conductance, and increased excitability are simulated by cAMP and correlate with closure of the S-K^+ channel (Klein and Kandel, 1980; Klein et al., 1982, 1986; Ghirardi et al., 1992). The spike broadening is correlated with modulation of both I_{Kv} and the S-K^+ current (Baxter and Byrne, 1990; Goldsmith and Abrams, 1992; Hochner and Kandel, 1992). Our results, therefore, suggest that at these concentrations (10–100 nM) PDBu does not modulate the S-K^+ current. We cannot completely rule out a modulation of the I_{Kv} current by phorbol esters, as suggested by Sugita et al. (1992). However, insofar as I_{Kv} contributes to spike broadening, the negligible

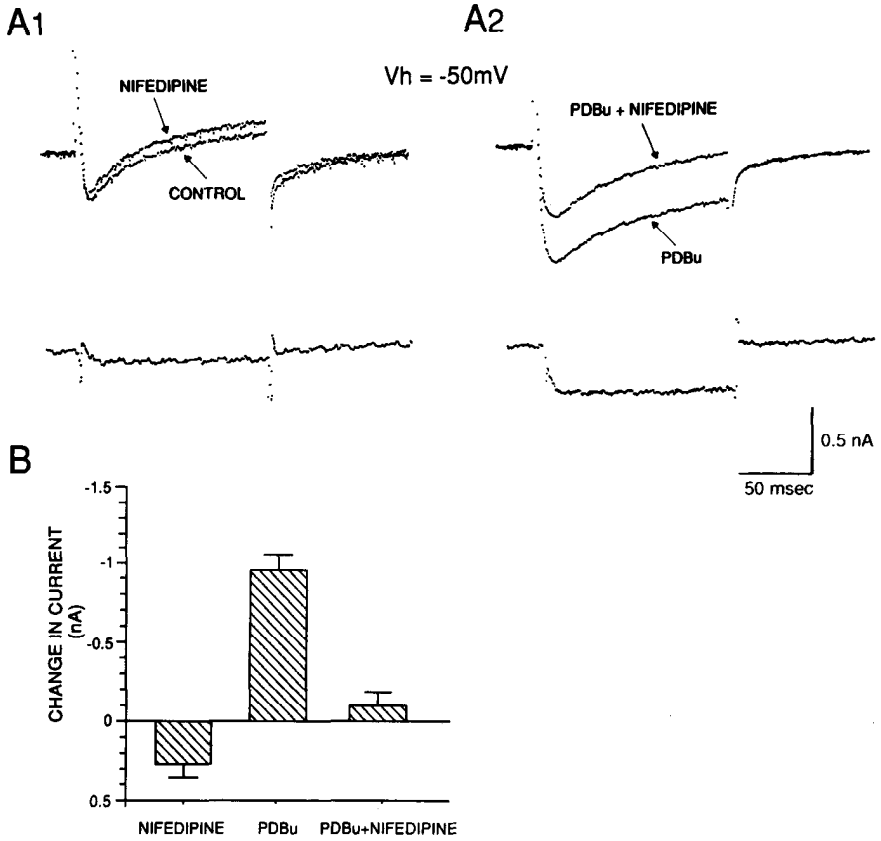

Figure 3. *A*, PDBu enhances a slowly inactivating, nifedipine-sensitive current. *1*, Superimposition of leak-subtracted membrane currents before (*CONTROL*) and after application of 5 μM nifedipine. Nifedipine causes a decrease in inward current (difference current is shown *below*). *2*, The membrane current from the same cell shown in *1*, after nifedipine was washed out, 20 nM PDBu applied, and nifedipine reapplied in the presence of PDBu. The difference current (*below*) is larger than the difference current produced by nifedipine in control. *B*, Group data showing the change in peak current produced by application of 5 μM nifedipine, by 20 nM PDBu applied after nifedipine washout, and by a second application of 5 μM nifedipine in the presence of PDBu. *Upward histograms* represent increases in inward (negative) current, while the *downward histogram* represents a decrease in inward current (n = 4).

broadening we observed suggests that at these concentrations and times of measurement (3–5 min after application) any effect of phorbol esters on I_{KV} must be small.

To examine more directly the effect of PDBu on the S-K$^+$ current, we also studied the membrane currents under voltage clamp. In normal *Aplysia* saline the outward currents produced by voltage-clamp steps to +10 mV, elicited every 10–20 sec from a holding potential of −50mV, were not affected by PDBu (Fig. 5*A1*). In the same cell, 5-HT produced a characteristic reduction in outward current, with a larger change at the end of the pulse (at 100 msec) than near the beginning. Under these conditions the I_{KV} current shown to be also modulated by 5-HT (Baxter and Byrne, 1990) is inactivated, and only the effect on the S-K$^+$ current should be expressed (see Materials and Methods). Indeed, isolation of the current decreased by 5-HT (Fig. 5*A2*) reveals a kinetically distinct S-K$^+$ current, described by Klein et al. (1982) and by Baxter and Byrne (1989). It has a slow activation; it does not reach maximum within 100 msec and shows no inactivation during the pulse. In one of five such experiments, PDBu caused a 5 nA increase in the outward current, which could be due to Ca^{2+}-activated K$^+$ current (Critz and Byrne, 1990); in the other four experiments, application of PDBu caused no significant change in the current, while 10 μM 5-HT reduced the outward current (measured at the end of a 120 msec pulse) by an average of 7.23 nA (SEM = 1.041, $p <$ 0.01).

In the presence of extracellular K$^+$ channel blockers (460 mM TEA and 0.1 mM 3,4-DAP), which block I_{KV}, the Ca^{2+}-activated K$^+$ current, and the early K$^+$ currents but do not completely suppress the S-K$^+$ current (Klein et al., 1982; Shuster and Siegelbaum, 1987), PDBu caused an increase in the nifedipine-sensitive Ca^{2+} current (Fig. 5*B1*). The increase in the slowly inactivating nifedipine-sensitive Ca^{2+} current by PDBu appears as an inward movement of the membrane current that is kinetically different from the change in membrane current due to blocking of the S-K$^+$ current: the increase at the end of a 100–150 msec pulse is equal to or smaller than the increase at the peak of the current (at around 10 msec from the beginning of the pulse). The fact that application of 5-HT in the presence of PDBu produced an additional inward movement of the current with kinetics similar to the S-K$^+$ current and accompanied by a characteristic decrease in the tail current (Fig. 5*B2*) is an additional indication that PDBu does not affect the S-K$^+$ current.

While the increase in the Ca^{2+} current produced by PDBu does not cause a change in the shape of the action potential as recorded in normal seawater, it does produce a change in the shape of the action potential when examined in the presence of K$^+$ channel blockers. In ASW solution containing 60 mM TEA and 0.1 mM 3,4-DAP, PDBu (20 nM) caused a gradual broadening of the action potential, reaching a steady state within 5–20 min. However, even here the maximal broadening was small, only 32% (SEM = 6.3, n = 11, $p < 0.005$), compared to the 260% broadening (SEM = 14.2, n = 4, $p < 0.005$) produced by 10 μM 5-HT under the same conditions, which reflects the closure of the S-K$^+$ channel. Application of 100–1000 nM α-PDBu, an isomer that does not activate PKC, did not cause any broadening.

The Ca^{2+} current increased by PDBu is reversed by H-7

In cell homogenates, vertebrate PKA and PKC are inhibited by H-7 to about the same extent (K_i = 3 μM and 6 μM, respectively; Hidaka et al., 1984). By contrast, in several intact *Aplysia* neu-

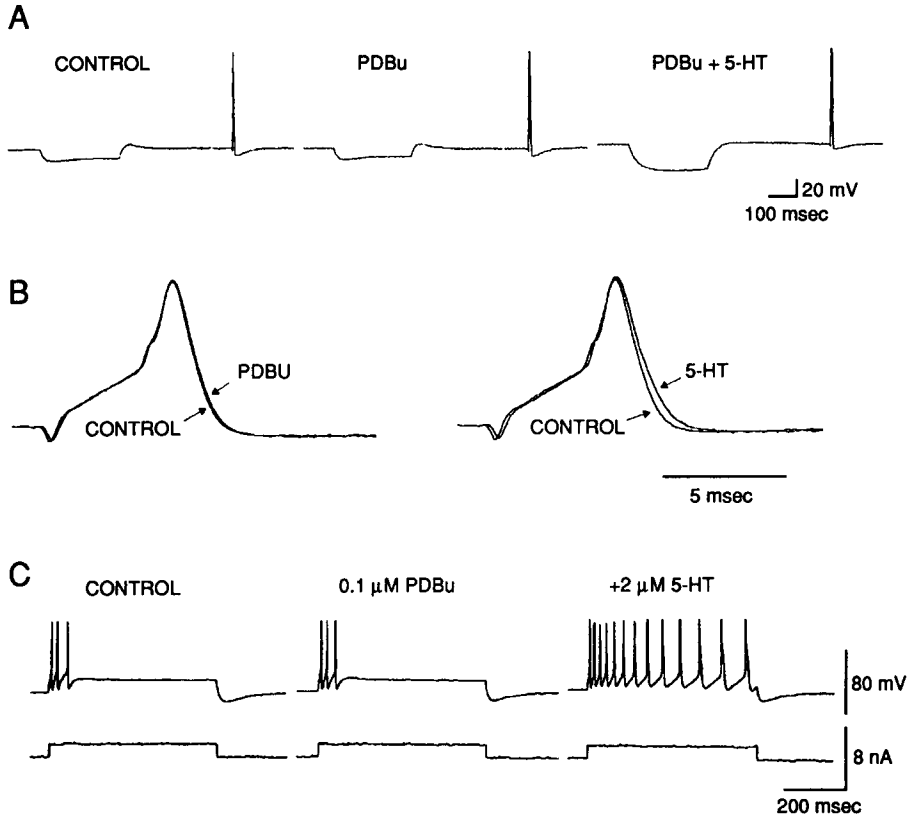

Figure 4. Comparison of the effects of PDBu with the effects of 5-HT on membrane properties of the sensory neuron in normal *Aplysia* saline. *A*, A constant, 300 msec hyperpolarizing pulse was delivered to a sensory neuron in culture to monitor changes in input resistance; a 5 msec depolarizing pulse was delivered to evoke an action potential. *Left trace* is in normal *Aplysia* saline (*CONTROL*), *middle trace* was taken 5 min after application of 100 nM PDBu, and *right trace* was taken after addition of 10 μM 5-HT in the presence of PDBu. Unlike 5-HT, PDBu did not increase the membrane resistance, did not cause a depolarization, and did not decrease the afterhyperpolarization following the action potential. The increase in input resistance produced by 5-HT was independent of the changes in the resting membrane potential. In experiments where 5-HT produced larger depolarization (than the 2 mV in the figure shown), the increase in input resistance still occurred when hyperpolarizing currents were passed to bring the membrane potential to the control levels. *B*, The action potentials from *A*, at a faster sweep speed. *Left*, Superposition of the action potential in control and in the presence of PDBu. *Right*, The action potentials in control and after application of 5-HT. The baselines were aligned for superpositioning. *C*, Constant 500 msec depolarizing pulses (*lower traces*) were delivered to the sensory neuron in culture to monitor excitability (number of action potentials produced by the depolarizing pulse). PDBu (100 nM; *middle trace*) did not increase the number of action potentials compared with control (*left traces*). Application of 2 μM 5-HT to the same cell produced a large increase in excitability.

rons, H-7 preferentially inhibits the physiological responses to activation of PKC without affecting the effects mediated by PKA (Conn et al., 1989; Taussig et al., 1989; Ghirardi et al., 1992).

We first examined the effect of H-7 on PKA and PKC in homogenates of *Aplysia* neuronal tissue and found that, similar to vertebrates, H-7 inhibits PKC with a K_i (obtained from Dixon plots) of 2.5 μM and 1.94 μM and PKA with a K_i of 4.8 μM (SEM = 0.52, $n = 3$). These experiments as well as those performed by Hidaka et al. (1984) used 10 μM ATP in the assay. According to Hidaka et al. (1984), H-7 competes with ATP for the free enzyme. Within *Aplysia* neurons the ATP concentration is more likely to be in the range of 1–3 mM (J. D. Sweatt, personal communication). We therefore assayed the inhibition of PKC produced by a constant concentration (200 μM) of H-7 and varying concentrations of ATP, and found the inhibition to be around 97% at 10 μM ATP and only 50% at 1 mM ATP (which corresponds to K_i of 3.2 μM). For PKA we found that 200 μM H-7 at 1 mM ATP caused 37% inhibition, with IC_{50} of 400 μM, which corresponds to K_i value of 6.3 μM. These results suggest that in contrast to the results in intact *Aplysia* neurons (Conn et al., 1989; Taussig et al., 1989), in homogenates the effects of H-7 on PKA and PKC are very similar. Consistent with the findings in the intact *Aplysia* neurons, we found that 200–400 μM H-7 did not reverse the increase in input resistance or the increase in excitability produced by application of CPTcAMP, a membrane-permeable analog of cAMP, suggesting that H-7 does not affect PKA in intact sensory cells (Fig. 6*A*). H-7 also did not reverse the depolarization produced by CPTcAMP, but because H-7 alone caused some depolarization in the sensory cell, we did not find this parameter suitable for assessing the interactions between PKA-mediated effects and H-7.

In contrast, 200 μM H-7 partially blocked the increase of the Ca^{2+} current in response to PDBu (Fig. 6*B*). In whole-cell clamp experiments, with patch electrodes containing Cs, 200 μM H-7 applied alone caused no change in current in two cells, and a small increase in the inward current in a third cell (0.1 nA; Fig. 6*B*). In the same three cells, application of 20 nM PDBu after washout of H-7 caused an increase of 0.99 nA (SEM = 0.01, $p < 0.001$) in the inward current. When 200 μM H-7 was then reapplied in the presence of PDBu, it reduced the inward current by 0.65 nA (SEM = 0.15, $p < 0.05$; Fig. 6*C*), which corresponds to about 66% inhibition. The current increased by PDBu and decreased by H-7 resembles the slowly inactivating nifedipine-sensitive Ca^{2+} current (Fig. 6*C*, bottom trace). Thus, H-7 causes

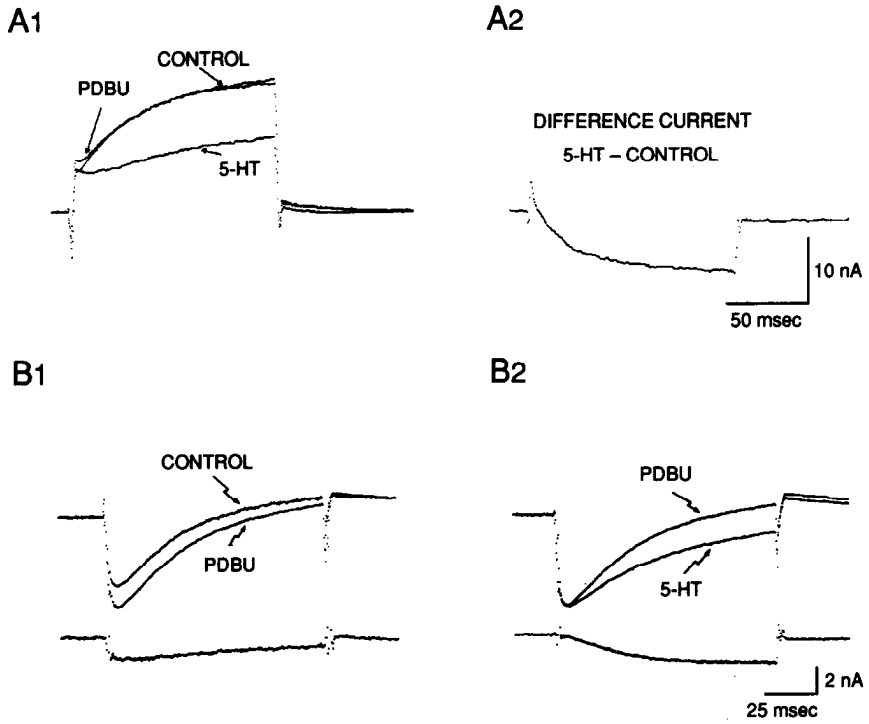

Figure 5. PDBu does not affect the S-K^+ current. *A*, The extracellular solution contained ASW. The cell was held at −50 mV and stepped to +10 mV every 15 sec. *1*, Superimposition of the membrane currents in control, after application of 20 nM PDBu and in the presence of 5-HT. PDBu did not cause a significant change in the outward current, while application of 5-HT caused a decrease in the outward current as well as a decrease in the outward tail current. *2*, A computer subtraction of the membrane current in control from the current in the presence of 5-HT. The kinetics of the 5-HT–sensitive current are similar to the kinetics of the S-K^+ current described by Klein et al. (1982) and by Baxter and Byrne (1989). *B*, The extracellular solution contained 460 mM TEA and 0.1 mM 3,4-DAP, pharmacologically blocking all K^+ currents except the S-K^+ current, which is only partially blocked. The cell was voltage clamped at a holding potential of −50 mV and stepped to +20 mV, using two-electrode voltage clamp with KCl-filled electrodes. *1:Upper traces*, Superimposition of the current before (*CONTROL*) and after application of 20 nM PDBu. *Bottom trace*, The difference current is similar in its kinetics to the nifedipine-sensitive Ca^{2+} current. *2:Upper traces*, Superimposition of the membrane currents in the presence of PDBu and in the presence of PDBu + 10 μM 5-HT (*5-HT*). *Bottom trace*, The difference current is similar in its kinetics to the S-K^+ current. The smaller magnitude of this current compared to the S-K^+ current shown in *A2* is due to the partial blockade of the S-K^+ current by high concentrations of TEA.

partial block of the effect of PDBu, suggesting that the increase in the slowly inactivating nifedipine-sensitive Ca^{2+} current produced by PDBu involves activation of a protein kinase. Also, in intact cells, H-7 reversed synaptic facilitation and the increase in spontaneous miniature synaptic potentials produced by PDBu (Braha et al., 1990; Ghirardi et al., 1992). These findings are consistent with the idea that H-7 inhibits PKC in intact sensory cells. We therefore used H-7 in an attempt to separate the effects of PKA and PKC in the response of intact cells to 5-HT.

The Ca^{2+} current increased by 5-HT is also partially blocked by H-7

As mentioned earlier, unlike most K^+ currents the S-K^+ current is not totally suppressed by high concentrations of TEA (Klein et al., 1982; Shuster and Siegelbaum, 1987), unless Cs^+ is applied intracellularly (Edmonds et al., 1990). We thus used K^+-containing pipette solutions in experiments in which both the Ca^{2+} and the K^+ currents were to be monitored. As shown in Figure 6, the slow activation of the S-K^+ current results in small changes near the onset of the current and more substantial changes at the end of the pulse. By contrast, changes in the nifedipine-sensitive Ca^{2+} current cause a change early in the step that is comparable to or larger than that at the end. Therefore, a change in current at the early peak would consist predominantly of a change in the Ca^{2+} current, while a change at the end of the pulse would consist of a mixture of the Ca^{2+} and the S-K^+ currents.

When H-7 was applied in the presence of 5-HT (using a K^+-containing pipette solution), it decreased the inward current (Fig. 7*A1*). Application of 10 μM 5-HT to four cells caused an increase of 6.3 nA in peak inward current (SEM = 1.38) and an increase of 7.5 nA at end of the pulse (SEM = 0.73). Application of 200 μM H-7 to the same four cells in the presence of 5-HT caused a decrease in peak inward current of 4.88 nA (SEM = 0.59) and a decrease of 3.9 nA at the end of the pulse (SEM = 0.414) (Fig. 7*A2*), corresponding to 81% inhibition of the effect of 5-HT at peak current (SEM = 9.3%, $p < 0.01$) and 54% inhibition at the end of the pulse (SEM = 9.5%, $p < 0.02$). Figure 7*A1* shows an example of one experiment. H-7 reversed the current by a similar or smaller magnitude at the end of the pulse than at the peak, as would be expected for an effect on a slowly inactivating, nifedipine-sensitive Ca^{2+} current.

To examine the effect of H-7 on the increase in Ca^{2+} current, we used Cs^+-containing pipette solutions. In two experiments H-7 reversed the 5-HT–induced increase in peak inward current by 35% and 86%, respectively. We also tested the effect of H-7 on the S-K^+ current directly by using a K^+-containing pipette solution in the presence of the extracellular Ca^{2+} channel blocker Cd^{2+} (0.2 mM). In two experiments, 100 μM H-7 caused reversal of 13.2% and 6.2% of the effect of 5-HT on the membrane

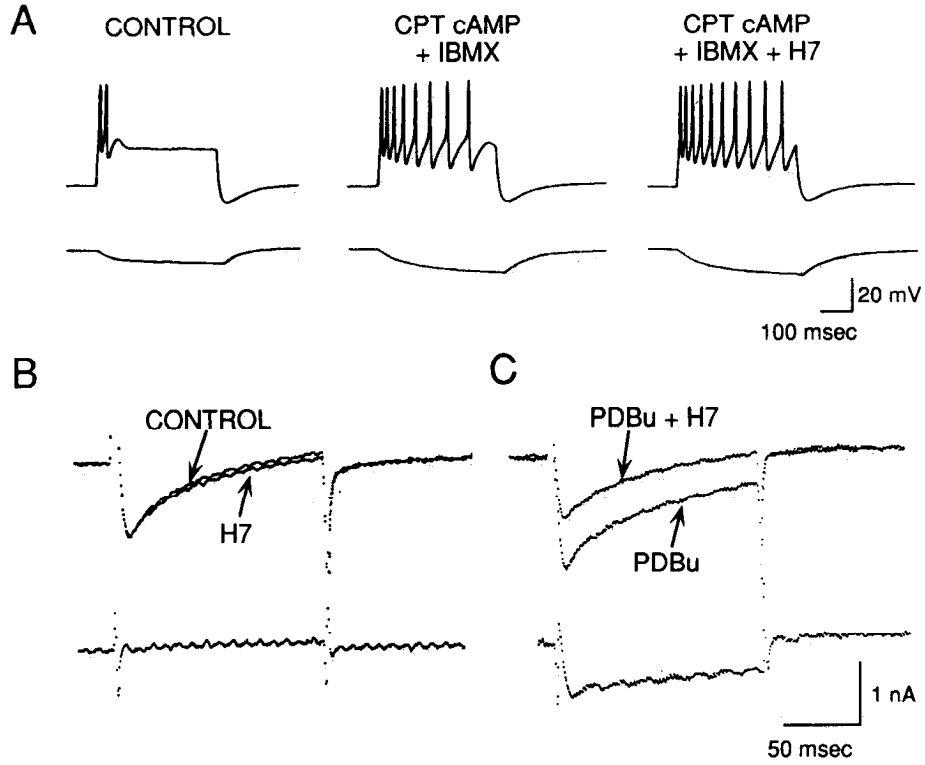

Figure 6. *A*, In intact sensory cells, H-7 does not reverse cAMP-mediated effects. Increases in excitability and membrane resistance (*bottom traces*) of a sensory neuron were monitored for expression of cAMP-mediated effects produced by application of the membrane-permeable cAMP analog CPTcAMP (100 μM) in the presence of the phosphodiesterase inhibitor IBMX (100 μM). The analog produced an increase in excitability as well as an increase in the membrane resistance (*middle traces*), both independent of changes in membrane potential. Application of 400μM H-7 in the presence of CPTcAMP and IBMX did not reverse those effects (*right traces*); H-7 usually produced a further increase in excitability when applied in the presence of cAMP. When applied alone (not shown), H-7 caused some depolarization but no increase in excitability. *B* and *C*, The kinase inhibitor H-7 reverses the increase in Ca^{2+} current produced by PDBu. *B*, Membrane currents were elicited in the presence of 460 mM TEA and 0.1 mM 3,4-DAP in the extracellular medium and a pipette solution containing Cs^+ (holding potential −50 mV, step to +10 mV). Application of 200 μM H-7 caused a small increase in the inward current (in two other such experiments it had no effect). The *bottom trace* is the computer difference of the current in the presence of H-7 and the control. *C*, Second application of 200 μM H-7 in the same cell after wash and reapplication of 20 nM PDBu. H-7 in the presence of PDBu reduced the inward current. The difference current (*bottom trace*) has the same kinetics as the nifedipine-sensitive Ca^{2+} current. Leak was subtracted from the membrane currents.

current (measured at the end of the pulse), in contrast to the 81% reversal seen when Ca^{2+} channels are not blocked. These experiments suggest that H-7 has little or no effect on the decrease in the S-K^+ current caused by 5-HT.

A peptide inhibitor of PKC also decreases the Ca^{2+} current increased by 5-HT

Because of the potential nonspecificity of H-7 as demonstrated by our biochemical results, it was important to study the effect of other PKC inhibitors. We thus studied the effect of a 13 amino acid peptide inhibitor (PKCI) synthesized by House and Kemp (1987) that acts as a pseudosubstrate and has a high affinity for PKC relative to other kinases in mammals. Specifically, it has 1000 times higher affinity for PKC than for PKA (House and Kemp, 1987). We assayed the effect of PKCI in a homogenate preparation of pleural-pedal ganglia of *Aplysia*. Five micromolar PKCI inhibited the *Aplysia* PKC by 60%, and 100 μM PKCI caused 80% inhibition of PKC, similar to the amount of inhibition found in the mammalian PKC by House and Kemp (1987).

We monitored both the S-K^+ current and Ca^{2+} currents. We whole-cell clamped the neurons using a pipette solution containing KCl. 5-HT (10 μM) caused an increase in inward current. After the effect reached steady state, the cell was penetrated with a microelectrode containing 200–500 μM PKCI in 0.5 K-acetate and 0.2% fast green. The PKCI was injected into the cell by applying repeated 20 msec pulses of 10 psi, causing a decrease in the inward current (Fig. 7*B*).

Pressure injection ($n = 6$) of PKCI caused a decrease of 2.15 nA in the peak current (SEM = 0.371), corresponding to a 62.5% inhibition of the effect of 5-HT (SEM = 7.86, $p < 0.001$), and a decrease of 2.16 nA in the current measured at the end of the pulse (SEM = 0.408), corresponding to 54.1% inhibition of the effect of 5-HT (SEM = 4.81, $p < 0.001$). The current blocked by the injections resembles the nifedipine-sensitive Ca^{2+} current. Control injections from electrodes containing 0.5 K-acetate and 0.2% fast green with no inhibitor also caused a decrease in the inward current that resembled the nifedipine-sensitive Ca^{2+} current, but this (measured at the peak) was smaller (0.95 nA, SEM = 0.25 nA, $n = 5$; corresponding to a 30.5% decrease of the effect of 5-HT, SEM = 9.53%) and statistically different from the experimental results ($p < 0.025$ for absolute current and $p < 0.001$ for percentage of 5-HT inhibition). The decrease in the inward current as a result of the pressure injection may be due to damage to the cell that could have led to an increase in the intracellular Ca^{2+} concentration. Nonetheless, the difference between the control and the experimental data suggests that the PKCI injection caused a net inhibition of the 5-HT effect on the Ca^{2+} current. The similarity between the effects of

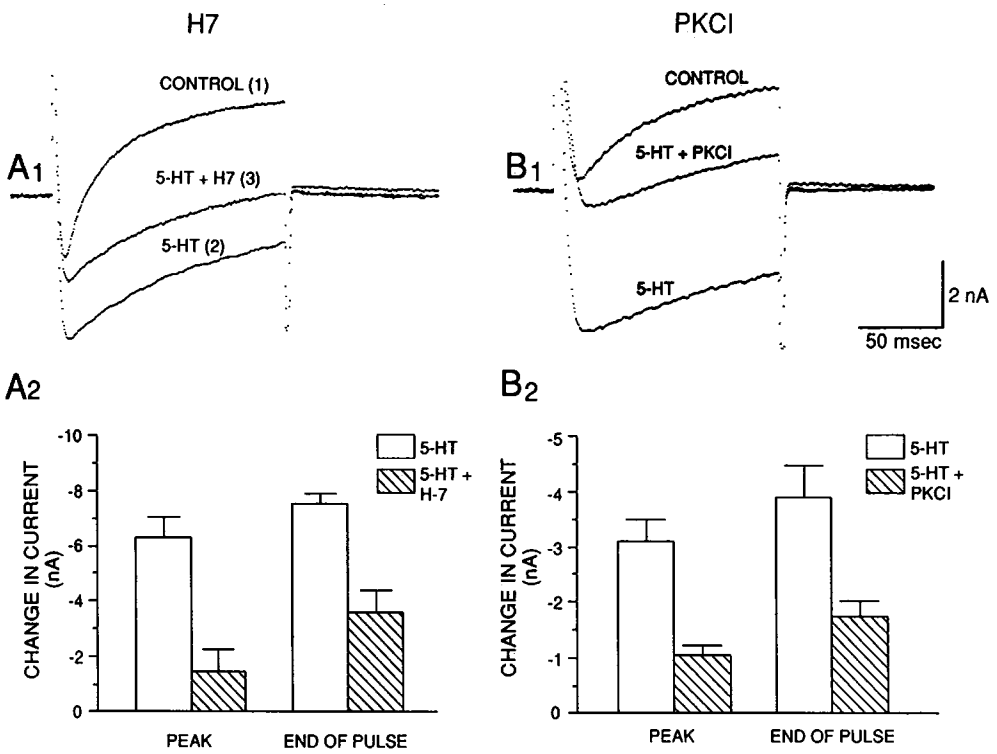

Figure 7. *A*, H-7 reverses the increase in Ca^{2+} current by 5-HT but does not reverse the effect on the S-K^+ current. *1*, The cell was voltage clamped (in the presence of 460 mM TEA and 0.1 mM 3,4-DAP) with a KCl-containing pipette. Membrane currents in control (*CONTROL, 1*), 2 min after application of 10 μM 5-HT (*5-HT, 2*), and 2 min after addition of 200 μM H-7 in the presence of 5-HT (*5-HT+H-7, 3*) are superimposed. *2*, Average of four experiments, showing the difference from control of inward (negative) current after application of 10 μM 5-HT and after subsequent application of 200 μM H-7. Measurements at the peak consist predominantly of changes in the Ca^{2+} current, while measurements at the end of the pulse consist of changes in both the Ca^{2+} current and the S-K^+ current. H-7 reverses the effect of 5-HT. Unlike the effect of 5-HT, the effect of H-7 on the peak current is not significantly different from the effect on the current at the end of the pulse ($p > 0.2$), suggesting that H-7 affects the nifedipine-sensitive Ca^{2+} current without affecting the S-K^+ current. *B*, Pressure injection of the peptide inhibitor of PKC (*PKCI*) reverses the increase in the Ca^{2+} current by 5-HT. The cell was voltage clamped with a KCl-containing pipette at a holding potential of −50 mV, and the current responses to 100–500 msec voltage steps to +10 mV were recorded. *1*, Superimposition of the membrane currents from one cell before application of any drug (*CONTROL*), 2 min after application of 10 μM 5-HT (*5-HT*), and after intracellular injection of PKC. *2*, Average of six experiments, showing the changes in inward current produced by application of 10 μM 5-HT and subsequent pressure injection of PKCI. Changes at the peak and at the end of the pulse were of similar magnitude, suggesting that like H-7, PKCI affects the nifedipine-sensitive Ca^{2+} current.

the two different inhibitors, H-7 and PKCI, suggests that they both exert their effect by inhibiting PKC.

Inhibition of PKA does not decrease the Ca^{2+} current increased by 5-HT

As we have seen (Fig. 1), the Ca^{2+} current is also increased by cAMP. We therefore examined the possible involvement of PKA in the Ca^{2+} current response to 5-HT using Rp-cAMPS, a PKA inhibitor that competes with cAMP for the binding site on the regulatory subunit of the kinase. The inhibitor (500 μM) was added to the Cs^+-containing pipette solution and sensory neurons were voltage clamped in the whole-cell clamp configuration (Hamill et al., 1981). In paired control cells tested at the same time, the inhibitor was omitted. 5-HT was applied at 5 μM and the effect on the current elicited by a 150 msec step to +10 mV or +20 mV was examined at both the peak of the inward current and at the end of the step. In 10 such experiments (one of which is shown in Fig. 8*A*), there was no significant difference between the 5-HT responses of the control cells and those into which the inhibitor had been introduced [increase in peak current (nA ± SEM) was 0.94 ± 0.26 with inhibitor vs 0.67 ± 0.18 for control; increase at 150 msec was 0.33 ± 0.10 with inhibitor vs 0.35 ± 0.15 for control; $p > 0.2$ for both; Fig. 8*B*]).

To verify that the inhibitor entered the cells and was effective in blocking PKA, a set of control experiments was carried out using K^+-containing pipette solution in conjunction with 95% normal ASW extracellularly and the response of the membrane potential to 5-HT was examined (Fig. 8*C*). Application of 5-HT caused depolarizations of 4.38 ± 0.54 mV (±SEM) in controls cells and 1.66 ± 0.49 mV in cells with the inhibitor (18 pairs, $p < 0.001$), a reduction of approximately 62% of the response to 5-HT.

In a further set of experiments on the Ca^{2+} current response to 5-HT, 1 μM 5-HT was used in order to ensure that the response was far from saturation. Again, in nine pairs of cells using the Cs^+-containing pipette solution, there was no significant difference between the responses of control cells and those with the inhibitor, despite the fact that the average 5-HT response was much smaller than with 5 μM [increase in peak current (nA ± SEM) was 0.09 nA ± 0.04 for cells with inhibitor vs 0.08 ± 0.05 for controls; increase at 150 msec was 0.07 ± 0.2 for cells with inhibitor vs 0.12 ± 0.07 for controls; $p > 0.2$ for both].

The effects of 5-HT on the K^+ and Ca^{2+} currents develop in two temporally distinct phases

When we bath applied 10 μM 5-HT and studied the effects on the membrane current (with K^+-containing pipettes), we noticed that the inward movement of the current caused by 5-HT oc-

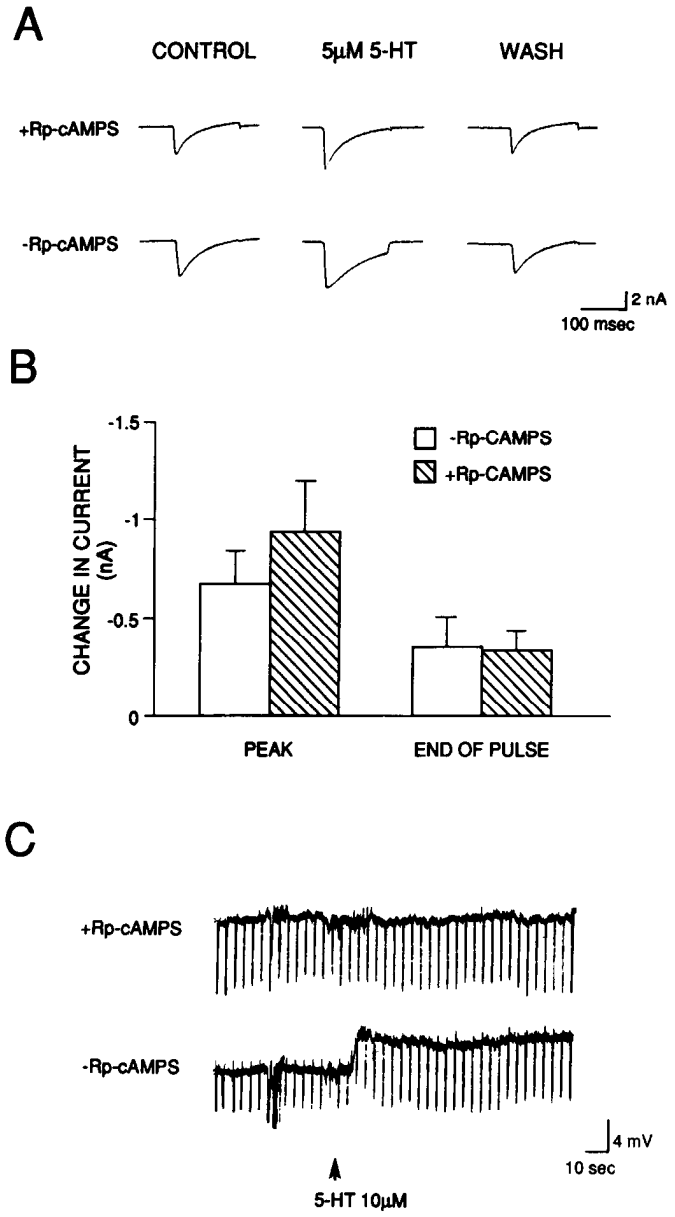

Figure 8. *A*, Intracellular introduction of the PKA inhibitor Rp-cAMPS does not reduce the increase in Ca^{2+} current caused by 5-HT in sensory neurons. Two cells less than 200 μm apart were voltage clamped in the whole-cell configuration at −50 mV and stepped to +20 mV for 150 msec every 30 sec; the pipette used to clamp the cell of the *top traces* contained Cs^+ intracellular solution with 500 μM Rp-cAMPS, while the cell of the *bottom traces* contained the same solution without Rp-cAMPS. *B*, Average of 10 experiments showing the change in current produced by 5 μM 5-HT in pairs of cells with and without Rp-cAMPS (as described in *A*). The increase in inward current by 5-HT is not blocked by the PKA blocker. *C*, Intracellular Rp-cAMPS blocks the depolarization caused by 5-HT. Two cells were monitored at the same time with K^+ intracellular solution in the recording pipettes. The pipette solution used in the cell at the top contained 500 μM Rp-cAMPS; the *bottom records* are from the paired control cell. Ten micromolar 5-HT was applied in this experiment. The downward deflections are responses to hyperpolarizing current pulses used to monitor membrane resistance.

curred in two temporally distinct phases (Fig. 9*A*,*B*). A first inward movement in current (marked as "1st in 5-HT") occurred within 10 sec after application of 5-HT. This change is accompanied by an inward movement of the holding current and reduction in the amplitude of the outward tail current, which are also characteristic expressions of reduction in the S-K^+ current induced by 5-HT (Klein et al., 1982; Baxter and Byrne, 1989). Moreover, the difference current at this time resembles the S-K^+ current in its kinetics (Fig. 9*A*, bottom trace; Klein et al., 1982). A second movement in inward current occurs over the next 30 sec (Fig. 9*B*, "2nd in 5-HT"). This additional change in current can be isolated by subtracting the membrane current of the "1st in 5-HT" from the "2nd in 5-HT" (Fig. 9*B*, lower trace). The second movement in the current is usually not accompanied by a change in the holding current or a change in the tail current; it activates more rapidly and resembles the slowly inactivating nifedipine-sensitive Ca^{2+} current.

While the effect of 5-HT on the S-K^+ current reaches its maximum within 10–30 sec, the increase in the later current is gradual, reaching a steady state within 2–4 min. Figure 9*C* shows the time course of development of the inward current elicited in response to 5-HT, measured as difference current at 10 msec from the beginning of the pulse. The first change was small (first point on the graph) and resembled the S-K^+ current. The other points represent difference currents that resembled the nifedipine-sensitive Ca^{2+} current.

Further evidence that the secondary inward movement was due to Ca^{2+} current modulation comes from experiments with the inorganic Ca^{2+} channel blocker Cd^{2+}. Figure 10*A* (top traces) shows that in the presence of 200 μM Cd^{2+}, only the first inward movement in the current occurs, the current that resembles the S-K^+ current (Fig. 10*A*, bottom trace). The second phase never developed in the presence of Cd^{2+}, providing evidence that the second phase is a Ca^{2+} current. Application of the membrane-permeable cAMP analog BTcAMP (200 μM) in the presence of Cd^{2+} simulated the decrease in the slowly activating current (the S-K^+ current, not shown). In the absence of Cd^{2+}, BTcAMP also appears to affect the slowly activating (S-K^+) current with little or no effect on the rapidly activating Ca^{2+} current (Fig. 10*B*). In three of six experiments, the cAMP analog caused what appears to be a small increase in the Ca^{2+} current, manifested as the inward movement at the peak current (compare Fig. 10*A*,*B*, top traces). Application of 10 μM 5-HT in the presence of BTcAMP caused an additional increase in the inward current (the second phase, Fig. 10*C*), with comparable changes at the peak of the current and the end of the pulse, resembling the rapidly activating Ca^{2+} current.

As described above, direct injection of cAMP into the sensory neurons produces a large increase in a slowly inactivating Ca^{2+} current. The discrepancy between the results shown here and the direct introduction of cAMP may imply that the effect of cAMP on the Ca^{2+} channel requires higher concentrations of cAMP than does the modulation of the S-K^+ channel. In agreement with the possibility that the analog application does not reach an effective concentration comparable to the direct injection of cAMP, we find that in most of our experiments the membrane-permeable analog was not as potent as 5-HT in producing its effect on the S-K^+ current. This conclusion, together with our results with Rp-cAMPS, suggests that the modulation of the Ca^{2+} channels by cAMP may not be mediated by PKA, but rather may involve direct modulation of the Ca^{2+} channels by cAMP (DiFrancesco and Tortora, 1991), or perhaps activation by cAMP of other kinases.

Discussion

This study indicates that the effects of 5-HT on the currents of the sensory cells seem to involve at least two different second

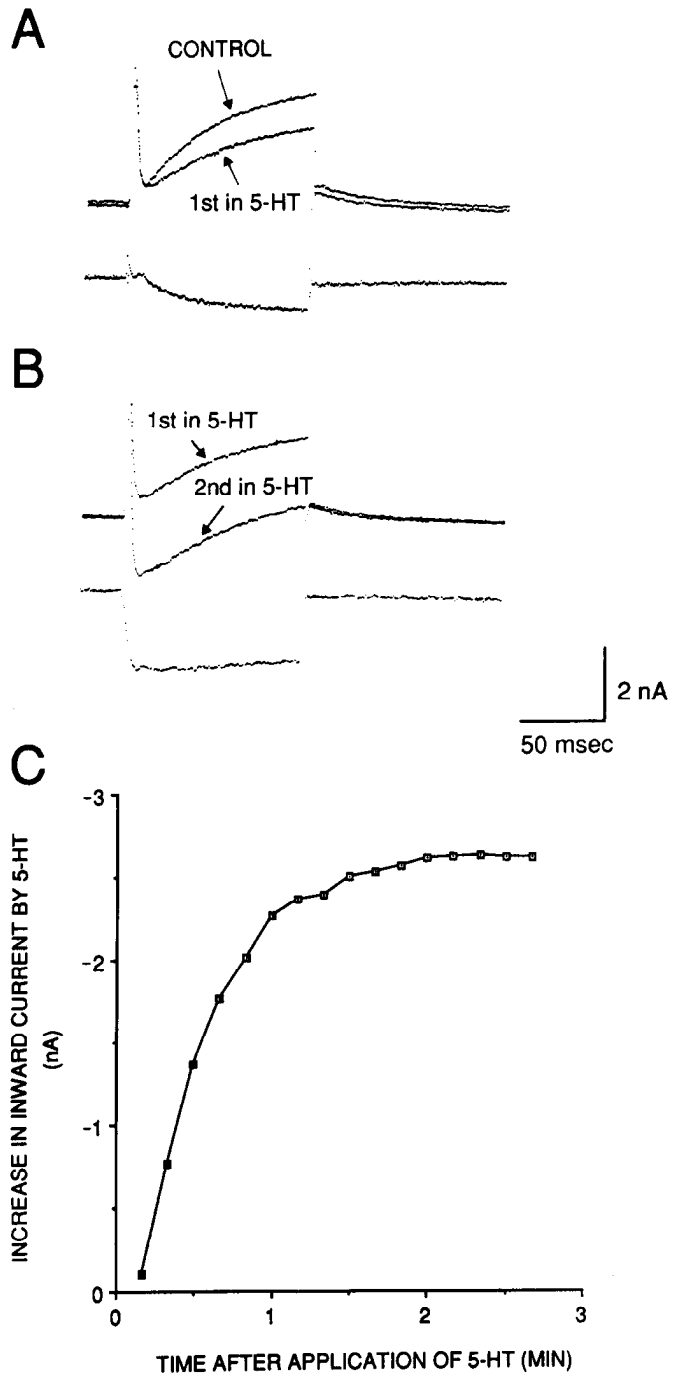

Figure 9. The increase in the inward current in response to 5-HT shows two kinetically different current changes that can be separated on the basis of the time of their appearance. *A* and *B*, The cell was voltage clamped with a KCl-containing pipette (whole-cell configuration), held at −50 mV, and stepped for 120 msec to +10 mV every 30 sec. *A: Upper traces*, Superimposition of the membrane currents before (*CONTROL*) and within the first 30 sec after application of 10 μM 5-HT (*1st in 5-HT*). *Bottom trace*, Difference current; shows the first current change caused by 5-HT (within less than 30 sec from 5-HT application). This change is accompanied by an inward movement of the holding current and a decrease in the tail current. *B*, Superimposition of the membrane currents after the first and after the second inward movement in response to 5-HT. *Bottom trace*, The difference current depicts the second phase of the current change produced by 5-HT. The current change in the first phase (*A, bottom trace*) resembles the S-K^+ potassium current, while the current change in the second phase (*B, bottom trace*) resembles the nifedipine-sensitive Ca^{2+} current. *C*, Time course of the effect of 5-HT on the currents. The sensory cell was voltage clamped with a potassium-containing pipette and depolarized every 10 sec. Ten micromolar 5-HT was applied and the difference current, measured at 10 msec from the beginning of the pulse, was plotted as a function of time after the application of 5-HT. The first change was small (*first point on the graph*) and represents the first inward movement that resembled the S-current (it occurred around 10 sec after the application of 5-HT). The other points were of current change that resembled the nifedipine-sensitive Ca^{2+} current; the effect reaches a steady state within 2–4 min.

messengers. The cAMP-dependent PKA, which mediates the effect of 5-HT on the S-K^+ channel and on I_{Kv} (Goldsmith and Abrams, 1992; Hochner and Kandel, 1992), seems not to be importantly involved in the increase in the nifedipine-sensitive Ca^{2+} current. By contrast, an important part of the increase in the Ca^{2+} current produced by 5-HT appears to be mediated by PKC. In turn, PKC does not affect the S-K^+ current, nor does it affect membrane excitability. In contrast to the results of Sugita et al. (1992), who found that long exposure to high concentrations of phorbol esters (1–5 μM) caused spike broadening in the intact ganglion, we find that low concentrations of PDBu (1–10 nM) and shorter periods of exposure do not produce significant spike broadening, yet these low concentrations of PDBu produce maximal facilitation of transmitter release and substantial modulation of the nifedipine-sensitive Ca^{2+} currents.

The effect of cAMP injection on the Ca^{2+} current and the effect of PKA inhibition on the 5-HT response

While direct injection of cAMP increases the Ca^{2+} current, inhibition of PKA does not reduce the increase in this current caused by 5-HT. Since 5-HT is known to increase intracellular cAMP in the sensory neurons (Bernier et al., 1982), these two findings appear to be contradictory. There are at least three possible ways of explaining this apparent contradiction. First, it is possible that modulation of the Ca^{2+} channels may require higher concentrations of cAMP than the other processes modulated by cAMP in the sensory neurons. This possibility could be a result of the Ca^{2+} channels being modulated by an isoform of PKA that has a lower affinity for cAMP than the isoform that modulates the S-K^+ channel. Alternatively, the affinity of the kinase for the different substrates may differ, or it may be necessary for a number of sites to be phosphorylated on the substrate that mediates the effect on the Ca^{2+} channel. If this is the case, then the effect of cAMP injection may not be directly related to the physiological modulation of the Ca^{2+} current by 5-HT. The second possibility is that both PKA and PKC modulate the Ca^{2+} channel to give the same final effect (perhaps even phosphorylate the same site on the protein), but with PKC being more potent, or with higher affinity for the phosphorylation site. Thus, full activation of PKC will produce most of the modulation and will dominate the effect, whereas inhibition of PKA will not have a significant effect. This possibility implies that modulation of Ca^{2+} channels by 5-HT is mediated primarily by PKC. The third possibility is that cAMP may modulate the Ca^{2+} current, even at low concentrations, but that it does not act through PKA. cAMP may either activate some other molecule that modulates the channel, or it may act directly on the channel—as has been shown in the olfactory system, for example (Nakamura and Gold, 1987), and in rabbit cardiac pacemaker channels (DiFrancesco and Tortora, 1991). On the basis of our experiments, we cannot distinguish among these possibilities.

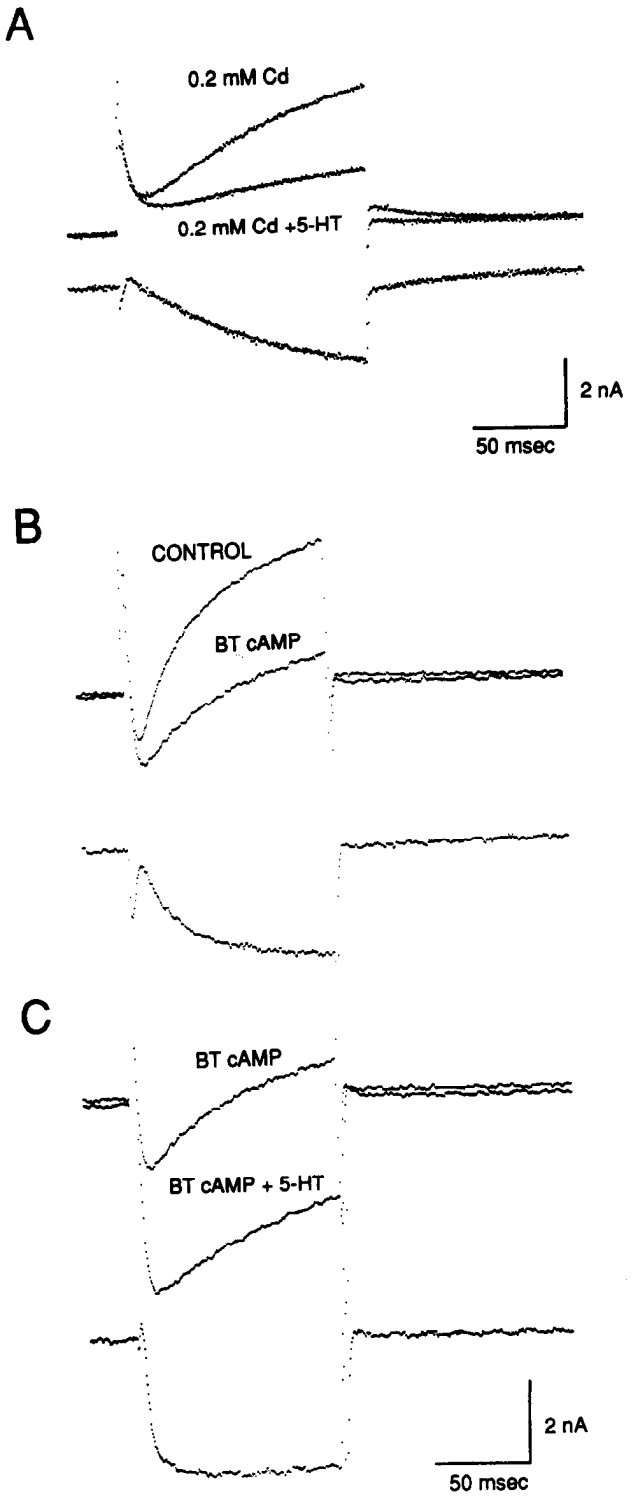

Figure 10. *A*, The second phase of the current change by 5-HT is blocked by Cd^{2+}. The cell was voltage clamped with a KCl-containing pipette (at holding potential of −50 mV and stepped to +20 mV for 120 msec). *Upper traces*, Superimposition of the membrane currents elicited in the presence of the Ca^{2+} channel blocker Cd^{2+} (0.2 mM) and a few minutes after application of 5-HT in the presence of Cd^{2+}. Only the first phase of change in current occurred in response to 5-HT: there was a small change near the beginning of the current and a large change at the end of the pulse. *Bottom trace*, The difference current showing the effect of 5-HT in the presence of Cd^{2+} resembles the $S\text{-}K^+$ current. *B*, The membrane-permeable cAMP analog BTcAMP mimics the effect of 5-HT on the first phase of the current change. The cell was voltage clamped with a KCl-containing pipette at a holding potential of −50 mV and stepped for 100 msec to +10 mV. *Upper traces*, Superimposition of the membrane currents before (*CONTROL*) and after application of 200 μM BTcAMP. *Bottom trace*, Difference current depicting the current change in response to BTcAMP; it has the kinetics of the $S\text{-}K^+$ current. *C*, Application of 5-HT in the presence of BTcAMP causes a second movement in the inward current, which did not occur in response to the cAMP analog. *Upper traces*, Superimposition of the membrane currents in the presence of BTcAMP and after application of 10 μM 5-HT. *Bottom trace*, The difference current has fast activation kinetics, like the nifedipine-sensitive Ca^{2+} current.

The time course of the effect of 5-HT

We found that the increase in the nifedipine-sensitive Ca^{2+} current caused by 5-HT appears later than the effect on the $S\text{-}K^+$ current. Similar results were described by Boyle et al. (1984), who studied depolarization-induced Ca^{2+} transients in the sensory neurons using arzenazo III. They found that the increase in Ca^{2+} transients in response to 5-HT reached its peak later than the peak effect on the $S\text{-}K^+$ current. The delay in the onset of the effect on the Ca^{2+} current may be due to (1) a longer time required for the production of diacylglycerol (DAG) and activation of PKC (in comparison with PKA activation by cAMP), or (2) additional steps between kinase activation and the final effect on the Ca^{2+} channel (an indirect effect of the kinase on the Ca^{2+} channel). The first possibility is consistent with the detailed measurements of cAMP, inositol trisphosphate (IP_3), and DAG accumulation in rat pancreatic acini (Trimble et al., 1986). In that study, cAMP accumulation was nearly maximally stimulated at 15 sec after application of secretin, while IP_3 and DAG accumulation reached a peak within 2–19 min after the application of the stimulating hormone. A lag in DAG accumulation also occurs when it originates by the action of phospholipase D on phosphatidylcholine, which is first hydrolyzed to phosphatidic acid (PA) and then dephosphorylated by PA phosphatase to DAG (Exton, 1990). Qian and Drewes (1990) measured the accumulation of PA and DAG in synaptic membranes of cerebral cortex in response to ACh and found PA to accumulate first, with a lag in the accumulation of DAG (about 30 sec), and a further fourfold increase in the accumulation of DAG between the first and the fifth minutes after the stimulation by ACh. Such a lag is similar to the time course of the effect of 5-HT on the Ca^{2+} current, and together with the apparent absence of IP_3 turnover in the sensory neurons (J. D. Sweatt, personal communication), may imply that DAG arises from the hydrolysis of phosphatidylcholine in the sensory neurons. From the work of Trimble et al. (1986), it also appears that the time course of accumulation of a second messenger is a function of the ligand concentration, and that various second messengers accumulate with different time courses. They reported a concentration-dependent induction (and accumulation) of second messengers in rat pancreatic acini, where low concentrations of secretin induced a rise in cAMP, while high concentration of the peptide induced a rise in DAG and IP_3. The situation is reversed in the response of adrenal cells to ACTH (Farese et al., 1986) or of hepatocytes to glucagon (Wakelam et al., 1986), where low concentrations of the hormone induced production of IP_3 and higher concentrations induced production of cAMP. It would be interesting to study whether the sensory neurons of *Aplysia* have a similar differential response to different concentrations of 5-HT.

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