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MODULATION OF THE SEROTONIN-SENSITIVE POTASSIUM CHANNEL IN *APLYSIA* SENSORY NEURONE CELL BODY AND GROWTH CONE

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SUMMARY

Using single-channel recording, we have been able to obtain some insight into the molecular mechanism of a modulatory transmitter action in *Aplysia* sensory neurones. Our results show that serotonin produces a slow EPSP and increases action potential duration in the sensory neurones by producing prolonged closures of the S potassium channel. Such closures appear to be mediated by cyclic AMP-dependent phosphorylation of a membrane protein which may be the channel. Modulation of S channels by serotonin also occurs in sensory neurone growth cones. This provides the first direct evidence that channel modulation occurs in nerve processes and increases the likelihood of channel modulation at the nerve terminal.

TWO TYPES OF TRANSMITTER ACTIONS

Neurotransmitter actions can be divided into two classes based on the duration of transmitter action, molecular mechanism and the nature of the transmitter-sensitive ion channel (Kupferman, 1979; Siegelbaum & Tsien, 1983). Mediatory transmitter actions are perhaps the more familiar and are exemplified by the effects of acetylcholine at the neuromuscular junction (Karlin, 1980). Here the transmitter action is rapid and short lived; a brief application of acetylcholine causes the endplate membrane to depolarize for at most a few milliseconds. This rapid postsynaptic effect results from a direct action of transmitter binding to its receptor in the postsynaptic membrane to open an ion channel that is tightly coupled to the receptor. In the case of the nicotinic acetylcholine receptor, the channel has been shown to be an integral part of the receptor macromolecule (Karlin, 1980). The channels that participate in such rapid transmitter actions, in general open only in the presence of transmitter. As a result, the channels normally play no role in generating the cell resting potential or propagated action potential. Rather, the channels are only involved in generating the short-lived postsynaptic potential change, which in turn either inhibits or elicits an all-or-none action potential.

Key words: modulation, ion channel, cyclic AMP.

In contrast, modulatory transmitter actions are in general much slower than mediatory actions (Kehoe & Marty, 1980; Hartzell, 1981). A short pulse of transmitter can produce postsynaptic potential changes that persist for tens of seconds up to several minutes. Modulatory transmitter effects also rely on quite different molecular mechanisms and are often mediated by various intracellular messenger systems including calcium (Putney, 1979; Trautmann & Marty, 1984), the products of phospholipid metabolism (Berridge & Irvine, 1984) and cyclic nucleotides (Greengård, 1976; Kupferman, 1980). One final difference concerns the nature of the ion channel involved in the transmitter response. As opposed to the conventional effects, modulatory transmitter actions often alter the activity of ion channels that normally are open in the absence of transmitter (including both Ca^{2+} and various K^+ channels; Siegelbaum & Tsien, 1983).

As these channels often play important roles in generating the cell resting and action potentials, modulation of such channels is frequently accompanied by a series of changes in the active and passive electrical properties of the postsynaptic cell. These include changes in action potential duration and amplitude, threshold potential, resting potential, and the space and time constants. Changes in action potential configuration can influence Ca^{2+} influx into the cell and thus alter a variety of cellular processes, including transmitter release from the nerve cell terminals. Such a change in synaptic effectiveness is thought to underlie a simple form of learning and short-term memory in the marine snail *Aplysia* (Kandel & Schwartz, 1982). Thus, while the conventional type of synaptic actions are ideally suited for rapid electrical signalling, modulatory transmitter actions tend to be associated with longer term changes in a wide variety of cellular and integrative neuronal processes.

With the development of single-channel recording by Neher & Sakmann and their colleagues (Hamill *et al.* 1981), it is now possible to study transmitter actions on current flow through single ion channels. Recently, much attention has been directed to studying the mode of action of modulatory transmitters at the single-channel level (Kaczmarek & Levitan, 1986). How might a modulatory transmitter action alter current flow through single ion channels? In general, the magnitude of a given type of macroscopic ionic current, $<I>$, will depend on the number of functional channels in the membrane, N_f, the magnitude of the current flow through a single open channel, i, and the probability that a given channel is open, p, according to: $<I> = N_f \times i \times p$. In principle, a neurotransmitter could alter the net current flow through a channel by modulating any one of these parameters. This is illustrated in Fig. 1. To some extent the distinction between mechanisms is arbitrary since a decrease in the number of functional channels is just the limiting case where single-channel current amplitude or open probability is reduced to zero.

SLOW MODULATORY EFFECTS OF SEROTONIN IN *APLYSIA* SENSORY NEURONES

A well studied example of a modulatory transmitter action is the slow decreased conductance EPSP produced by serotonin in the sensory neurones of the abdominal

ganglia of the marine snail, *Aplysia*. This slow EPSP is accompanied by an increase in the duration of the action potential, measured in the cell body, and an increase in transmitter release from the sensory neurone terminals. As these sensory neurones mediate the gill-withdrawal reflex in *Aplysia*, the presynaptic facilitation of transmitter release results in facilitation of the reflex and is thought to provide the cellular basis for a simple form of learning, behavioural sensitization.

The ionic basis of the membrane effects of serotonin has been investigated using the voltage-clamp technique (Klein & Kandel, 1980; Klein, Camardo & Kandel, 1982). These studies have shown that the primary effect of serotonin is to decrease a specific outward potassium membrane current (S current) that is distinct from the previously identified K^+ currents in molluscan neurones, including the early outward A current, delayed rectification and the calcium-activated potassium current (Adams, Smith & Thompson, 1980). The mechanism of presynaptic facilitation is, however, less clear. Klein & Kandel (1980) proposed that facilitation itself could also be explained by modulation of the S current. If such modulation were to occur at the terminals, the resultant broadening of the action potential would allow for greater Ca influx and hence produce greater transmitter release. More recently Boyle, Klein, Smith & Kandel (1984) have shown that serotonin also leads to an increase in internal calcium by a mechanism that is independent of S channel modulation. This increase in calcium concentration may also contribute to facilitation.

There is now good evidence that the modulatory effects of serotonin on the S current are mediated by the second messenger, cyclic AMP, according to the following general sequence of events (see e.g. Krebs, 1972; see Fig. 2A). (1) The binding of serotonin (5-HT) to its receptor leads to activation of membrane-bound adenylate cyclase. (2) This causes a rise in intracellular cyclic AMP concentration. (3) Cyclic AMP then activates cyclic-AMP-dependent protein kinase. (4) The catalytic subunit of protein kinase phosphorylates several substrate proteins. (5) This leads to the decrease in S current by some unknown mechanism. Evidence in support of this scheme includes the finding that intracellular injection of cyclic AMP mimics the electrophysiological effects of serotonin (see Kandel & Schwartz, 1982). Serotonin, but not other transmitters, has been shown to produce a specific increase in cyclic AMP levels in single identified sensory neurones (Bernier, Castellucci, Kandel & Schwartz, 1982). Finally, intracellular injection of the purified catalytic subunit of protein kinase also mimics the effects of serotonin (Castellucci *et al.* 1980) and intracellular injection of a protein inhibitor of protein kinase blocks the effects of serotonin (Castellucci *et al.* 1982).

SEROTONIN CLOSES A SPECIFIC POTASSIUM CHANNEL

For several years, we have used the patch-clamp technique to study the mechanism of action of serotonin on single-channel function (Siegelbaum, Camardo & Kandel, 1982; Camardo, Shuster, Siegelbaum & Kandel, 1983; Shuster, Camardo, Siegelbaum & Kandel, 1985). In the experiments summarized below we first identify the serotonin-sensitive potassium channel and characterize the mode of action

of serotonin on single-channel function. Next we investigate the role of second messengers in serotonin's action as well as the effects of the purified catalytic subunit of cyclic-AMP-dependent protein kinase on channel activity in cell-free patches of membrane. Finally, we consider the cellular distribution of the S channel, its presence and modulation in sensory neurone growth cones, and the implications of such findings for presynaptic facilitation.

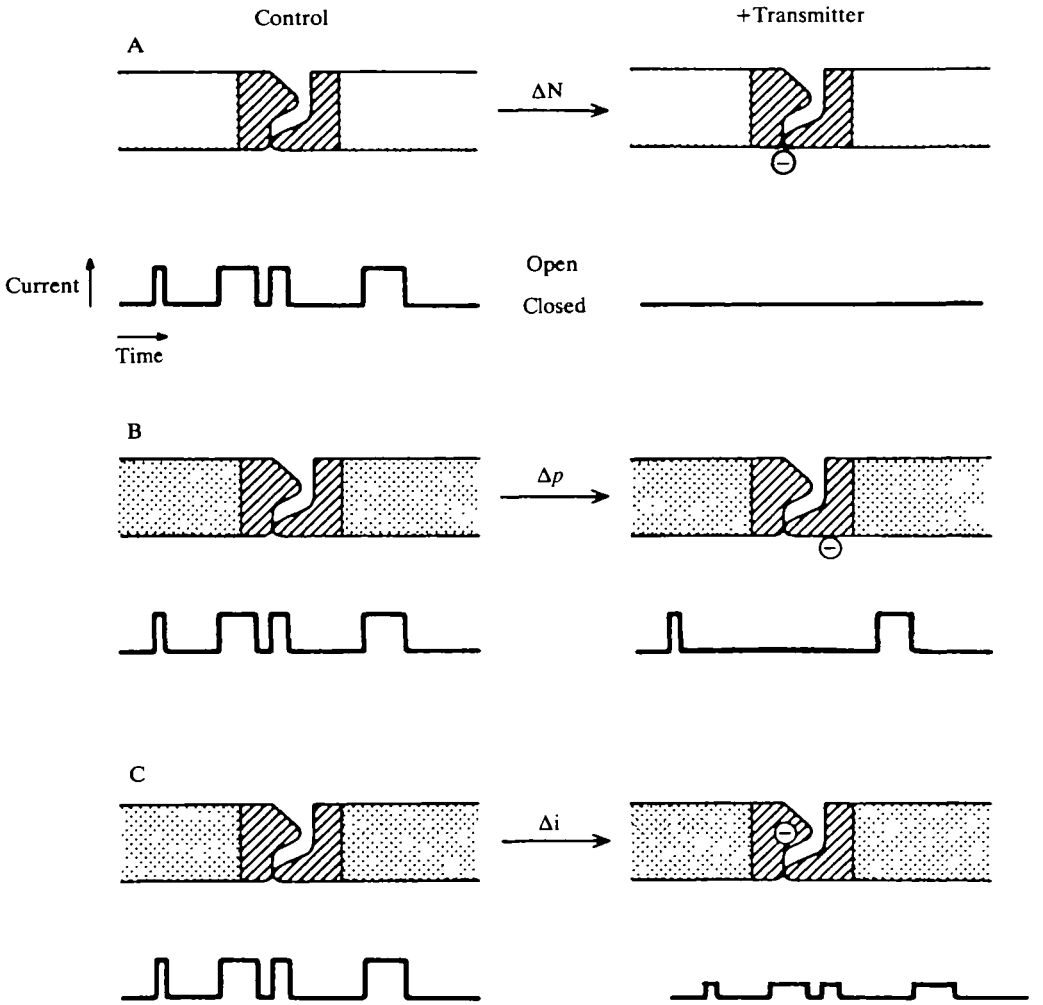

Fig. 1. Possible modes of modulatory transmitter action on single-channel currents. The drawings depict channels as integral membrane proteins with an aqueous pore for ion permeation and a gate for controlling channel opening and closing. Idealized current records show channel openings as an upward current deflection. Transmitter could, in principle, lead to a decrease in the average current carried by a population of such channels by modulating the number of functional channels (A, ΔN), the probability of channel opening (B, Δp) or the amplitude of the single channel current (C, Δi) giving rise to different changes in single-channel function. (Modified from Siegelbaum & Tsien, 1983.)

The experimental protocol that was used first to identify the serotonin-sensitive channel is illustrated in Fig. 2A. Cell membrane potential and resting input resistance were measured with a conventional intracellular microelectrode. This allowed us to identify sensory neurones on the basis of their electrophysiological properties and monitor the whole cell's response to serotonin. At the same time, a high resistance giga-ohm seal was obtained between the sensory cell membrane and an extracellular fire-polished glass pipette. Single-channel currents were recorded from the small patch of membrane under the pipette and the response of the channels to bath application of serotonin could then be examined.

S channel properties

Fig. 2B illustrates a single-channel current record from a patch that contains only a single active S channel. The channel contributes square pulses of outward current over a wide range of membrane potentials. At any potential the channel is in the open configuration for a large fraction of time. Upon depolarization of the patch, the size of the unit current step increases, due to the increase in outward driving force on ion movement through the open channel. However, at a fixed membrane potential, the histogram of channel amplitudes reveals a single peak with a Gaussian distribution, indicating that there is only one type of channel in the patch and that the channel undergoes transitions between two distinct conductance levels, a fully closed state and a fully open state. At a membrane potential of 0 mV, the amplitude of current flow through the open channel is around 3 pA.

In addition to being open for a large fraction of time, the channel is open at voltages negative to the resting potential of the cell (−40 mV in Fig. 2B). This channel therefore contributes to setting the normal level of resting potential and resting membrane conductance in the sensory neurones. Turning off this channel by serotonin could thus account for the slow depolarization in the resting potential and the observed decrease in resting membrane conductance and increase in excitability.

As the channel is open over a wide range of potentials, it will also contribute outward repolarizing current during the entire time course of the action potential and a turning off of such a channel will necessarily slow repolarization and lead to a broadening of the spike duration. Thus this channel has many of the expected properties for a channel underlying the S current. However, the final identification of this class of channel with the S channel depends on the demonstration that it is modulated by serotonin.

Mode of action of serotonin on potassium channel current

Serotonin consistently caused prolonged all-or-none closures of this background potassium conductance channel. Fig. 3 shows an example of serotonin's action on single-channel current from a patch that initially displayed four active channels. Soon after addition of serotonin to the bath, there was a small depolarization of the resting potential, a decrease in cell membrane conductance (data not shown) and a progressive closure of three of the four active channels (Fig. 3A). The channels are seen to close in an all-or-none steplike manner: the channels are either completely closed

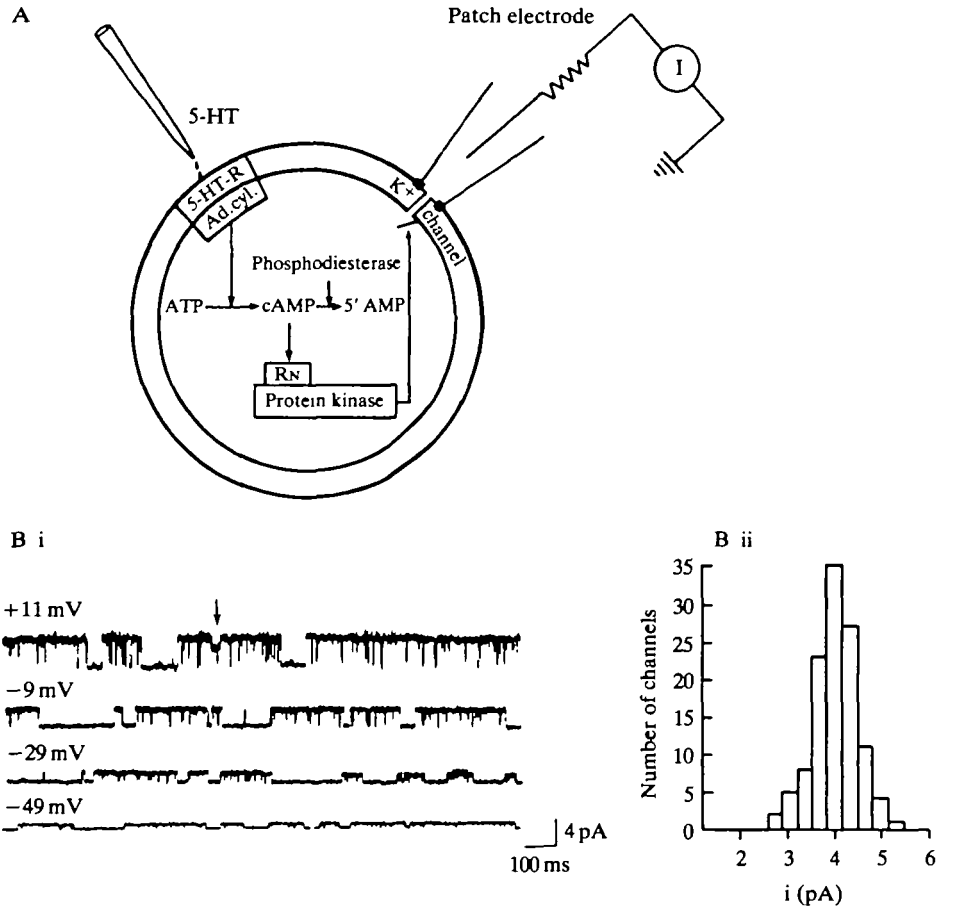

Fig. 2. Patch-clamp recording of single S channel currents. (A) Schematic illustration of the experimental recording protocol and the cyclic-AMP-dependent model for the action of 5-HT (serotonin). A high-resistance giga-ohm seal is obtained between the extracellular patch electrode and the cell membrane to record single-channel currents in the small patch of membrane under the electrode. This is the cell-attached recording configuration. If the patch electrode is withdrawn from the cell, the seal remains intact and single-channel currents can be recorded from the cell-free patch of membrane where the intracellular surface of the membrane faces the bath solution, termed the inside-out patch configuration. Serotonin is applied to the cell outside the membrane patch but may still alter channel activity in the patch *via* the cyclic AMP cascade, which is illustrated diagrammatically. The substrate protein for the kinase may or may not be the S channel itself. (Bi) Serotonin-sensitive K^+ channel. Single-channel current records from the mechanoreceptor sensory neurones (LE cluster) in the abdominal ganglion of *Aplysia californica*. Both the bath and recording pipette contain artificial sea water. The patch membrane potential (left of each trace) was altered by changing the potential inside the recording pipette while the intracellular resting potential (−39 mV) was constant. Channel openings appear as step increases in outward current (outward current plotted in upward direction). The current fluctuates between two levels corresponding to the fully closed and fully open channel, and channel size increases with depolarization. At all potentials, the channel shows both brief closures (downward flickers) and longer closures but is in the open configuration for most of the time. The arrow indicates a rare event representing a closure of the channel to an intermediate conductance level. Addition of 10 μmol l^{-1} serotonin in the bath caused this channel to close (record not shown) (from SCK82). (Bii) A histogram of channel current amplitudes (i) at a potential of +11 mV obtained from the experiment in Bi. The channel current sizes cluster around 4 pA with a single peak in the histogram, which indicates that there is a single type of channel. (From SCK82.)

in the presence of serotonin or continue to open and close normally (Fig. 3B,C). Serotonin does not reduce the conductance of the S channels nor does it cause small changes in the rate of channel opening and closing. Rather, the transmitter appears to cause a decrease in the number of active channels in the membrane by causing prolonged and complete channel closures (see Fig. 1).

This result also provides indirect support that the effects of serotonin are mediated by an intracellular messenger. In these experiments, 5-HT is applied to the cell only after formation of the high resistance giga-ohm seal. The contact between the glass pipette and the cell membrane is thought to be so tight that it prevents the diffusion of even small molecules from the bath into the pipette. Since bath application of 5-HT consistently closes channels under the pipette in the patch, it is likely that 5-HT acts to mobilize some intracellular messenger, which can then modulate the channels from the inside of the membrane. Direct support for this scheme has been obtained in experiments where injection of cyclic AMP into sensory neurones from cyclic-AMP-filled microelectrodes has been shown to mimic the effects of serotonin in closing the channels (see Fig. 5).

CYCLIC-AMP-DEPENDENT PROTEIN KINASE ALSO CLOSES THE POTASSIUM CHANNEL

The experiments of Castellucci *et al.* (1980, 1982) provide compelling evidence that the effects of serotonin on the sensory neurone's channel are mediated by cyclic-AMP-dependent phosphorylation. Protein phosphorylation has now been implicated in modulatory transmitter actions in a number of different systems (see Kennedy, 1983; Nestler & Greengard, 1983; Levitan, 1985 for review). However, the nature of the coupling mechanism between protein phosphorylation and channel modulation is not known. While the most direct mechanism for channel closure involves the direct phosphorylation of the channel by protein kinase, it is possible that the effect of the kinase is indirect and that channel closure is mediated by some other regulatory protein. One alternative mechanism involves changes in ion pump activity due to phosphorylation (e.g. Tada & Katz, 1982) which might directly alter channel activity or lead to activation of some other enzyme (e.g. calcium-calmodulin-dependent protein kinase). We have therefore studied the action of the catalytic subunit of cyclic-AMP-dependent protein kinase on S channel activity in inside-out patches of membrane. These experiments allow us to determine whether in cell-free conditions (where presumably cytoplasmic constituents and ion pumps do not play a role) the kinase is capable of modulating channels by a mechanism similar to that seen in the intact cell with serotonin.

Our results show that kinase consistently produced all-or-none closures of the S channels, in a manner that is similar to the effects of serotonin in the intact cell. An example of the effect of the catalytic subunit on channel currents in an inside-out patch is shown in Fig. 4. In this patch there were initially four channels active. Upon addition of the catalytic subunit, one channel closes relatively quickly. After a somewhat longer delay, a second channel closes. The channels do not remain closed,

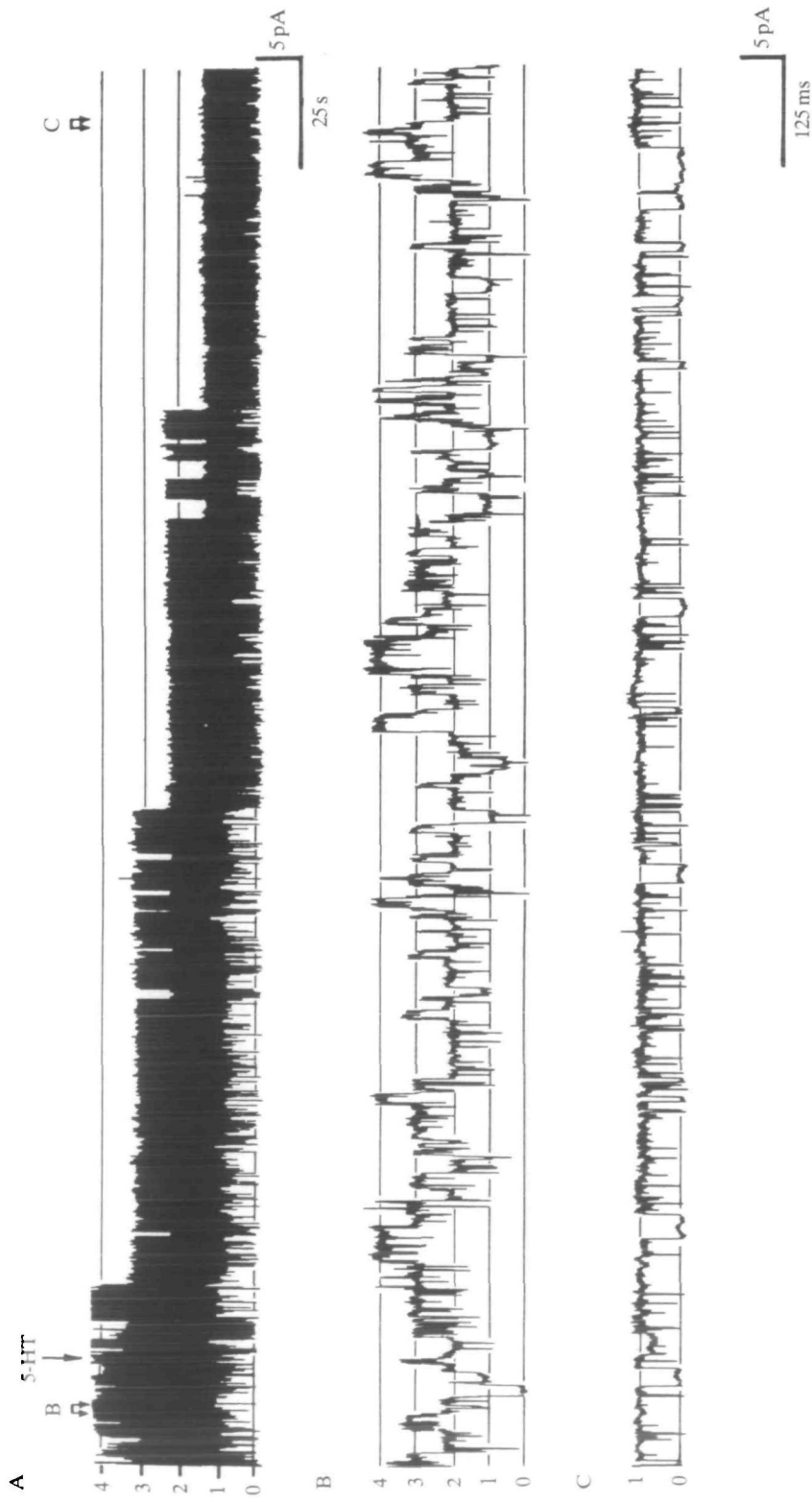

Fig. 3. Modulation of single-channel currents by serotonin. Patch-clamp current records from a sensory neurone membrane patch that initially contained four active channels. (A) Channel current on a slow time base. At the arrow, 100 μmol l^{-1} serotonin was added to the bath causing all-or-none closure of three out of four channels. The numbers on the left-hand ordinate indicate current levels corresponding to the number of open channels. Before addition of serotonin there is a maximum of four open channels. Regions labelled B and C indicate regions of trace in A from which expanded records of lower two traces were obtained. The cell resting potential depolarized from −41 mV to −37 mV after application of serotonin (5-HT) and there was a 33% increase in input resistance. Patch membrane potential was held at 0 mV before 5-HT and +4 mV in the presence of 5-HT. (From Siegelbaum & Tsien, 1983.)

even in the maintained presence of kinase, but rather reopen. There is then one more long-lasting channel closure and this channel does not reopen until after the kinase is washed out of the bath. These effects of protein kinase appear to be mediated by a phosphorylation reaction since they depend on the presence of MgATP. In the absence of this source of high-energy phosphate, there is a fourfold reduction in the frequency of observing prolonged closures in response to the enzyme.

The similarity of the action of the catalytic subunit of cyclic-AMP-dependent protein kinase to the effects of serotonin on channels in cell-attached patches suggests that most of the important molecular components for channel modulation are present in the cell-free patch. However, our results do not allow us to distinguish whether it is the channel itself that is being phosphorylated, or whether the primary substrate is some other protein that remains attached to the patch. Moreover, there are some important differences between the effects of kinase and those of 5-HT on channel activity. In general, the kinase-induced closures are shorter than those produced by high concentrations of serotonin. Serotonin also closes a somewhat greater total fraction of active ion channels (46 %) than does protein kinase (34 %).

A possible explanation for these differences between kinase and 5-HT, is that the cell-free patches contain an endogenous membrane-bound phosphoprotein phosphatase which is capable of cleaving the phosphate from the substrate protein, thus terminating the channel closure. Some support for this view is provided by experiments where protein kinase was applied to the patch in the presence of fluoride ions, a non-specific inhibitor of phosphatase activity (Revel, 1963). We find that 20–50 mmol l^{-1} KF is capable of potentiating the effects of protein kinase both by prolonging the periods of closure and by increasing the total percentage of channels closed. On average, 49 % of channels in the patches are closed by kinase in the presence of fluoride compared with a percentage of closure of only 34 % in the absence of fluoride. Biochemical experiments also show that isolated *Aplysia* neuronal membranes contain substantial phosphoprotein phosphatase activity and that this activity is partly inhibited by fluoride. However, since fluoride is a rather non-specific inhibitor, an important line of future experiments should involve use of more specific phosphatase inhibitors, such as those recently isolated from mammalian cells (Ingebritsen & Cohen, 1983; Hemmings, Greengard, Tung & Cohen, 1984). Although no specific phosphatase inhibitors have been isolated from molluscs, preliminary experiments show that inhibitor II (an inhibitor of type I phosphoprotein phosphatase), can inhibit over 80 % of the phosphatase activity in the *Aplysia* neuronal membrane preparation.

If phosphoprotein phosphatase is present in *Aplysia* neuronal membranes, why does it not also limit the duration of channel closure in the intact cell? Our present hypothesis is that *Aplysia* neurones contain cytoplasmic inhibitor proteins, similar to those described in mammalian cells, that normally keep the phosphatase activity low. In the cell-free patches, such inhibitor proteins would be lost so that the rate of dephosphorylation would be much greater and the extent of channel closure would be correspondingly decreased.

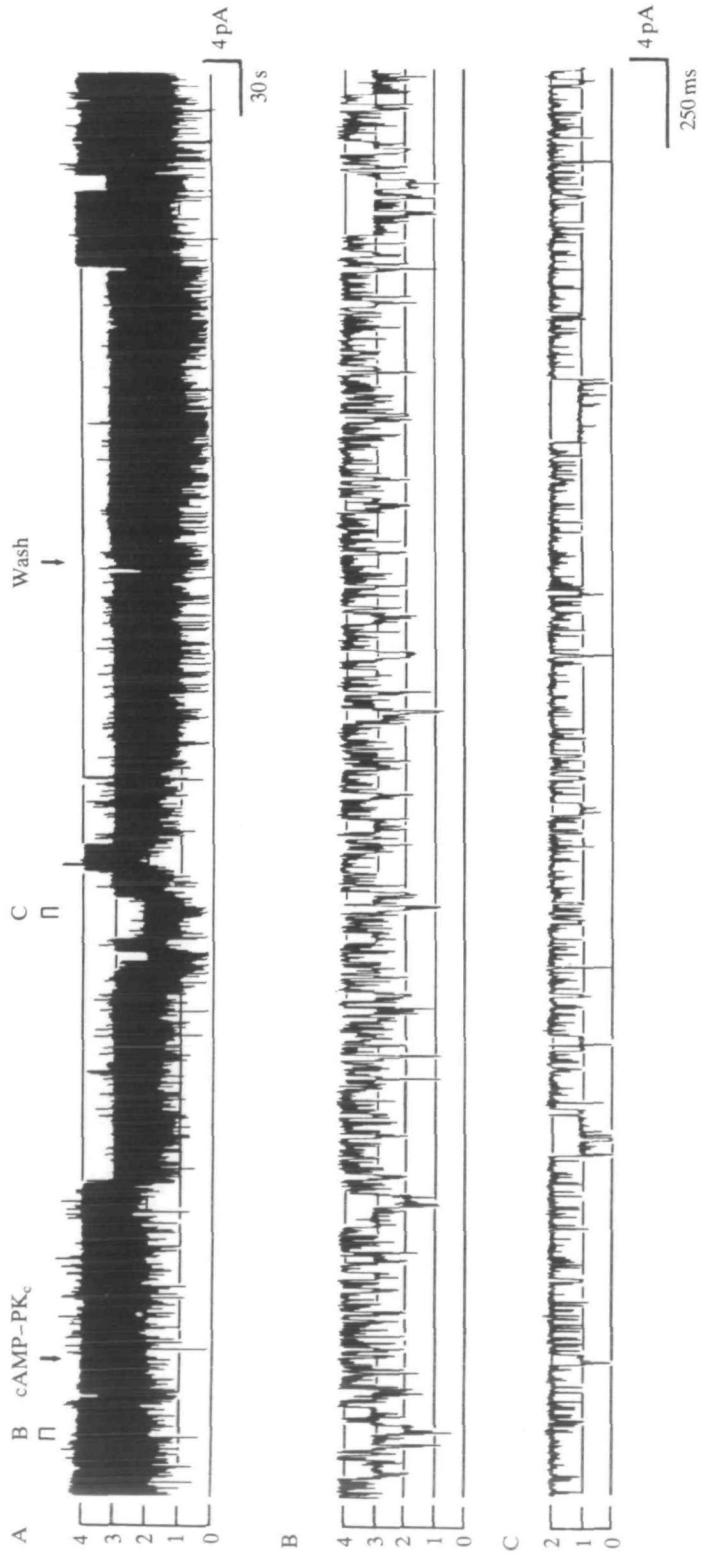

Fig. 4. (A) Catalytic subunit (cAMP–PK$_c$) closes single S channels in inside-out membrane patches. (B),(C) Expanded current records showing S channel activity taken at times indicated in trace A. (B) Before addition of 0·1 μmol l^{-1} kinase, four channels are active. (C) In the presence of kinase, a maximum of two out of four channels are closed. Membrane held at 0 mV. Free calcium in bath was 0·1 μmol l^{-1}. (From Shuster, Camardo, Siegelbaum & Kandel, 1985.)

Table 1 presents a summary of our findings with protein kinase and compares the modulation produced in cell-free patches with the modulation seen in cell-attached patches with 5-HT and cyclic AMP. There is rather good quantitative agreement among the three treatments in terms of fraction of channels closed and percentage of patches where closure was observed. A consistent finding of our results with both serotonin and protein kinase is that not all of the S channels in a patch can be closed. On average we find that only about half of all channels close in response to 5-HT, cyclic AMP or protein kinase. One explanation for the lack of response of some channels is that modulation is not due to channel phosphorylation but rather is mediated by the phosphorylation of a regulatory protein present in the membrane in a molar ratio that is approximately one-half the S channel density. Alternatively, there could be two populations of S channels that have identical conductances and gating properties but that differ in the presence or absence of an exposed phosphorylatable site for modulation.

One final point concerns the latency to channel closure with protein kinase. The results of Table 1 indicate that the latency to channel closure is rather slow with all three treatments and that the latency is longer with cyclic AMP injections and protein kinase than with serotonin application. This is somewhat surprising since cyclic AMP and protein kinase should be closer to the final reaction step in the modulation sequence than serotonin and might be expected to produce more rapid modulation than 5-HT. One possible explanation for the long latency with cyclic AMP injections has to do with the geometry of the cell. During the injections, cyclic AMP is injected at a point source within the cell from a microelectrode using repetitive iontophoretic current pulses. To produce channel modulation in the membrane under the patch electrode, the injected cyclic AMP must diffuse from the injection electrode, across the cell and up into the membrane patch. Although free diffusion within the cell is

Table 1. *Comparison of modulatory effects in cell-attached and cell-free membrane patches*

Treatment	*N*	Experiments with closures (%)	Channels closed (%)	Latency to first closure (min)	Closed* time (min)
			Cell-attached		
Serotonin	33	76·5	45·8	1·7 ± 1·3	>5
Cyclic AMP	7	71·4	52·2	4·5 ± 1·6	>5
			Cell-free		
cAMP–PK† +ATP‡	42	81·0	42·0	4·5 ± 4·6	3·0 ± 2·7

* Closed times in serotonin and cyclic AMP generally outlasted experiment so 5 min is a lower limit. Average closed time with cAMP–PK was calculated using data from experiments where channels showed recovery from closure ($N = 18$).

† cAMP–PK, cyclic-AMP–protein-kinase.

‡ Results are combined (±) fluoride results.

likely to be rapid considering the short distances involved, cyclic AMP is rapidly hydrolysed by phosphodiesterase so that relatively large amounts of cyclic AMP need to be injected to achieve an effective final cyclic AMP concentration at the membrane patch. Thus, in this case, the long latency probably reflects the time required to build up the cyclic AMP concentration at the inside of the membrane patch.

At least part of the long latency with the kinase is due to incomplete mixing in our small experimental chamber following addition of an aliquot of enzyme. Under conditions where the bath is well stirred, the latency to closure with kinase is reduced to around 2·5 min with a range from 30 s up to 10 min. This is comparable to the latency in response to serotonin, although the serotonin response is still somewhat faster. One possible explanation for the relative speed of the serotonin response is that in the intact cell, the 5-HT receptor, adenylate cyclase, protein kinase and channel are grouped together as a complex in the membrane, allowing efficient coupling between transmitter binding and channel activation. Such tight localization could also explain why cyclic-AMP-dependent transmitter actions modulate different channel types in different molluscan nerve cells. Specificity of transmitter action would be controlled by the localization of the kinase and/or cyclase next to the appropriate target channels.

How does the latency we observe with protein kinase compare with expected rates for channel phosphorylation? Published kinetics for phosphorylation reactions mediated by cyclic-AMP-dependent protein kinase show a large variability depending on the substrate protein. Huganir & Greengard (1983) have shown for the nicotinic acetylcholine receptor that the γ and δ subunits are very good substrates for the kinase, with a K_m of around 4 μmol l^{-1} and a V_{max} of around 2 μmol min^{-1} mg^{-1} enzyme. These constants, determined from biochemical enzyme kinetics, can be translated into expected rates of phosphorylation of a single substrate protein (which is what is measured in our experiments) using the following kinetic scheme:

$$E + S \underset{}{\overset{K_m}{\rightleftharpoons}} E\text{–}S \xrightarrow{k_i} E + S \sim P,$$

where E is the catalytic subunit, S is the substrate protein and $S \sim P$ is the phosphorylated protein. Assuming that the rate of binding and unbinding is rapid compared to the rate of catalysis, the effective rate, k_f, of phosphorylation of a single substrate protein will be given by: $k_f = k_i/(1+K_m/[E])$. k_i is a true rate constant that can be obtained from V_{max} by converting from minutes to seconds and normalizing V_{max} by the number of moles of catalytic subunit (relative molecular mass, 40 000) per mg protein. For the nicotinic receptor γ and δ subunits, k_i is around 1·3 s^{-1}. Since our enzyme concentrations are of the order of 0·1–1 μmol l^{-1}, if the S channel were phosphorylated at the rate of the nicotinic receptor, we would expect an effective rate of phosphorylation around 0·3–0·03 s^{-1} or a mean latency of 4–30 s. Our observed latencies are somewhat longer than this estimate, although in favourable circumstances we have observed closures as soon as 30 s after kinase application. Recent experiments on modulation of single Ca-dependent potassium channels from *Helix* neurones by catalytic subunits of cyclic-AMP–protein-kinase (cAMP–PK)

show approximately similar latencies to modulation, ranging from 12 s to 4 min (Ewald, Williams & Levitan, 1985).

CONVERGENT ACTIONS OF TRANSMITTERS ON A SINGLE CHANNEL

Second messenger-dependent transmitter actions have an advantage over more conventional mediatory synaptic actions in that they allow different transmitters to modulate the same ionic channel. Any transmitter or treatment that leads to a rise in cyclic AMP or an increase in activation of the cyclic-AMP-dependent protein kinase in the sensory neurones will lead to closure of the S channel. Thus Abrams *et al.* (1984) have shown that the small cardioactive peptide (SCP) increases cyclic AMP concentration and produces presynaptic facilitation, a slow EPSP, action potential broadening and S channel closure in the sensory neurones (Fig. 5B).

Fig. 5 compares S channel closures produced by four very different treatments that all recruit cyclic-AMP-dependent protein phosphorylation. There is a marked similarity in the closures produced by serotonin (Fig. 5A), SCP (Fig. 5B), cyclic AMP (Fig. 5C) and the catalytic subunit of cAMP–PK (Fig. 5D), lending support to the view that all treatments modulate S channel activity through a common final pathway. It seems likely that, over the next several years, new transmitters will be identified that increase cyclic AMP concentration and thus produce S channel closure in the sensory neurones. Such convergent mechanisms greatly increase the range of chemical inputs available to the sensory neurones for producing channel modulation. This allows the sensory neurones to respond both to conventional synaptic inputs in response to transmitters such as serotonin as well as to neurohormones and peptides that may be released from distant nerve terminals.

ION CHANNEL MODULATION IN SENSORY NEURONE GROWTH CONES

So far our experiments have concentrated on the action of serotonin on S channels in the cell body and do not provide any information about whether a similar modulation occurs in sensory neurone processes such as nerve branches, synaptic terminals or growth cones. Channel modulation in such structures is of great potential importance since it could influence a number of neuronal properties, including integration, spike propagation and transmitter release. Control of transmitter release by channel modulation in synaptic terminals, was in fact postulated by Klein & Kandel (1980) as a mechanism for presynaptic facilitation (see above). However, there is no direct evidence to support the view that S channel modulation does in fact occur at the terminal or in any neuronal structure outside the cell body.

To investigate whether S channel modulation takes place in regions of the neurone in addition to the cell body, in collaboration with Sam Schacher at Columbia, we have used the patch-clamp technique to record action potentials, ionic currents and single channels from the growth cones of *Aplysia* sensory neurones in culture. The growth cones are of particular interest because they provide a model for the synaptic terminal, which itself is too small to study. Growth cones are the immediate

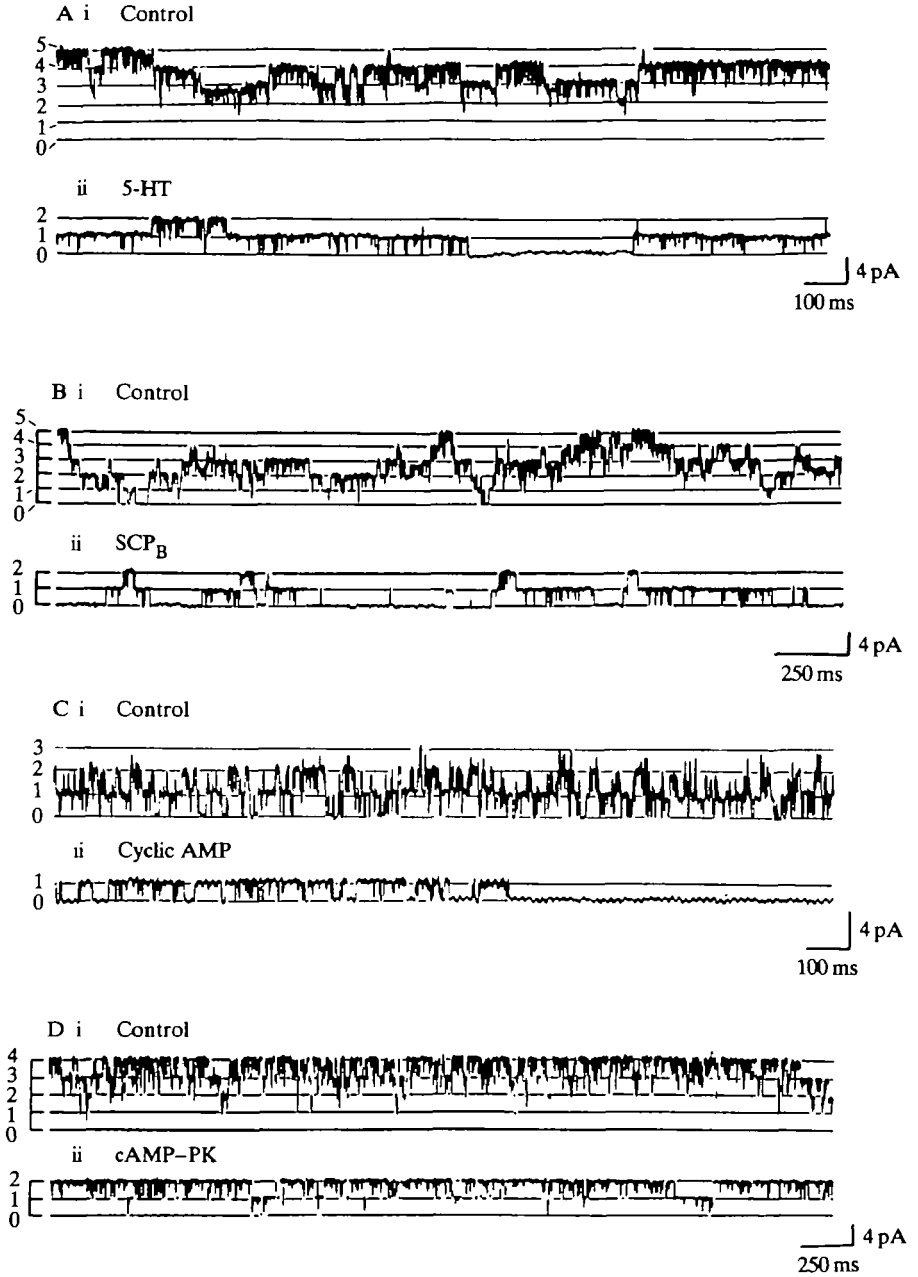

Fig. 5. Convergent actions of transmitters, second messengers and enzymes for channel modulation. (A) Closure of S channels by serotonin in patch containing five active channels. Addition of 30 μmol l^{-1} serotonin (5-HT) closes three of the active channels in an all-or-none manner. (B) SCP_B (small cardioactive peptide, 1 μmol l^{-1}) also closes three of five active channels from another patch in an all-or-none manner. (C) Intracellular injection of cyclic AMP also closes S channels. (D) S channel closure produced by catalytic subunit of cyclic-AMP–protein-kinase (cAMP–PK). Similarity of action of all agents results from final common step of protein phosphorylation mediated by cyclic-AMP-dependent protein kinase. (From Castellucci *et al.* 1986.)

precursor to the synaptic terminal and share a number of biochemical and morphological properties with the terminals, including the ability to release transmitter (Hume, Role & Fischbach, 1983; Young & Poo, 1983). Beyond their use as a model for the terminal, modulation of channels in growth cones is also of interest due to the recent finding that transmitters can alter their movement (Haydon, McCobb & Kater, 1984).

Identified sensory neurones from the pleural ganglion of *Aplysia* were isolated and grown in culture (Schacher & Proshansky, 1983). Pleural sensory neurones were used since they respond to serotonin in an identical manner to the abdominal ganglia sensory neurones (Walters, Byrne, Carew & Kandel, 1983; Pollock, Camardo & Bernier, 1985), but are more numerous and thus more convenient to isolate and plate in culture. To compare the action of 5-HT on the growth cone and cell body, membrane potentials were recorded simultaneously from the cell body and growth cone (Fig. 6, inset). The sensory cell body was impaled with a conventional intracellular microelectrode for recording membrane voltage. Stimulating current pulses were delivered to the cell body through the voltage electrode using a bridge circuit. Membrane potential from the growth cone was recorded using the whole cell patch configuration. A giga-ohm seal was first obtained between a patch pipette (filled with an intracellular-like solution; see legend to Fig. 6) and the growth cone membrane. The membrane in the patch was then ruptured by applying extra suction to obtain an intracellular recording of membrane potential from the growth cone.

Under these conditions we record mean resting potentials of around -40 to -50 mV in both cell body and growth cone. In response to a brief application of serotonin onto the cell body from a puffer pipette, the cell body resting potential depolarizes by 4–10 mV, there is a 15–20% increase in input resistance and a 14–30% increase in action potential duration (Fig. 5A,B). Recordings from the growth cone show a similar action of serotonin (Fig. 5C,D), with a 2–7 mV depolarization, a 3–14% increase in input resistance and a 25% increase in action potential duration ($N = 4$).

These effects seen in the growth cone could be due to the modulation of growth cone ionic conductances by 5-HT or might simply be a passive reflection of the potential changes produced in the cell body. To determine whether the growth cone itself responds to 5-HT, we recorded from growth cones that had been mechanically isolated from the rest of the neurone by cutting the axon near the end of the growth cone with a fine microelectrode. Most growth cones survived this surgery and displayed regenerative action potentials in response to a depolarizing current pulse during whole growth cone current clamp. Application of 5-HT to these isolated growth cones ($N = 6$) produces effects similar to those observed in the cell body and intact growth cone (Fig. 7). There is a slow depolarization of the resting potential by 2–5 mV, a 12–50% increase in membrane resistance and an increase in action potential duration of 11–22%.

As a first step to determining the ionic basis of this modulation, we have studied the action of 5-HT on the isolated growth cones under voltage clamp. Serotonin produces a decrease in net outward current that is similar to that previously described

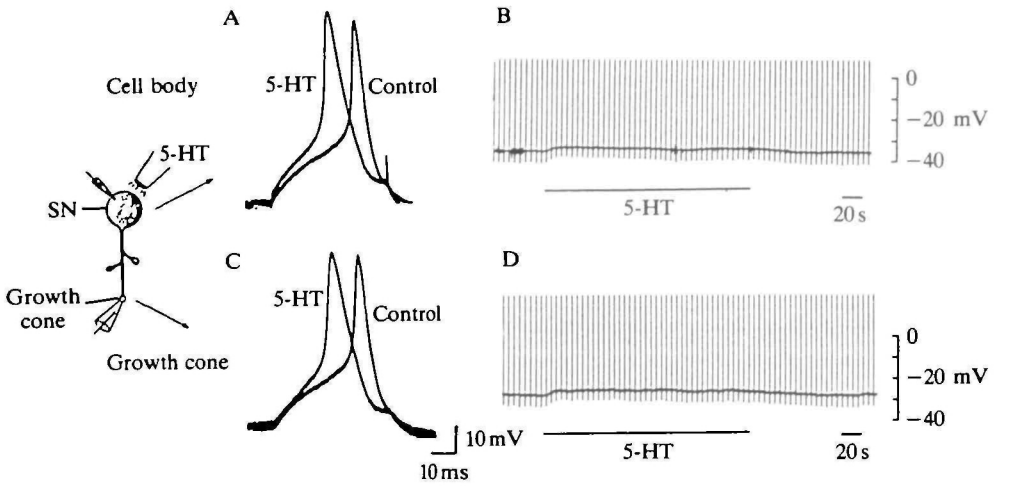

Fig. 6. Effect of serotonin application on the resting potential and action potentials recorded from the cell bodies and growth cones of *Aplysia* sensory neurones (SN) in culture. (A),(B) Recordings from cell body. (C),(D) Recordings from the growth cone. (A),(C) Simultaneous records of membrane potential from chart recorder showing a small, slow depolarization in response to serotonin (5-HT) application to the cell body (duration indicated by bar). Brief spikes are action potentials elicited by constant current pulses. (B),(D) Superimposed action potentials at a faster sweep photographed from the oscilloscope. 5-HT causes a 31 % increase in action potential duration in the cell body (B) and a 29 % increase in the growth cone (D). The drawing (inset) depicts the experimental protocol. (From Belardetti, Schacher, Kandel & Siegelbaum, 1986.)

in the cell body. These results are thus consistent with the idea that serotonin's action in the growth cone is also due to closure of the S potassium channels. Preliminary single-channel current recordings from growth cones demonstrate the presence of S channels and show that the channels close in response to 5-HT.

These findings demonstrate that serotonin modulates the action potential duration and resting potential in growth cones of *Aplysia* sensory neurones in a similar manner to its effects in the cell body. In view of the similarities between growth cones and nerve terminals, these results support the view that action potential broadening may also occur in the mature sensory neurone terminal, and thus may contribute to presynaptic facilitation. In addition to action potential broadening, serotonin also has a direct action on Ca^{2+} handling within the sensory neurone, leading to an increase in internal Ca^{2+} concentration that is independent of broadening (Boyle *et al.* 1984). Thus presynaptic facilitation is likely to result from the synergistic action of a number of mechanisms including channel modulation, changes in Ca^{2+} metabolism, and possibly changes in availability of vesicles for release (Hochner, Schacher, Klein & Kandel, 1985).

In the future it will be interesting to explore the role that other second messenger systems may play in regulating synaptic efficacy in the sensory neurones. Recent experiments show that the peptide FMRFamide produces a slow hyperpolarization, a decrease in action potential duration and presynaptic inhibition in the sensory neurones (Abrams *et al.* 1984). There is some evidence that this effect may involve

the opening of S channels (Erxleben, Brezina & Eckert, 1985; Belardetti *et al.* 1986) by a cyclic-AMP-independent mechanism (J. H. Byrne & T. W. Abrams, unpublished results). This system thus offers the exciting prospect of studying the

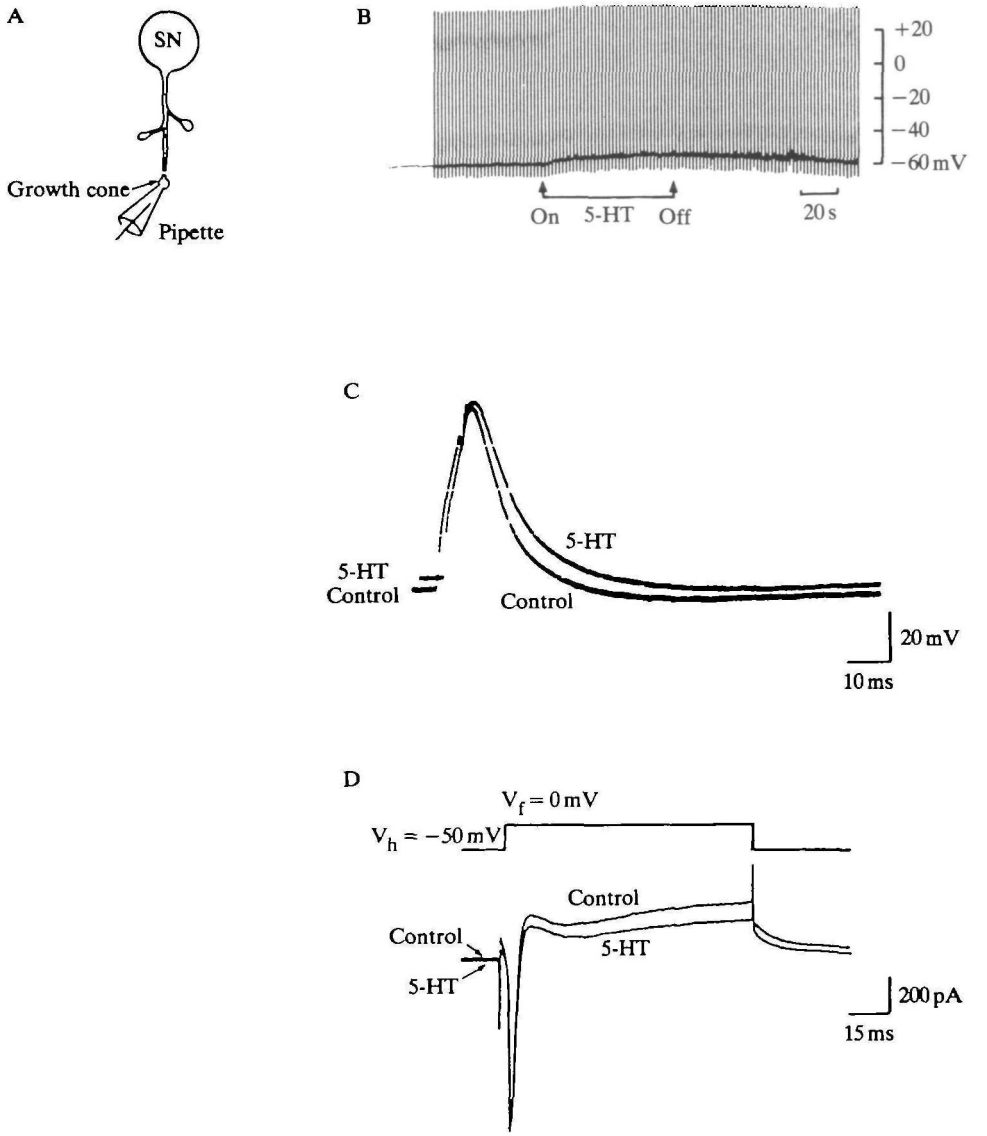

Fig. 7. Effect of serotonin (5-HT) on the resting potential, action potential and ionic currents from an isolated growth cone. (A) Experimental protocol. (B) Slow chart recording of membrane potential showing slow depolarization with 5-HT. The bar marks the duration of serotonin application. (C) Two superimposed action potentials from the oscilloscope at a faster sweep before and after serotonin application. The action potential duration is increased by 21% in response to 5-HT. (D) Superimposed voltage-clamp current records in response to a depolarization from −50 mV to 0 mV. The pulse elicits a brief inward current followed by a delayed outward current. 5-HT decreases the net inward current both at the holding potential and during the depolarization. (From Belardetti, Schacher, Kandel & Siegelbaum, 1986.)

up-and-down modulation of channel activity by different second messenger systems and the relationship of such channel modulation to the control of transmitter release.

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REFERENCES

ABRAMS, T. W., CASTELLUCCI, V. F., CAMARDO, J. S., KANDEL, E. R. & LLOYD, P. E. (1984). Two endogenous neuropeptides modulate the gill and siphon withdrawal reflex in *Aplysia* by presynaptic facilitation involving cAMP-dependent closure of a serotonin-sensitive potassium channel. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7956–7960.

ADAMS, D. J., SMITH, S. J. & THOMPSON, S. H. (1980). Ionic currents in molluscan soma. *A. Rev. Neurosci.* **3**, 141–167.

BELARDETTI, F., ABRAMS, T. W., CASTELLUCCI, V. F., KANDEL, E. R. & SIEGELBAUM, S. A. (1986). FMRFamide depresses transmitter release and increases the opening probability of the S K^+ channel in *Aplysia* sensory neurons. *Soc. Neurosci. Abstr.* (in press).

BELARDETTI, F., SCHACHER, S., KANDEL, E. R. & SIEGELBAUM, S. A. (1986). Serotonin modulates the action potential in growth cone precursors of sensory neuron terminals in *Aplysia*. In *Molecular Aspects of Neurobiology* (ed. Rita Levi Montalcini *et al.*). Berlin: Springer-Verlag.

BERNIER, L., CASTELLUCCI, V. F., KANDEL, E. R. & SCHWARTZ, J. H. (1982). Facilitatory transmitter causes a selective and prolonged increase in adenosine 3′:5′-monophosphate in sensory neurones mediating the gill and siphon withdrawal reflex in *Aplysia*. *J. Neurosci.* **2**, 1682–1691.

BERRIDGE, M. J. & IRVINE, R. F. (1984). Inositol triphosphate, a novel second messenger in cellular signal transduction. *Nature, Lond.* **312**, 315–321.

BOYLE, M. B., KLEIN, M., SMITH, S. J. & KANDEL, E. R. (1984). Serotonin increases intracellular Ca^{2+} transients in voltage-clamped sensory neurones of *Aplysia californica*. *Proc. natn. Acad. Sci. U.S.A.* **81**, 7642–7646.

CAMARDO, J. S., SHUSTER, M. J., SIEGELBAUM, S. A. & KANDEL, E. R. (1983). Modulation of a specific potassium channel in sensory neurones of *Aplysia* by serotonin and cAMP-dependent protein phosphorylation. *Cold Spring Harb. Symp. quant. Biol.* **48**, 213–220.

CASTELLUCCI, V. F., KANDEL, E. R., SCHWARTZ, J. H., WILSON, F. D., NAIRN, A. C. & GREENGARD, P. (1980). Intracellular injection of the catalytic subunit of cyclic AMP-dependent protein kinase simulates facilitation of transmitter release underlying behavioural sensitization in *Aplysia*. *Proc. natn. Acad. Sci. U.S.A.* **77**, 7492–7496.

CASTELLUCCI, V. F., NAIRN, A. C., GREENGARD, P., SCHWARTZ, J. H. & KANDEL, E. R. (1982). Inhibitor of adenosine 3′:5′-monophosphate-dependent protein kinase blocks presynaptic facilitation in *Aplysia*. *J. Neurosci.* **2**, 1673–1681.

CASTELLUCCI, V. F., SCHACHER, S., MONTAROLO, P. G., MACKEY, S., GLANZMAN, D. L., HAWKINS, R. D., ABRAMS, T. W., GOELET, P. & KANDEL, E. R. (1986). Convergence of small molecule and peptide transmitters on a common molecular cascade. *Progr. Brain Res.* **68** (in press).

ERXLEBEN, C., BREZINA, V. & ECKERT, R. (1985). A voltage-insensitive potassium conductance in *Aplysia* neurones is enhanced by FMRFamide and depressed by elevation of cAMP. *Soc. Neurosci. Abstr.* **11**, 710.

EWALD, D. A., WILLIAMS, A. & LEVITAN, I. B. (1985). Modulation of single Ca^{2+} dependent K^+-channel activity by protein phosphorylation. *Nature, Lond.* **315**, 503–506.

GREENGARD, P. (1976). Possible role for cyclic nucleotides and phosphorylated membrane proteins in postsynaptic actions of neurotransmitters. *Nature, Lond.* **260**, 101–108.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Arch. ges. Physiol.* **391**, 85–100.

HARTZELL, H. C. (1981). Mechanisms of slow postsynaptic potentials. *Nature, Lond.* **291**, 539–544.

HAYDON, P. G., MCCOBB, D. P. & KATER, S. B. (1984). Serotonin selectivity inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science* **226**, 561–564.

HEMMINGS, H. C., GREENGARD, P., TUNG, H. Y. L. & COHEN, P. (1984). DARP-32, a dopamine-regulated neuronal phosphoprotein, is a potent inhibitor of protein phosphatase-1. *Nature, Lond.* **310**, 503–505.

HOCHNER, B., SCHACHER, S., KLEIN, M. & KANDEL, E. R. (1985). Presynaptic facilitation in *Aplysia* sensory neurones: A process independent of K^+ current modulation becomes important when transmitter release is depressed. *Soc. Neurosci. Abstr.* **11**, 29.

HUGANIR, R. L. & GREENGARD, P. (1983). cAMP-dependent protein kinase phosphorylates the nicotinic acetylcholine receptor. *Proc. natn. Acad. Sci. U.S.A.* **80**, 1130–1134.

HUME, R. I., ROLE, L. W. & FISCHBACH, G. D. (1983). Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membrane. *Nature, Lond.* **305**, 632–634.

INGEBRITSEN, T. S. & COHEN, P. (1983). Protein phosphatases: Properties and role in cellular regulation. *Science* **221**, 331–338.

KACZMAREK, L. & LEVITAN, I. (1986). *Neuromodulation*. Oxford: Oxford University Press.

KANDEL, E. R. & SCHWARTZ, J. H. (1982). Molecular biology of learning: Modulation of transmitter release. *Science* **218**, 433–443.

KARLIN, A. (1980). Molecular properties of nicotinic acetylcholine receptors. In *Cell Surface Reviews: The Cell Surface and Neuronal Recognition,* vol. 6 (ed. C. W. Cotman, G. Poste & G. L. Nicolson). Amsterdam: Elsevier/North-Holland.

KEHOE, J. & MARTY, A. (1980). Certain slow synaptic responses: Their properties and possible underlying mechanisms. *A. Rev. Biophys. Bioeng.* **9**, 437–465.

KENNEDY, M. (1983). Experimental approaches to understanding the role of protein phosphorylation in the regulation of neuronal function. *A. Rev. Neurosci.* **6**, 493–525.

KLEIN, M., CAMARDO, J. S. & KANDEL, E. R. (1982). Serotonin modulates a specific potassium current in the sensory neurones that show presynaptic facilitation in *Aplysia. Proc. natn. Acad. Sci. U.S.A.* **79**, 5713–5717.

KLEIN, M. & KANDEL, E. R. (1980). Mechanism of calcium current modulation underlying presynaptic facilitation and behavioural sensitization in *Aplysia. Proc. natn. Acad. Sci. U.S.A.* **77**, 6912–6916.

KREBS, E. G. (1972). Protein kinases. *Curr. Topics Cell Reg.* **5**, 99–133.

KUPFERMAN, I. (1979). Modulatory actions of neurotransmitters. *A. Rev. Neurosci.* **2**, 447–465.

KUPFERMAN, I. (1980). Role of cyclic nucleotides in excitable cells. *A. Rev. Physiol.* **42**, 629–641.

LEVITAN, I. (1985). Phosphorylation of ion channels. *J. Membrane Biol.* **87**, 177–190.

NESTLER, E. J. & GREENGARD, P. (1983). Protein phosphorylation in the brain. *Nature, Lond.* **305**, 583–588.

OCORR, K. A. & BYRNE, J. H. (1985). Membrane responses and changes in cAMP levels in *Aplysia* sensory neurones produced by serotonin, tryptamine, FMRFamide and small cardioactive peptide B (SCPB). *Neurosci. Letts* **55**, 113–118.

POLLOCK, J., CAMARDO, J. S. & BERNIER, L. (1985). Serotonin and cyclic adenosine 3′:5′-monophosphate modulate the potassium current in tail sensory neurons in the pleural ganglion of *Aplysia. J. Neurosci.* **5**, 1862–1871.

PUTNEY, J. W., JR (1979). Stimulus permeability coupling: Role of calcium in the receptor regulation of membrane permeability. *Pharmac. Rev.* **30**, 209–245.

REVEL, H. R. (1963). Phosphoprotein phosphatases. *Meth. Enzym.* **6**, 211–214.

SCHACHER, S. & PROSHANSKY, E. (1983). Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J. Neurosci.* **3**, 2403–2413.

SHUSTER, M. J., CAMARDO, J. S., SIEGELBAUM, S. A. & KANDEL, E. R. (1985). Cyclic-AMP dependent protein kinase closes the serotonin-sensitive K^+ channels of *Aplysia* sensory neurones in cell-free membrane patches. *Nature, Lond.* **313**, 392–395.

SIEGELBAUM, S. A., CAMARDO, J. S. & KANDEL, E. R. (1982). Serotonin and cyclic AMP close single potassium channels in *Aplysia* sensory neurones. *Nature, Lond.* **299**, 413–417.

SIEGELBAUM, S. A. & TSIEN, R. W. (1983). Modulation of gated ion channels as a mode of transmitter action. *Trends Neurosci.* **6**, 307–313.

TADA, M. & KATZ, A. M. (1982). Phosphorylation of the sarcoplasmic reticulum and sarcolemma. *A. Rev. Physiol.* **44**, 401–423.

TRAUTMANN, A. & MARTY, A. (1984). Activation of Ca-dependent K channels by carbamoylcholine in rat lacrimal glands. *Proc. natn. Acad. Sci. U.S.A.* **81**, 611–615.

WALTERS, E. T., BYRNE, J. H., CAREW, T. J. & KANDEL, E. R. (1983). Mechanoafferent neurones innervating the tail of *Aplysia*. II. Modulation by sensitizing stimuli. *J. Neurophysiol.* **50**, 1543–1559.

YOUNG, S. J. & POO, M. M. (1983). Spontaneous release of transmitter from growth cones of embryonic neurons. *Nature, Lond.* **305**, 634–637.