Second messengers involved in the two processes of presynaptic facilitation that contribute to sensitization and dishabituation in *Aplysia* sensory neurons

(cAMP/protein kinase C/serotonin/learning/memory)

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ABSTRACT   Presynaptic facilitation of transmitter release contributes to behavioral sensitization and dishabituation, two simple forms of learning in *Aplysia*. This enhancement of transmitter release can be simulated by the facilitatory transmitter serotonin and has been shown to result from two types of mechanisms. The first facilitating process involves broadening of the presynaptic action potential in the sensory neurons of the reflex and is maximally effective when the synapse has not been depressed by repeated stimulation, as during sensitization. The second process is independent of changes in spike duration and can enhance release even when the synapse is quite depressed, as during dishabituation. Earlier work suggests that the first process is mediated by an increase in the intracellular level of cyclic AMP in the sensory neurons. We show here that release of free cyclic AMP from a photolyzable analogue introduced into sensory neurons can enhance release even at depressed synapses, indicating that cyclic AMP can activate the second as well as the first process. In addition, we find that phorbol esters, activators of protein kinase C, enhance release at depressed synapses. This is consistent with the report in the accompanying paper [Sacktor, T. C. and Schwartz, J. H. (1990) Proc. Natl. Acad. Sci. USA 87, 2036–2039] that serotonin and sensitizing stimuli translocate protein kinase C from cytoplasm to membrane. Our findings suggest that the cyclic AMP-dependent phosphorylation system can mediate more than one facilitatory process and that both cyclic AMP-dependent kinase and protein kinase C may be involved in facilitation of depressed synapses.

The gill- and siphon-withdrawal reflex of *Aplysia* can be modified by three forms of nonassociative learning: habituation, dishabituation, and sensitization (1, 2). Habituation is a decrease in reflex strength with repeated stimuli. Dishabituation is the enhancement of a habituated reflex response by a noxious stimulus, whereas sensitization is an enhancement of a nonhabituated reflex response by that stimulus. Habituation is associated with depression of transmitter release at the connections between the siphon sensory neurons and the motor cells that mediate the gill- and siphon-withdrawal reflex. Dishabituation and sensitization involve an increase in transmitter release at these same synapses (3). Two distinct cellular mechanisms in the sensory neurons contribute to the facilitation of transmitter release produced by serotonin (4, 5), a major facilitating transmitter released by natural dishabituating and sensitizing stimuli applied to the tail (6) and by stimulation of the connective that carries the neural information from the tail.

The first facilitatory mechanism is a decrease in outward K^+ current due to a closure of the "S"-type [serotonin

(5-HT) sensitive] K^+ channel (4, 7, 8, 20). The modulation of this ion channel is mediated by cAMP-dependent protein phosphorylation (8–10). Closure of the S channel broadens the action potential and enhances transmitter release by increasing the Ca^{2+} influx through voltage-gated N-type (41) rapidly inactivating Ca^{2+} channels (4, 7, 11). This facilitatory mechanism, referred to as the "first process," enhances transmitter release at nondepressed synapses (5) and parallels the behavioral process of sensitization.

The second facilitatory mechanism (5) becomes evident when the sensory motor cell connection becomes radically depressed by repeated activation of the sensory neuron, as in habituation. Under these circumstances spike broadening alone becomes less effective in facilitating the synaptic connection (4). 5-HT now produces a reversal of synaptic depression that is independent of spike broadening (5). This reversal of synaptic depression parallels the behavioral process of dishabituation. Hochner *et al.* (5) proposed that this second facilitatory mechanism, referred to as the "second process," might represent a direct modulation of transmitter release—either an increase in the availability of transmitter for release or a modulation of the release mechanism itself. A second facilitatory process independent of ionic currents was also suggested by Gingrich and Byrne (12) and by the experiments of Dale and Kandel (13) who found that 5-HT enhances spontaneous release of transmitter from the terminals of the sensory neurons by a mechanism independent of changes in the intracellular Ca^{2+}.

Although the first process is a major contributor to facilitation in nondepressed synapses and the second process is critical when the synapse is highly depressed (5), both mechanisms can contribute to both components of facilitation. Thus, once synaptic depression is reversed by the second facilitatory mechanism, the first process, spike broadening, will facilitate the depressed synapse further. Similarly, the second facilitatory process may contribute to facilitation at nondepressed synapses, albeit modestly (5).

In this paper, we investigate further the mechanisms involved in triggering the second process by exploring whether the two processes of facilitation previously distinguished physiologically can also be separated biochemically. Specifically, we have asked whether cAMP, the second messenger that mediates the first process, also triggers the second process or whether another second messenger is involved. We find that both cAMP and phorbol ester can facilitate depressed synapses, suggesting that although cAMP can contribute to the second process as well as to the first, the second process may, in addition, involve the protein kinase

C (PKC) pathway. In addition, we have found that H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride], an inhibitor of protein kinases, does not affect the facilitation by 5-HT of nondepressed synapses but selectively blocks a component of the synaptic facilitation of depressed synapses. These results provide independent evidence that facilitation of nondepressed and depressed synapses represents separate molecular processes. (For preliminary reports, see refs. 14 and 15.)

METHODS

Most experiments were done on primary cell cultures (16). Sensory cells were taken from pleural ganglia of adult *Aplysia* (80–150 g) and plated together with either siphon motor neuron LFS from adult *Aplysia* or gill motor neuron L7 from juvenile *Aplysia* (1–3 g). Intracellular microelectrodes containing 3 M KCl were used for stimulation and recording. The follower cell was hyperpolarized by about 40 mV from rest to prevent it from spiking. Data were recorded on tape and analyzed by using the "Spike" program (Hilal Associates). Recordings were made in culture medium with the following salt composition: 460 mM NaCl, 11 mM $CaCl_2$, 10 mM KCl, 55 mM $MgCl_2$. 5-HT (serotonin creatinine sulfate; Sigma) was dissolved in water at 10 mM. H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride; Seikagaku America, Saint Petersburg, FL] was dissolved in water at 20 mM, and aliquots were lyophilized and stored at 4°C. Both 5-HT and H-7 were diluted to the required concentration with recording medium. They either were applied directly to the bath or, when fast exchange of solution was desirable, were applied by using a multibarrel microperfusion system (17) whose tip was located 100–200 μm from the cells. Active (β) and inactive (α) isomers of phorbol dibutyrate and active forms of phorbol 12-myristate 13-acetate (LC Services) and phorbol 12,13-diacetate (Sigma) were dissolved in 100% dimethyl sulfoxide at 0.05 M and kept at −20°C. Before an experiment, they were diluted in recording medium and applied directly to the bath.

In the intact ganglion phorbol dibutyrate produced only weak effects that were delayed in onset by 30–40 min, perhaps because of poor penetration. In cell culture, phorbol dibutyrate produced brisk and reliable effects. The cAMP analogues 8-(4-chlorophenylthio)-cAMP and 8-benzylthio-cAMP, when present extracellularly, were unreliable in facilitating either depressed or nondepressed synapses and in modulating certain cAMP-dependent currents, perhaps because they cause only a relatively slow rise in cAMP-dependent protein phosphorylation. This problem is reduced by the procedure of Schacher *et al.* (18), which produces reliable facilitation with 8-benzylthio-cAMP but requires that the analogue be washed out of the bathing solution before the synapse is tested. Intracellular injections also require time for cAMP to reach the terminals (22). We therefore used the light-sensitive cAMP analogue dimethoxynitrobenzyl-cAMP (19) (Molecular Probes). Photolysis of "caged" cAMP allowed us to raise rapidly the level of cAMP throughout the sensory neurons. The caged cAMP was dissolved in dimethyl sulfoxide at 40 mM and incubated with neurons at a concentration of 50 μM for 30–60 min prior to the start of an experiment to allow maximal loading of cells. To photolyze the caged cAMP, we used a modified xenon arc flash lamp (Chadwick–Helmuth, model 238); the light was focused onto the neurons with an elliptical mirror. Flash durations were either 0.5 or 5 msec.

The effects of various treatments were calculated as the percent change from control—i.e., [(treatment−control)/control] × 100. Significance was calculated by Student's *t* test. To assess the action of H-7, we calculated the amount of depression of a given excitatory postsynaptic potential (EPSP) by using the following equation: Depression index = 1 − (EPSP$_i$/EPSP$_o$), where EPSP$_i$ is the average of the

control EPSP amplitude just before the application of 5-HT and the EPSP amplitude after washout of H-7 and 5-HT, and EPSP$_o$ is the amplitude of the first EPSP at the beginning of the experiment (which is assumed to be nondepressed). Calculating the depression index in this manner takes into account the ongoing depression of the sensory–motoneuron synapse with repeated stimulation. The effect of H-7 on the EPSP facilitated by 5-HT was measured as the EPSP amplitude in the presence of 5-HT and H-7 divided by the EPSP amplitude in the presence of 5-HT alone. Again, to take into account the ongoing depression, the EPSP amplitude in 5-HT alone was taken as an average of the EPSP before application of H-7 and the EPSP after washout of H-7.

RESULTS

5-HT can act through the cAMP-dependent protein kinase (PKA) to close K^+ channels in sensory neurons. Bath application of 5-HT produces four characteristic changes in these cells: (*i*) a decrease in the resting conductance; (*ii*) a depolarization of the resting membrane potential; (*iii*) a broadening of the action potential; and (*iv*) a reduction in spike frequency accommodation (3, 9, 20, 21). In addition, 5-HT produced facilitation of the synaptic potential that was accompanied by an increase in both the time to peak (33.5%; SD = 9.9, n = 8) and rate of rise of the EPSP (165%; SD = 145%, n = 8; Fig. 1A). Broadening of the presynaptic action potential alone primarily increased the time to peak of the EPSP without affecting the rate of rise; presumably this increase in time to peak results from prolonged influx of Ca^{2+} into the presynaptic neuron and prolonged transmitter release (4). Therefore, Hochner *et al.* (4) proposed that the increase in the rate of rise of the EPSP in response to 5-HT reflects the contribution of a second facilitatory process. Moreover, Hochner *et al.* (4) found that at nondepressed synapses, the change in time to peak of the EPSP contributed more to facilitation than did the increase in rate of rise. At depressed synapses the reverse was true: the increase in the rate of rise of the EPSP contributed more than the increase in time to peak. This raises the questions: Does cAMP, which mediates the first process, also mediate the second process, or is another second messenger system involved?

Phorbol Esters Enhance Transmitter Release Without Producing Changes in Presynaptic Input Resistance or Excitability. Consistent with the findings of Hochner *et al.* (4), we observed that depressed sensory neuron synapses in culture can be facilitated by addition of phorbol esters. Of three phorbol esters that we have tried (phorbol 12,13-dibutyrate, phorbol 12,13-diacetate, and phorbol 12-myristate 13-acetate), we found phorbol dibutyrate to be the most potent. It caused facilitation at concentrations ranging from 1 to 50
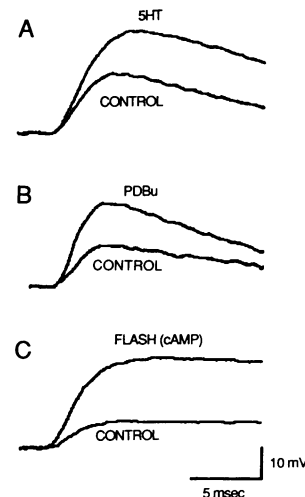

Fig. 1. Facilitation of depressed synaptic potentials by 5-HT, phorbol esters, and cAMP. (*A*) Facilitation by 10 μM 5-HT of a depressed EPSP involves an increase in both the rate of rise and the time-to-peak of the EPSP. (*B*) Facilitation by 10 nM phorbol dibutyrate (PDBu) of a depressed EPSP involves an increase in the rate of rise with no change in the time to peak. (*C*) Facilitation of a depressed EPSP by release of intracellular cAMP involves an increase in the rate of rise; the increase in the time to peak of the EPSP after the flash seen in this example was not typically observed.

nM (Fig. 2B). At 10 nM this facilitation was 5-fold, had an onset within 30 sec, and reached a peak within 1–5 min. The low concentrations needed to obtain facilitation suggest that the effect is likely due to the specific activation of PKC. This conclusion is supported by the finding that the isomer 4α-phorbol 12,13-dibutyrate, which is ineffective in activating PKC, did not cause any facilitation; it caused only a small decrease in the EPSP (−7%; SD = 18%, n = 4; P > 0.25). In preliminary experiments, we found phorbol esters also facilitated nondepressed synapses.

Whereas 5-HT and cAMP cause a decrease in the resting conductance of the sensory neurons, phorbol esters caused no change or a small increase in resting conductance (13%; SD = 19%, n = 11). Phorbol esters also did not change the resting potential, the excitability, or the S-type K⁺ current of the sensory neuron. Moreover, the facilitation of the EPSP caused by phorbol esters was entirely due to an increase in rate of rise of the EPSP (262%; SD = 165%, n = 9) with no significant change in time to peak (3.2%; SD = 8.4%, n = 9; P > 0.25; Fig. 1B). Thus, the facilitation induced by phorbol esters does not resemble that caused by the first process produced by 5-HT at nondepressed synapses. Rather, phorbol esters cause facilitation like that caused by the second process, the reversal of synaptic depression produced by 5-HT.

cAMP Can Enhance Transmitter Release in Depressed Synaptic Connections. We have previously found that intracellular injection of either cAMP or the catalytic subunit of the cAMP-dependent protein kinase A (PKA) enhances both nondepressed and moderately depressed synapses (9, 18, 22). However, the relationship between synaptic depression and the efficacy of cAMP in inducing facilitation was not examined previously. Therefore, we studied the ability of cAMP to produce facilitation in greatly depressed synapses where spike broadening alone would be ineffective.

To stimulate cAMP-dependent phosphorylation, we used light-induced release of cAMP from a cell-permeant "caged" cAMP compound, dimethoxynitrobenzyl-cAMP. This allowed us to rapidly elevate the level of cAMP throughout a sensory neuron, including in presynaptic terminals. We estimated that single light flashes raised intracellular cAMP levels transiently to between 5 and 10 μM, which is likely to be within or near the physiological range. We found that cAMP released by light flashes can facilitate even synapses that are extremely depressed (Fig. 1C). The facilitation due to the cAMP released by a single flash was 1.8-fold, about one-third of the increase caused by a 2-min exposure to 1–3

μM 5-HT. A single flash was also substantially less effective than 5-HT in producing spike broadening in tetraethylammonium, where the S current closed by cAMP is the main current that repolarizes the action potential. This latter result suggests that the total amount of cAMP-dependent phosphorylation produced by a single flash is less than that produced by 2-min exposures to 5-HT. By contrast, with a series of four to eight flashes, facilitation was substantially larger, averaging 3.8-fold (Fig. 2A). As was the case with phorbol esters, the increase produced by cAMP at depressed synapses resembled the action of 5-HT on the second process in that the facilitation occurred primarily through an increase in the rate of rise of the EPSP. The light flashes had no effects on synaptic transmission in preparations that were not exposed to the caged cAMP or that were exposed to "caged" acetate. (In four of six experiments with caged acetate, flashes produced a small decrease in EPSP amplitude; in the other two instances, increases of 8% and 10% were observed.)

H-7 Preferentially Reduces Facilitation of Depressed Synapses. Since both cAMP and phorbol esters are capable of simulating the second process, we wanted to see whether PKC actually has a physiological role in the second process. Therefore, we used H-7, a blocker that has been reported to preferentially inhibit PKC in intact *Aplysia* neurons (23), although it inhibits both PKC and PKA *in vitro* (24). When applied at 200–400 μM to sensory and motor neurons in culture, H-7 caused a small depolarization (2.4 mV; SD = 1.1, n = 6) and conductance increase (13%; SD = 9%, n = 5) in the sensory neuron. Similarly, H-7 also depolarized the LFS motor cell by 5.2 mV (SD = 4.4, n = 5). The amplitude of the EPSP was also reduced by H-7 alone (22.7%; SD = 14.1%, n = 29; see Fig. 4B Upper). In addition to these effects on the resting properties of the neurons, H-7 at 200–400 μM reduced the EPSP facilitated by phorbol dibutyrate substantially, on average by 62% (SD = 18%; n = 6; Fig. 3). Since H-7 reduced facilitation produced by phorbol dibutyrate, suggesting it could inhibit PKC, we examined the effects of H-7 on the facilitation by 5-HT of evoked release. In each experiment, the sensory neuron was stimulated at 50-sec intervals one or more times prior to application of 10 μM 5-HT. After addition of 5-HT, the EPSP was retested; 400 μM H-7 was added for less than 2 min, the EPSP was tested, and H-7 was washed out with a 5-HT-containing solution. The EPSP was then tested once more.

The efficacy of H-7 in blocking facilitation depended on the state of depression of the synapse. Fig. 4A shows examples from one experiment, where different depression levels were achieved at the same synapse, while Fig. 4B Lower is the summary of 18 applications of H-7 to nine cultures. There is a direct relationship between the reduction in the facilitated EPSP amplitude caused by H-7 and the degree of depression of the EPSP prior to addition of 5-HT. The amount of synaptic facilitation evoked by 5-HT was strongly correlated with the degree to which the synapses were depressed. In synapses that were depressed to 80–100% of control, 5-HT
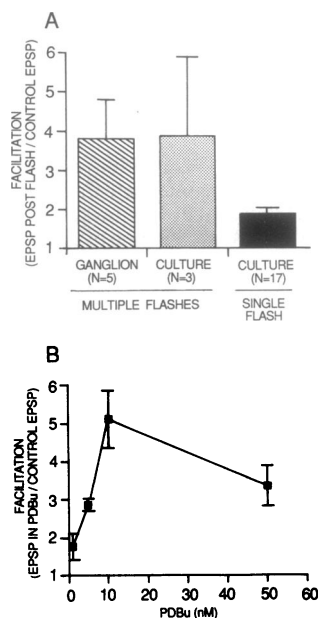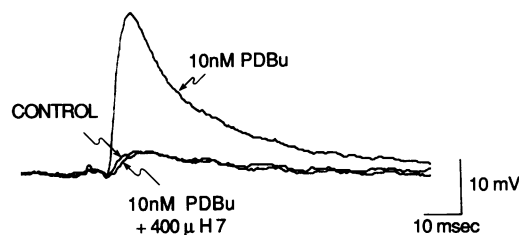
A

B

FIG. 2. Summary of facilitation caused by light-released cAMP and by phorbol dibutyrate (PDBu). (A) Average of facilitations of depressed synapses produced by light-release of caged cAMP. Multiple flashes of light produce larger facilitation than does a single flash. Facilitation can be produced in intact ganglia as well as in synapses in culture (error bars represent SEM). (B) Dose-dependence of the facilitation of depressed synapses by PDBu; mean of five experiments for each dose. PDBu caused facilitation at concentrations as low as 1 nM, with an optimal dose of 10 nM (error bars represent SEM). All experiments with PDBu were performed in culture.

FIG. 3. H-7 reverses facilitation by phorbol dibutyrate (PDBu). Application of 10 nm PDBu caused facilitation of a depressed EPSP; 400 μM H-7 applied in the presence of PDBu reversed the facilitation within <2 min.

caused a mean facilitation of 1.9-fold (SD 0.53, $n = 4$). With greater depression (45–55% of control), the amount of facilitation caused by 5-HT was 2.5-fold (SD 0.98, $n = 5$), while in synapses depressed to <20% of control, 5-HT elicited facilitation of 9.05-fold (SD 7.96, $n = 4$). When a synapse was not depressed, H-7 had no effect on facilitation. However, as a synapse became depressed with repeated activation, H-7 caused a progressively greater reduction in the facilitated EPSP. Thus, when the synaptic potential was depressed by 50%, H-7 reduced the facilitated EPSP by 42%. When the synaptic potential was depressed by 80%, H-7 reduced the facilitated EPSP by 70%.

Whereas *in vitro* assays indicate that H-7 inhibits PKA and PKC equally effectively (24), in the bag cells and in B15 cells of *Aplysia* (23, 25), H-7 blocks the effects of phorbol esters with cAMP-mediated effects. Consistent with a preferential inhibition of protein kinase C, we found that H-7 did not affect the decrease in resting sensory neuron conductance produced by 8-(4-chlorophenylthio)-cAMP. On the other hand, H-7 reduced the facilitation of depressed synapses by

"caged" cAMP by an amount comparable to its reduction of facilitation by 5-HT, suggesting that H-7 might interfere with a process affected by both PKA and PKC, perhaps a common substrate. Regardless of its specificity for PKA or PKC or its site of action, the selective blockade by H-7 provides independent evidence for the existence of at least two processes in facilitation.

DISCUSSION

We here provide independent biochemical and pharmacological evidence for the distinction between facilitation at depressed and nondepressed synapses proposed by Hochner *et al.* In addition, our experiments indicate that both PKA and PKC are capable of reversing the depression of synaptic transmission caused by repeated activations of the synapse.

Two Kinases Are Capable of Simulating the Second Process. Experiments on the second messengers involved in the second component of facilitation have led to two findings. First, cAMP can cause facilitation of depressed as well as nondepressed synapses. Thus, a single second messenger, cAMP, can mediate more than one facilitatory process in the same neuron. Since broadening of the sensory neuron action potential, as occurs with reduction in the S-type K^+ current, is insufficient to facilitate depressed synapses, cAMP must also act at a second step distal to Ca^{2+} entry, where it can modulate some property of the synaptic vesicle mobilization or the release process. This double function of PKA is consistent with the work of Sweatt *et al.* (26), who found that both cAMP and 5-HT act on not one but on a family of common substrate proteins, including cytoskeleton proteins like actin that could be important in vesicle mobilization. Consistent with these results, S. Schacher, P. G. Montarolo, and E.R.K. (unpublished results) found that the peptide transmitter SCP_B (small cardioactive peptide B) normally facilitates nondepressed, but not depressed, sensory neuron synapses. However, raising the level of cAMP by means of inhibitors of cAMP phosphodiesterase (3-isobutylmethyl-xanthine or Ro-20-1724) enables SCP_B to enhance transmitter release of even depressed synapses. Second, although activators of PKC do not cause spike broadening or modulation of K^+ currents and therefore are not likely to contribute to the first process, these activators can facilitate transmission at depressed synapses. Moreover, the biochemical observations that 5-HT causes translocation of PKC from cytosol to membrane in sensory neurons (15, 27) suggest that activation of PKC may contribute to the facilitation by 5-HT at depressed synapses.

H-7 Provides Independent Pharmacological Evidence for Two Processes in Facilitation. H-7, a general inhibitor of protein kinases, selectively inhibits the second process, removal of synaptic depression, without affecting the first process, the facilitation of the nondepressed synapse produced by the broadening of the presynaptic action potential. We do not know whether this blockade of synaptic facilitation of depressed PSPs results from inhibition of one or both protein kinases or from an interaction of H-7 with some other molecule involved in reversal of synaptic depression. Regardless of its site of action, H-7 is a useful pharmacological tool for separating the two facilitatory processes and provides additional evidence for their independence. A more direct molecular and morphological characterization of the two processes will be required, however, before it is possible to relate these processes to specific steps in transmitter release.

Relative Contributions of Different Kinases to the Second Process. The relative contributions of the two second-messenger systems to the second process remain to be determined. It is possible that one or the other kinase alone triggers the second process. Alternatively, two kinases, PKA and PKC, could act together in series to trigger the second process. More likely, however, facilitation in the depressed

A

B EFFECT OF H7 ON CONTROL EPSP

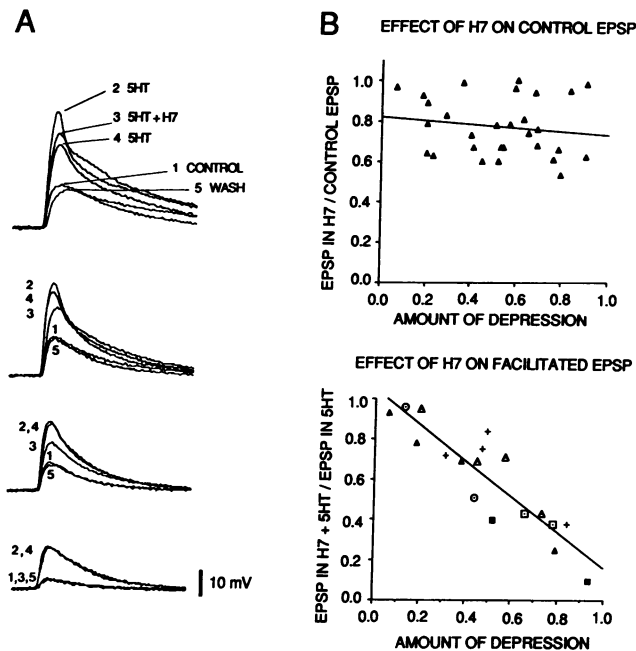EFFECT OF H7 ON FACILITATED EPSP

FIG. 4. The effect of H-7 on the EPSP facilitated by 5-HT depends on the state of depression of the synapse: the more depressed the synapse, the larger the effect of H-7. The sensory cell was fired once every 50 sec, and the following protocol was carried out. (*i*) a control EPSP was elicited prior to application of 5-HT; (*ii*) 5-HT (10 μM) was applied for two consecutive stimuli; (*iii*) H-7 (400 μM, in the presence of 5-HT) was applied for two additional stimuli; (*iv*) H-7 was washed out with a 5-HT-containing solution; and (*v*) 5-HT was washed out. (*A*) EPSPs from an experiment in a cell where the protocol described above was repeated four times, at different levels of depression. Each set of five superimposed EPSPs represent an experiment at a different level of depression. (*B*) The effect of H-7 on control EPSPs (*Upper*) and on EPSPs facilitated by 5-HT (*Lower*) as a function of the amount of depression of the EPSP (calculations were done as described in *Methods*). (*Upper*) H-7 alone caused some reduction in the EPSP amplitude; this effect does not seem to correlate with the amount of depression of the EPSP (summary of 29 applications of 400 μM H-7 in 13 cultures). (*Bottom*) The reduction by H-7 of EPSPs facilitated by 5-HT shows a direct relationship with the amount of depression of the synapse. The straight line is the best least-squares fit to the data ($r = 0.90$). Eighteen data points were pooled from nine experiments (nine different cultures). The crosses represent four experiments in which only one datum point was obtained; the other symbols denote data points in five other experiments where the effect of H-7 was examined at more than one level of depression.
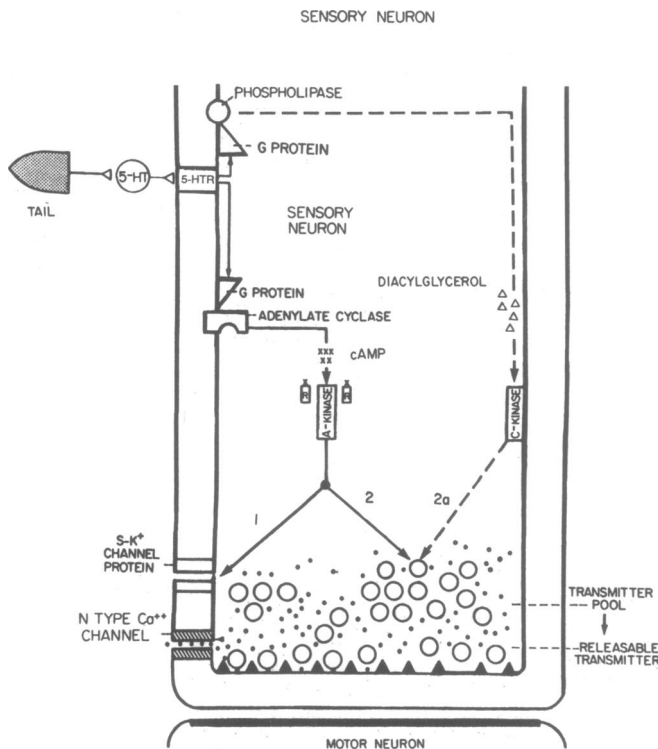
FIG. 5. Schematic summary of proposed second-messenger pathways involved in presynaptic facilitation. Sensitizing stimulation of the tail (upper left; 5-HTR = 5 HT receptor) causes release of at least three facilitating transmitters; 5-HT, the small cardioactive peptide (SCP), and a still unidentified transmitter released by the L29 cells. Of these, only 5-HT is illustrated here. 5-HT activates adenylate cyclases in the membrane of the sensory neuron, leading to the synthesis of cAMP and the activation of the A kinase. The cAMP pathway leads to two detectable effects (arrows 1 and 2) that contribute to release: (1) an effect on the S-type K^+ channel leading to its closure (described in earlier work; see refs. 3 and 8) and (2) an effect on transmitter mobilization or on availability (described in this paper). In addition, the activation of a PKC (dashed lines) is suggested by results described in this report and by the translocation studies of Sacktor and Schwartz (27). The PKC pathway does not affect the S-type K^+ channel but appears to affect availability (2a dashed arrow) of transmitter.

state may be mediated by two modulatory systems, PKA and PKC, converging to phosphorylate a common set of substrates and thus jointly trigger the second process. This possibility is illustrated schematically in Fig. 5. According to this view, H-7 may produce its selective effects by interfering with the action of both kinases on the common substrate important for overcoming synaptic depression.

Multiple Kinases in Cellular Regulation and Synaptic Plasticity. Several recent studies suggest that in other cellular processes, independent second messenger systems can act synergistically (see refs. 28–32). This synergism also may be important for some forms of synaptic plasticity. For example, studies of long-term potentiation in the hippocampus, an attractive candidate for a mechanism of associative learning, suggest a critical role for Ca^{2+} influx (33, 34) for PKC (35, 36), for Ca^{2+}/calmodulin-dependent kinase (37), and for the arachidonic acid cascade (38). Similarly, studies of classical conditioning in *Hermissenda* indicate that the changes in membrane excitability during learning may involve Ca^{2+}/ calmodulin-dependent kinase, PKC, and perhaps also PKA (39, 40). Thus, in diverse neural systems several second-messenger processes may act in concert to produce plastic changes important for learning.

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