Temporal asymmetry in activation of *Aplysia* adenylyl cyclase by calcium and transmitter may explain temporal requirements of conditioning

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ABSTRACT   Cellular experiments have suggested that during classical conditioning of the gill and siphon withdrawal reflex of *Aplysia*, adenylyl cyclase may serve as a molecular site of convergence for Ca^{2+} and serotonin (5-hydroxytryptamine; 5-HT), the cellular representations of the conditioned and unconditioned stimuli (CS and US). We explored the possible molecular basis of the behavioral requirement that the CS and US be paired within a narrow time window and in the appropriate order. To examine the temporal interactions of brief pulses of Ca^{2+} and 5-HT in stimulating *Aplysia* neural cyclase, we used a perfused-membrane cyclase assay. When brief pulses of Ca^{2+} and 5-HT were paired, cyclase activation depended upon the sequence of the pulses: peak cyclase activation was greater when the Ca^{2+} pulse immediately preceded the 5-HT pulse. Examination of the rising phase of 5-HT stimulation suggested that a Ca^{2+} prepulse might accelerate the onset of activation by 5-HT, without affecting the final level of activation achieved with prolonged 5-HT exposure. The observed interactions between Ca^{2+} and transmitter in activating cyclase could contribute importantly to the temporal requirements of conditioning for CS–US pairing.

To function effectively, animals need to learn relationships between stimuli or events within their environment. Recent cellular studies of neural plasticity in both molluscs and mammals suggest that during associative learning, dually regulated proteins, such as the N-methyl-D-aspartate receptor, serve as the sites where inputs from behavioral stimuli converge and critical associative interactions occur (1, 2). For example, in conditioning of the defensive gill and siphon withdrawal reflex of *Aplysia*, the Ca^{2+}/calmodulin (CaM)-sensitive adenylyl cyclase may play an associative role (1). In this conditioning paradigm, animals learn to increase their withdrawal response to the conditioned stimulus (CS), a light siphon touch, when it is paired with the unconditioned stimulus (US), a tail shock (3). The US triggers release of modulatory transmitters, including serotonin (5-HT) (4, 5). These modulatory transmitters facilitate synaptic transmission from the siphon mechanosensory neurons, which are activated by the CS, to postsynaptic neurons that produce the withdrawal response. This synaptic facilitation is substantially due to activation of adenylyl cyclase in the sensory neurons (6, 7, 8). When sensory neuron activity or depolarization immediately precedes the arrival of facilitatory transmitter, as during pairing of the CS and US in associative training, both the rise in cAMP levels and the synaptic facilitation are enhanced (1, 9–13). Ca^{2+} influx during sensory neuron activity is critical for this activity-dependent enhancement of presynaptic facilitation (13). Thus, Ca^{2+} may

serve as the cellular signal representing the CS, while facilitatory transmitter is the signal from the US.

These cellular studies suggested that elevated intracellular Ca^{2+} might potentiate the activation of cyclase by facilitatory transmitter; this interaction may underlie activity-dependent facilitation and, thus, contribute to conditioning (1). Consistent with this hypothesis, *Aplysia* nervous system was found to contain Ca^{2+}/CaM-sensitive cyclase that is also activated by the stimulatory GTP-binding protein, G_s, which couples receptors to cyclase (14). This analysis of associative synaptic plasticity in *Aplysia*, together with studies of the *Drosophila* mutant rutabaga, which is deficient in learning and memory and which lacks Ca^{2+}/CaM-sensitive cyclase (15–18), suggested a functional role for this dually regulated enzyme. Moreover, in mammalian brain, Ca^{2+}/CaM-sensitive cyclase is relatively abundant in areas implicated in learning (19).

If the Ca^{2+}/CaM-sensitive cyclase is the site of associative convergence between the inputs from the CS and US, then the activation properties of the enzyme might explain the temporal requirements of the associative learning in this system. In many forms of classical conditioning, animals learn that the CS predicts the arrival of the US if the two stimuli occur within a specific time window and in the appropriate order (20). In conditioning of the defensive withdrawal reflex, *Aplysia* learn to increase their response to the CS if the CS and US are presented within a 1-sec interval during training. There is also a sequence requirement: associative learning does not result from backward pairing in which the US begins first during training, even when the two stimuli overlap temporally (21). Activity-dependent presynaptic facilitation has similar sequence requirements for pairing: sensory neuron activity is most effective in enhancing the response to facilitatory transmitter if it precedes the transmitter (22).

To further explore the hypothesis that Ca^{2+} potentiates activation of cyclase by transmitter and that this interaction contributes to activity-dependent facilitation and conditioning of the defensive withdrawal reflex, we have asked two questions. First, do Ca^{2+} and 5-HT act synergistically in stimulating adenylyl cyclase? Second, does the cyclase display a sequence requirement for optimal activation by Ca^{2+} and transmitter that parallels the temporal requirements for CS–US pairing in the conditioning of the withdrawal reflex? In the experiments described here, which employed a perfused-membrane methodology to transiently apply Ca^{2+} and 5-HT, we found that cyclase activation depended upon the precise temporal relationship of the Ca^{2+} and 5-HT exposures. By analyzing the conditions necessary for optimal activation to occur, we were able to gain some insight into the nature of the mechanisms by which Ca^{2+} and 5-HT interact.

METHODS

Steady-State and Perfused-Membrane Cyclase Assays. Cyclase activity under constant, steady-state conditions was assayed as described (14, 23). Continuous assay of cyclase stimulated with transient exposures to 5-HT and Ca^{2+} was accomplished by using a modification of a perfused-membrane method (24) in which neural membranes are retained on a low-density fibrous filter (Millipore AP25, 13 mm) and perfused with assay solution. A valve (General Valve 9-453-900) upstream from the filter, with five input ports gated by computer-activated solenoids, selected among four assay solutions: (*i*) low Ca^{2+}, (*ii*) high Ca^{2+}, (*iii*) 20 μM 5-HT and low Ca^{2+}, and (*iv*) 20 μM 5-HT and high Ca^{2+}. The fifth port enabled washing of a filter prior to each assay to remove cytosol. Assay solution was drawn at 2.5 ml/min by a peristaltic pump downstream from the filter and collected in 6-sec fractions. For each perfused membrane experiment, five or six nervous systems of *Aplysia californica* were homogenized (14, 24); 25% of the homogenate was applied to a filter for each of four assays. Forward vs. backward pairing was compared within preparations, with each assayed twice in an A–B–B–A sequence.

Solutions. All buffers contained 50 mM K-Hepes (pH 7.6), 75 mM KCl, 1 mM dithiothreitol, and protease inhibitors (14, 32). Homogenization buffer contained, in addition, 3 mM EGTA. Assay solutions contained, in addition, 10 μM [α-^{32}P]ATP ($\approx$5 $\times$ 10^7 cpm/ml), 50 μM [^{3}H]cAMP (5 $\times$ 10^4 cpm/ml; for normalization of column recovery and fraction volume in perfused-membrane assays), 2 μM CaM, 10 μM GTP, 0.5 mM isobutylmethylxanthine, creatine phosphokinase at 2.5 units/ml, 5 mM creatine phosphate, and one of eight Ca^{2+}/EGTA buffers with Mg^{2+}. Three pairs of Ca^{2+} buffers were used in perfused-membrane assays and a fourth pair was used in steady-state assays. Final total concentrations of EGTA/Ca^{2+}/Mg^{2+} in low- and high-Ca^{2+} buffers were (in mM): in A$_{lo}$ and B$_{lo}$, 3.0/0.015/1.45; in A$_{hi}$, 3.0/5.0/1.0; in B$_{hi}$, 3.0/4.5/1.0; in C$_{lo}$, 0.1/0.065/2.0; in C$_{hi}$, 0.1/0.12/2.0; in D$_{lo}$, 2.5/0.6/2.5; and in D$_{hi}$, 2.5/2.48/2.0. Free Mg^{2+} was 1 mM in buffers A and B and 2 mM in buffers C and D.

Estimates of Stimulating Ligand Concentration in a Pulse. While the valve had an internal volume of <6 μl, the filter itself contained $\approx$170 μl of solution; thus during and after a pulse of Ca^{2+} or 5-HT, the ligand concentration changed gradually at the membranes (Fig. 1*A*). For example, a "1-sec pulse" of

5-HT produced an exposure to 5-HT of >6-sec width at half-maximal concentration. Although we attempted to achieve briefer exposures, the low rate of cAMP synthesis precluded faster flow rates. The 5-HT concentration is simply proportional to the ratio of assay solutions with and without transmitter at the membranes (Fig. 1*A*). In contrast, the free Ca^{2+} transient is partially determined by chelation—i.e., the Ca^{2+} profile is influenced by the EGTA and Ca^{2+} concentrations in the Ca^{2+} buffers. Profiles of "relative effective concentrations" of 5-HT and Ca^{2+} were estimated from solution change vs. time curves (Fig. 1*A*) in combination with data from steady-state assays on cyclase stimulation with various concentrations of 5-HT or various ratios of high- and low-Ca^{2+} buffers (Fig. 1*B* and *C*). It should be emphasized that although these curves are expressed as "% stimulation above basal," they are not predictions of instantaneous cyclase activation. (They do not correspond to cyclase activation because binding and unbinding of ligand and activation and inactivation of cyclase all require time; moreover, in the case of Ca^{2+} stimulation, the presence of GTP in perfused membrane assays would reduce the percent stimulation by Ca^{2+} compared with Fig. 1*C*.). In addition, because mixing occurred within the filter, these curves correspond to average relative effective ligand concentrations at the membranes at each time.

RESULTS

Ca^{2+} and 5-HT Do Not Activate Cyclase Synergistically in the Steady State. To test whether *Aplysia* neural adenylyl cyclase is activated synergistically by Ca^{2+} and transmitter, we measured cyclase activity with free Ca^{2+} at either low or stimulatory levels, with or without 5-HT. In *Aplysia* neural membranes in the presence of physiological concentrations of CaM and Mg^{2+}, increasing free Ca^{2+} from <0.1 to 1–3 μM produces modest cyclase stimulation, typically ranging from 1.5- to 2-fold in the absence of GTP (half-maximal stimulation at about 0.3 μM) (14, 25). In the presence of GTP, which increases basal activity 3- to 5-fold, the relative stimulation by Ca^{2+} is substantially smaller (Fig. 2). 5-HT with GTP produces relatively powerful stimulation, ranging from 2- to 10-fold. Dual activation of the cyclase by Ca^{2+} and 5-HT was not even minimally synergistic (i.e., dual activation was not greater than the sum of the two separate stimulations) (Fig. 2). This lack of synergism, which was observed in >12 experiments, does not represent a ceiling effect because synergism was also absent in four experiments examining
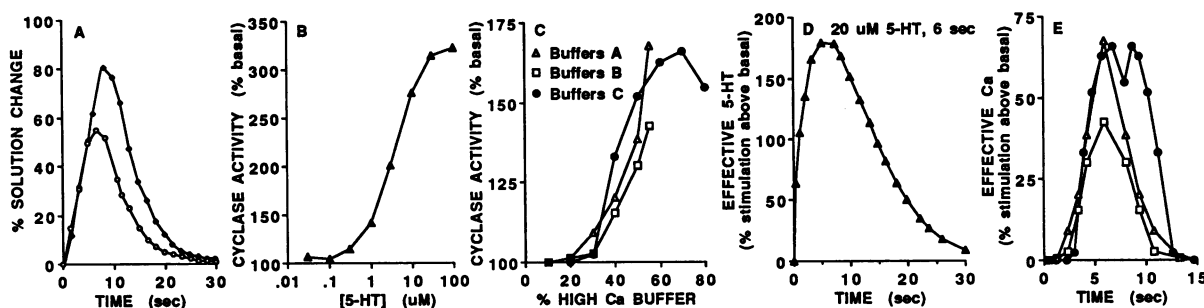

FIG. 1. Estimation of 5-HT and Ca^{2+} pulse profiles in perfused membrane experiments. (*A*) Percent of stimulus solution vs. time for stimulus pulses of 6 (o) and 8 (♦ or ●) sec. Measurements were made with a radioactivity-detector flow cell immediately downstream from the filter and with ^{32}P in the stimulus solution. (*B*) Cyclase activity vs. 5-HT concentration measured in steady-state assays, expressed as the percent of basal activity, which was measured with 10 μM GTP and without 5-HT. (*C*) Cyclase activity vs. the ratio of high-Ca^{2+}/low-Ca^{2+} buffers in steady-state assays, expressed as the percent of basal activity in the low-Ca^{2+} buffer. Because GTP increases basal cyclase activity (14) thereby reducing relative Ca^{2+} stimulation these assays were done without GTP to more accurately measure Ca^{2+} sensitivity. The highest level of free Ca^{2+} tested gave submaximal stimulation because of the biphasic Ca^{2+} dependence of CaM-sensitive cyclase (14). Data in *B* and *C* are the means of three experiments on different preparations, each assayed in quadruplicate. (*D*) Calculated "relative effective concentration" of 5-HT during and after a 6-sec 5-HT pulse. This effective 5-HT profile was generated from the steady-state data on 5-HT stimulation (from *B*) in combination with the solution change profile (from *A*). (*E*) Calculated "relative effective concentration" of Ca^{2+} during and after a Ca^{2+} pulse for a 6-sec pulse with buffer pairs A (△) and B (□) and an 8-sec pulse with buffer pair C (●). These estimates of Ca^{2+} pulse profiles were generated from the data on steady-state Ca^{2+} stimulation (from *C*) in combination with the solution change profile (from *A*). Note that *D* and *E* are estimated profiles of effective concentrations of 5-HT and Ca^{2+} at the membranes and not instantaneous cyclase stimulation.
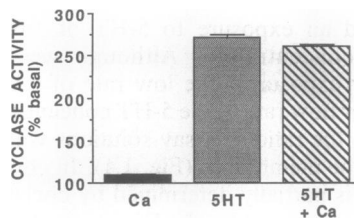
FIG. 2. Ca^{2+} and 5-HT do not interact synergistically in activating *Aplysia* neural adenylyl cyclase under steady-state conditions. Stimulating ligands (either 3 μM free Ca^{2+} or 10 μM 5-HT or both) were present throughout the 5-min assay. Activity is expressed as the percent of basal activity (assayed with 10 nM free Ca^{2+}). All assays contained 2 μM CaM, 10 μM GTP, and 2 mM free Mg^{2+}. Basal activity was 28.7 ± 2.03 pmol/min per mg of protein. Data are means ± SD of four replicate assays on membranes from two nervous systems.

effects of Ca^{2+}/CaM on 5-HT dose–response curves; furthermore, *Aplysia* neural cyclase can be stimulated more powerfully by artificial activators (14, 32). Similar absence of synergism in activation of cyclase by Ca^{2+} and transmitter has been observed in *Drosophila* cyclase (17) and some (26, 27), though not all (28, 29), studies of mammalian brain cyclase. During conditioning and activity-dependent facilitation, Ca^{2+} (30) and facilitatory transmitter are likely to be at stimulatory levels only transiently. This suggested that brief pulses of Ca^{2+} and 5-HT might act synergistically in activating cyclase, even though prolonged stimuli do not.

Perfused-Membrane Assays with Transient Stimuli Reveal Temporal Asymmetry in Cyclase Activation. Previous studies of temporal requirements for cyclase activation by Ca^{2+} and 5-HT (14) were limited by the conventional assay methodology in which transmitter, once added, is present for the remainder of the assay. We adapted a perfused-membrane cyclase assay (24) that allows the delivery of brief pulses of stimulating ligands while cyclase activity is assayed continuously. In this method, homogenized neural membranes are embedded in a filter and perfused with assay solution containing $[\alpha\text{-}^{32}P]ATP$ (the substrate for the cyclase), GTP, CaM, and a Ca^{2+} buffer. The $[\alpha\text{-}^{32}P]cAMP$ synthesized by the immobilized membranes during 6-sec intervals is chromatographically separated (23) and quantified. The perfusate could be switched from control assay solution with low Ca^{2+} to assay solution with either high Ca^{2+} or 5-HT. In each assay, a 6-sec Ca^{2+} pulse was paired with a 6-sec 5-HT pulse (see Fig. 1 for pulse profiles at the membranes). With forward pairing (where Ca^{2+} is followed by 5-HT), the Ca^{2+} exposure ended before the peak of the 5-HT exposure; with backward pairing (where 5-HT precedes Ca^{2+}), free Ca^{2+} increased after the 5-HT concentration began to decline (Fig. 3).

With this configuration of Ca^{2+} and 5-HT pulses, a substantial difference between forward and backward pairing was observed. Peak cyclase stimulation was significantly greater when Ca^{2+} preceded 5-HT than when 5-HT came first [$P < 0.001$¶; Fig. 3; the overall stimulation profiles were also significantly different ($P < 0.001$)ǁ]. In principle, this differential activation might have resulted if there were greater overlap between independent Ca^{2+} and 5-HT stimulations with forward pairing as compared with backward pairing. However, the sequence effect cannot be explained by better summation of the two separate stimulations with forward pairing since Ca^{2+} stimulation in the presence of GTP is extremely modest (e.g., see Fig. 2). This brief Ca^{2+} pulse, when delivered alone in the presence of GTP, produced no detectable stimulation (Fig. 3A); in the absence of GTP, it
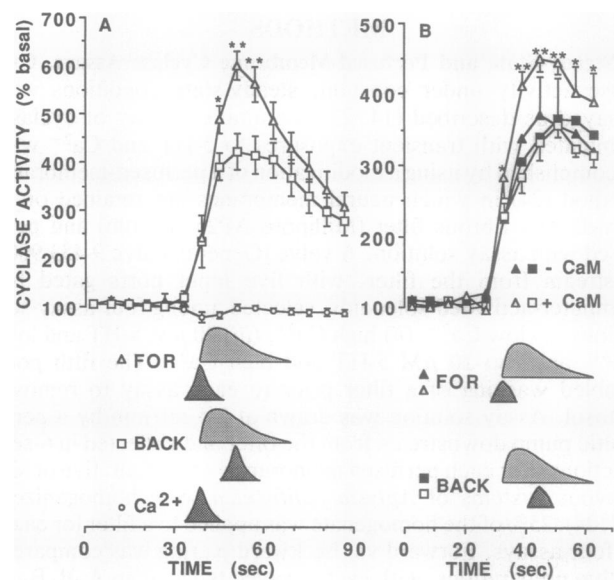

FIG. 3. Responses of adenylyl cyclase in perfused neural membranes to forward or backward pairing of Ca^{2+} and 5-HT pulses or to a Ca^{2+} pulse alone. ($\triangle$ ▲) Forward sequence, Ca^{2+} pulse followed by 5-HT. ($\square$ ■) Backward sequence, 5-HT pulse followed by Ca^{2+}. (○) Ca^{2+} pulse alone. Profiles of "relative effective concentrations" of Ca^{2+} and 5-HT (from Fig. 1 *D* and *E*) are shown below. (*A*) Forward vs. backward pairing and Ca^{2+} pulse alone. Cyclase activity during each 6-sec interval is expressed as the percent of average basal activity before stimuli. Data are means of eight separate experiments ± SEM. Error bars may be smaller than symbols. (*B*) Dependence of forward vs. backward sequence effect on CaM. Responses to forward ($\triangle$ ▲) and backward ($\square$ ■) pairing are shown for assay solution with ($\triangle$ $\square$, $n = 4$) and without (▲ ■, $n = 6$) exogenous CaM. Error bars (± SEM) are shown for all points during 5-HT responses with CaM and only for peak stimulation points without CaM. Note that with CaM, forward pairing gave significantly greater peak stimulation than did backward pairing. Note also that during the rising phase of 5-HT stimulation, forward pairing produced significantly more activation at one time point in *A* and at two time points in *B*. (∗, $P < 0.05$; ∗∗, $P < 0.01$; one-tailed *t* test calculated on within-preparation differences between forward and backward pairing; in *B*, statistical comparisons shown were between forward and backward pairing in experiments with CaM; no significant differences were observed between forward and backward pairing in experiments without CaM.) Ca^{2+} buffers used were A_{lo} and A_{hi} in *A*, and B_{lo} and B_{hi} in *B*. Free Ca^{2+} when "low" was ≤1 nM; at the peak of the 6-sec Ca^{2+} pulse, free Ca^{2+} increased to ≈1 μm in *A* and ≈0.3 μM in *B*; (free Ca^{2+} was calculated with an iterative computer program). Each individual perfused membrane experiment consisted of two duplicate assays on one preparation. Basal activity was 4–5 pmol/min per mg of protein.

caused stimulation of 30–40% (31). Thus, the order dependence observed in these experiments indicates that Ca^{2+} and 5-HT pulses must interact in activating the cyclase. The difference between forward and backward pairing was dependent upon the presence of exogenous CaM in the assay solution (Fig. 3*B*). Since CaM normally modulates proteins by binding to their cytosolic domains, this suggests that Ca^{2+} influences transmitter activation of cyclase by acting at an intracellular site in these homogenized membranes, rather than by affecting the extracellular domain of the receptor.

A Ca^{2+} Prepulse May Enhance the Rate of Cyclase Activation by Transmitter. An initial insight into the nature of the interaction between transient Ca^{2+} and 5-HT stimuli came from perfused membrane experiments in which the Ca^{2+} pulse was relatively broad and overlapped more extensively with the 5-HT peak. With the broad Ca^{2+} pulse, during forward pairing, the effective Ca^{2+} exposure extended ≈1 sec past the 5-HT peak; with backward pairing, the Ca^{2+} exposure began ≈1 sec before the 5-HT concentration began to decline. With these pulse configurations, no difference was

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¶Paired one-tailed *t* test on the differences within preparations between forward and backward (or between forward and 5-HT alone).
ǁNormalized data were analyzed with a two-way ANOVA with one repeated measure and with paired within-preparation comparisons.
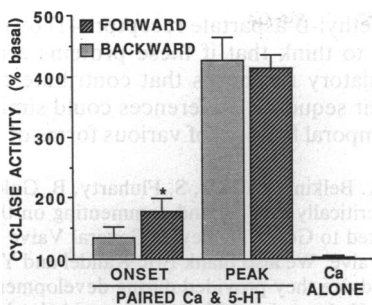
FIG. 4. Responses of cyclase to forward or backward pairing of Ca^{2+} and 5-HT pulses with increased pulse overlap. Mean cyclase activity in two individual 6-sec time periods during responses to paired Ca^{2+} and 5-HT pulses or Ca^{2+} alone. Exposure to Ca^{2+} (8 sec pulse with buffer C_{hi}) was longer than in Fig. 3 (see Fig. 1E). With forward pairing, the Ca^{2+} exposure continued past the peak of the 5-HT exposure. Free Ca^{2+} was $\approx$30 nM when low and $\approx$9 μM at the peak of the pulse. PEAK is the 6-sec interval of maximal stimulation by 5-HT. ONSET is the first 6-sec interval in which 5-HT stimulation occurred. Ca^{2+} ALONE is the response to a Ca^{2+} pulse delivered with the same timing as in forward pairing; the response is shown for the same interval as in ONSET. For forward and backward pairing, $n = 8$; for Ca^{2+} pulse alone, $n = 3$. Error bars are $\pm$ SEM.

observed in peak stimulation between forward and backward pairing (Fig. 4). However, during the 6-sec interval in which 5-HT stimulation first began to rise, there was greater cyclase activation with forward pairing than with backward pairing (initial stimulation was 77 $\pm$ 23% for forward and 34 $\pm$ 18% for backward pairing, where stimulation is expressed as the percent above basal activity; $n = 8$; $P < 0.05^{¶}$). Since the enhancement of cyclase stimulation with forward pairing was seen only in the first interval in the activation curve, it could have reflected a random difference. Therefore, we specifically examined this initial activation point in a second series of experiments comparing forward pairing with a 5-HT pulse alone; this comparison was identical to the forward vs. backward pairing comparison since at this initial activation point with backward pairing, the Ca^{2+} pulse had not yet begun. Ca^{2+} followed by 5-HT produced significantly greater initial activation than did 5-HT alone (initial stimulation was 97 $\pm$ 23% for forward pairing vs. 60 $\pm$ 33% for 5-HT alone, where stimulation is expressed as the percent above basal; $n = 5$; $P < 0.02^{¶}$). As in the previous experiment with these broad Ca^{2+} pulses (Fig. 4), forward pairing did not increase the peak stimulation of the cyclase. The enhancement of initial activation with forward pairing could not have been simply due to summation of separate Ca^{2+} and 5-HT stimulations, since the same Ca^{2+} pulse delivered alone in the presence of GTP gave no detectable stimulation (Fig. 4).

With the briefer Ca^{2+} pulses (Fig. 3), during the rising phase of cyclase activation, forward pairing also produced significantly greater stimulation than did backward pairing; this difference was observed at either one or two of the initial activation points depending upon the size of the Ca^{2+} pulse (Fig. 3).** These results from perfused membrane assays together with those from steady-state assays suggest that while 5-HT alone can effectively activate cyclase in the absence of Ca^{2+}, the binding of Ca^{2+}/CaM to the cyclase may cause a conformational change that facilitates coupling of activated G_s to the enzyme; thus, a Ca^{2+} prepulse would enhance the rate of cyclase activation. This same hypothe-

sized CaM-dependent conformation of the cyclase could explain the absence of enhanced peak activation in forward pairing with a prolonged Ca^{2+} pulse (Fig. 4); it is possible that as 5-HT washout began, G_s uncoupling from cyclase was accelerated by CaM that remained bound. Although CaM could act on other proteins in the receptor–G_s–cyclase system, the cyclase itself is a likely site since it binds, and is modulated by, CaM (14, 32).

5-HT Alone May Produce Cyclase Stimulation Intermediate Between Forward and Backward Pairing of Ca^{2+} and 5-HT. Abrams *et al.* (14) had observed that pairing a 10-sec increase in Ca^{2+} with the onset of a prolonged exposure to 5-HT resulted in enhancement of the stimulation of cAMP synthesis by 5-HT under assay conditions in which 5-HT alone produced very modest cyclase stimulation (only 1.3- to 1.4-fold).[††] The experiments presented above that examined the onset of cyclase activation suggested that a prepulse of Ca^{2+} could enhance the rate of activation by a 5-HT pulse. In general, we avoided comparing all three conditions (forward pairing, 5-HT alone, and backward pairing) within the same neural preparation because cyclase activity and transmitter stimulation declined over time in the homogenate (which included cytosol and endogenous proteases). In the one series of experiments in which all three were directly compared, forward pairing resulted in significantly greater peak activation (3.96 $\pm$ 0.25-fold) than both backward pairing (2.41 $\pm$ 0.47-fold) and 5-HT alone (2.54 $\pm$ 0.30-fold) ($n = 3$; $P < 0.01$). Stimulation with 5-HT alone was examined in three additional series of experiments that had different Ca^{2+} pulse profiles and in which forward vs. backward differences were robust. In these four series of experiments, with two configurations of Ca^{2+} and 5-HT pulses, forward pairing produced significantly greater peak cyclase activation than did 5-HT alone; in contrast, with two other pulse configurations, there was no significant difference between forward pairing and 5-HT alone. The observation that differences between forward pairing and 5-HT alone were relatively sensitive to the precise timing and shape of the paired Ca^{2+} pulse, compared with differences between forward and backward pairing, suggested that a backward pulse of Ca^{2+} may actually be inhibitory; thus, 5-HT alone may produce stimulation that is intermediate between forward and backward pairing (see *Discussion*).

DISCUSSION

Cellular studies of intact *Aplysia* mechanosensory neurons (9, 11, 13, 33, 34) suggested that the Ca^{2+}/CaM-sensitive adenylyl cyclase may be an important site of stimulus convergence, contributing to activity-dependent enhancement of presynaptic facilitation in these neurons and to associative modification of the withdrawal reflex. In both conditioning of this reflex and in activity-dependent facilitation, the associative change depends upon the sequence of pairing (21, 22); therefore, it seemed possible that activation of cyclase by Ca^{2+} and transmitter would depend upon the order of their arrival. In the present study, we found that when brief exposures to Ca^{2+} and 5-HT were paired, they interacted in stimulating the cyclase in a temporally asymmetric manner. A Ca^{2+} pulse that preceded the arrival of 5-HT resulted in greater activation than a Ca^{2+} pulse that began after the peak of the 5-HT exposure.

Several observations suggest that the greater peak cyclase activation with forward pairing might be due to effects of Ca^{2+} on the rate of cyclase activation by transmitter, rather than to actual synergism between the two inputs once activation has occurred. First, Ca^{2+} and 5-HT did not produce

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**With forward pairing, this first time point with 5-HT stimulation includes both cAMP synthesis during the Ca^{2+} pulse before 5-HT's arrival and cAMP synthesis as 5-HT arrives; since a Ca^{2+} pulse alone may have a small inhibitory effect in the presence of GTP (ref. 31 and Fig. 3A), enhanced initial activation by 5-HT may be masked by earlier exposure to Ca^{2+}.

[††]It is unclear why 5-HT stimulation of *Aplysia* neural cyclase was unusually weak in the protocol used in these earlier experiments.

synergistic activation under steady-state conditions. Second, in perfused-membrane assays with longer Ca^{2+} pulses, where no difference was observed in peak 5-HT activation, forward pairing of Ca^{2+} and 5-HT produced enhanced initial activation compared with both backward pairing and 5-HT alone (Fig. 4). The temporal resolution of these assays did not allow us to clearly resolve the rapid time course of cyclase activation in order to definitively distinguish effects on activation rate from effects on peak activation. However, it is possible to accurately measure the slower decay of cyclase activation once free 5-HT has been washed out. We recently found that a late pulse of Ca^{2+} accelerated the decay of cyclase activation (31), suggesting that Ca^{2+} may increase the rate of both cyclase activation and cyclase deactivation.

The striking order dependence in activation of cyclase by paired Ca^{2+} and 5-HT would be expected to contribute to the temporal requirements for stimulus pairing both during activity-dependent enhancement of presynaptic facilitation in the sensory neurons and in classical conditioning of the withdrawal reflex. If Ca^{2+}/CaM affects the rate of activation of cyclase by receptor and G_s, then this interaction should be particularly important in situations where the facilitatory transmitter is available for only a few seconds. Thus, the effects of a preceding rise in Ca^{2+} on cyclase activation by 5-HT might be more dramatic with briefer 5-HT exposures, as would occur if serotonergic facilitator interneurons were active for only a few seconds, primarily during the 1- or 1.5-sec US (3, 21). The actual pattern of activity of the neurons that trigger synaptic facilitation during conditioning is not known. To date, two patterns of facilitator neuron response have been observed. The serotonergic CB1 neurons are active for minutes after a tail shock (5). In contrast, the nonserotonergic L29 neurons remain active for <0.5 sec after a tail shock (35). Most probably other groups of yet unidentified facilitator neurons also contribute to synaptic plasticity during conditioning. We suggest that most of the facilitatory transmitter may be released in a temporally restricted manner, given the narrow time window required for CS–US pairing during conditioning. In general, the activation properties of the cyclase would enable it to play an associative role in forms of conditioning where the input from the US is quite brief. Other cellular mechanisms, such as convergence at downstream loci, are likely to mediate associative interactions where the US input is more prolonged or the optimal interval for stimulus pairing is substantially longer; one such potential downstream convergence site that shows synergistic activation is the CRE binding protein (36). Alternatively, under circumstances where cyclase activation is extremely modest during prolonged exposure to facilitatory transmitter (e.g., ref. 14), pairing a Ca^{2+} pulse with 5-HT could enhance cyclase activation, though we would expect order dependence to be absent.

As described above, the difference in peak activation between forward pairing (Ca^{2+} followed by 5-HT) and 5-HT alone was not as robust as the difference between forward and backward pairing of Ca^{2+} and 5-HT. The finding that a late pulse of Ca^{2+} accelerates cyclase deactivation (31) suggests that a late pulse of Ca^{2+} may have inhibitory effects, resulting in decreased stimulation as compared with 5-HT alone. Thus, the difference between forward and backward pairing observed in the present experiments may be due to a combination of enhanced activation with forward pairing and reduced stimulation with backward pairing. These results raise the possibility that backward pairing might result in less enhancement of the response elicited by the CS than completely unpaired presentation of the CS and US. Indeed, in a number of vertebrate conditioning paradigms, the response to a CS that is presented at a short interval after a US shows inhibitory conditioning (37–39). Sequence preferences have also been observed in the activation of a protein kinase (40)

and the N-methyl-D-aspartate receptor (41) by dual inputs. It is appealing to think that if these proteins are involved in gating modulatory responses that contribute to associative learning, their sequence preferences could similarly contribute to the temporal features of various forms of conditioning.

1. Abrams, T. W. & Kandel, E. R. (1988) Trends Neurosci. 11, 128–135.
2. Gustafsson, B. & Wigstrom, H. (1988) Trends Neurosci. 11, 156–162.
3. Carew, T. J., Walters, E. T. & Kandel, E. R. (1981) J. Neurosci. 1, 1426–1437.
4. Glanzman, D. L., Mackey, S. L., Hawkins, R. D., Dyke, A. M., Lloyd, P. E. & Kandel, E. R. (1989) J. Neurosci. 9, 4200–4213.
5. Mackey, S. L., Kandel, E. R. & Hawkins, R. D. (1989) J. Neurosci. 9, 4227–4235.
6. Bernier, L., Castellucci, V. F., Kandel, E. R. & Schwartz, J. H. (1982) J. Neurosci. 2, 1682–1691.
7. Castellucci, V. F., Nairn, A., Greengard, P., Schwartz, J. H. & Kandel, E. R. (1982) J. Neurosci. 2, 1673–1681.
8. Goldsmith, B. A. & Abrams, T. W. (1991) Proc. Natl. Acad. Sci. USA 88, 9021–9025.
9. Hawkins, R. D., Abrams, T. W., Carew, T. J. & Kandel, E. R. (1983) Science 219, 400–405.
10. Walters, E. T. & Byrne, J. H. (1983) Science 219, 405–408.
11. Ocorr, K. A., Walters, E. T. & Byrne, J. H. (1985) Proc. Natl. Acad. Sci. USA 82, 2548–2552.
12. Abrams, T. W., Bernier, L., Hawkins, R. D. & Kandel, E. R. (1984) Soc. Neurosci. Abstr. 10, 269.
13. Abrams, T. W. (1985) Cell. Mol. Neurobiol. 5, 123–145.
14. Abrams, T. W., Karl, K. A. & Kandel, E. R. (1991) J. Neurosci. 11, 2655–2665.
15. Dudai, Y. & Zvi, S. (1984) Neurosci. Lett. 47, 119–124.
16. Livingstone, M. S., Sziber, P. P. & Quinn, W. G. (1984) Cell 37, 205–215.
17. Livingstone, M. S. (1985) Proc. Natl. Acad. Sci. USA 82, 5992–5996.
18. Levin, L. R., Han, P.-L., Hwang, P. M., Feinstein, P. G., Davis, R. L. & Reed, R. R. (1992) Cell 68, 479–489.
19. Xia, Z. G., Refsdal, C. D., Merchant, K. M., Dorsa, D. M. & Storm, D. R. (1991) Neuron 6, 431–443.
20. Hearst, E. (1988) Fundamentals of Learning and Conditioning: Steven's Handbook of Experimental Psychology, eds. Atkinson, R. C., Herrnstein, R. J., Lindzey, G. & Luce, R. D. (Wiley, New York).
21. Hawkins, R. D., Carew, T. J. & Kandel, E. R. (1986) J. Neurosci. 6, 1695–1701.
22. Clark, G. A. (1984) Soc. Neurosci. Abstr. 12, 268.
23. Salomon, Y. (1979) Adv. Cyclic Nucleotide Res. 10, 35–55.
24. Yovell, Y., Kandel, E. R., Dudai, Y. & Abrams, T. W. (1987) Proc. Natl. Acad. Sci. USA 84, 9285–9289.
25. Yovell, Y., Dudai, Y. & Abrams, T. W. (1986) Soc. Neurosci. Abstr. 12, 400.
26. Salter, R. S., Krinks, M. H., Klee, C. B. & Neer, E. J. (1981) J. Biol. Chem. 256, 9830–9833.
27. Treisman, G. J., Bagley, S. & Gnegy, M. E. (1983) J. Neurochem. 41, 1398–1406.
28. Malnoe, A., Stein, E. A. & Cox, J. A. (1983) Neurochem. Int. 5, 65–72.
29. Natsukari, N., Hanai, H., Matsunaga, T. & Fujita, M. (1990) Brain Res. 534, 170–176.
30. Blumenfeld, H., Spira, M. E., Kandel, E. R. & Siegelbaum, S. A. (1990) Neuron 5, 487–499.
31. Jarrard, H. E., Yovell, Y. & Abrams, T. W. (1991) Soc. Neurosci. Abstr. 17, 1301.
32. Eliot, L. S., Dudai, Y., Kandel, E. R. & Abrams, T. W. (1989) Proc. Natl. Acad. Sci. USA 86, 9564–9568.
33. Hawkins, R. D. & Abrams, T. W. (1984) Soc. Neurosci. Abstr. 10, 268.
34. Walters, E. T. & Byrne, J. H. (1983) Brain Res. 280, 165–168.
35. Hawkins, R. D. & Schacher, S. (1989) J. Neurosci. 9, 4236–4245.
36. Dash, P. K., Karl, K. A., Colicos, M. A., Prywes, R. & Kandel, E. R. (1991) Proc. Natl. Acad. Sci. USA 88, 5061–5065.
37. Kaplan, P. S. (1984) J. Exp. Psychol. 10, 113–126.
38. Ewing, M. F., Larew, M. B. & Wagner, A. R. (1985) J. Exp. Psychol. 11, 537–547.
39. Williams, D. A. & Overmier, J. B. (1988) J. Exp. Psychol. 14, 26–35.
40. Cohen, P. (1982) Nature (London) 296, 613–620.
41. Holmes, W. R. & Levy, W. B. (1990) J. Neurophysiol. 63, 1148–1168.