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Biochemical correlates of short-term sensitization in *Aplysia*: Temporal analysis of adenylate cyclase stimulation in a perfused-membrane preparation

(cAMP/synaptic facilitation/serotonin/short-term learning)

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ABSTRACT **During short-term sensitization, a simple form of nonassociative learning in *Aplysia*, the presentation of a single brief noxious stimulus results in enhancement of the defensive withdrawal reflex lasting minutes to tens of minutes. This behavioral plasticity involves presynaptic facilitation of synaptic transmission from the mechanosensory neurons that mediate the reflex to their central target cells. This facilitation is due to cAMP-dependent protein phosphorylation. To determine whether the time course of presynaptic facilitation might be due to a persistent increase in activity of adenylate cyclase (EC 4.6.1.1) itself, persistence of the transmitter, or yet other processes, we developed a perfused-membrane method to analyze the time course of activation of adenylate cyclase by transient stimuli. After stimulation by a pulse of stimulatory transmitter, activation of adenylate cyclase decayed within 60 sec. This finding indicates that the enzyme does not remain persistently active in the absence of transmitter and suggests that short-term retention is likely to be due to other mechanisms. Possible additional mechanisms include continued activation of the cyclase by transmitter, cellular factors extrinsic to the cyclase that prolong the time course of its activation, and persistence of processes downstream from the cyclase.**

Adenylate cyclase (EC 4.6.1.1) is activated by extracellular stimuli, such as hormones and neurotransmitters, via an intermediary guanyl nucleotide-binding G_s protein that couples receptors to the catalytic unit of the cyclase (1, 2). It is also activated by intracellular messengers, such as Ca^{2+} (3, 4). In the nervous system, these stimuli are usually transient and brief. With existing methods for the measurement of adenylate cyclase activity *in vitro*, it is difficult to follow the response of the cyclase enzymatic complex to transient stimuli. However, such time-dependent responses may be important for regulation of neuronal function. An interesting example of the role of adenylate cyclase in the nervous system is its function in neural plasticity during learning. Both in the marine mollusk *Aplysia californica* (5–7) and in *Drosophila* (7–11) adenylate cyclase has been implicated in elementary forms of learning and short-term memory. In such cases temporal properties of interactions of the cyclase complex with transmitters and ions may be important for the enzyme's function in neuronal plasticity (7, 12–15).

In *Aplysia*, the defensive gill and siphon withdrawal reflex undergoes sensitization, an elementary form of learning in which the reflex is enhanced after presentation of a noxious stimulus (5). In sensitization, the noxious stimulus results in facilitatory input to the mechanosensory neurons that mediate the reflex, thereby causing enhancement of their synaptic connections with gill and siphon motoneurons. In the short term, this synaptic facilitation is mediated by cAMP-dependent protein phosphorylation in the sensory neurons (5). Noxious stimuli and at least three facilitatory transmitters, serotonin and the neuropeptides SCP_A and SCP_B (small cardioactive peptides A and B), activate adenylate cyclase in the sensory neurons, causing an increase in cAMP synthesis (16, 17).

Short-term memory for sensitization lasts several minutes to tens of minutes, the duration depending upon the intensity of the sensitizing stimulus. Both electrophysiological and biochemical evidence suggested that in the monosynaptic component of the reflex, the duration of short-term memory of the learned response is primarily due to sustained elevation of cAMP in the sensory neurons (12). Following facilitatory stimuli, the increase in the synaptic connection between sensory neurons and motoneurons and the prolongation of the presynaptic action potential (a presynaptic change that contributes to synaptic facilitation) were substantially reduced by either injection of the protein inhibitor of the cAMP-dependent kinase (18) or injection of guanosine-5′-*O*-(2-thiodiphosphate) (GDP[βS]) (19), which inhibits adenylate cyclase activation (20, 21). Thus, continuous production of cAMP and continuous activation of cAMP-dependent kinase may be essential components for maintenance of the short-term form of the facilitation response. Consistent with this possibility, elevations in cAMP were seen to last at least 10 (16) and up to 30 min (22) after ganglia had been exposed to serotonin (5-HT) for several minutes.

What accounts for the time course of cAMP production? Does the adenylate cyclase enzyme itself remain persistently active after stimulation by transmitter has ceased? Or is continued transmitter stimulation required for the continued activity of the cyclase? With conventional biochemical methods it is difficult to explore this issue. We have, therefore, developed a method that enables adenylate cyclase activity to be assayed continuously, while transmitter stimuli are delivered in a transient manner. In this procedure, a homogenized-membrane preparation is retained on a filter and continuously perfused with cyclase assay solution. The continuous-perfusion method allows repeated monitoring of adenylate cyclase activity at short time intervals and rapid initiation and termination of ligand stimuli, such as neurotransmitters. We describe here this method and its application to the analysis of the time course of activation of adenylate cyclase from *Aplysia* nervous system.

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Abbreviations: 5-HT, serotonin; GDP[βS], guanosine-5′-*O*-(2-thiodiphosphate); CNS, central nervous system; SCP_A and SCP_B, small cardioactive peptides A and B; GTP[γS], guanosine-5′-*O*-(3-thiotriphosphate).

MATERIALS AND METHODS

Continuous-Perfusion Methodology. The experimental apparatus (Fig. 1) included a reaction chamber, containing a filter to which a membrane preparation was applied at the onset of each experiment. The chamber was continuously perfused by solutions from one of three reservoirs, which contained either (*i*) washout solution, unlabeled cyclase assay solution; (*ii*) assay solution, cyclase assay solution with [^{32}P]ATP and [^{3}H]cAMP; or (*iii*) stimulus solution, the assay solution plus transmitter or guanosine-5′-*O*-(3-thiotriphosphate) (GTP[γS]). After stimulus application, the stimulus was rapidly terminated by switching to the washout solution and increasing flow rate through the filter from 310 μl/min per cm^2 (assay mode) to 6.5 ml/min per cm^2 (washout mode) for 20 sec, thereby washing away the stimulating ligand, and then switching back to assay solution. This procedure effectively terminated a ligand stimulus: after a 4-min pulse of 5-HT, its concentration in the effluent, as monitored with [^{3}H]5-HT, was reduced to 0.1% of its original value in 90 sec (Fig. 2). The effluent during washout mode was discarded because the high flow rate diluted cAMP synthesized during this brief period. To avoid including the small amount of washout effluent that remained in the system, washout was made with a solution lacking [^{32}P]ATP and [^{3}H]cAMP. Basal cyclase activity in the system was stable for at least 20 min and was not affected by the rapid washout procedure (see Fig. 4*A*). Assays were at room temperature (≈23°C).

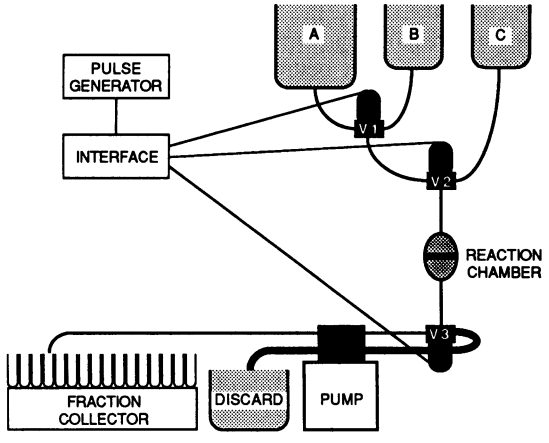

FIG. 1. Perfusion apparatus for the continuous assay of adenylate cyclase response to transient stimuli. Homogenized membranes from *Aplysia* central nervous system (CNS) were retained on a filter (Millipore AP) in the reaction chamber (Millipore SX fitting, 13 mm i.d.; the internal volume of which had been reduced to 60 μl by a Sylgard cast). Membranes were continuously perfused with solution from one of three reservoirs: (A) containing unlabeled cyclase assay solution (without [^{32}P]ATP or [^{3}H]cAMP) (washout solution); (B) containing cyclase assay solution with [^{32}P]ATP and [^{3}H]cAMP; or (C) containing the assay solution plus transmitter (5-HT). Teflon tubing (0.4 mm i.d.) was used for all connections. Selection of solutions and their rate of flow was made by electronically switching three-way valves (V_{1-3}; "isolatch" Teflon-lined valves; internal volume 60 μl; response time <10 msec; General Valves, Fairfield, NJ). An 8-channel programmable pulse generator (A.M.P.I., Jerusalem) controlled the valves. Each individual pulse from the pulse generator was converted by an interface to two 30-msec pulses, one at the onset and one at the offset of each original pulse. After application of a transmitter stimulus, rapid washout was achieved by increasing the flow by switching the outflow with valve three (V3) from narrow (0.38 mm i.d.) to wide (2.3 mm i.d.) tubing on the two-channel peristaltic pump (Gilson). During assay mode, 175-μl fractions of the effluent were continuously collected for 30 sec each in tubes that contained stop solution (23), whereas the effluent during the washout mode was discarded. The [^{32}P]cAMP in each fraction was separated (23) and counted.

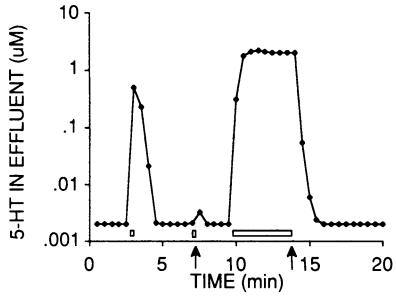

FIG. 2. 5-HT retention in the perfusion apparatus, as monitored by elution of [^{3}H]5-HT. At times indicated by horizontal bars, pulses of 2 μM [^{3}H]5-HT (10^5 cpm/ml) were applied to a membrane preparation under standard assay conditions. Radioactivity was assayed in 30-sec fractions of effluent. The first pulse was terminated without rapid washout; the second and third pulses were terminated by a 20-sec rapid washout with a 20-fold increase in flow rate (marked by arrows). Two nanomolar was the lowest detectable concentration of 5-HT. Note that after switching to the 5-HT solution (at the onset of the long pulse), the effluent was completely changed within 60 sec.

Temporal Resolution. Whereas temporal resolution of stimulus administration in the perfusion apparatus depends upon valve response time and mixing/washout kinetics alone, temporal resolution of measurement of cyclase activity also depends upon the duration over which each fraction is collected. Valve response times were <10 msec and thus did not limit temporal resolution. Mixing and washout kinetics are a function of the flow rate of the perfusate and the volume of the reaction chamber. Flow rate during assay mode was limited by the activity of the enzyme, which is rather low; less than 0.1% of the radiolabeled ATP is converted to cAMP in this preparation. A flow of 350 μl/min allowed us to reliably assay basal and stimulated cyclase activity. Under these flow conditions, when perfusion solutions were switched, a complete change in effluent occurred within 60 sec (Fig. 2). Therefore consecutive aliquots of effluent were collected for durations of 30 sec each.

Preparation of Membranes. For each experiment, the cerebral, pleural, pedal, and abdominal ganglia from two *Aplysia californica*, weighing 100–200 g, were dissected and homogenized as described (24). The homogenate was centrifuged for 1 min at 3000 × *g* to remove large particles, and the supernatant (containing ≈5 mg of protein) was applied to the filter. Using a syringe connected to the reaction chamber, the membranes were then rapidly flushed with 20 ml of washout solution to remove cytosolic components and membrane particles not retained by the filter.

Solutions. Assay solution consisted of 50 mM K-Hepes (pH 7.6), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.01 mg of leupeptin per ml, 0.01 mg of aprotinin per ml, 0.1 mg of phenylmethylsulfonyl sulfate per ml, [α-^{32}P]ATP (15 μCi/ml; 1 Ci = 37 GBq), [^{3}H]cAMP (10^5 cpm/ml), 20 μM ATP, 50 μM cAMP, 10 μM GTP, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 5 mM creatine phosphate, and 50 units of creatine phosphokinase per ml. Washout solution was identical but lacked the radiolabeled ATP and cAMP. cAMP-dependent protein kinase, both peak I and peak II from rabbit muscle, were purchased from Sigma. Phorbol 12,13-dibutyrate was obtained from LC Services (Woburn, MA). SCP_B was obtained from Peninsula Laboratories (Belmont, CA).

RESULTS

To investigate whether the time course of short-term presynaptic facilitation in intact siphon sensory neurons might be due to persistent activation of adenylate cyclase after free facilitatory transmitter has disappeared, we have examined the time course of decay of cyclase activation *in vitro* after a pulse of the facilitatory transmitter 5-HT. 5-HT elevates

cAMP levels in intact sensory neurons, as well as in the entire abdominal ganglion, and causes presynaptic facilitation in the sensory neurons (16, 25). We first studied the time course of the response of adenylate cyclase to 5-HT in membranes from the entire CNS. Exposing a membrane preparation to 5-HT resulted in rapid activation of the cyclase, within 30 sec (Fig. 3*A*). Activity remained elevated, with little or no desensitization, as long as transmitter was present (up to 4 min in these experiments). The magnitude of activation was comparable to that observed under conventional steady-state assay conditions (26). Upon rapid removal of transmitter, cyclase activity decreased to nearly basal levels within 60 sec (Fig. 3*A*); usually, a small "tail" of activation (from 5% to 15% over basal and less than 10% of peak stimulation level) could still be detected for several minutes.

It is possible that adenylate cyclase is heterogeneous with respect to its off-rate after transmitter removal; the cyclase capable of remaining persistently active could comprise only a small fraction of all CNS cyclase activity, possibly being relatively abundant in sensory neurons, where it would mediate prolonged synaptic plasticity. To test this possibility, we repeated these experiments on membranes from clusters of pleural ganglion sensory neurons (28). After removal of 5-HT, activation of the cyclase decayed with the same rapid time course as it did in membranes from the whole CNS (Fig. 3*B*).

Aplysia nervous system contains other facilitatory neurotransmitters that activate cyclase in sensory neurons in addition to 5-HT, including the neuropeptides SCP_A and SCP_B (17). We found that SCP_B, like 5-HT, activates adenylate cyclase and that this activation decays rapidly after removal of the peptide (Fig. 3*C*).

Because cyclase activity in these experiments declined rapidly after removal of transmitter, we wanted to verify that the enzyme in the continuous-perfusion apparatus could display persistent activation. We therefore examined the response of cyclase to pulses of GTP[γS]. GTP[γS], a synthetic nonhydrolyzable GTP-analogue, persistently activates adenylate cyclase by binding to the stimulatory G protein, G_s (1, 2). We found that activation of adenylate cyclase in the perfused membrane preparation persisted after removal of the GTP analogue (Fig. 3*D*).

Because the continuously perfused membrane preparation could undergo persistent activation, we asked whether circumstances existed in which transient exposure to transmitter resulted in prolonged cyclase activation. It is possible that in intact cells, persistent activation of the cyclase after exposure to transmitter depends on interaction with a cytosolic factor, such as a cytosolic protein kinase (29–31), and such factors may have been depleted from the membrane during homogenization and washing. We therefore did a series of experiments to determine whether addition of cytosol would prolong activation of the cyclase. Supernatant from homogenized nervous tissue (Fig. 4) was added to the perfusion solutions. No evidence was detected for prolongation by cytosol of cyclase stimulation (Fig. 4*B*). We also tested the specific possibilities that phosphorylation by cAMP-dependent protein kinase or by the Ca^{2+}/phospholipid-dependent kinase (C kinase) alters the properties of cyclase, slowing its turn-off after removal of stimulatory transmitter. In two experiments, cAMP-dependent protein kinase was added to the perfusion solutions. In two other experiments, 100 nM phorbol 12,13-dibutyrate, an activator of C kinase, was added. Neither of these procedures influenced the decay of cyclase activation after a transmitter pulse (data not shown).

Results of these studies demonstrated that *in vitro*, cyclase activity decays rapidly (within 60 sec) after removal of transmitter. In contrast, experiments on intact ganglia have shown a prolonged increase in cAMP synthesis following exposure to 5-HT. Cedar and Schwartz (22) and Bernier *et al.* (16) exposed whole abdominal ganglia to 200 μM 5-HT for 5 min and then washed with transmitter-free medium; they found that cAMP levels then remained elevated for some time, returning to basal over a time course of 10–30 min. The difference between these results and those in the present study suggested that the maintained activation of adenylate cyclase seen in intact ganglia might have resulted from continued presence of exogenous stimulatory transmitter. We therefore examined the rate of removal of 5-HT from intact ganglia by incubating abdominal ganglia with 200 μM ^{3}H-5-HT for 5 min and then thoroughly washing the transmitter away, following the protocol of Bernier *et al.* (16). The ganglia were immediately placed in the reaction chamber, and the chamber was continuously perfused with saline. We found that 5-HT was released by the ganglion throughout the 20-min perfusion period. The free 5-HT in the extracellular space of the ganglion was estimated assuming (*i*) that half the ganglionic volume is extracellular space and (*ii*) that the 5-HT that eluted in any one fraction represented all free 5-HT in the ganglionic extracellular space at the time of collection (Fig. 5). Because of the second assumption, these calculated values probably represent an underestimate of the free 5-HT within the ganglion at any single time.

DISCUSSION

Both electrophysiological and biochemical studies in *Aplysia* have suggested that during sensitization of the defensive

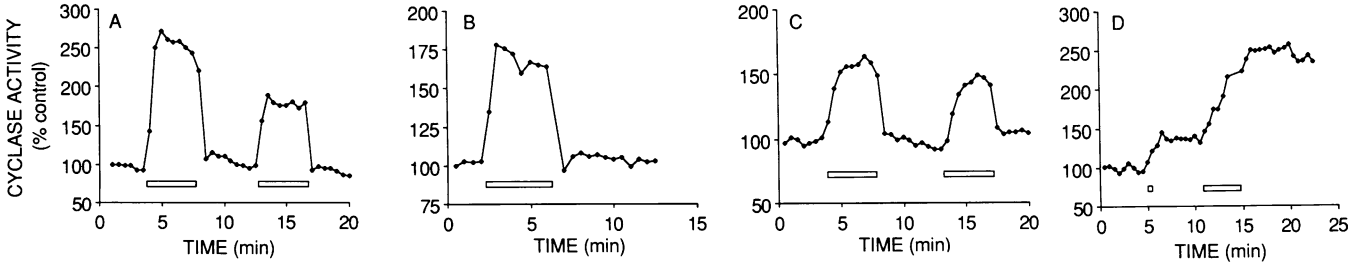

FIG. 3. Response of adenylate cyclase in the perfused membrane preparation to transients of activating stimuli. Application of stimuli is indicated by bars. (*A*) Response to 5-HT. At times indicated by bars, 200 μM 5-HT was delivered to membranes. In this and other experiments, stimuli were terminated by a 20-sec rapid washout, and a single time point is missing during the period of rapid washout when the effluent is discarded. All experiments were replicated at least three times with similar results. Decreased response to the second stimulus may be, at least partially, due to desensitization because the decrease was less pronounced with lower concentrations of transmitter or shorter stimuli. Rapid washout affected neither basal activity nor transmitter sensitivity. (*B*) Response to 100 μM 5-HT in membranes from pleural ganglion sensory cell clusters pooled from nine animals. (*C*) Response to pulses of 100 μM SCP_B. (*D*) Response to 100 mM GTP[γS] in the absence of transmitter. Only the second stimulus was terminated by rapid washout. Note that the activation by GTP[γS], in the absence of transmitter, occurred gradually, and that the increased cyclase activity persisted after termination of the GTP[γS] stimuli. Basal activity was 3.0, 1.2, 4.6, and 3.3 pmol of cAMP per min in the experiments in *A*, *B*, *C*, and *D*, respectively.

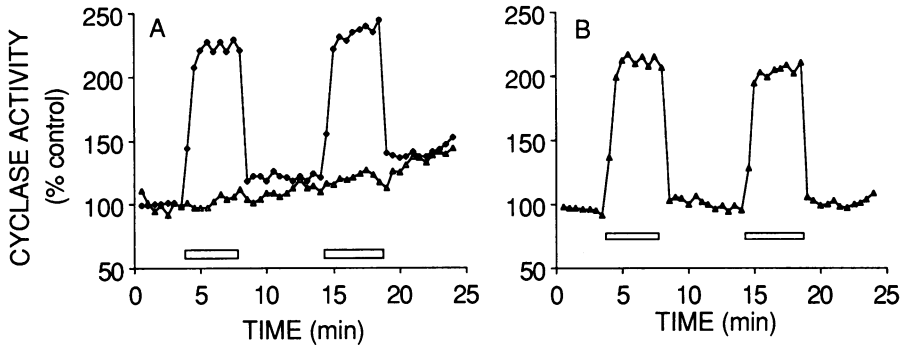

FIG. 4. Response to pulses of 5-HT in the presence of "cytosol." Cytosol was obtained by homogenization of the CNS tissue of 20 *Aplysia* in an equal volume of buffer, followed by centrifugation at 100,000 × *g* for 30 min at 2°C. The supernatant was further diluted 5-fold in all three perfusion solutions; this dilution was necessary to reduce viscosity so as not to impede flow. (*A*) A membrane preparation from four CNS's was divided to two equal aliquots, and one (◆) was exposed to 20 μM 5-HT in the presence of cytosol. Supernatant added to perfusate has some cyclase activity of its own. This soluble enzyme causes cAMP to accumulate in the reservoirs before the perfusate reaches the membranes, resulting in an apparent sustained increase in cyclase activity. Therefore, as a control, the other aliquot of the membranes (△) was treated with the same protocol, including washout, but without exposure to transmitter. Control activity appeared to increase steadily over time, reflecting cAMP synthesis by soluble cytosolic cyclase. Note that rapid washout alone had no effect on basal cyclase activity assayed without transmitter. (*B*) Corrected cyclase activity in membranes perfused with cytosol. The cAMP synthesized in the perfusate, estimated by fitting a line to the control data in *A*, was subtracted from the cAMP collected from membranes exposed to transmitter. Basal activity was 2.9 pmol of cAMP/min.

withdrawal reflex, short-term memory in the monosynaptic component of the reflex may reside in the activity of the adenylate cyclase complex (12). We have therefore investigated whether activation of *Aplysia* neuronal cyclase is autonomous and persists after removal of facilitatory transmitter. We developed a method that allowed us to characterize the time course of adenylate cyclase response to transient applications of the facilitatory transmitter 5-HT. Under a variety of different assay conditions, we observed that stimulation of adenylate cyclase activity declined rapidly, nearly to basal levels, within 60 sec after transmitter was removed (Figs. 3 and 4).

These results on perfused homogenized-membrane preparations contrasted with those of earlier studies on intact cells: Biochemical experiments on intact cells indicated that elevated cAMP levels in intact ganglia declined with a time course of many minutes following removal of exogenous 5-HT (16, 22). Moreover, physiological experiments suggested that continued cAMP synthesis and continued cAMP-dependent protein phosphorylation may be required for maintenance of the full presynaptic facilitation response in the short term (20, 21). We consider below several possible reasons for the differences in time course between the results of the present and the previous studies.

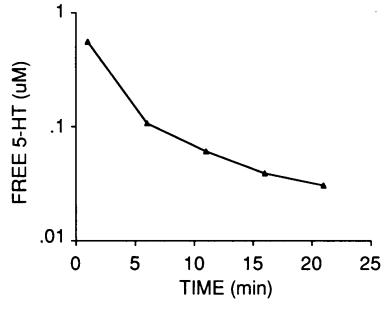

FIG. 5. 5-HT retention by a nondesheathed abdominal ganglion. A ganglion was incubated with 200 μM [^{3}H]5-HT (that had been purified by HPLC, as described below) for 5 min, and the transmitter was removed with three complete bath replacements. The ganglion was then placed in the reaction chamber without a filter and perfused with saline at 350 μl/min. Effluent was collected in 60-sec fractions, and the [^{3}H]5-HT in selected fractions was separated by HPLC using a C_{18} column (Waters μBondapak) and isocratic elution at 1 ml/min, with a mobile phase of 25 mM formic acid/50% acetonitrile/30 mM EDTA. (HPLC separation of 5-HT was critical, because 5-HT in the perfusate went from 75% of the total ^{3}H counts in the initial fraction, to 12% of total counts after 20 min.) Free 5-HT in the ganglion was estimated as described in the text.

Time Course of Removal of Exogenous 5-HT from the Ganglion. One possible cause for the persistent elevation of cAMP in *in vivo* studies is persistent action of facilitatory transmitter. Studies in intact ganglia have looked at the duration of elevation in cAMP levels after exposures to 200 μM 5-HT (16, 22). To assess the contribution of residual stimulatory transmitter to the time course of cAMP elevation, we monitored release of [^{3}H]5-HT from abdominal ganglion after incubation with 5-HT. Free 5-HT in the extracellular space of the ganglion was estimated to decay to ≈50 nM after 13 min (Fig. 5). This concentration, although a 4000-fold decrease in the original 5-HT concentration, is still above threshold for stimulating the cyclase in *Aplysia* nervous tissue (T.W.A. and K. A. Karl, unpublished observations) and for producing presynaptic facilitation (20). Such residual 5-HT may explain the continued elevation of cAMP levels observed by Bernier *et al.* (16) for 10 min after transmitter washout. Moreover, in the 5-HT retention experiments, the ganglion was continuously superfused at a rate of 6 chamber volumes per min (Fig. 5); more residual 5-HT is likely to have remained in studies done with bath application of 5-HT without high rates of superfusion. Thus, persistent activation of the cyclase after bath application of 5-HT possibly is due to slow removal of facilitatory transmitter.

Time Course of Presynaptic Facilitation in Intact Ganglia After Natural or Afferent Nerve Stimulation. Average concentrations of facilitatory transmitter within the ganglion are unlikely to approach 200 μM in response to physiological stimuli, such as noxious stimuli to the skin or nerve stimulation. Therefore, transmitter disappearance is likely to occur more rapidly than over the 10- to 15-min time course seen with bath-applied transmitter. Moreover, we doubt that residual concentrations of facilitatory transmitter, such as we have seen with exogenous transmitter, could account for presynaptic facilitation lasting 20 min to 1 hr (27). This leaves several alternative hypotheses for the localization of short-term memory lasting more than 10 min produced by physiological stimuli. These hypotheses, which have not been addressed experimentally in this study, include the following: (*i*) After noxious stimuli to the skin, or nerve stimuli, some facilitator neurons may show increased activity over many minutes, resulting in a prolonged release of facilitatory transmitter. (*ii*) Receptors for modulatory transmitter may be

heterogeneous, with the receptor molecules at synapses differing from extrasynaptic receptors. Thus, the synaptic receptors could show more persistent binding of modulatory transmitter and more persistent activation of the adenylate cyclase complex than do extrasynaptic receptors. The response to the endogenous transmitter, released by modulatory neurons, is most likely dominated by synaptic receptors, whereas that to exogenous transmitter is likely to be dominated by extrasynaptic receptors. The two responses could therefore have different time courses. Consistent with this possibility [but also with possibilities (*i*) and (*iv*)] is the observation that a single brief train of nerve stimuli (10 or 15 sec in duration) tends to produce longer-lasting presynaptic facilitation (27) than a single, brief, rapidly terminated, application of 5-HT (6, 17). (*iii*) Small amounts of residual cyclase activation may be sufficient to mediate persistence of short-term memory. Although cyclase activity declined rapidly after washout of transmitter in the perfused membrane preparation, a residual "tail" of activation amounting to <10% of peak stimulation was seen in most experiments. These small tails might be physiologically significant; such residual cyclase activation might maintain cAMP-dependent processes at a level sufficient to underlie synaptic facilitation lasting many minutes. (*iv*) A cytosolic factor, a membrane-associated component, or some aspect of ultrastructure may be required for persistence of cyclase activation, but this factor may have been depleted or disrupted in membrane preparation. Although the present results indicate that in homogenized *Aplysia* nervous tissue the cyclase enzyme itself exhibits little or no memory, the situation may be different in intact cells. Despite the fact that addition of either cytosol or, in limited experiments, the A or C kinase, did not prolong activation of cyclase by transmitter, we cannot be certain that such persistent activation does not occur in intact neurons. A critical cytosolic factor may not have been effectively replaced when we added dilute cytosol. (*v*) More persistent processes may be triggered by a brief rise in cAMP, but these processes might then become independent of the level of cAMP. Such mechanisms, representing an intermediate form of synaptic plasticity important for learning, could be interposed between more transient, cAMP-mediated processes and long-term processes; one such medium-term form of synaptic facilitation has now been described by Greenberg *et al.* (33).

At present, it is difficult to distinguish among these hypotheses. Our results indicate, however, that should a persistence mechanism exist, it probably is not intrinsic to the cyclase complex (i.e., a receptor coupled to the cyclase, the G_s stimulatory unit, and the catalytic unit) because this complex is functional in the perfused-membrane preparation, and yet does not show persistent activation.

Applications of the Perfused-Membrane Method. The method described offers two main advantages over existing methods for adenylate cyclase assay: (*i*) the ability to assay cyclase activity of a single membrane preparation repeatedly over time and (*ii*) the ability to apply and terminate ligand stimuli to the cyclase complex in a temporally controlled manner. These advantages may prove useful in analysis of the function of cyclase in plasticity in the nervous system. For example, evidence concerning the role of adenylate cyclase in learning has given rise to several predictions about the temporal properties of the enzyme complex. Among these is the proposal that the enzyme serves as a molecular integrator of converging stimuli during associative learning (6, 7, 15). Testing these predictions requires the ability to make continuous measurements of the response of the cyclase to multiple transient stimuli. This is now possible with the continuously perfused-membrane preparation, which offers significant analytical advantages over approaches that were previously used in studying cyclase under dynamically changing conditions (26, 32). Responses of cyclase to temporal interactions of multiple stimuli have recently been analyzed (Y.Y. and Y.D., unpublished work) using this method. The perfused-membrane method should also enable better understanding of interactions between components of the cyclase complex. Finally, this method should apply to studies of other membrane-bound enzymes with time-dependent regulatory functions, like guanylate cyclase and phospholipase C.

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