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Biochemical Studies of Stimulus Convergence during Classical Conditioning in *Aplysia*: Dual Regulation of Adenylate Cyclase by Ca^{2+}/Calmodulin and Transmitter

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Activity-dependent facilitation is a mechanism of associative synaptic plasticity that contributes to classical conditioning in *Aplysia*. Previous studies of activity-dependent facilitation in the mechanosensory neurons of *Aplysia* suggested that the Ca^{2+} influx during paired spike activity enhances the transmitter-stimulated, cAMP-dependent, presynaptic facilitation in these cells. Moreover, paired activity was found to potentiate the activation of the adenylate cyclase by transmitter. It was therefore proposed that the Ca^{2+}/calmodulin-sensitive cyclase may serve as a site of interaction between the inputs from the conditioned and unconditioned stimuli. These studies were carried out to test whether a Ca^{2+}/calmodulin-sensitive adenylate cyclase in the *Aplysia* CNS has the properties necessary to mediate such an associative interaction.

Three lines of evidence indicate that the same cyclase molecules that are sensitive to Ca^{2+}/calmodulin are also stimulated by receptor to facilitatory transmitter via the stimulatory G-protein, G_s: First, calmodulin inhibitors reduced stimulation of the cyclase by facilitatory transmitter. When membranes had been preexposed to one of these inhibitors, trifluoperazine, the addition of exogenous calmodulin partially reversed the inhibition. Second, when G_s had been activated by GTPγS, so that it persistently activated the catalytic unit of the cyclase, stimulation of the cyclase by Ca^{2+} was greatly amplified, suggesting that the two inputs interact in activating a common population of the enzyme. Third, solubilized cyclase activity that bound to calmodulin-Sepharose in a Ca^{2+}-dependent manner was stimulated by G_s, which had been partially purified from *Aplysia* CNS, as well as by Ca^{2+}/calmodulin. Having demonstrated dual activation of the cyclase, we have explored the dependence of cyclase activation on the temporal pattern of Ca^{2+} and transmitter addition. Optimal activation required that a pulse of Ca^{2+} temporally overlap the addition of facilitatory transmitter.

These several results suggested that the dually regulated adenylate cyclase might underlie the temporal requirements for effective classical conditioning in this system.

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During associative learning, memories are formed and future behavior is altered as a result of an association made between two or more events. Perhaps the simplest form of associative learning is classical conditioning, where the temporal pairing of one event, the conditioned stimulus, with a second event, the unconditioned stimulus, alters the response to the first event. Thus, the animal learns that the conditioned stimulus predicts the unconditioned stimulus (Rescorla, 1988).

Where in the nervous system do the associative interactions between the conditioned and unconditioned stimuli occur during learning? Are these associative interactions an emergent property of complex neural circuits specifically organized for learning, or do they take place among small groups of neurons or perhaps even within individual cells? Recent analysis of mechanisms of learning in *Aplysia* and *Drosophila* have suggested that associative changes can result from allosteric interactions at particular molecular loci within single neurons (Dudai and Zvi, 1984; Livingstone et al., 1984; Abrams and Kandel 1985, 1988; Ocorr et al., 1985; Yovell and Dudai, 1987). In classical conditioning of the defensive withdrawal reflex of *Aplysia,* the convergent regulation of adenylate cyclase by Ca^{2+}/calmodulin and modulatory transmitter has been proposed to underlie associative changes in the monosynaptic component of the reflex (Abrams, 1985; Abrams and Kandel, 1985, 1988). In this training paradigm, the conditioned stimulus, a weak siphon touch which produces only a small reflex withdrawal, is presented immediately before the unconditioned stimulus, a moderate tail shock which elicits a strong defensive withdrawal response. After a series of these pairings, the response to that same weak conditioned stimulus is substantially enhanced, as if the animal has learned that the siphon touch predicts the noxious tail stimulus (Carew et al., 1981; Hawkins et al., 1986).

At the cellular level, the unconditioned stimulus activates modulatory input to the siphon sensory neurons, the neurons that comprise the afferent pathway for the conditioned stimulus. This modulatory input triggers presynaptic facilitation of synaptic transmission from the siphon sensory neurons to postsynaptic interneurons and motoneurons, thus strengthening the afferent input to the reflex. The response of these sensory neurons to the facilitatory input is enhanced if the sensory neurons are

caused to fire action potentials immediately prior to their receiving the facilitatory input (Hawkins et al., 1983; Walters and Byrne, 1983a). Because activity-dependent facilitation in the siphon sensory neurons is likely to be an important contributor to the associative changes produced by conditioning (Hawkins et al., 1983), we probed the underlying associative interactions on both a cellular and a biochemical level.

Conventional presynaptic facilitation of nondepressed synaptic connections from siphon sensory neurons, which occurs when facilitatory input arrives in the absence of paired spike activity, is mediated by an increase in cAMP levels (Brunelli et al., 1976; Bernier et al., 1982; Castellucci et al., 1982). During activity-dependent enhancement of presynaptic facilitation, paired spike activity in the sensory neurons enhances the cAMP-dependent changes, including the modulation of presynaptic membrane currents that contributes to synaptic facilitation (Hawkins et al., 1983; Walters and Byrne, 1983b; Hawkins and Abrams 1984). Ocorr et al. (1983, 1985) and Abrams et al. (1984) found that, in response to brief applications of the facilitatory transmitter 5-HT, the rise in cAMP in sensory neurons is enhanced if the sensory neurons were caused to fire action potentials or were depolarized just before they were exposed to transmitter. Electrophysiological studies revealed that Ca^{2+} influx during a train of action potentials was critical for enhancement of the facilitation response by the paired spike activity (Abrams, 1985). These several findings suggested that activity-dependent enhancement might occur because the brief elevation of intracellular Ca^{2+} during a paired train of action potentials increases the synthesis of cAMP stimulated by facilitatory transmitter. According to this view, the conditioned stimulus is represented within the sensory neuron by Ca^{2+} influx, and the unconditioned stimulus is represented by the action of facilitatory transmitters, such as 5-HT; the interaction of Ca^{2+} influx and facilitatory transmitter might occur within the adenylate cyclase system.

The possibility that Ca^{2+} was modulating the activation of adenylate cyclase by transmitter seemed plausible in light of the finding of Brostrom et al. (1975) and Cheung et al. (1975) that, in mammalian brain, some of the adenylate cyclase is stimulated by Ca^{2+}, via the Ca^{2+}-dependent regulatory protein calmodulin. Eliot et al. (1989) demonstrated that the majority of *Aplysia* neural cyclase can be stimulated by Ca^{2+}/calmodulin. Yovell et al. (1986) examined the sensitivity of the cyclase to Ca^{2+}/calmodulin and found that this enzyme would be stimulated by the normal rise in intracellular Ca^{2+} that occurs during and immediately after a brief train of action potentials. To explore whether the Ca^{2+}/calmodulin-sensitive adenylate cyclase in *Aplysia* neurons provides a molecular site of convergence between the conditioned and the unconditioned stimuli, we have asked whether the same adenylate cyclase molecules that are stimulated by Ca^{2+}/calmodulin are also activated by transmitter.

When the calmodulin-sensitive cyclase in mammalian brain was first described, there was disagreement about whether the same cyclase that is stimulated by Ca^{2+}/calmodulin is also activated by a receptor for transmitter or hormone. Activation of adenylate cyclase by a receptor has been shown to be mediated by a coupling protein, the stimulatory G-protein, G_s, which binds GTP when a receptor becomes occupied with agonist; G_s, in turn, activates the catalytic unit of the cyclase (Schramm and Selinger, 1984). Studies that have tested for dual activation of the cyclase by attempting to determine whether the stimulatory G-protein interacted with calmodulin in activating the enzyme have given inconsistent results. While a number of studies of cyclase in mammalian brain membranes suggested that the stimulatory G-protein activates the same cyclase molecules that are stimulated by Ca^{2+}/calmodulin (Brostrom et al., 1977, 1978a,b; Seamon and Daly, 1982; Malnoe et al., 1983; Gnegy et al., 1984; Harrison et al., 1988, 1989), other studies suggested a lack of convergence (MacDonald, 1975; Lynch et al., 1977; Salter et al., 1981). Sano and Drummond (1981) reported that solubilized cyclase from cerebral cortex could be separated into two fractions, one that was Ca^{2+}/calmodulin sensitive and unaffected by activators of G_s, and a second fraction that was stimulated by G_s but was not stimulated by Ca^{2+}. More recently, however, several studies of solubilized, partially purified mammalian brain cyclase have indicated that the calmodulin-sensitive catalytic unit is also activated by G_s (Heideman et al., 1982; Yeager et al., 1985; Rosenberg et al., 1987). In mammals, whether the cyclase is dually activated may depend upon the specific tissue; for example, mammalian sperm cyclase is stimulated by calmodulin but is not known to be activated by either hormones or G_s (Gross et al., 1987). In invertebrates, the issue of dual activation remains unresolved; the only relevant analysis was on *Drosophila* by Livingstone (1985), who concluded that the calmodulin-sensitive population of cyclase was transmitter insensitive. Convergent activation is an essential requirement for the hypothesis, described above, that Ca^{2+} influx and facilitatory transmitter interact at the level of the cyclase. We therefore carried out experiments designed to determine whether the same adenylate cyclase in *Aplysia* CNS that is activated by Ca^{2+}/calmodulin is also stimulated by facilitatory transmitter. We show here that convergent, dual activation of the cyclase does occur. We also describe initial experiments designed to explore whether activation of the cyclase is affected by temporal overlap between a transient elevation in Ca^{2+} and arrival of transmitter.

Materials and Methods

Preparation of tissue. Aplysia californica, weighing 100–200 gm, were anesthetized by injection of isotonic $MgCl_2$, and their central ganglia were removed. Most experiments were done on desheathed pleural ganglia, the ganglia with the largest population of mechanosensory neurons (Walters et al., 1983). A small number of experiments were done on isolated pleural ganglion sensory neuron clusters. In both cases, the neurons (without sheath) were homogenized on ice in a glass–Teflon homogenizer. In contrast, in experiments on the entire CNS, the abdominal, cerebral, pleural, and pedal ganglia were trimmed to reduce the amount of non-neural tissue (sheath) and homogenized on ice in a glass–glass homogenizer. The homogenate, without the large pieces of sheath, was rehomogenized in a glass–Teflon homogenizer as above. With all three types of material, the homogenate was then centrifuged at 1000 × *g* for 2 min to remove large particles. The supernatant was centrifuged at 30,000 × *g* for 20 min. In experiments using the entire CNS, the pellet was rehomogenized in homogenization buffer (see below) and centrifuged again. The final pellet was rehomogenized in a small volume, 20 μl per desheathed pleural ganglion and 500 μl per CNS. In most experiments, homogenization was done in the absence of EGTA so that membranes retained substantial endogenous calmodulin (Lynch et al., 1977).

Assay of calmodulin inhibitors. Potential calmodulin inhibitors were screened by testing for inhibition of Ca^{2+}/calmodulin activation of phosphodiesterase in *Aplysia* neural tissue. Desheathed pleural ganglia were homogenized in a glass–glass homogenizer and centrifuged at 13,000 × *g*. Phosphodiesterase activity in the supernatant was assayed (Kauvar, 1982) with free Ca^{2+} at 10 nM or 1 mM to assess Ca^{2+} stimulation. Ca^{2+} (in the presence of endogenous calmodulin) stimulated phosphodiesterase activity by approximately 60%. Compounds tested for their inhibition of Ca^{2+} stimulation included melitin, trifluoperazine (TFP; Sigma), R24571 (calmidazolium; Boehringer Mannheim), and W7 (Seikagaku America, St. Petersburg, FL). R24571, trifluoperazine, and

W7 were maximally effective at concentrations of 5 μM, 50–100 μM, and 1 mM, respectively; these efficacies are consistent with those for inhibition of *Aplysia* calmodulin-dependent protein kinase reported by DeRiemer et al. (1984). Melitin (Sigma) was ineffective in the range of concentrations that we tested [up to 10 μM, which is 10 times the maximally effective concentration in vertebrate studies (Barnette et al., 1983)]. The efficacies of R24571 and trifluoperazine in inhibiting Ca^{2+}/calmodulin stimulation of adenylate cyclase were then verified. (A small number of experiments were also conducted using W7; W7 produced similar effects as the other two compounds, though it required substantially higher concentrations.)

Solutions. All buffers contained 50 mM K-HEPES, 1 mM dithiothreitol (DTT), and protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotonin, and 50 μg/ml PMSF). Except when indicated, homogenization buffer was pH 8.0 and contained no additional components. Other buffers contained additional components, listed below. Cyclase solubilization buffer consisted of 1 mM EDTA and 250 mM sucrose, pH 7.5. G-protein solubilization buffer consisted of 1 mM EDTA, 250 mM sucrose, 10 mM $MgCl_2$, and 800 mM NaCl, pH 8.0. CHAPS chromatography buffer consisted of 10 mM CHAPS, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mg/ml human serum albumin (HSA; Schwarz-Mann), and 10 μM ATP, pH 8.0. Calmodulin-Sepharose binding buffer consisted of 8 mM CHAPS, 30 mM KCl, 2 mM $CaCl_2$, 5 mM $MgCl_2$, and 0.1 mg/ml HSA, pH 7.5. Calmodulin-Sepharose elution buffer consisted of 25 mM KCl, 8 mM CHAPS, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM EDTA, and 0.1 mg/ml HSA, pH 7.5. *cyc*$^-$ reconstitution buffer consisted of 10 mM $MgCl_2$, 1 mM EDTA, 180 mM NaCl, 0.1 mg/ml HSA, and 10 μM ATP, pH 8.0. Reconstitution buffer consisted of 10 mM $MgCl_2$ and 1 mM EDTA, pH 8.0. In the temporal pairing experiments, pleural ganglia were homogenized in 50 mM K-HEPES, pH 7.5 (with 1 mM DTT and protease inhibitors), plus 200 μM EGTA, 60 μM $CaCl_2$, 6 mM $MgCl_2$, and 10 μM GTP and resuspended after centrifugation in 2.5 mM EGTA, 160 μM $CaCl_2$, and 6.8 mM $MgCl_2$, pH 7.5 (free Ca^{2+}, approximately 5 nM). In these pairing experiments, elevations in Ca^{2+} were achieved by adding buffer with $CaCl_2$ and $MgCl_2$ to bring the total concentrations of Ca^{2+} to 800 μM, of Mg^{2+} to 6.8 mM, and of EGTA to 1.8 mM (free Ca^{2+}, approximately 0.1–0.2 μM, after an initial transient of >100 μM free Ca^{2+}); free Mg^{2+} remained approximately 5 mM throughout the experiment. To terminate the Ca^{2+} pulse, buffer with EGTA and $MgCl_2$ was added to bring the total concentrations of Ca^{2+} to 640 μM, of Mg^{2+} to 8.3 mM, and of EGTA to 10 mM (free Ca^{2+}, approximately 5 nM). For the trifluoperazine preexposure experiments, Ca^{2+}/EGTA buffers that yielded a free-Mg^{2+} concentration of 2 mM and a range of free Ca^{2+} concentrations were prepared according to the method developed by Yovell et al. (1986).

Reconstitution. A fraction from *Aplysia* CNS containing stimulatory G-protein was prepared using a modification of the protocol described by Hanski et al. (1981). Nervous systems from 50 animals were homogenized in homogenization buffer with 1 mM EDTA, pH 8.0. The membranes were centrifuged (30,000 × *g* for 20 min) and rehomogenized and recentrifuged two more times. The membranes were then resuspended in G-protein solubilization buffer and centrifuged once again. The pellet was suspended in 1 ml of the same buffer with 1.25% cholate and 0.1 mg/ml HSA and shaken at 0°C for 70 min, with occasional vortexing. After solubilization, 10 mM $MgCl_2$ and 1 mM ATP were added, and the material was centrifuged at 100,000 × *g* for 1 hr. The supernatant was diluted in CHAPS chromatography buffer and concentrated to approximately 1 ml by centrifugation in a Centricon 30 tube. The concentrated material was fractionated by size-exclusion FPLC at 4°C in CHAPS chromatography buffer using a Superose 6 column and a Superose 12 column, in series (each column was 30 cm in length; Pharmacia). Fractions were assayed for cyclase activity, and those containing cyclase were discarded. The fractions were also assayed for the presence of $G_s\alpha$, by its fluoride-dependent stimulation of the cyclase activity in membranes from the *cyc*$^-$ strain of the *S49* lymphoma cell line, a mutant lacking $G_s\alpha$ (Ross et al., 1978). Reconstitution of G_s with *cyc*$^-$ membranes was done as follows: 3 μl of each fraction were added to 12 μl of *cyc*$^-$ membranes (0.35 mg/ml protein), then diluted with 10 μl of *cyc*$^-$ reconstitution buffer and incubated for 15 min at 0°C. A second incubation was done with 25 μM ATP, with and without fluoride (50 mM NaF with 60 μM AlF_3 as an activator) for 10 min at 30°C. Cyclase activity was then assayed by addition of ^{32}P-ATP and other assay components. The peak fractions containing $G_s\alpha$ (see Fig. 4) were then preactivated by incubating them with 100 μM GTPγS in the presence of 60 mM Mg^{2+} for 100 min at 30°C. The preactivated material was desalted by ultrafiltration on Centricon 30 filters in CHAPS chromatography buffer (with 8 mM CHAPS) to remove free GTPγS, then aliquoted and frozen.

Calmodulin-binding adenylate cyclase was isolated by calmodulin-Sepharose affinity chromatography (Westcott et al., 1979) using the procedure described by Eliot et al. (1989) with minor modifications. Prior to each reconstitution experiment, pleural ganglia from 50 animals were desheathed and homogenized in homogenization buffer with 5 mM EGTA. The material was centrifuged and rehomogenized twice in the EGTA buffer to deplete endogenous calmodulin. The final pellet was resuspended in cyclase solubilization buffer and centrifuged once again. The pellet was suspended in 2 ml solubilization buffer with 0.1 mg/ml HSA and 1.5% Lubrol and shaken at 0°C for 70 min, with occasional vortexing. After solubilization, 10 mM $MgCl_2$ and 1 mM ATP were added, and the material was centrifuged 1 hr at 100,000 × *g*. The supernatant was diluted with 1 ml binding buffer, brought to 2 mM $CaCl_2$ and 30 mM KCl, and incubated, with rotation, with 1 ml of calmodulin-Sepharose (Pharmacia) for 3 hr at 4°C. The resin was then transferred to a column and washed with 80 ml of binding buffer. The calmodulin-binding cyclase was then eluted with 4 ml of elution buffer. The eluted material from calmodulin-Sepharose was thoroughly desalted and concentrated by ultrafiltration on Centricon 30 filters in CHAPS chromatography buffer, without ATP, that contained 10 μM forskolin (Behring Diagnostics) to stabilize the enzyme (Smigel, 1986).

Reconstitution of the fraction containing G_s with the fraction containing calmodulin-binding cyclase was achieved with a protocol adapted from Smigel (1986). Aliquots of 50 μl of this material were added to equal volumes (50 μl) of 1.5 mg/ml dioleolylphosphatidylcholine (in reconstitution buffer). This mixture was shaken with 20 mg of SM-2 beads (Bio-Rad) for 15 min at 4°C, and then diluted with 300 μl of the reconstitution buffer with 1 μM forskolin and again shaken 20 min at 4°C. The material was centrifuged at 13,000 × *g* for 1 min, and the supernatant (without SM-2 beads) was added in 125-μl aliquots to tubes with 5.5 mg of SM-2 beads each. To these tubes, either 13 μl of the activated G_s or an equal volume of 8 mM CHAPS chromatography buffer was added; the tubes were shaken for 20 min at 4°C. Ca^{2+}/calmodulin was then added to selected tubes. The cyclase in each tube was then assayed in triplicate.

Adenylate cyclase assays. Synthesis of cyclic AMP was assayed by a modification of the method of Salomon (1979). The reaction was carried out with membranes or solubilized cyclase in a 50-μl volume containing the following: 50 mM K-HEPES (pH 7.6), 50 U/ml creatine phosphokinase, 5 mM creatine phosphate, 0.1 mg/ml HSA, 1 mM 3-isobutyl-1-methylxanthine (IBMX), 2 × 10^4 dpm ^{3}H-cyclic AMP (New England, Nuclear, #TRK304), and 2–30 × 10^6 dpm α^{32}P-ATP (Amersham, #PB171). Concentrations of nucleotides, divalent cations, and transmitters varied among experiments and are noted elsewhere. Because *Aplysia* neural tissues yielded limited amounts of membrane protein and low total cyclase activity, ^{32}P-cAMP synthesized per assay tube was increased by using low concentrations of cold ATP, typically 20 μM. Assay mixtures were incubated 2–6 min at 25°C. Assays of solubilized cyclase were carried out for 10 min. In preliminary experiments, synthesis of cyclic AMP was found to be linear with time over the durations used. Cyclic AMP synthesized was isolated by successive cation exchange (Dowex 50W-X4, Bio-Rad) and neutral alumina (Sigma) chromatography. ^{3}H-cyclic AMP included in the assay solution permitted normalization for recovery in calculating cyclic AMP synthesized.

Results

Exogenous calmodulin can overcome effects of calmodulin inhibitors in reducing stimulation of the cyclase by transmitter

As an initial approach to determining whether, in the *Aplysia* CNS, calmodulin influences activation of adenylate cyclase by transmitter, we tested the effects of calmodulin inhibitors on transmitter stimulation. Two inhibitors, R24571 and TFP, were found in preliminary experiments to be effective in blocking Ca^{2+} stimulation of calmodulin-dependent phosphodiesterase and cyclase in *Aplysia* (see Materials and Methods). In further preliminary experiments, the stimulation of the cyclase by the facilitatory transmitter 5-HT in the presence of 100 μM free Ca^{2+} was found to be decreased by either 4 μM R24571 or 100 μM TFP.

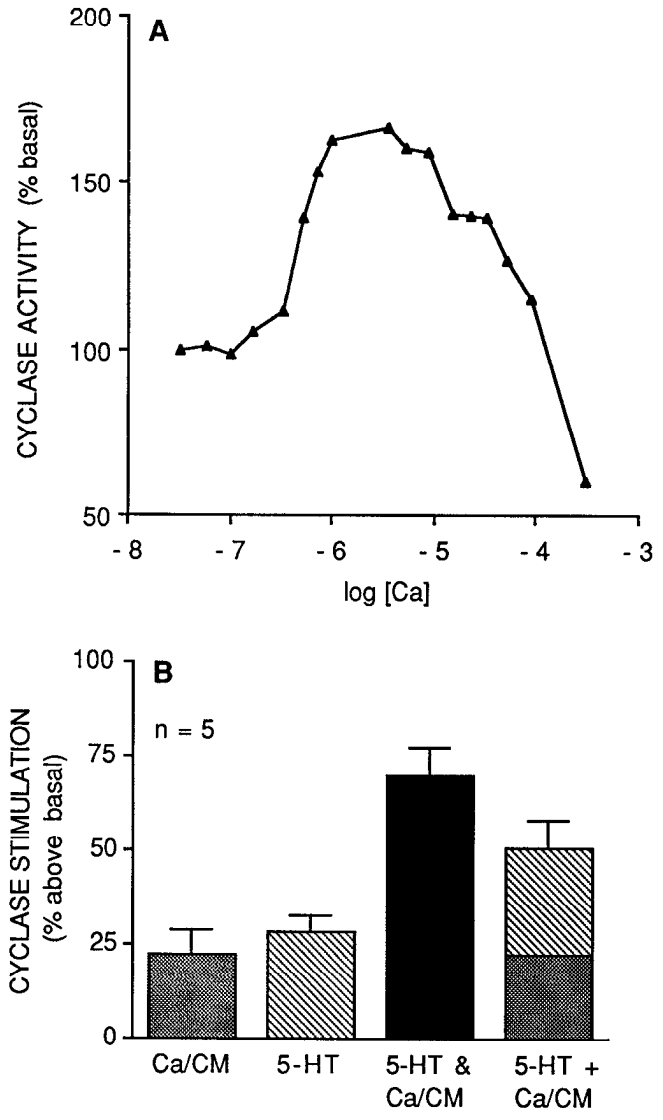

Figure 1. Exogenous calmodulin partially reverses effects of TFP. *A*, Ca^{2+} sensitivity of cyclase. To determine optimal levels of Ca^{2+} to use in these experiments, membranes from *Aplysia* CNS were assayed in a series of buffers prepared according to the methods of Yovell et al. (1988). Free Mg^{2+} in assays was 2 mM; added calmodulin was 3 μM. Data are from a single experiment. Cyclase activity is expressed as percentage of basal with 10 nM free Ca^{2+} in the presence of calmodulin. *B*, Ca^{2+}/calmodulin and 5-HT stimulation of membranes preexposed to TFP. After exposure to 200 μM TFP, membranes from *Aplysia* CNS were centrifuged and resuspended in buffer without the calmodulin inhibitor (it was necessary to remove the TFP from the buffer before adding exogenous calmodulin so that the inhibitor would not saturate the exogenous calmodulin). Assays were conducted in either 30 nM free Ca^{2+} (basal conditions) or 3 μM free Ca^{2+} (high Ca^{2+} conditions). Stimulation of cyclase by Ca^{2+} with exogenous calmodulin (Ca/CM), by 10 μM 5-HT, or by 5-HT simultaneously with Ca^{2+}/calmodulin (5-HT & Ca/CM) are expressed as percentage above basal activity. The sum of the separate stimulations by 5-HT and Ca^{2+}/calmodulin is also shown (*5-HT + Ca/CM*). Average basal cyclase activity was approximately 15 pmol cAMP/mg·min. Data are means ± SEM for five separate experiments, each assayed in quadruplicate. The simultaneous stimulation by the two ligands was significantly greater than the sum of the independent stimulations by the two ligands separately ($p < 0.02$, two-tailed *t* test). (In the absence of added calmodulin, the increase in free Ca^{2+} from 30 nM to 3 μM resulted in a 14.3 ± 3.9% inhibition of cyclase activity; data not shown.) Assays were conducted with 20 μM ATP in the presence of 10 μM GTP.

One explanation of these results is that the transmitter-activated cyclase in *Aplysia* CNS could also be regulated by Ca^{2+}/calmodulin. Alternatively, these inhibitors might have interfered with transmitter stimulation in some nonspecific manner. To explore further whether calmodulin influences the stimulation of cyclase by transmitter, we examined whether the effect of TFP could be reversed by the addition of exogenous calmodulin. To determine whether exogenous calmodulin can restore transmitter stimulation, membranes were preexposed to 200 μM TFP prior to the assay, then centrifuged and resuspended in buffer without TFP. Because TFP is lipophilic, this procedure leaves substantial amounts of the inhibitor in the membrane (see below); this TFP in the membranes effectively reduced Ca^{2+} stimulation of the cyclase even after they were resuspended in buffer lacking TFP.

If the TFP in the membranes were exerting its effects nonspecifically by inhibiting some component of the adenylate cyclase complex other than calmodulin, we would expect that exogenous calmodulin would not improve stimulation by transmitter. On the other hand, if the TFP were influencing transmitter stimulation by binding to and inhibiting the endogenous calmodulin, we would expect that exogenous calmodulin, added to the TFP-free buffer, might interact with the cyclase and overcome the effects of the inhibitor in the membranes (Gross et al., 1987).

Two Ca^{2+}/EGTA buffers were selected for these experiments, one in which free Ca^{2+} was 3×10^{-8} M, which gave no Ca^{2+} stimulation, and one in which free Ca^{2+} was 3×10^{-6} M, which gave optimal Ca^{2+} stimulation (Fig. 1*A*). In contrast to the stimulation observed in untreated membranes with Ca^{2+} concentrations of 1–100 μM, cyclase activity in TFP-pretreated membranes showed modest inhibition by 3 μM Ca^{2+} (data not shown). After addition of 3 μM exogenous bovine brain calmodulin, cyclase activity in pretreated membranes showed stimulation by 3 μM Ca^{2+}, though less than in untreated membranes (30% vs 67% stimulation in one experiment in which membranes with and without TFP pretreatment were compared; also cf. Fig. 1*A,B*). In the absence of exogenous calmodulin, stimulation by 10 μM 5-HT in pretreated membranes was invariably weak. Addition of Ca^{2+}/calmodulin improved this 5-HT stimulation. In five experiments on separate preparations, the simultaneous stimulation of the cyclase by the two ligands was consistently greater than the sum of the separate stimulations by Ca^{2+}/calmodulin and by transmitter (Fig. 1*B*). Although this difference was small (19.6 ± 4.3%, mean ± SEM), it was significant ($p < 0.02$, two-tailed *t* test), suggesting that Ca^{2+}/calmodulin and facilitatory transmitter interact in activating some of the same cyclase molecules.

An alternative explanation for this reversal of inhibition by exogenous calmodulin could be that the added Ca^{2+}/calmodulin binds and removes TFP located within the membrane. Rather than substituting for the endogenous calmodulin in interacting with the cyclase, the calmodulin added might merely decrease the effective concentration of the inhibitor. To test for this possibility, CNS membranes were preexposed to 200 μM ^{3}H-TFP, centrifuged, and resuspended in buffer without TFP, with the identical procedure used with the experimental cyclase preparation. By centrifuging these membranes once more and counting the pellets and supernatants, we calculated the relative amount of inhibitor remaining in the membranes after incubation with and without Ca^{2+} and calmodulin. The membranes concentrated

Table 1. Interactions between Ca^{2+}/calmodulin and G_s in stimulating adenylate cyclase

	Control	+ GTPγS	GTPγS stimulation
Low Ca^{2+}	3.6	137	133
High Ca^{2+}	6.7	188	181
Ca^{2+} stimulation	3.1	51	—

Membrane pellets from whole CNS were resuspended in homogenization buffer with 10 mM $MgCl_2$ and divided into two equal portions. One portion (+ GTPγS) was preactivated by incubating it for 3 min at room temperature with 100 μM GTPγS plus 100 μM 5-HT. The other portion (control) was similarly incubated without transmitter or guanyl nucleotide. Both portions of membrane were then centrifuged twice more before resuspension for assay. Values are pmol cAMP/mg·min.

the inhibitor so that, after resuspension in TFP-free buffer, they contained a minimum of 5 mM TFP (based on an estimate of the volume of the pellets obtained by centrifuging the resuspended membranes). Exposure to calmodulin in the presence of 3 μM Ca^{2+} resulted in a 14–20% decrease in the amount of the inhibitor remaining within the membranes. Such a small decrease in the TFP concentration within the membrane fraction is unlikely to reduce effectively any nonspecific effects the inhibitor may have, unless the TFP is selectively removed from a site at which it acts nonspecifically to inhibit the cyclase. While not conclusive, the reversal of the TFP inhibition of 5-HT stimulation by exogenous calmodulin suggests that the TFP is exerting its action specifically, by inhibiting endogenous calmodulin.

Interaction between G_s and Ca^{2+}/calmodulin in activating cyclase

These TFP preexposure experiments suggested that the same adenylate cyclase can be influenced by two signals, Ca^{2+}/calmodulin and transmitter. Another way to test whether such dual activation occurs is to determine whether the Ca^{2+}/calmodulin-sensitive cyclase can also be activated by the stimulatory G-protein, G_s. In those systems that have been studied, receptor has been found to be coupled to the catalytic unit of cyclase by means of G_s (Schramm and Selinger, 1984). Although G_s has not been directly shown to mediate receptor activation of the cyclase catalytic unit in *Aplysia,* Castellucci et al. (1983) found that, in sensory neurons, cyclase-mediated effects of transmitter were blocked by GDPβS, and Volterra et al. (1987) found that GTPγS mimicked these effects; moreover, Vogel et al. (1989) have identified immunoreactive G_s in *Aplysia* CNS. Indeed, we found transmitter stimulation of cyclase from *Aplysia* CNS required the presence of the guanyl nucleotide GTP, which is necessary for G_s-mediated activation (Fig. 2). These several results suggest that, as in other systems, receptor activation of cyclase occurs via an intermediary G-protein that binds GTP.

To examine whether the same Ca^{2+}/calmodulin sensitive cyclase is also activated by G_s, we determined whether G_s alters the Ca^{2+} stimulation of the enzyme. For this purpose, we preactivated G_s by incubating membranes with the nonhydrolyzable GTP analog GTPγS. As shown in Table 1, after activation by GTPγS, the Ca^{2+} stimulation increased dramatically, by more than 17-fold. The stimulation by GTPγS was also greater, by about 36%, in the presence of Ca^{2+} than in its absence. It should be noted that, though the dual activation by G_s together with Ca^{2+}/calmodulin was greater than the sum of the two separate

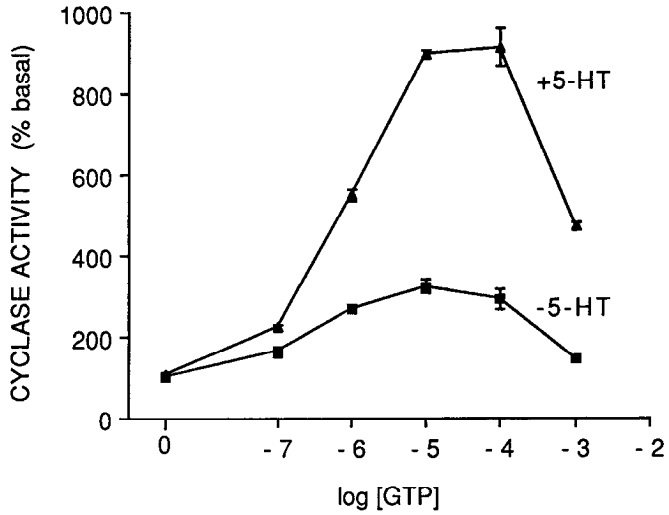

Figure 2. GTP dependence of cyclase stimulation by transmitter. Cyclase activity in membranes from the entire CNS was assayed with and without 20 μM 5-HT at four different concentrations of GTP. Note that 5-HT stimulation is negligible with 100 nM GTP and improves with increasing GTP. Cyclase activity is expressed as percent of basal activity (without added guanyl nucleotides or 5-HT), which was 5.1 pmol cAMP/mg·min. Data are the mean ± SD for three replicates from a single experiment. A similar dependence of transmitter stimulation on GTP was observed in two other experiments. Assays were conducted with 20 μM ATP.

stimulations by G_s and Ca^{2+}/calmodulin, the dual stimulation was less than multiplicative. Nevertheless, these results suggest dual activation of a single species of cyclase. If G_s and Ca^{2+}/calmodulin were activating separate cyclases, then the absolute stimulation of cAMP synthesis by Ca^{2+} would be identical whether or not an independent, calmodulin-insensitive species of cyclase had been activated by G_s. Clearly the two stimulations interact and are not independent, consistent with convergent activation of the Ca^{2+}/calmodulin-sensitive adenylate cyclase.

Reconstitution of stimulatory G-protein with the calmodulin-binding cyclase

The possibility of dual activation can be tested more directly by isolating the cyclase that binds to calmodulin and determining whether this same enzyme can be activated by the stimulatory G-protein. The calmodulin-sensitive cyclase was isolated from other species of adenylate cyclase that may exist in *Aplysia* CNS by calmodulin-Sepharose affinity chromatography (Westcott et al., 1979; Yeager et al., 1985; Eliot et al., 1989). In an attempt to enrich the experimental preparation for the mechanosensory neurons that show activity-dependent facilitation, we carried out these experiments on desheathed pleural ganglia, the ganglia with the greatest abundance of these neurons. Solubilized material from pleural ganglion membranes was incubated with calmodulin-Sepharose in the presence of Ca^{2+}, the resin was washed to remove unbound material, and calmodulin-binding cyclase activity was then eluted with EGTA-containing buffer (Fig. 3; see also Materials and Methods). As expected, the calmodulin-binding cyclase activity was stimulated by addition of 200 μM Ca^{2+} plus bovine brain calmodulin (see Fig. 5). To determine whether this calmodulin-binding adenylate cyclase interacted with the stimulatory G-unit, we prepared a crude G_s-containing fraction from solubilized *Aplysia* CNS membranes.

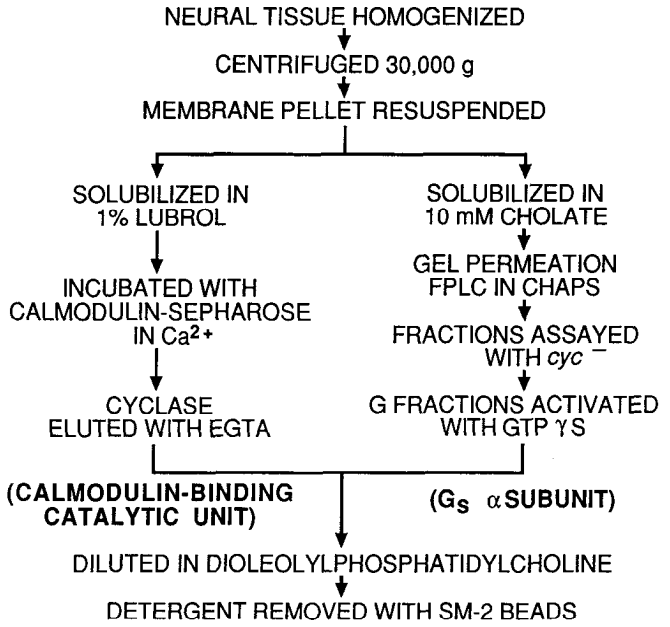

Figure 3. Protocol for reconstitution of G_s with calmodulin-binding adenylate cyclase in phospholipid. Starting material for partial purification of calmodulin-sensitive adenylate cyclase was desheathed *Aplysia* pleural ganglia. Starting material for partial purification of G_s was entire *Aplysia* CNS. Additional details are described in Materials and Methods.

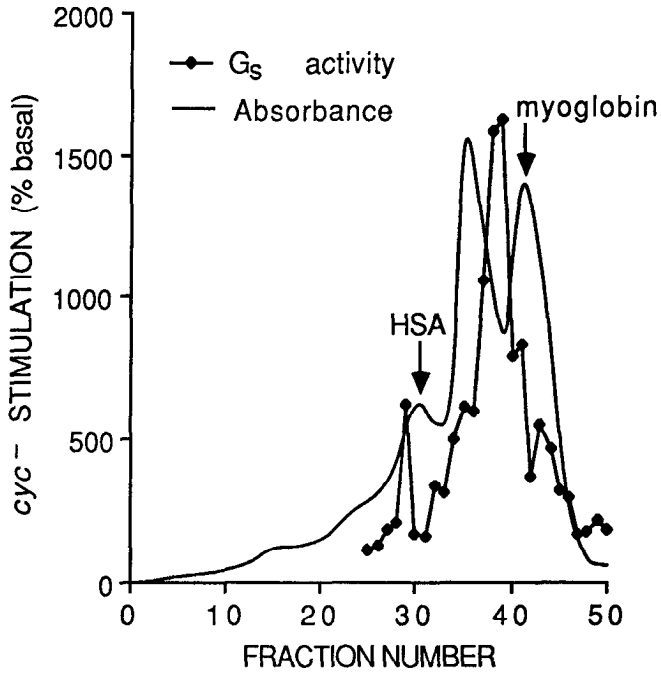

Figure 4. Partial purification of G_s from *Aplysia* CNS membranes. CNS membranes were solubilized in cholate, diluted into CHAPS buffer, and fractionated by gel-permeation FPLC. Fractions (400 μl vol) were assayed for the presence of $G_s\alpha$ by reconstituting aliquots of each with membranes from S49 lymphoma mutant *cyc*⁻, which lacks $G_s\alpha$. Reconstituted membranes were then assayed with or without fluoride, an activator of $G_s\alpha$. *cyc*⁻ stimulation is the cyclase stimulation by fluoride, as percent above activity in the absence of fluoride; no fluoride stimulation occurs in the absence of G_s. The *continuous curve* indicates absorbance at 280 nm. *Arrows* indicate the elution times of peaks representing HSA and myoglobin, which were included with injection of solubilized material as molecular-weight markers. Some G_s activity eluted, together with cyclase activity, in the first 8 ml; these early fractions were not assayed in the experiment shown in this figure. Cyclase activity was negligible in those fractions that were assayed, fractions 25–50. The two peaks of G_s activity may represent the intact $\alpha\beta\gamma$ heterotrimer, which would be expected to elute before HSA, and the dissociated G_s unit, which would be expected to elute between HSA and myoglobulin.

The solubilized material was fractionated by size-exclusion chromatography. Fractions containing $G_s\alpha$ were identified using the *cyc*⁻ lymphoma mutant, which lacks $G_s\alpha$ (Fig. 4; see also Materials and Methods). The fractions containing the peak $G_s\alpha$ activity were preactivated using the nonhydrolyzable GTP analog GTPγS (Fig. 3). Without such preactivation (or without an alternative activator, fluoride), these fractions were unable to activate cyclase (data not shown). The preactivated, partially purified G_s was reconstituted with the calmodulin-Sepharose eluate in phospholipid (Smigel, 1986). By itself, the G_s fraction had no detectable cyclase activity. However, addition of the preactivated G_s fraction stimulated the calmodulin-binding cyclase 3.6 ± 1.1–fold (mean ± SEM) in five experiments on separate preparations (Fig. 5). In all of these reconstitution experiments, while cyclase stimulation was always observed, the quantitative stimulation by the G_s-containing fraction and by Ca^{2+}/calmodulin was quite variable; this was possibly a consequence of either inconsistent reconstitution into lipid or varying amounts of degradation of the solubilized enzyme. Whatever the cause of this variability, it prevented us from reliably assessing whether dual stimulation by the G_s fraction combined with Ca^{2+}/calmodulin was greater or less than the sum of the separate stimulations. While we do not yet know which factors affected whether or not synergistic stimulation was observed, we did note that the synergism tended to be stronger when both individual stimulations were weaker.

In these reconstitution experiments, because of the limited amount of membrane proteins available from the *Aplysia* CNS, neither component was a purified protein; for example, the pleural ganglion membranes from which we purified the calmodulin-binding cyclase provided only approximately 20 μg of solubilized membrane protein. Although neither $G_s\alpha$ nor the catalyst was fully purified, these experiments do provide evidence that the Ca^{2+}/calmodulin-sensitive cyclase from *Aplysia* neural tissue can be activated by a stimulatory G-protein. The cyclase activity that we have examined was the activity that binds specifically to calmodulin in a Ca^{2+}-dependent manner (Eliot et al., 1989). Moreover, while the G_s-containing fraction was not fully purified, stimulation of the calmodulin-binding cyclase by this fraction required its preactivation with GTPγS; this argues that the observed stimulation of the calmodulin-binding cyclase is mediated by a guanyl nucleotide binding protein, probably some species of the stimulatory G-protein, G_s.

Temporal requirements for effective Ca^{2+}–transmitter pairing

During classical conditioning of the defensive withdrawal reflex, the conditioned stimulus must be temporally paired with the unconditioned stimulus for associative learning to result (Carew et al., 1981, 1983; Hawkins et al., 1986). Similarly, in cellular experiments, action potentials in the sensory neurons must occur immediately before the cells receive facilitatory input for activity-dependent enhancement of the facilitation response to occur (Hawkins et al., 1983; Clark, 1984). We therefore wanted to examine whether convergent activation of the dually activated cyclase by Ca^{2+} and transmitter could underlie this temporal specificity. Does the elevation in Ca^{2+} need to overlap

temporally with the addition of 5-HT for it to enhance stimulation of cyclase?

In order to explore these issues, initial experiments on temporal interactions were conducted with traditional methods in test tubes, in which it was not possible to terminate the exposure to stimulatory transmitter once the transmitter had been added. We tested the effects of brief Ca^{2+} pulses that were paired or unpaired with 5-HT addition. Experiments were done on membranes from desheathed pleural ganglia that were resuspended in a Ca^{2+}/EGTA buffer with a low level of free Ca^{2+}, approximately 10 nM. To pulse Ca^{2+} in an assay tube, we pipetted in additional Ca^{2+} to bring the free Ca^{2+} in the buffer to approximately 0.1–0.2 μM and, 10 sec later, pipetted in additional EGTA to return the free Ca^{2+} to the initial concentration of approximately 10 nM. (Free Mg^{2+} was maintained at 5 mM throughout.) For paired stimulation, we added 5-HT in the middle of the Ca^{2+} pulse; for unpaired stimulation, we added 5-HT 5 sec after the end of the pulse. Tubes were vortexed within 0.5 sec after each addition. Assays were initiated at the same time as the addition of the stimulating ligands and terminated 2.5 min later.

Although the differences were modest, we consistently observed differences in cyclase stimulation between paired and unpaired Ca^{2+} and 5-HT addition (Fig. 6). Membranes that received Ca^{2+} paired with addition of 5-HT showed significantly greater stimulation of cyclase activity than did membranes that received unpaired Ca^{2+} and 5-HT (55.6 ± 10.0% stimulation above basal for paired vs. 32.6 ± 5.8% stimulation above basal for unpaired; the mean difference, with stimulation compared within experiments, was 23.0 ± 5.9%; $p < 0.02$, two-tailed t test; data are means ± SEM). In other experiments, cyclase stimulation when a Ca^{2+} pulse was paired with 5-HT addition was compared with stimulation with 5-HT alone. Again, the paired Ca^{2+} pulse significantly enhanced activation by 5-HT (e.g., in one series of experiments, stimulation was 62.9 ± 5.2% for paired Ca^{2+} and 5-HT vs. 37.4 ± 4.1% for 5-HT alone; $p < 0.05$).

One possible explanation for the apparent enhancement of stimulation when Ca^{2+} and 5-HT overlapped temporally is that there was simply summation of the two separate stimulations by 5-HT and Ca^{2+}. However, such summation cannot account for the differential cyclase stimulation observed because, in the presence of GTP, Ca^{2+} stimulation is extremely modest (Yovell et al., 1986); these brief, 10-sec pulses of Ca^{2+} produced no detectable stimulation when delivered alone. In fact, in similar experiments without 5-HT in which free Ca^{2+} was increased (as in the pulse experiments), but then left elevated throughout the remainder of the 2.5-min assay, stimulation was only 6.4 ± 1.5% of basal activity. Such minimal stimulation by Ca^{2+} could not explain the enhanced cyclase stimulation observed with pairing.

Discussion

The cyclase as a molecular site of stimulus convergence

Activity-dependent presynaptic facilitation is an associative form of synaptic plasticity that has been implicated in classical conditioning in *Aplysia*. Studies of activity-dependent facilitation have suggested a specific molecular locus for the associative changes in the sensory neurons. Investigations on intact mechanosensory neurons indicated that the associative enhancement of synaptic transmission might result from interactions between inputs from the conditioned and unconditioned stimuli converging at the Ca^{2+}/calmodulin-sensitive adenylate cyclase. Eliot et al. (1989) and Yovell et al. (1986) demonstrated

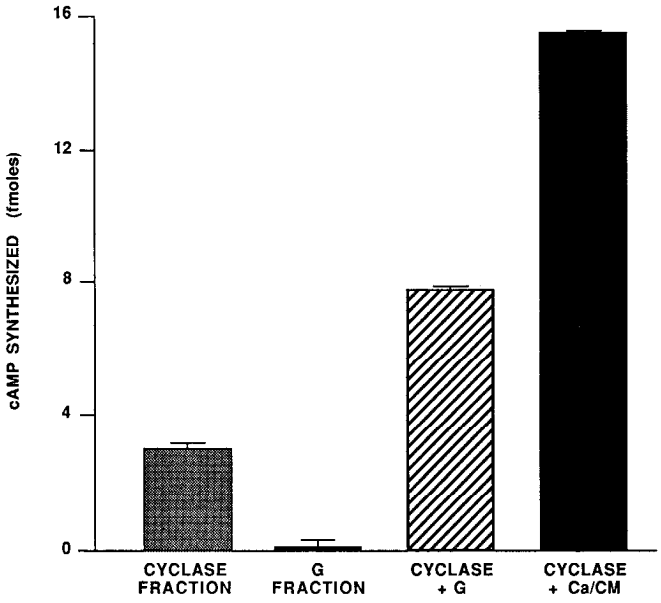

Figure 5. Stimulatory G-protein activates the calmodulin-binding cyclase catalytic unit. EGTA eluate from calmodulin-Sepharose was desalted and reconstituted into phospholipid vesicles, and cyclase activity was assayed alone or with addition of activated G_s-containing fraction or 200 μM Ca^{2+} plus 0.3 μM calmodulin. Calmodulin-binding cyclase was stimulated both by activated G_s and by Ca^{2+}/calmodulin (*Ca/CM*). The *G fraction,* containing activated G_s, was reconstituted into dioleolylphosphatidylcholine vesicles to which the calmodulin-Sepharose eluate had not been added. Note that the *G_s fraction* by itself had negligible cyclase activity. Data for cAMP synthesized are means ± SD of four replicates from a single, representative reconstitution experiment. Qualitatively similar results were obtained in a total of five other experiments. Assays were without free guanyl nucleotides. [Because total cyclase activity from these solubilized pleural ganglion membranes was quite low, assays were conducted with relatively large amounts of ^{32}P-ATP (up to 3×10^7 cpm per tube) and reduced cold ATP concentrations (8 μM ATP). Under these assay conditions, basal cyclase activity yielded more than 1500 cpm ^{32}P-cAMP per tube; recovery was approximately 80%, and blanks were less than 100 cpm.]

that *Aplysia* CNS contains a species of adenylate cyclase that is stimulated by Ca^{2+} via calmodulin; the existence of a Ca^{2+}/calmodulin-sensitive cyclase was confirmed by the experiments described above (see, e.g., Figs. 1*B*, 5).

A critical requirement for the hypothesis that the Ca^{2+}/calmodulin-sensitive cyclase serves an associative role during learning is that the enzyme can be dually regulated. It was therefore essential to determine whether the same adenylate cyclase molecules that can be modulated by Ca^{2+} influx into the sensory neurons during activity elicited by the conditioned stimulus can also be activated by facilitatory transmitter released by the unconditioned stimulus. To address this issue, we have reconstituted in phospholipid the Ca^{2+}/calmodulin-sensitive cyclase, isolated using calmodulin-Sepharose, together with a fraction containing stimulatory G-protein, G_s, from *Aplysia* CNS. These reconstitution experiments demonstrated that the catalytic unit of cyclase that binds to calmodulin in a Ca^{2+}-dependent manner is effectively activated by G_s. Addition of Ca^{2+}/calmodulin also stimulated this same cyclase. This stimulation by calmodulin is most likely direct, given that the enzyme binds to calmodulin-Sepharose; alternatively, it could be mediated by a Ca^{2+}/calmodulin-dependent kinase present both in the membrane preparation and in the eluate from calmodulin-Sepharose.

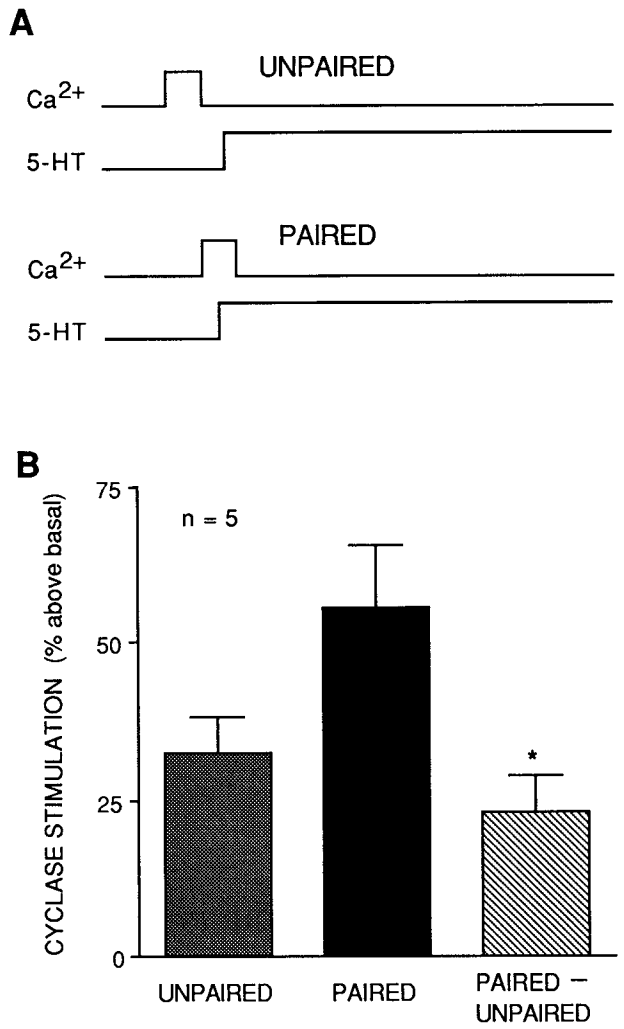

Figure 6. Temporal requirements for effective Ca^{2+}–transmitter pairing. *A*, Protocol for Ca^{2+} pulses paired and unpaired with facilitatory transmitter. Homogenized desheathed pleural ganglia were resuspended in a Ca^{2+}-EGTA buffer with 5 nM free Ca^{2+}. During the assay, each tube contained the membranes from approximately one pleural ganglion. Ca^{2+} transients were achieved by pipetting Ca^{2+} into a tube, followed 10 sec later by addition of EGTA to reduce the free Ca^{2+} to the initial low level (see Materials and Methods). Addition of 20 μM 5-HT was done either in the middle of the Ca^{2+} pulse (*PAIRED*) or 5 sec after the EGTA addition (*UNPAIRED*). Assay of cyclase activity was terminated 2.5 min after 5-HT addition. Tubes were vortexed immediately after each addition. Assays included 20 μM ATP and 10 μM GTP, and no added calmodulin (membranes were homogenized and centrifuged in a buffer designed to retain endogenous membrane-bound calmodulin). *B*, Cyclase stimulation by paired and unpaired Ca^{2+} pulses: results of experiments carried out according to protocol in *A*. Mean of the difference, within each experiment, between stimulation with paired Ca^{2+} pulse and stimulation with unpaired Ca^{2+} pulse (*right bar*) was significantly greater than 0 (*, $p < 0.02$, two-tailed t test). Data are means ± SEM for five experiments, consisting of four or five replicates each. Average basal cyclase activity was approximately 16 pmol cAMP/mg·min.

We have not yet carried out the complete reconstitution with receptor, G-protein, and catalyst. However, in all systems that have been sufficiently studied, the stimulatory G-protein serves as a coupling protein, binding GTP and activating the catalyst when agonist binds to the appropriate receptor (Schramm and Selinger, 1984). As in other systems, stimulation of *Aplysia* neural cyclase by the facilitatory transmitter 5-HT requires the presence of GTP (Fig. 2). Thus, the observation that the Ca^{2+}/calmodulin-sensitive catalyst can be activated by a G_s-containing fraction suggests that it can be also activated by transmitter via the stimulatory receptors linked to G_s. The Ca^{2+}/calmodulin-sensitive adenylate cyclase similarly isolated from bovine brain is also activated by the stimulatory G-protein; Heideman et al. (1982) and Yeager et al. (1985) found that, after separation with calmodulin-Sepharose, the bovine catalytic unit remains coupled to G_s and is stimulated by ligands that activate G_s. In contrast, the solubilized *Aplysia* cyclase appears to separate readily from G_s, because it usually shows little or no sensitivity to ligands that activate G_s after calmodulin-Sepharose chromatography (L. S. Eliot, unpublished observations). Thus, it was necessary in this study to reconstitute the catalyst with a G_s fraction to demonstrate activation by the guanyl nucleotide–binding protein.

Additional evidence that the Ca^{2+}/calmodulin-sensitive adenylate cyclase is also activated by transmitter and stimulatory G-protein comes from the observation that, under some conditions, transmitter and G_s interact with Ca^{2+}/calmodulin in stimulating the cyclase in homogenized neural membranes. If the two stimuli were activating separate enzymes, the absolute stimulation by one would be unaffected by activation by the other. As discussed below, the magnitude of this interaction, or synergism, varied substantially, depending upon experimental conditions. Nevertheless, the fact that under some circumstances these interactions occurred must mean that Ca^{2+} and transmitter converge in stimulating a single species of cyclase.

Because the *Aplysia* enzyme is dually regulated by calmodulin and by the stimulatory G-protein, it provides a potential site of convergence between the conditioned stimulus, which causes activity and Ca^{2+} influx in the sensory neurons, and the unconditioned stimulus, which causes release of facilitatory transmitter. This dual regulation of the cyclase contrasts with the conclusion by Livingstone (1985), studying *Drosophila* cyclase, that a single species of cyclase cannot by itself mediate stimulus convergence during conditioning and that more complex molecular cascades must be postulated to account for the critical roles of Ca^{2+} influx and the Ca^{2+}/calmodulin-sensitive cyclase in associative changes during learning.

Can Ca^{2+} and facilitatory transmitter act synergistically in activating the calmodulin-sensitive cyclase?

If Ca^{2+} influx and facilitatory transmitter act associatively to produce an enhanced facilitation response by converging on the cyclase, they may be expected to stimulate the adenylate cyclase synergistically. We consider an activation to be synergistic when the absolute stimulation by two ligands acting simultaneously is greater than the sum of the two separate stimulations produced by each ligand acting alone; thus, synergistic stimulation is greater than additive stimulation. Synergistic activation by two stimulating inputs that overlap in time could underlie the requirement for the pairing of the conditioned and unconditioned stimuli. Alternatively, in the absence of synergism, an associative requirement for dual activation of the cyclase could result from a nonlinear step in the facilitation response downstream from the synthesis of cAMP (e.g., a process with a discrete threshold) that necessitated the coincidence of the two stimuli to achieve a minimum required level of cAMP (see discussion by Yovell and Dudai, 1987). Studies that have sought to identify synergism between the stimulatory G-protein and Ca^{2+}/calmodulin in activating cyclase in mammalian brain

membranes have yielded inconsistent results (MacDonald, 1975; Brostrom et al., 1977, 1978b; Treisman et al., 1983). Similarly, in the CNS of *Aplysia,* we have found that Ca^{2+}/calmodulin and either transmitter or guanyl nucleotides may produce stimulation that is either greater than or less than additive stimulation, depending upon assay conditions. For example, when solubilized cyclase and a G_s-containing fraction were reconstituted in phospholipid, dual stimulation by the preactivated G_s fraction and Ca^{2+}/calmodulin was greater or less than additive, depending upon the individual experiment; our preliminary observations suggested that the synergism tended to be stronger when the individual effects of Ca^{2+}/calmodulin and G_s were weak. In contrast, in experiments on intact membranes, GTPγS and Ca^{2+}/calmodulin consistently produced more than additive stimulation of the cyclase (Table 1). Eliot et al. (1989) also observed synergistic activation of solubilized bovine brain cyclase by nonhydrolyzable guanyl nucleotides and Ca^{2+}/calmodulin. Reliable, though weak, synergism was also observed when TFP preexposure was used to inhibit endogenous calmodulin (Fig. 1*B*). The parameters that affect the extent to which synergism is observed under different assay conditions must still be elucidated. Nevertheless, these experiments, and those in which pulses of Ca^{2+} were paired with 5-HT, suggest that at least under some conditions Ca^{2+}/calmodulin may regulate the activation of cyclase by transmitter. As discussed below, under normal conditions (where G_s is activated by the natural guanyl nucleotide GTP, and in the absence of calmodulin inhibitors), synergistic activation may be most dramatic when the activating stimuli are transient.

Temporal requirements for effective stimulus pairing

Perhaps the most interesting prediction from behavioral and electrophysiological studies about associative activation of adenylate cyclase is that there should be precise temporal requirements for the pairing of Ca^{2+} and transmitter to achieve optimal cyclase activation: (1) Ca^{2+} and transmitter must overlap temporally, and (2) activation by Ca^{2+} must begin before activation by transmitter.

Ideally, the interaction between stimuli would be explored with transient increases in Ca^{2+}, comparable to the rise in intracellular Ca^{2+} during a brief ($\leq$0.5 sec) train of action potentials, and with transient exposures to facilitatory transmitter, such as would be produced by a short (1–1.5 sec) unconditioned stimulus. The techniques used in the present study were inadequate for a thorough temporal analysis. Nevertheless, our preliminary attempts to test the effects of temporal coincidence indicated that Ca^{2+} pulses that overlap in time with 5-HT addition were more effective in activating cyclase than Ca^{2+} pulses that ended before the addition of transmitter (Fig. 6). Thus, this initial study suggests that Ca^{2+}/calmodulin may serve to prime the cyclase for optimal stimulation by facilitatory transmitter. There are, however, two important limitations to the procedure used in these initial experiments: First, durations of stimuli and intervals between stimuli were long compared with physiological stimuli. For instance, the 10-sec Ca^{2+} rise in the test tube is probably much longer than the Ca^{2+} transient produced by a 0.5-sec train of action potentials in the sensory neuron (M. Spira, H. Blumenfeld, and S. Siegelbaum, personal communication). Second, in our experiments, 5-HT, once added, remained in the membranes for the duration of the assay. In contrast, in the ganglion, the concentration of free facilitatory transmitter probably begins to decline once the unconditioned stimulus has ended. Ideally, exposure to facilitatory transmitter should be transient; in electrophysiological experiments, no activity-dependent enhancement of the facilitation response is observed when 5-HT exposures last more than a few sec (Abrams, 1985; T. W. Abrams, unpublished observations). Recent studies have used a modification of the perfused membrane cyclase assay methodology of Yovell et al. (1987) to examine the activation of *Aplysia* neural cyclase by temporally paired transient stimuli (Yovell and Abrams, 1988). These results are consistent with the results of the Ca^{2+} pulse experiments described here in suggesting that, whereas there is no synergism when Ca^{2+} and transmitter are present for the duration of an assay, Ca^{2+}/calmodulin may interact to enhance activation by 5-HT when the stimuli are sufficiently brief (Yovell and Abrams, unpublished observations).

Despite the substantial temporal limitations in the present protocol, one interesting observation has emerged: a Ca^{2+} pulse that ended 5 sec before 5-HT was added did not noticeably enhance the stimulation of the cyclase, suggesting that Ca^{2+}/calmodulin effects on cyclase activation decay in less than 5 sec (Fig. 6, unpaired pulse). Such a rapid decay of the effects of Ca^{2+} is consistent with the narrow interstimulus interval ($<$2 sec) for effective stimulus pairing that has been observed behaviorally (Hawkins et al., 1986).

In addition to requiring temporal proximity for effective pairing of conditioned and unconditioned stimuli, a molecular mechanism for associative plasticity during conditioning would be expected to display the sequence requirement observed in behavioral and physiological studies of classical conditioning; that is, the conditioned stimulus, or activity, and Ca^{2+} influx must precede the unconditioned stimulus, or facilitatory transmitter. Results indicating a possible Ca^{2+}–5-HT sequence requirement for optimal cyclase activation have recently been obtained using the perfused membrane cyclase methodology mentioned above (Yovell and Abrams, 1988).

Possible additional sites of stimulus convergence

Although we have identified one molecular site at which activity and Ca^{2+} influx can enhance the cAMP-mediated facilitation response, undoubtedly there are other sites of interaction. For example, in several cell types, phosphorylation by the Ca^{2+}/phospholipid-dependent kinase (kinase C) increases transmitter activation of the cyclase by inactivating the inhibitory G-protein, G_i (Jakobs et al., 1985; Katada et al., 1985). Thus, this is a second possible mechanism by which Ca^{2+} could enhance cAMP synthesis. Downstream from adenylate cyclase and cAMP synthesis, there are additional possible sites at which Ca^{2+} could modulate the facilitation response. Such interactions could diminish, as well as potentiate, cAMP-mediated effects, as Kramer and Levitan (1988) and Kramer et al. (1988) have described in neuron R15 in *Aplysia.* In this cell, Ca^{2+} influx antagonizes modulation by cAMP by activating the Ca^{2+}/calmodulin-sensitive phosphodiesterase. Similarly, *Aplysia* sensory neurons contain a calmodulin-sensitive phosphodiesterase (T. W. Abrams and K. A. Karl, unpublished observations) as well as a calmodulin-sensitive cyclase. The predominant effect of Ca^{2+} elevations on cAMP-mediated processes may depend on the relative abundance of the two enzymes and on the distribution of Ca^{2+} within the cell; a small influx Ca^{2+} may be localized near the membrane and may primarily activate the cyclase. The role played by other sites of interaction between activity and the facilitation response in activity-dependent facilitation must be explored in future cellular and biochemical studies.

Allosteric interactions and associative learning

Following the initial characterization of proteins that had central roles in neuronal function, such as the ACh receptor, the suggestion was made that allosteric interactions at a single molecule could underlie associative changes during learning (Stent, 1973; Heidmann and Changeux, 1982; Changeux and Heidmann, 1987). Recently, two particular examples of such allosteric interactions have been described in studies of learning and synaptic plasticity in simple neural systems. First, the analysis of activity-dependent facilitation gave rise to the hypothesis that convergence of the conditioned stimulus and unconditioned stimulus at the Ca^{2+}/calmodulin-sensitive adenylate cyclase could be responsible for the associative synaptic changes. The present study indicates that, in *Aplysia,* the Ca^{2+}-sensitive cyclase has some of the properties required for it to play such an associative role. Additional support for an associative role for the cyclase comes from the finding that the *Drosophila* mutant *rutabaga,* which shows poor learning and memory, has a defect in the function of its Ca^{2+}/calmodulin-sensitive adenylate cyclase (Dudai and Zvi, 1984; Livingstone et al., 1984; Yovell et al., 1986). Second, recent studies of the mechanism of associative long-term synaptic potentiation in hippocampus suggest that associative activation of the glutamate-gated channel may be responsible for strengthening the synaptic connections (Collingridge et al., 1983; Harris et al., 1984; Kelso et al., 1986; Sastry et al., 1986; Wigstrom et al., 1986). Temporal coincidence of glutamate binding and postsynaptic depolarization is required for effective activation of the NMDA-gated Ca^{2+} conductance (Nowak et al., 1984; MacDermott et al., 1986); the resulting postsynaptic Ca^{2+} influx may serve to trigger synaptic plasticity (Lynch et al., 1983; Malenka et al., 1988). Thus, in both *Aplysia* sensory neurons and hippocampus, associative changes in synaptic connections may not require a complex neuronal circuit, but may result from allosteric interactions at molecules that gate second-messenger-induced plasticity (Abrams and Kandel, 1988).

In conclusion, it should be pointed out that, though the calmodulin sensitivity of adenylate cyclase in mammalian brain was reported more than a decade ago (Brostrom et al., 1975; Cheung et al., 1975), until recently there had been no suggestion as to its functional role. The results reported here are consistent with the possibility suggested by recent cellular studies of learning in *Aplysia* (Abrams, 1985; Ocorr et al., 1985) and genetic studies of learning in *Drosophila* (Dudai and Zvi, 1984; Livingstone et al., 1984; Yovell et al., 1986), that the dually regulated calmodulin-sensitive cyclase may play a critical associative role in learning.

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