Analysis of Sequence-Dependent Interactions Between Transient Calcium and Transmitter Stimuli in Activating Adenylyl Cyclase in *Aplysia*: Possible Contribution to CS–US Sequence Requirement During Conditioning

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Abstract

An important recent insight in a number of neurobiological systems is that during learning, individual dually regulated proteins with associative properties function as critical sites of stimulus convergence. During conditioning in *Aplysia*, the Ca^{2+} /calmodulin-sensitive adenylyl cyclase (AC) in mechanosensory neurons serves as a molecular site of interaction between Ca^{2+} and serotonin [5-hydroxytryptamine (5-HT)]—two signals that represent the CS and US in these cells. Conditioning requires that the CS and US be paired within a narrow time window and in the appropriate sequence. AC shows an analogous sequence preference: It is more effectively activated when a pulse of Ca^{2+} precedes a pulse of 5-HT than when the 5-HT precedes Ca^{2+} . One mechanism that contributes to this sequence preference is that Ca^{2+} /calmodulin binding to AC accelerates the rate of AC activation by receptor- G_s . We have identified two additional properties of AC activation that would cause pairing with Ca^{2+}

preceding 5-HT to be more effective than simultaneous pairing or pairing with the reciprocal sequence: (1) Activation of *Aplysia* AC by a Ca^{2+} pulse rose with a delay compared with activation by a 5-HT pulse. (2) A late pulse of Ca^{2+} , which arrived after 5-HT, acted, via calmodulin, to accelerate the decay of AC activation by receptor- G_s . Together, these activation properties of AC may contribute to the CS–US sequence requirement of classical conditioning.

Introduction

Through experience, animals learn relationships between stimuli or events in their environment or between these external events and their own behavior. A fundamental goal of the study of learning in simple neural systems is to identify general molecular mechanisms relevant to various types of learning in diverse species. A number of studies have suggested that during simple forms of associative learning, dually regulated proteins serve as the locus at which inputs from multiple stimuli or behaviors converge. According to this proposal, these proteins, such as the NMDA receptor (Collingridge 1987; Madison et al. 1991; Davis et al. 1992; Tsien et al. 1996) and the Ca^{2+} /

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calmodulin-sensitive adenylyl cyclase (AC) (Abrams and Kandel 1988), serve an associative role: When two stimuli are paired with the appropriate temporal relationship, the protein responds to the pairing by triggering a second-messenger cascade that initiates alterations of neuronal properties. The type I and type VIII forms of AC, which are stimulated by Ca^{2+} via calmodulin (CaM), are expressed at relatively high levels in regions of mammalian brain believed to be involved in learning (Xia et al. 1991; Cali et al. 1994). Moreover, genetic deletion of Ca^{2+} /CaM-sensitive AC in *Drosophila* (Dudai and Zvi 1984; Livingstone et al. 1984; Livingstone 1985; Levin et al. 1992) or mice (Wu et al. 1995) is associated with learning deficits.

Evidence that Ca^{2+} /CaM-sensitive AC has an associative function comes from cellular studies of associative synaptic plasticity in *Aplysia*. During conditioning of the defensive gill and siphon withdrawal reflex of *Aplysia*, there is an associative enhancement of the synaptic connections from the siphon sensory neurons, which mediate the input from the conditioned stimulus (CS), to interneurons and motoneurons that produce the withdrawal response. During this associative facilitation of the sensory neuron synapses, activity of the sensory neurons, which is triggered by the CS, is temporally paired with facilitatory transmitter, which is released by the unconditioned stimulus (US) (Hawkins et al. 1983; Walters and Byrne 1983; Abrams 1985). One important site of interaction between sensory neuron activity and facilitatory transmitter is the Ca^{2+} /CaM-sensitive AC. When Ca^{2+} influx, which accompanies sensory neuron activity, is paired with facilitatory transmitter, there is a greater increase in cAMP levels than that produced by facilitatory transmitter alone (Ocorr et al. 1985). Elevations in cAMP within the sensory neurons initiate both short-term and long-term facilitation of their synaptic connections (Brunelli et al. 1976; Braha et al. 1990; Dash et al. 1990; Goldsmith and Abrams 1991, 1992; Ghirardi et al. 1992; Hochner and Kandel 1992; Klein 1993). Other cAMP-independent processes, including Hebbian plasticity, may also contribute to associative enhancement of transmitter release at these synapses (Murphy and Glanzman 1996, 1997); however, Abrams and Galun (T.W. Abrams and J.E. Galun, unpubl.) and Bao et al. (1998) have found that pre-synaptic cAMP-dependent phosphorylation plays a necessary role in associative synaptic facilitation.

Generally, in classical conditioning, an animal learns to associate the CS and US when the two

stimuli are temporally paired during training so that the CS predicts the occurrence of the US. Most forms of conditioning show a sequence requirement in which the animal only learns the associative relationship if the CS begins before the US (Hearst 1988; Rescorla 1988). Conditioning of the defensive withdrawal reflex in *Aplysia* exhibits a number of features typical of mammalian conditioning paradigms, including the CS-US sequence requirement (Hawkins et al. 1986; Colwill et al. 1988). Activity-dependent facilitation displays a parallel sensitivity to the sequence of paired cellular events; optimal facilitation occurs when activity and Ca^{2+} influx, triggered by the CS, precedes facilitatory transmitter, which is released by the US (Clark et al. 1994).

If the Ca^{2+} /CaM-sensitive AC serves as a coincidence detector during classical conditioning, one would expect that activation of this enzyme might also show an analogous sequence requirement. Yovell and Abrams (1992) explored whether activation of the Ca^{2+} /CaM-sensitive AC is affected by the order in which paired stimuli arrive. They found that although this enzyme is activated by both Ca^{2+} and facilitatory transmitter, the dual activation is not synergistic under normal steady-state assay conditions, where the two activating stimuli are present for the duration of the assay. However, perfused membrane experiments, in which the neural membranes are retained on a filter and exposed transiently to activating stimuli, revealed that AC shows the predicted sequence preference analogous to the sequence requirements of the conditioning and the associative synaptic plasticity: Ca^{2+} preceding transmitter gave more powerful activation than Ca^{2+} following transmitter or than transmitter without Ca^{2+} (Yovell and Abrams 1992). Although the mechanism of this sequence effect was not definitively identified, comparisons of the rising phase of the activation curve suggested that a prepulse of Ca^{2+} may increase the rate of activation by transmitter. In *Aplysia* neural membranes, as in most other systems, neurotransmitter receptors stimulate AC via a guanine nucleotide-binding stimulatory G protein, G_s (Vogel 1989; Abrams et al. 1991). Because we knew that Ca^{2+} /CaM binds to AC (Yeager et al. 1985; Eliot et al. 1989), we hypothesized that this binding might induce a conformational change in AC that accelerates activation of AC by receptor- G_s .

In the present study we have identified two additional characteristics of activation of *Aplysia* neural AC by Ca^{2+} and 5-hydroxytryptamine (5-HT)

that would contribute to the observed sequence preference. Comparison of the time courses of AC activation by transient 5-HT and Ca^{2+} stimuli revealed that activation by a Ca^{2+} pulse exhibits a small delay, such that interactions between the two stimulus pulses should be temporally coincident if the Ca^{2+} pulse begins first. Second, we observed that a transient Ca^{2+} stimulus that follows a 5-HT stimulus, as occurs with backward pairing, actually accelerates the decay of AC stimulation by transmitter; thus, a late pulse of Ca^{2+} has an inhibitory effect on overall AC activation.

Materials and Methods

PREPARATION OF MEMBRANES

Aplysia californica, weighing 100–200 grams, were obtained from Alacritty (Redondo Beach, CA) or Marinus, Inc. (Long Beach, CA). For each experiment, six animals were anesthetized by injection of isotonic MgCl_2 , and abdominal, cerebral, and pleural-pedal ganglia were removed. The ganglia were trimmed to reduce non-neural tissue (i.e., sheath) and then homogenized in a glass-glass homogenizer in ~0.4 ml per CNS of homogenization buffer [50 mM K-HEPES at pH 7.6, 75 mM KCl, 3 mM EGTA, 1 mM dithiothreitol (DTT), and protease inhibitors (1 mM benzamidine, 10 $\mu\text{g}/\text{ml}$ of leupeptin, 10 $\mu\text{g}/\text{ml}$ of aprotinin, and 25 μM p-nitrophenyl-p'-guanidinobenzoate hydrochloride)]. Any residual pieces of sheath were removed, and the material was rehomogenized in a glass-Teflon homogenizer. The homogenate was diluted to 5 ml with homogenization buffer and centrifuged at 1000g for 2 min to remove any particulate material. All processing of membranes was at 0–4°C.

PERFUSED MEMBRANE AC ASSAYS

Continuous assay of AC activity stimulated with transient exposures to 5-HT and Ca^{2+} was done using the perfused membrane method of Yovell et al. (1987), as modified by Yovell and Abrams (1992), in which neural membranes are retained on a low-density fibrous filter and perfused with assay solution containing [α - ^{32}P]ATP. Upstream from the filter, a valve with five input ports gated by computer-controlled solenoids (General Valve no. 9-453-900, internal volume of 6 μl) enabled switching of the incoming solution from among three assay solutions: (1) low Ca^{2+} , (2)

high Ca^{2+} , and (3) low Ca^{2+} with 20 μM 5-HT. A fourth input port enabled washing with 6 ml of unlabeled low Ca^{2+} assay solution immediately before the start of an assay to equilibrate membranes and to remove any residual cytosol or free membranes. Assay solution was drawn at 2.5 ml/min by a peristaltic pump downstream from the filter and collected in 6-sec fractions in tubes containing 100 μl of assay stop solution (Salomon 1979). Assay solutions contained 50 mM K-HEPES (pH 7.6), 75 mM KCl, 1 mM DTT, 10 μM [α - ^{32}P]ATP (~25 $\mu\text{Ci}/\text{ml}$), 50 μM [^3H]cAMP (5×10^4 cpm/ml, for normalization of column recovery and fraction volume), 1 μM CaM, 10 μM GTP (unless otherwise noted), protease inhibitors (described above), and one of two $\text{Ca}^{2+}/\text{Mg}^{2+}/\text{EGTA}$ buffers. EGTA was 3.0 mM in both low and high Ca^{2+} assay solutions; total Ca^{2+} was 25 μM in the low Ca^{2+} assay solution and 4.5 mM in the high Ca^{2+} assay solution (i.e., the Ca^{2+} pulse solution); and total Mg^{2+} was 1.45 mM in the low Ca^{2+} solution and 1.0 mM in the high Ca^{2+} solution. Free Mg^{2+} was 1 mM in both solutions. Free Ca^{2+} in the low Ca^{2+} solution was <1 nM. During a 6-sec “pulse,” the maximum solution change is ~80%; thus, free Ca^{2+} transiently reached ~600 μM ; during a 6-sec pulse of 5-HT, the peak 5-HT concentration reached ~16 μM (Fig. 1A). In some experiments, 0.5 mM IBMX was included in the assay solution. However, the phosphodiesterase inhibitor did not affect cAMP recovery; this may reflect either effective protection of synthesized cAMP by the 50 μM cAMP present in the assay solution or the low level of phosphodiesterase remaining in the membranes.

In each experiment, 25% of the homogenate from six CNSs was injected onto a prewet, 10-mm-diam., borosilicate glass filter (Millipore #AP25); a separate filter was used for each of four 2-min assays. Cytosol was immediately removed by high-pressure washing with 20 ml of ice-cold buffer before connecting the filter to the valve. All comparisons, either of Ca^{2+} -before-5-HT pairing versus 5-HT-before- Ca^{2+} pairing or of decay of AC stimulation with and without a late Ca^{2+} pulse, were made within preparations; in each preparation, the two conditions were both assayed in duplicate in a counterbalanced A-B-B-A sequence. Because the magnitude of stimulation by 5-HT and Ca^{2+} pulses varies among preparations, it was critical to make all comparisons within experiments. AC stimulation by Ca^{2+} alone that is shown in Figures 4 and 5 (below) was assayed with and without GTP in the same preparations.

AC ACTIVATION BY TRANSIENT Ca^{2+} AND 5-HT STIMULI

Figure 1: Responses of AC in perfused *Aplysia* neural membranes to forward or backward pairing of Ca^{2+} and 5-HT pulses. (A) Profiles of 5-HT and Ca^{2+} pulses in perfused membrane experiments. Concentration of 5-HT in the neural membranes during and after a 6-sec 5-HT pulse (■) was calculated from measurements of the time course of solution change (which was determined with a radioactivity detector flow cell immediately downstream from the filter and ^{32}P in the stimulus solution). Free Ca^{2+} concentration during and after a 6-sec Ca^{2+} pulse (●) calculated from measurements of the time course of solution change. The narrower Ca^{2+} stimulus profile is a consequence of Ca^{2+} chelation by the EGTA in the low Ca^{2+} assay solution. Note that 12 sec after the termination of perfusion with 5-HT, free 5-HT at the membranes had dropped below 100 nM, a level that would not have produced measurable AC stimulation in these assays. (B) Temporal relationship of Ca^{2+} and 5-HT stimuli when paired in forward and backward sequences. Profiles of Ca^{2+} (dark stippled area) and 5-HT (light stippled area) are from A. With forward pairing, a 6-sec Ca^{2+} pulse was given immediately before a 6-sec 5-HT pulse; with backward pairing, the 6-sec Ca^{2+} pulse was given immediately following the 6-sec 5-HT pulse. Thus, in both sequences when the valve controlling one stimulus solution was switched off, the valve controlling the second stimulus solution was switched on. The overlapping temporal profiles of the two stimuli result from the fact that ligand concentration increased and decreased gradually within the filter chamber. (C) AC stimulation with paired Ca^{2+} and 5-HT pulses in forward (Ca^{2+} followed by 5-HT) and backward (5-HT followed by Ca^{2+}) sequences. Peak AC activity during response to paired Ca^{2+} and 5-HT pulses is expressed as percent of basal AC activity. AC activity was determined by collecting perfusate during consecutive 6-sec intervals and determining ^{32}P -labeled cAMP synthesized from $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. The difference between peak stimulation with forward and backward pairing was highly consistent and significant within preparations ($P < 0.001$, two-tailed paired t -test; $n = 6$); moreover, the profiles of stimulation over time were significantly different $F(9,45) = 17.2$, $P < 0.001$). (Because both basal activity and AC stimulation vary substantially among preparations, all comparisons are made within preparations. Each stimulus condition in this and subsequent figures was assayed in duplicate on each preparation.)

ESTIMATES OF TIME COURSE OF EXPOSURE TO 5-HT AND Ca^{2+}

Although the valve had an internal volume of $<6 \mu\text{l}$ and produced a relatively square pulse of Ca^{2+} or 5-HT, the filter itself contained $\sim 100 \mu\text{l}$ of solution; thus, with the flow rate used, the ligand concentration increased and decreased gradually at the membranes. To determine the rate of solution change, the ratio of two solutions during and after a pulse was measured with radioactivity detector flow cell immediately downstream from the filter

chamber and ^{32}P in one of the solutions. The 5-HT concentration during and after a pulse of transmitter is directly proportional to the ratio of assay solutions with and without transmitter at the membranes (Fig. 1A). The free Ca^{2+} concentration during and after a Ca^{2+} pulse (Fig. 1A) was calculated from the rate of solution change data and the concentrations of EGTA, Ca^{2+} and Mg^{2+} in the high and low Ca^{2+} solutions, by using an iterative computer program and published association constants for Ca^{2+} -EGTA, Mg^{2+} -EGTA, and H^{+} -EGTA (Blinks et al. 1982).

Figure 2: Ca^{2+} and 5-HT produce less than additive stimulation of AC. AC activity was assayed in *Aplysia* neural membranes under steady-state conditions with a range of concentrations of 5-HT, from 100 nM to 100 μM , in either 10 nM or 1.5 μM free Ca^{2+} with 1 μM CaM. (A) AC activity expressed as percent of AC basal activity [(○) with 5-HT; (●) with 5-HT + Ca^{2+}]. (B) Ca^{2+} stimulation vs. 5-HT concentration. Ca^{2+} stimulation is expressed as either percent of basal AC activity as in A (■) or as percent of AC activity in low Ca^{2+} with the same concentration of 5-HT (▲). Note, as 5-HT stimulation of AC increases, net Ca^{2+} stimulation decreases. As suggested in Discussion, this reduction in Ca^{2+} stimulation may actually represent a decrease in GTP stimulation caused by Ca^{2+} /CaM.

DATA ANALYSIS

Statistical analysis of peak AC stimulation or of total cAMP synthesis was done with within-preparation comparisons and a paired *t*-test. Comparisons of profiles of AC stimulation over time were done with a repeated measures ANOVA with 2 within-preparation factors (treatment and time) testing treatment $\times$ time interactions. Rate constants for decay of AC stimulation were estimated by fitting exponential functions to the falling phase of data with Igor software (Wavemetrics, Lake Oswego, OR). All data are presented as mean $\pm$ S.E.M.

Results

FORWARD VS. BACKWARD AC STIMULATION WITH BRIEFER 5-HT AND Ca^{2+} STIMULI

To better study the effect of Ca^{2+} /CaM on 5-HT activation of AC by transient transmitter stimuli, we improved the flow characteristics of the chamber that houses the filter with the neural membranes and reduced the diameter of the filter; the 5-HT pulse now terminated more rapidly, without the long tail present in previous experiments (Fig. 1A) (Yovell and Abrams 1992). As in the previous study (Yovell and Abrams 1992), activation of AC in perfused neural membranes was substantially greater when a Ca^{2+} pulse immediately preceded the 5-HT pulse (forward pairing) than when Ca^{2+} immediately followed the transmitter pulse (backward pairing) (Fig. 1C) (peak stimulation above basal = $263 \pm 32\%$ vs. $129 \pm 3\%$, $P < 0.001$; these and all other data are expressed as mean $\pm$ S.E.M.). Because of differences of several

fold in both basal activity and amount of transmitter stimulation among groups of animals, all comparisons are made within experiments (see Materials and Methods). In the present experiments, because the improved rate of solution change, the Ca^{2+} pulse reached a substantially higher peak level ($\sim 600 \mu\text{M}$) than in previous experiments (0.3–10 μM) (Yovell and Abrams 1992). Thus, the sequence preference of AC for Ca^{2+} preceding 5-HT is relatively insensitive to the precise peak level of free Ca^{2+} during a pulse.

LACK OF SYNERGISTIC INTERACTIONS BETWEEN Ca^{2+} AND TRANSMITTER IN ACTIVATING AC WHEN EXPOSURES TO THE TWO STIMULI ARE CONTINUOUS OR SIMULTANEOUS

Yovell and Abrams (1992) had reported a lack of synergism when Ca^{2+} /CaM and 5-HT are paired in steady-state assays in which both stimuli are present for the duration of the assay. In experiments in which a range of 5-HT concentrations were tested with or without stimulatory Ca^{2+} , we now observed that at maximally effective concentrations of 5-HT, the stimulatory effect of Ca^{2+} /CaM actually disappeared. Thus, 30 μM 5-HT plus Ca^{2+} produced no greater activation of *Aplysia* neural AC than 30 μM 5-HT alone (Fig. 2A). Indeed, absolute stimulation by Ca^{2+} decreased in a graded manner as the concentration of 5-HT increased (Fig. 2B). [Less-than-additive stimulation by 5-HT plus Ca^{2+} was present in the earlier results of Yovell and Abrams (1992) although it was not considered to be a real effect because of limited data.] This lack of enhancement of 5-HT stimulation of AC was also observed when transient Ca^{2+} and 5-HT stimuli

were given simultaneously or with substantial overlap; for example, in one series of experiments, simultaneous onset did not produce significantly different peak AC stimulation as compared with backward pairing [$415 \pm 28\%$ vs. $392 \pm 29\%$, peak stimulation as percent of basal, $n = 6$ (L. Lee, unpubl.)].

THE RESPONSE OF *APLYSIA* NEURAL AC TO A Ca^{2+} TRANSIENT IS DELAYED, OCCURRING AFTER Ca^{2+} HAS DECLINED

One reason that simultaneous transient Ca^{2+} and 5-HT stimuli may not show effective synergism is that the response to a Ca^{2+} pulse is actually delayed. Figure 3 compares the time course of the responses to separate 6-sec Ca^{2+} and 5-HT pulses.

Figure 3: Time course of activation of *Aplysia* neural AC by a 6-sec pulse of either Ca^{2+} or 5-HT. To allow comparison of the time courses of the responses, different vertical axes were used for the two stimuli; thus, stimulation with the Ca^{2+} pulse ($\square$) was much smaller than with the 5-HT pulse ($\triangle$): 1.54 ± 0.17 -fold ($n = 6$) vs. 6.01 ± 0.56 -fold ($n = 3$). At its peak the Ca^{2+} pulse (dark stippled area) reached $600 \mu\text{M}$, and the 5-HT pulse (light stippled area) reached $16 \mu\text{M}$. To facilitate comparison of the rising phase of the two responses, we have aligned the two sets of data so that the effective onsets of the two stimuli coincide in the graph (in contrast, in Fig. 1A, it is evident that after the beginning of a pulse, Ca^{2+} actually rises more slowly than 5-HT). (Because the magnitude of 5-HT and Ca^{2+} stimulation varies among preparations, the level of peak activation should not be compared across experiments.)

Because there is no reliable stimulation by a Ca^{2+} pulse in the presence of GTP, the Ca^{2+} responses were measured in the absence of GTP. Under these conditions, the 5-HT stimulation rose earlier than the Ca^{2+} stimulation. Because the 6-sec fraction duration, over which cAMP synthesis is integrated, limits the temporal resolution of these experiments, it is not possible to determine precisely the delay between the peaks of the responses. However, given that the interval in which stimulation first appeared was two fractions earlier with 5-HT than with Ca^{2+} (Fig. 3) and given the difference between the rising phases of the stimuli in Figure 1A, there must be a temporal shift of at least 2.7 sec in the onset of the two responses. The peaks of the two responses appear to be shifted by ~ 1.9 sec (Fig. 3); this estimate, though, has substantial uncertainty because of the fraction size. In another series of experiments, we studied the response to steps of Ca^{2+} and 5-HT. The onset of stimulation by the 5-HT step occurred significantly earlier than the initial stimulation by the Ca^{2+} step ($P < 0.01$, $n = 9$, two-tailed t -test). A comparison of the rising phase of the responses to these steps suggested that 50% activation with 5-HT occurred ~ 1.0 sec earlier than 50% activation by Ca^{2+} (data not shown). In summary, these results indicate that 5-HT stimulation rises faster than Ca^{2+} /CaM stimulation of AC. Although little or no Ca^{2+} stimulation is detectable in the presence of GTP, we suggest that the relevant modulatory effect of Ca^{2+} /CaM on the activation of AC by receptor- G_s (which occurs in the presence of GTP) is likely to occur with the same time course as the Ca^{2+} /CaM stimulation (characterized here in the absence of GTP) (see Discussion).

A LATE Ca^{2+} PULSE ACCELERATES THE DECAY OF AC ACTIVATION

On the basis of several lines of evidence, Yovell and Abrams (1992) proposed that a prepulse of Ca^{2+} may accelerate the rate of AC activation by a pulse of 5-HT. However, direct determination of the effect of Ca^{2+} on the rate of AC activation proved difficult, primarily because the time course of activation was rapid compared with the temporal resolution of the perfused membrane assay (Yovell and Abrams 1992). If elevated Ca^{2+} increases the rate of receptor- G_s activation of AC, we hypothesized that it might also accelerate the decay of AC activation when the transmitter pulse

ends. Consistent with this possibility, the difference between pairing with Ca^{2+} -before-5-HT and pairing with 5-HT-before- Ca^{2+} tended to be greater than the difference between pairing with Ca^{2+} -before-5-HT and a 5-HT pulse alone (Yovell and Abrams 1992). To independently test the hypothesis that Ca^{2+} /CaM binding influences the rate of receptor- G_s interactions with AC, we examined the effects of a late Ca^{2+} pulse on the decay phase of AC stimulation by a 5-HT pulse. Because the decay phase of 5-HT activation is substantially slower than the rising phase, it is possible to more accurately determine changes in the decay of activation.

To separate effects of Ca^{2+} on peak AC activation from effects on the decay of activation, the Ca^{2+} pulse was delivered after 5-HT available to the membranes had decreased below the threshold concentration for stimulating AC (Figs. 1A, 2, and 4). The Ca^{2+} exposure began ~4 sec after 5-HT

dropped below 100 nM, which was <0.6% of the peak concentration during the transmitter pulse; 100 nM 5-HT is the minimal concentration for detectable stimulation of AC in *Aplysia* neural membranes (Jarrard et al. 1993; Fig. 2) and one order of magnitude lower than the EC_{50} for the AC-coupled 5-HT receptor (J.E. Cohen and T.W. Abrams, unpubl.). The decay of 5-HT stimulation could be measured more accurately than previously possible because the reduced volume of the valve and filter; we were now able to observe some persistent AC activation after 5-HT elimination, which had not been detected by Yovell et al. (1987). In the absence of a late Ca^{2+} pulse, AC activation decayed to basal levels ~1 min after free 5-HT was washed from the membranes. Consistent with the prediction that Ca^{2+} would accelerate the decay of AC activation by receptor- G_s , we found that the decay of 5-HT stimulation was substantially faster when a pulse of Ca^{2+} was given after 5-HT washout (Fig.

Figure 4: Effect of a late Ca^{2+} pulse on the decay of AC stimulation after 5-HT exposure. (A) A 6-sec 5-HT pulse was delivered either alone ($\square$) or followed by a Ca^{2+} pulse ($\blacksquare$). The profiles below show timing of 5-HT (light stippled area) and Ca^{2+} (dark stippled area) exposures. Perfusion with the high Ca^{2+} assay solution began 12 sec after the 5-HT pulse was terminated; the Ca^{2+} concentration rose into a stimulatory range ~4 sec later. Ca^{2+} alone ($\circ$) is the response to the same Ca^{2+} pulse (in the presence of GTP) without prior exposure to 5-HT ($n = 6$). AC activity during each 6-sec interval is expressed as percent of basal activity. Note that AC activity throughout the decaying phase of 5-HT stimulation was significantly decreased by the brief Ca^{2+} pulse $F(8,40) = 10.6$, $P < 0.001$. Whereas Ca^{2+} alone has a transient inhibitory effect, this reduction in activity lasted only during the Ca^{2+} pulse and therefore cannot directly account for the sustained reduction in AC activity produced by the Ca^{2+} pulse after a 5-HT exposure. 5-HT and Ca^{2+} buffer concentrations were as in Fig. 1. [Error bars (S.E.M.) are frequently smaller than symbols.] (B) Total stimulation of cAMP synthesis after washout of 5-HT, with and without a late Ca^{2+} pulse. To emphasize the overall effect of the late Ca^{2+} pulse on cAMP synthesis during the decay phase of stimulation, the total stimulated synthesis of cAMP (minus basal synthesis) is plotted from the time of onset of the Ca^{2+} pulse. Data from experiments in A.

4A). In the first 6 sec after 5-HT was eliminated, AC stimulation declined $49.6 \pm 1.6\%$ when a late Ca^{2+} pulse was given as compared with a decrease of $35.8 \pm 1.8\%$ without a Ca^{2+} pulse (the overall decay phase of the stimulation was significantly altered by the Ca^{2+} pulse; $F(8,40) = 10.6$, $P < 0.001$, $n = 6$; repeated measures ANOVA testing the treatment $\times$ time interaction). In the two 6-sec fractions during which the Ca^{2+} elevation occurred, the rate constant for decay of AC stimulation increased significantly (from $0.098 \pm 0.004/\text{sec}$ with 5-HT alone to $0.170 \pm 0.007/\text{sec}$ with a late Ca^{2+} pulse; $P < 0.002$, two-tailed paired t -test). This effect on the decay rate of AC activation was transient; by the first 6-sec fraction after free Ca^{2+} had declined, the decay rate was no longer increased. Although the acceleration of the decay rate occurs only during the Ca^{2+} pulse, its consequences are longer lasting. The decrease in AC activity with a late Ca^{2+} pulse compared with 5-HT alone remained significant until the activity had decayed to near basal levels, that is, >30 sec (Fig. 4A). Thus, Ca^{2+} appears to accelerate the decay of AC activation by 5-HT, rather than producing transient inhibition. In the absence of GTP, this same Ca^{2+} pulse was stimulatory, producing peak stimulation of $54 \pm 17\%$ above basal (Fig. 3). In contrast, in the presence of GTP, the Ca^{2+} pulse alone produced a transient decrease in AC activity (Fig. 4, Ca^{2+} ALONE); this brief effect cannot account quantitatively for the long-duration effect on the decaying phase of 5-HT stimulation, which is evident well after Ca^{2+} disappears. (The reason for the switch in the direction of the transient Ca^{2+} effect when GTP is present is considered in Discussion.)

Yovell and Abrams (1992) had found that the difference between forward versus backward pairing could be reduced or eliminated by omitting exogenous CaM. [It is often not possible to entirely eliminate CaM-mediated effects by omitting exogenous CaM because neural membranes contain appreciable endogenous CaM that is difficult to remove completely (Eliot et al. 1989).] In two experiments, we examined the effect of Ca^{2+} on the decay phase of 5-HT stimulation in the absence of exogenous CaM. Although the late Ca^{2+} pulse still accelerated the decay of 5-HT stimulation, the decay rate during the Ca^{2+} pulse (mean rate constant = $0.118 \pm 0.014/\text{sec}$) was significantly less than in the presence of exogenous CaM ($P < 0.05$, two-tailed t -test), suggesting that the acceleration in decay rate is mediated via CaM.

Although compared with the peak of 5-HT

stimulation, this effect of a brief Ca^{2+} pulse on AC activity during the prolonged decaying phase of stimulation appears modest (Fig. 4A), it could well be physiologically significant. In Figure 4B, we compare the integral of total stimulated cAMP synthesis over the 54-sec period in the decaying phase from the time the Ca^{2+} exposure occurs; the brief Ca^{2+} pulse resulted in a 1.96-fold reduction in total stimulated cAMP synthesis during this late phase of the response.

EFFECT OF THE Ca^{2+} PULSE ON G_s -INDEPENDENT ACTIVATION OF AC BY FORSKOLIN

If the acceleration by Ca^{2+} of the decay of AC activation is due to modulation of receptor- G_s interactions with AC, there should not be a Ca^{2+} -dependent increase in the rate of decay of activation of AC with compounds such as forskolin (Seamon and Daly 1986) that stimulate AC directly, rather than via G_s . To test this prediction, we substituted for 5-HT a pulse of the water-soluble forskolin analog deacetyl-*N*-methyl-piperazino-butyryl forskolin (dampb-Forsk). A Ca^{2+} pulse delivered after dampb-Forsk washout did not accelerate the decay of AC activation but instead produced synergistic stimulation of AC, compared with a Ca^{2+} pulse alone (in the absence of GTP) (Fig. 5). These assays with dampb-Forsk were conducted in the absence of GTP to allow direct comparison with the stimulation that can be measured by a Ca^{2+} pulse in the absence of GTP. We also conducted similar experiments with a dampb-Forsk pulse in the presence of GTP. Although the stimulation (as a percent of basal activity) by dampb-Forsk was substantially less than in the absence of GTP, the stimulation profiles were qualitatively the same: A peak of Ca^{2+} stimulation sat superimposed on the falling phase of dampb-Forsk stimulation, and the two stimuli activated AC synergistically (data not shown).

Discussion

In a number of experimental systems, associative interactions between stimuli within the CNS have been found to occur at the level of dually regulated molecules (Bourne and Nicoll 1993). In *Aplysia* sensory neurons, the dually regulated Ca^{2+} /CaM-sensitive AC provides one site of associative convergence between Ca^{2+} influx and facili-

Figure 5: Effect of a late Ca^{2+} pulse on the decay of AC stimulation after dampb–Forsk exposure. (A) A 6-sec pulse of $100\ \mu\text{M}$ dampb–Forsk was delivered either alone ($\square$) or followed by a Ca^{2+} pulse ($\blacksquare$) ($n = 4$). Perfusion with the high Ca^{2+} assay solution began 18 sec after the dampb–Forsk pulse was terminated. Ca^{2+} alone ($\circ$) is the response to this same Ca^{2+} pulse, in assays without GTP, plotted on the same scale axes (data are from Fig. 3) ($n = 6$). To enable comparison, the Ca^{2+} alone graph is shifted vertically so that basal activity (100%) aligns with the dampb–Forsk alone response at the time of the Ca^{2+} pulse (indicated by § and the broken line). Note that the late Ca^{2+} pulse during the decaying phase of the forskolin response did not accelerate the decay but rather activated AC synergistically. (It is difficult to compare stimulation across experiments; however, the Ca^{2+} stimulation during the forskolin response was 162% of basal, calculated by subtracting the activity at the same time point in the forskolin response without Ca^{2+} , which is more than twofold greater than has been seen with Ca^{2+} pulses alone.) A longer delay was used between the dampb–Forsk and Ca^{2+} pulses than with the 5-HT pulse (Fig. 4) because the forskolin response decays more slowly than the 5-HT response. Data for dampb–Forsk and dampb–Forsk plus late Ca^{2+} are from experiments on four preparations; data for Ca^{2+} alone without GTP were obtained in the same six preparations in which responses to Ca^{2+} pulses alone with GTP were studied (shown in Fig. 4). No GTP was present in the dampb–Forsk experiment assay solutions. [Error bars (S.E.M.) may be smaller than symbols.]

tatory transmitter. During conditioning of the defensive withdrawal response, these two cellular stimuli are triggered by the CS and US, respectively. Consistent with the proposal that convergence at this molecular site plays an important role in initiating neural plasticity during conditioning,

Aplysia neural AC shows a sequence preference analogous to the CS–US sequence requirement of conditioning: Ca^{2+} preceding transmitter produces more powerful activation than Ca^{2+} following transmitter (Fig. 1; Yovell and Abrams 1992).

The difference between forward and backward pairing of Ca^{2+} and 5-HT has now been observed with the peak Ca^{2+} concentration during the pulse ranging from $<1\ \mu\text{M}$ to $600\ \mu\text{M}$ (Fig. 1; Yovell and Abrams 1992). Thus, the sequence preference of AC occurs over a wide range of peak Ca^{2+} levels, though the forward versus backward difference was somewhat greater with larger Ca^{2+} pulses. This sensitivity to Ca^{2+} is in part a reflection of the Ca^{2+} affinity of CaM, in the micromolar range (Maune et al. 1992); however, because CaM concentrations are relatively high, both in these assays and in the cell, only a small percentage of CaM needs to bind Ca^{2+} for AC to be modulated (J.E. Galun, C.U. Onyike, and T.W. Abrams, in prep.; Tang et al. 1991). In neurons, Ca^{2+} levels at regions of influx can very briefly approach $1\ \text{mM}$ during an action potential (Adler et al. 1991; Roberts et al. 1991; Llinas et al. 1992; Heidelberger et al. 1994); in contrast, shortly after a series of action potentials, Ca^{2+} levels remain elevated in the range of hundreds of nM (e.g., Blumenfeld et al. 1990). Our data suggest that forward pairing would produce more effective AC activation than backward pairing over a broad range of increases in free Ca^{2+} levels beneath the membrane of neurons, both during and shortly after a train of action potentials.

ACCELERATION OF THE DECAY OF TRANSMITTER STIMULATION BY A LATE Ca^{2+} PULSE IS OWING TO MODULATION OF AC ACTIVATION BY G_s , RATHER THAN TO A DIRECT INHIBITORY EFFECT OF Ca^{2+}

Yovell and Abrams (1992) observed that forward pairing increased the initial activation of AC by transmitter compared with both 5-HT alone and backward pairing. They proposed that CaM binding to AC might accelerate the rate of AC activation by receptor- G_s , which would have the consequence of increasing the peak AC stimulation achieved when transmitter is present only briefly. We further hypothesized that CaM binding might increase the rates of transition in both directions between the activated and deactivated forms of the G_s –AC complex. Thus, if Ca^{2+} /CaM were bound to AC when a 5-HT exposure terminated, the rate of

decay of activated AC might be accelerated. We tested this prediction and observed that a Ca^{2+} pulse arriving after 5-HT washout produced a decrease in AC activity during the period in which activation persisted (Fig. 4).

It is important to distinguish whether this decrease in AC activity represents an acceleration of the decay of 5-HT stimulation or simply Ca^{2+} inhibition of AC. Ca^{2+} has a biphasic effect on CaM-sensitive AC; intermediate levels of Ca^{2+} stimulate AC activity, via CaM, and high levels of Ca^{2+} directly inhibit AC, independent of CaM (Brostrom et al. 1977; Yovell et al. 1992; Wu et al. 1993). Several observations suggest that the difference in the decay phase because of a late Ca^{2+} pulse does not result from direct inhibition of AC: (1) When the same Ca^{2+} pulse was delivered in the absence of GTP and transmitter, stimulation was observed, rather than inhibition (Fig. 3); thus, the Ca^{2+} pulse used must not have been large enough to produce net inhibition of AC. (2) When a late Ca^{2+} pulse was given following a pulse of dampb-Forsk (which, unlike transmitter, directly activates AC, independently of G_s), Ca^{2+} stimulation interacted synergistically with the decaying phase of the dampb-Forsk response, with no evidence of inhibition. (3) Typically, when CaM is absent and a Ca^{2+} pulse produces direct AC inhibition, the effect is transient, occurring in only one or two 6-sec fractions (J.E. Galun, C.U. Onyike, and T.W. Abrams, in prep.). In contrast, the late Ca^{2+} pulse reduced AC activity for as long as the 5-HT stimulation persisted. Thus, this Ca^{2+} effect appears to decrease the residual 5-HT stimulation after the transmitter has been removed from the membranes and is no longer available to reactivate the enzyme. In summary, these results suggest that a late increase in Ca^{2+} after the disappearance of 5-HT accelerates the loss of the residual AC activation.

If the Ca^{2+} pulse does not have a direct inhibitory effect on AC, why in the presence of GTP did this same pulse result in a transient decrease in AC activity (Fig. 4, Ca^{2+} ALONE)? In *Aplysia* neural membranes, exposure to GTP alone, in the absence of transmitter, commonly produces a three- to fourfold increase in AC activity, presumably by limited activation of G_s . The transient inhibition of GTP-activated AC by the Ca^{2+} pulse may be related to the less-than-additive AC stimulation by Ca^{2+} plus 5-HT that we observed under steady-state conditions (Fig. 2). We propose that in these steady-state assays, rather than transmitter and G_s block-

ing Ca^{2+} stimulation, Ca^{2+} /CaM may be producing a modest decrease in the effective activation of AC by G_s . Consistent with this interpretation, when *Aplysia* AC has been preactivated with 5-HT with the GTP analog GTP γ S, which eliminates GTP hydrolysis and G_s dissociation from AC, there is no decrease in the activation by Ca^{2+} /CaM (Abrams et al. 1991). In the case of the Ca^{2+} pulse in the presence of GTP, Ca^{2+} /CaM may also be acting to decrease AC stimulation by G_s ; because GTP is present continuously, after the Ca^{2+} pulse has ended G_s -GTP is able to reactivate AC, restoring the original level of activated AC (Fig. 4, Ca^{2+} ALONE).

A possible mechanism for this proposed Ca^{2+} /CaM-induced decrease in AC activation by G_s emerges from the hypothesis that CaM alters the rates of transition of the G_s -AC complex between the activated and deactivated states. Although the evidence suggests that Ca^{2+} /CaM binding increases the rates in both directions, there may be a slightly greater increase in the rate of deactivation. Thus, in the case of both steady-state assays with 5-HT and GTP, and perfused membrane assays with GTP present continuously, Ca^{2+} /CaM may shift the equilibrium slightly, decreasing the fraction of AC molecules in the activated state. Under steady-state conditions with all stimuli constant, such effects of Ca^{2+} /CaM on the net activation of AC are fairly modest. On the other hand, if the amount of activated G_s is increasing or decreasing (i.e., when transmitter arrives or disappears), a Ca^{2+} pulse appears to have a relatively dramatic effect on the rate at which AC activity changes.

CONSEQUENCES OF THE EFFECT OF LATE Ca^{2+} FOR ASSOCIATIVE INTEGRATION OF PAIRED STIMULI

Although the effects of the late Ca^{2+} pulse on total AC stimulation may appear modest (Fig. 4), they could well be physiologically significant. When activity in *Aplysia* sensory neurons is paired with facilitatory input, backward pairing produces significant facilitation of synaptic transmission from these cells, although forward pairing is ~75% more effective (Clark et al. 1994). Thus, the differences in AC activation between forward and backward pairing (Fig. 1; Yovell and Abrams 1992) may be sufficient to account for the sequence dependence of synaptic facilitation. The accelerated decay of AC activation with backward pairing (Fig. 4) could contribute substantially to a difference in to-

tal cAMP synthesis and, therefore, to the difference in synaptic facilitation between forward and backward pairing.

It is appealing that a single molecular mechanism, a change in the rates of activation and deactivation of G_s -stimulated AC, could both enhance AC stimulation with forward pairing and reduce stimulation with backward pairing; both of these effects would together contribute to a sequence preference where forward pairing is substantially more effective than backward. One expectation would be that behavioral training with backward pairing would be less effective in causing enhancement of subsequent responses to a CS than completely unpaired presentations of the US and CS. Indeed, studies of vertebrate conditioning paradigms have shown that backward presentation of a CS, which at other times is presented before the US, interferes with the animal's learning to recognize this CS as a predictor of the US (Kaplan 1984; Ewing et al. 1985). In the conditioning of the defensive withdrawal reflex of *Aplysia*, there is also a small (though not statistically significant) reduction in the response to a backward paired CS compared with an unpaired CS (Hawkins et al. 1986).

DELAYED Ca^{2+} RESPONSE MAY MAKE SIMULTANEOUS STIMULUS ONSET RELATIVELY INEFFECTIVE

In most forms of classical conditioning, simultaneous stimulus onset is substantially less effective than forward, CS-US pairing. For example, in conditioning of the rabbit eye-blink reflex, with simultaneous presentations of a tone and an air puff, the animal does not learn to blink to the tone (Smith et al. 1969). Similarly, simultaneous CS and US onset is relatively ineffective in the conditioning of the defensive withdrawal reflex of *Aplysia*. How might the dually regulated Ca^{2+} /CaM-sensitive AC discriminate against simultaneous stimulus onset? We identified a property of AC activation that would cause activation to be more effective if Ca^{2+} arrived before 5-HT: Activation by Ca^{2+} /CaM has a delayed onset (Fig. 3). This delayed activation could account for optimal activation with forward, Ca^{2+} -before-transmitter, pairing if Ca^{2+} /CaM effects on AC activation by G_s follow the same time course as the Ca^{2+} /CaM activation of the enzyme. We have made the assumption that these two effects of Ca^{2+} /CaM, AC stimulation and modulation of receptor- G_s interactions with AC, depend on the

same molecular interaction. We believe that this is a reasonable assumption; in the case of the mammalian type I AC, which has been cloned and analyzed in some detail, a single CaM binding site has been identified (Wu et al. 1993).

The relatively crude temporal resolution of the perfused membrane assay, because of the 6-sec-fraction duration, limits our ability to determine precisely how much the Ca^{2+} response is delayed compared with the 5-HT response. With Ca^{2+} and 5-HT pulses, we estimated that peaks of the two responses were shifted by ~1.9 sec; our estimate of the shift in the rising phases of the responses to steps of Ca^{2+} and 5-HT was ~1 sec. However, as we emphasized, given the limited temporal resolution, these are only estimates of the magnitude of the shift in the two responses. In classical conditioning of the defensive withdrawal reflex, stimulus pairing is most effective when the onset of the CS precedes the onset of the US by ~0.5 sec. The temporal shift observed here in the response of AC to a Ca^{2+} pulse is undoubtedly severalfold longer. There is, however, also a substantial difference in the duration of the Ca^{2+} stimulus used in these biochemical assays, ~6 sec, and the Ca^{2+} signals in sensory neurons of the CS pathway during a brief burst of spikes. In behavioral experiments, the weak siphon touch CS probably elicited only a very brief burst of a few spikes in sensory neurons (Illich and Walters, 1997); in electrophysiological studies of associative synaptic facilitation these sensory neurons were activated artificially for only 0.5 sec or less (Hawkins et al. 1983; Walters and Byrne, 1983; Clark et al. 1994). Thus, the Ca^{2+} signal in the sensory neurons during the CS is probably much briefer than the Ca^{2+} stimulus used in these assays. This difference in duration could underlie a difference in the optimal delay between the Ca^{2+} signal and 5-HT stimulus. It is unfortunate that with the present assay system, given the limited level of enzyme activity in *Aplysia* neural membranes, we can not increase the flow rate to produce briefer solution changes that would more closely approximate the timing of the Ca^{2+} and 5-HT signals that the sensory neurons experience. Perhaps, this will be possible with another assay system in the future.

Although the Ca^{2+} /CaM-sensitive AC is present in mammalian brain where it could play a role in conditioning, there are likely to be multiple molecular mechanisms in the CNS for the formation of associations. In addition to the NMDA receptor and the Ca^{2+} /CaM-sensitive AC, other molecules in-

volved in second-messenger cascades may function as convergence sites, gating associative plastic changes (Shibuki and Okada, 1991; Zhuo et al. 1993). Interestingly, other forms of AC may be able to be activated in an associative manner by subunits of multiple G proteins (Tang and Gilman, 1991; Federman et al. 1992). The particular mechanism discussed here in which a Ca^{2+} prepulse enhances the rate of activation of AC is probably only important with brief USSs. With prolonged USSs, activation of AC would reach a steady-state level even without pairing. However, even with a prolonged US, Ca^{2+} modulation of the G_s -AC complex might still reduce the efficacy of backward pairing because the decay phase of AC activation would be accelerated.

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AC ACTIVATION BY TRANSIENT Ca^{2+} AND 5-HT STIMULI

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