Role of cAMP in the Short-Term Modulation of a Neuromuscular System in Aplysia

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Fox, Lyle E. and Philip E. Lloyd. Role of cAMP in the short-term modulation of a neuromuscular system in Aplysia. J. Neurophysiol. 83: 1567–1579, 2000. Neuromuscular synapses in buccal muscle I3a of Aplysia are modulated by the small cardioactive peptide (SCP), a peptide cotransmitter that is intrinsic to the motor neurons, and by serotonin (5-HT) released from modulatory neurons that are extrinsic to the motor circuit. Although the modulation of excitatory junction potentials (EJPs) and contractions by 5-HT and SCP has been studied extensively in this muscle, little is known about the mechanisms that underlie the modulation. 5-HT and SCP, at 1 μM, were found to potently increase the level of cAMP in I3a. Therefore we investigated whether the activation of the cAMP pathway was sufficient to modulate EJPs and contractions. The direct activation of adenylyl cyclase with forskolin increased the level of cAMP, facilitated EJPs, and potentiated contractions. Indeed, the short-term effects of forskolin were very similar to all aspects of the short-term effects of 5-HT and SCP. Membrane-permeable cAMP analogues also mimicked the effects of 5-HT and SCP on EJPs and contractions. However, it seems likely that some effects of 5-HT are also mediated through other second-messenger pathways because low concentrations of 5-HT modulate EJPs and contractions but do not significantly increase cAMP levels in I3a. It is possible that lower concentrations of 5-HT function through receptors linked to protein kinase C (PKC) because phorbol, an activator of PKC, modulated EJPs and contractions without increasing the levels of cAMP. In conclusion, we provide evidence that pharmacological agents that activate the cAMP pathway mimicked most of the effects of 5-HT or SCP and that more than one second-messenger system appears to be involved in the modulation of the I3a neuromuscular system.

INTRODUCTION

One mechanism utilized by the nervous system to modify behavior is the modulation of synaptic transmission. There is increasing evidence that major sites for this modulation in both vertebrates and invertebrates are peripheral on neuromuscular synapses (Calabrese 1989; Worden 1998). Peripheral modulation has been examined extensively in the muscles that control feeding movements in Aplysia (Weiss et al. 1992, 1993; Whim et al. 1993). Many of the motor neurons that drive ingestive and egestive movements have been identified, and all of them express modulatory peptide cotransmitters (Church and Lloyd 1991, 1994; Cohen et al. 1978; Gardner 1971). In addition, buccal muscle is innervated by purely modulatory serotonergic neurons termed the metacerebral cells (MCCs) (Weiss et al. 1978). Therefore these muscles are modulated by neuropeptides intrinsic to the motor neurons and by serotonin (5-HT) released from modulatory neurons that are extrinsic to the motor circuit (Church and Lloyd 1994; Church et al. 1993; Cropper et al. 1987, 1990; Lloyd et al. 1984, 1987; Weiss et al. 1978; Whim and Lloyd 1989, 1990).

The preparation we have chosen to study consists of a muscle (termed the intrinsic anterior muscle 3, I3a) that participates in the closing of the jaws. It is innervated by two identified excitatory motor neurons B3 and B38, which use glutamate as their fast transmitter and also express modulatory peptide cotransmitters; B3 expresses FMRFamide, and B38 expresses the small cardioactive peptides (SCP). All the muscle fibers in I3a are functionally innervated by both motor neurons; however, we do not know whether individual fibers are anatomically innervated by both neurons because the muscle fibers are electrically coupled (Church and Lloyd 1991; Church et al. 1993; Fox and Lloyd 1999; Lotshaw and Lloyd 1990). I3a is also innervated by the two MCCs, a bilateral pair of large serotonergic neurons that have extensive central and peripheral synaptic outputs (Eisenstadt et al. 1973; Weinreich et al. 1973; Weiss and Kupfermann 1976). Release of peptides from the motor neurons and 5-HT from the MCCs acts peripherally to modulate excitatory junction potentials (EJPs) and contractions of I3 (Church et al. 1993; Fox and Lloyd 1998; Keating and Lloyd 1999; Lotshaw and Lloyd 1990). Specifically, these modulatory transmitters enhance the speed and strength of biting responses. EJPs and contractions of I3a muscle fibers are also modulated by application of exogenous neuropeptide cotransmitters and 5-HT (Church et al. 1993; Fox and Lloyd 1997; Lotshaw and Lloyd 1990). In previous studies, we have concentrated on the actions of 5-HT and SCP. Both modulators produce similar short-term effects including the following: the selective facilitation of B38-evoked EJPs; an increase in the amplitude and relaxation rate of contractions evoked by either B3 or B38; and a decrease in the latency between the onset of a motor neuron burst and the onset of the resulting contraction, which is larger for B38 than for B3.

We continue our study of the I3a neuromuscular system in an attempt to determine the second-messenger pathways that are involved in mediating the effects of 5-HT or SCP. Because they have similar short-term effects on EJPs and contractions, it is possible that they act through the same second-messenger system. We concentrated on the cAMP pathway because cAMP levels in I3a are increased by the exogenous application of 5-HT or SCP as well as by release of endogenous SCP from motor neurons when they are stimulated in a manner similar to their activity recorded during feeding-like motor patterns.
(Church and Lloyd 1994; Church et al. 1993; Keating and Lloyd 1999; Lotshaw and Lloyd 1990). In addition, cAMP working through protein kinase A (PKA) has been shown to mediate many, but not all, of the effects of 5-HT and SCP in another Aplysia buccal muscle preparation (Breznina et al. 1994a; Probst et al. 1994). Here we provide evidence that the cAMP pathway mediates most of the effects of SCP and some of the effects of 5-HT in I3a. However, it seems likely that some of the effects of 5-HT are also mediated through other pathways.

**Methods**

**Animals**

*Aplysia californica* (60–300 g) were obtained from Marinus (Long Beach, CA), maintained in circulating artificial sea water (ASW) at 16°C, and fed dried seaweed every 3 days.

**Neuron stimulation**

Detailed experimental methods have been described previously (Fox and Lloyd 1997). Briefly, animals were immobilized with an injection of isotonic MgCl₂, and the dissection carried out in high Ca²⁺ (33 mM; 3 times normal), high Mg²⁺ (165 mM; 3 times normal) ASW (termed high Ca, Mg ASW). The buccal mass and buccal ganglia were removed and the mass bisected along the midline. Buccal nerve 2, which contains the peripheral axons of B3 and B38, was left intact (nerve designations from Gardner 1971; muscle nomenclature from Howells 1942; also see Lloyd 1988). The ganglia were desheathed and superfused with low Ca²⁺ (0.5 mM; 0.05 times normal) high Mg²⁺ (110 mM; 2 times normal) ASW (termed low Ca ASW). Neurons were normally impaled with two microelectrodes (2–4 MΩ; filled with 3 M K acetate), one to inject current and one to monitor membrane potential. B3 and B38 were identified by their position, size, and muscle innervation patterns (Church et al. 1993). All experiments were performed at room temperature (≈22°C). Individual spikes in motor neurons were driven by brief (10–20 ms) depolarizing current pulses. The frequency of action potentials within a burst was usually 16 Hz. The burst durations were adjusted so that the compound EJPs or contractions evoked by B3 or B38 were similar in amplitude. Many experiments were carried out by alternatively stimulating bursts in B3 and B38 at 50-s intervals (100-s intervals for each neuron). These long interburst intervals were used to minimize release of endogenous peptide cotransmitters and posttetanic potentiation (Church et al. 1993; Lotshaw and Lloyd 1990; Whim and Lloyd 1990).

**Measurement of I3a contractions**

The bath containing the I3a muscle was superfused with ASW and was separated from the ganglia by a partition through which ran the intact nerve containing the axons of the motor neurons. Transmitters and/or pharmacological agents were dissolved in ASW and applied via the superfusion. Typical application periods were 20 min to ensure adequate penetration into the muscle. Note that the partition prevented the ganglia from being exposed to these substances. Contraction amplitudes were monitored with an isotonic transducer (Harvard Apparatus), and submaximal contractions were evoked by stimulating B3 or B38. Burst durations were adjusted (from 0.5 to 4 s) so that contractions evoked by B3 or B38 were similar in amplitude.

**Measurement of I3a EJPs**

EJPs were recorded with a perfusion electrode (Church et al. 1993; Fox and Lloyd 1997). The perfusion electrode consisted of a small chamber (100 μl) and aperture (~1.5 mm) that was positioned to press firmly down on a portion of the muscle (see Fig. 1 in Church et al. 1993). The inside of this electrode was perfused with ASW, whereas the rest of the preparation was superfused with low Ca ASW to suppress synaptic transmission and muscle contractions. This procedure confined the contractions to the small area of the muscle covered by the recording chamber and thus markedly reduced movement artifacts in the recordings. The earliest evoked muscle contractions occur after the sixth EJP so the early EJPs in a burst are recorded in the absence of any movement. EJPs were recorded by extracellular electrodes placed inside and just outside the wall of the perfusion apparatus. Signals were amplified using a Grass P15D AC amplifier. Transmitters and/or pharmacological agents were applied in ASW to the inner channel of the perfusion electrode so the ganglia and the remainder of the muscle were not exposed to these substances. Typical application periods were also 20 min. This procedure permits us to simultaneously record from a population of muscle fibers thereby reducing sampling bias. Burst durations were adjusted (from 0.15 to 2 s) so that the compound EJPs evoked by B3 or B38 were similar in amplitude.

**Measurement of cAMP**

Muscle segments were dissected in high Ca, Mg ASW, weighted (~3 mg each), and washed in several changes of ASW for 1 h. Segments were incubated in ASW, transmitter, or pharmacological agents for 20 min (the same period used in most physiological experiments), extracted immediately in 2% 2N HCl in ethanol at −30°C, homogenized, and centrifuged at 10,000 × g. The supernatants were used for duplicate cAMP determinations using a commercial radioimmunoassay (Biomedical Technologies, Norwood, MA).

**Results**

5-HT and SCP increase the level of cAMP in isolated I3a

Brief application (5.5 min) of both 5-HT and SCP potently increase cAMP levels in the I3a muscle (Lotshaw and Lloyd 1990). However, in subsequent physiological experiments, we found that 20-min applications of the modulators were necessary to maximally facilitate EJPs or potentiate contractions (Fox and Lloyd 1997, 1998). Accordingly, we repeated the cAMP measurements using 20-min applications. At 1 μM, both 5-HT and SCP dramatically increased the level of cAMP in I3a muscle segments more than 250-fold over control (Fig. 1). The cAMP levels were ~10-fold higher measured after 20 min than those reported by Lotshaw and Lloyd (1990) for the shorter incubation suggesting that the levels of cAMP continue to rise. Lower concentrations of SCP (0.1 μM) were also effective at increasing cAMP levels (46 ± 13-fold increase over control, this is equivalent to a 4,500% increase, n = 8), but 0.1 μM 5-HT had no significant effect on these levels (20 ± 11% increase, mean ± SE, n = 5; P > 0.05, t-test; Fig. 1). This steep dose response curve for 5-HT, in which 0.1 M has very small effect, and 1 μM essentially has no effect on cAMP levels and 1 μM has very large effects, has also been observed in a previous study (Lotshaw and Lloyd 1990). Because 0.1 μM 5-HT had large effects on EJPs and contractions (Fox and Lloyd 1998), this suggests that 5-HT may act through mechanisms other than the cAMP pathway. This preparation contains both muscle fibers and the axons and terminals of neurons that innervate them. The large increases in cAMP levels are of such a magnitude that they must occur in the muscle fibers because they constitute nearly the total volume of the muscle. It is unlikely that an
increase in cAMP exclusively in the axons and terminals of neurons could account for the measured increase in cAMP levels because of their small volume. This conclusion is supported by work on another buccal muscle in *Aplysia* (termed I5 or ARC). 5-HT and SCP increase the level of cAMP in dissociated I5 muscle fibers and modulate their whole cell currents recorded by voltage clamp (Brezina et al. 1994a,b). Although most of the increase in cAMP must be in the muscle fibers, it is possible that the cAMP levels are also increased in the axons or terminals of the motor neurons.

**Forskolin selectively facilitates B38-evoked EJPs and modulates contractions of both neurons**

Superfusion with 5-HT or SCP has a number of effects including facilitation of EJPs, potentiation of contractions, increased rate of muscle relaxation, and a decrease in the latency between the onset of a burst and the onset of the evoked contraction (Fox and Lloyd 1997). Although 5-HT and SCP had similar effects on the amplitude of contractions evoked by both motor neurons, they facilitated B38-evoked EJPs much more than those of B3. If cAMP mediates all of the actions of 5-HT and SCP, then activators of the cAMP pathway should also mimic these effects. We focused on the facilitation of EJPs to screen the effects of activators of the cAMP pathway. Because B38-evoked EJPs are potently facilitated by the 5-HT and SCP, they served as a sensitive assay for the effects of the reagents tested, whereas B3-evoked EJPs, which are only slightly facilitated, served as a control for possible nonspecific effects.

Elevation of cAMP levels by activation of adenylyl cyclase with forskolin selectively facilitated B38-evoked EJPs in a concentration-dependent manner. At 10 μM, forskolin slightly facilitated B3-evoked EJPs and potently facilitated those of B38 (Figs. 2 and 3). We believe that this selective facilitation was caused by the activation of adenylyl cyclase because 10 μM forskolin increased the levels of cAMP in I3a muscle segments by 191 ± 46% (n = 8) and a forskolin analogue that does not activate adenylyl cyclase but has many of forskolin’s nonspecific effects (10 μM 1,9-dideoxy-forskolin) (Laurenza...
et al. 1989) had a much smaller effect on EJPs (Figs. 2 and 3). Lower concentrations of forskolin (1 \( \mu \)M) also facilitated the B38-evoked EJPs more than those of B3 (Fig. 3). Two other active forskolin derivatives were tested: 7-deacetyl-6-(N-acetylglucyl)-forskolin (DAAG; 75 \( \mu \)M) because it has fewer nonspecific effects than forskolin (Baxter and Byrne 1990b) and 7-deacetyl-7-(O-(N-methylpipеразино)-γ-butyryl)-forskolin (DMPB; 100 \( \mu \)M) because it is more soluble and more stable in water (Laurenza et al. 1987). Both DAAG and DMPB facilitated B38-evoked EJPs much more than those of B3 (Fig. 3). Therefore forskolin and its analogues that activate adenylyl cyclase selectively facilitated B38-evoked EJPs, presumably by increasing cAMP levels.

Because forskolin mimicked the effects of 5-HT and SCP on EJPs, we next examined whether it also modulated evoked contractions. At 10 \( \mu \)M, forskolin potentiated both B3- and B38-evoked contractions (by 850 \( \pm \) 88% for B3, \( n = 4 \); by 1,455 \( \pm \) 420% for B38, \( n = 5 \); Fig. 4). It also increased the relaxation rate of the contractions and reduced the latency between the onset of the motor neuron burst and the onset of the evoked contraction. The relaxation of the contractions was well fitted to a single exponential, and the time constant was calculated only for regions in which the amplitude of control and potentiated contractions overlapped. Forskolin reduced the relaxation time constant by 32 \( \pm \) 8% (\( n = 4 \)) for contractions evoked by B3 and by 29 \( \pm \) 14% for those evoked by B38 (\( n = 5 \)). As for the effects of 5-HT and SCP, it is difficult to measure small changes in the relaxation rate because of the large change in the contraction amplitude. The effects of forskolin on the latency were similar to the application of 5-HT or SCP in that the latency was reduced more for B38-evoked contractions than those of B3. The latency of B3-evoked contractions were reduced by 20 \( \pm \) 7% (\( n = 4 \)), whereas the B38-evoked contractions were reduced by 54 \( \pm \) 5% (\( n = 5 \); Fig. 4). These effects of forskolin appeared to be caused by activation of adenylyl cyclase because 1,9-dideoxy-forskolin (10 \( \mu \)M) had little effect on the amplitude, latency, or relaxation rate of contractions (Fig. 4). Thus the effects of forskolin were similar to the effects of 5-HT and SCP: it increased the level of cAMP, it selectively facilitated B38-evoked EJPs, it potentiated B38-evoked contractions somewhat more than those evoked by B3, it reduced the latency more for B38 than B3, and it increased the relaxation rate of contractions for both neurons. These observations, combined with the effects of 5-HT and SCP on cAMP levels, suggest that these modulators act, at least in part, through the cAMP pathway.

Membrane-permeable cAMP analogues selectively facilitate B38-evoked EJPs and modulate contractions of both neurons

We next investigated whether membrane-permeable cAMP analogues selectively facilitated B38-evoked EJPs. We used a...
cAMP analogue, cpt-cAMP, that has been used extensively in Aplysia and other animals (Baxter and Byrne 1990a; Goldsmith and Abrams 1992; Rydel and Greene 1988; Weiss et al. 1979). Two concentrations (100 and 500 μM) were tested and found to facilitate EJPs evoked by B38 more than those evoked by B3 (Figs. 5 and 6). The facilitation does not appear to be mediated by the extracellular effects of cpt-cAMP because cAMP (500 μM), which poorly diffuses across the cell membrane (Sandberg et al. 1991), had no effect on EJPs. Because cpt-cAMP is hydrolyzed by phosphodiesterases (Sandberg et al. 1991) and high concentrations of it have nonspecific effects on ion channels (Brezina et al. 1994a,b; Lambert and Harrison 1990; Sugita et al. 1994a), we tried to reduce these effects by using the lower concentration while simultaneously slowing its degradation with phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). The facilitation of EJPs produced by 100 μM cpt-cAMP was significantly enhanced by IBMX. Simultaneous application of IBMX (100 μM) with cpt-cAMP selectively facilitated B38-evoked EJPs more much more effectively than either cpt-cAMP or IBMX alone (Fig. 6). We believe that this facilitation was caused by the activation of PKA and not the extracellular actions of cpt-cAMP or IBMX because the coapplication of structurally related molecules, cAMP and adenosine (each at 100 μM), had no effect on EJPs. In addition, the purinergic receptors agonists ATP and adenosine (each at 100 μM) did not facilitate EJPs.

FIG. 5. Effects of cAMP analogues on EJPs recorded extracellularly in I3a muscle with a perfusion electrode. A: cpt-cAMP (500 μM). B: mixture of cpt-cAMP (100 μM) and 3-isobutyl-1-methylxanthine (IBMX, 100 μM). C: Sp-5,6-dichloro-1-β-D-ribofuranosylbenzimidazole-3′,5′-monophosphate (BiMPS; 100 μM) facilitated EJPs evoked by B38 much more than those evoked by B3. EJPs were evoked by alternating stimulation of B3 or B38 (interburst interval of 50 s). Bursts were at 16 Hz, and the burst durations were adjusted so control compound EJPs evoked by stimulating both neurons were similar in amplitude. Recordings for A–C are from different experiments. Pooled results are presented in Fig. 6.

FIG. 6. Summary of the effects of cpt-cAMP, cpt-cGMP, and IBMX on EJPs. A: cpt-cAMP alone (n = 8 for 100 μM; n = 4 for 500 μM), IBMX alone (100 μM; n = 15) or a mixture of cpt-cAMP and IBMX (each at 100 μM; n = 8 for B3 and n = 9 for B38) facilitated B38-evoked EJPs more than those of B3. Note that the effects of cpt-cAMP were amplified by co-application with IBMX (100 μM), a phosphodiesterase inhibitor. B: the mixture of cpt-cAMP and IBMX was more effective at facilitating B38-evoked EJPs than IBMX alone or the mixture of cpt-cGMP and IBMX when compared on the same preparations (n = 4). The mean amplitude of the third EJPs in the bursts were used because the first EJP was often too small to measure reliably (e.g., Fig. 5). Increases were calculated using the EJP amplitude before the application of the test agent as the control.
Our working hypothesis was that cpt-cAMP and IBMX were activating PKA. However, it was possible that they were acting through the cGMP pathway because both cpt-cAMP and IBMX inhibit the cGMP-specific phosphodiesterases leading to an elevation of cGMP levels, and cpt-cAMP directly activates cGMP-dependent protein kinase (PKG) (Beavo and Reifsnyder 1990; Connolly et al. 1992; Gillespie and Beavo 1989; Sandberg et al. 1991). We tested for this by using a second more specific activator of PKA and by comparing the effects of PKA and PKG activators on the same preparations. Sp-5,6-dichloro-1-b-D-ribofuranosylbenzimidazole-3′,5′-monophosphorothioate (cBiMPS), is a potent and specific activator of PKA (Schultz et al. 1994). It is not necessary to use IBMX in these experiments because cBiMPS is resistant to hydrolysis by phosphodiesterases (Sandberg et al. 1991). Therefore the contribution of the cGMP pathway to the facilitation produced by cpt-cAMP can be determined by comparing the effects of cpt-cAMP to those of cpt-cGMP. In four experiments, the effects of IBMX alone, a cpt-cAMP/IBMX mixture, and a cpt-cGMP/IBMX mixture were compared on the same preparation and found to selectively facil-
iterate B38-evoked EJPs (Fig. 6B). Whereas the cpt-cAMP/IBMX mixture was much more effective at facilitating EJPs than IBMX alone, the effects of the cpt-cGMP/IBMX mixture on B38-evoked EJPs was not significantly different from IBMX alone. These results indicate that in this preparation cpt-cAMP appears to act through the cAMP pathway.

The membrane-permeable cAMP analogues also potentiated contractions evoked by B3 or B38. cpt-cAMP (500 μM) potentiated contractions evoked by both neurons to a similar degree (Figs. 7 and 8). The effects of cpt-cAMP on contraction latency and relaxation rate were inconsistent. The latency was significantly reduced only for the B3-evoked contractions and the relaxation time constant only for B38-evoked contractions (Fig. 8). The weak effects of cpt-cAMP on contractions might be due to limited diffusion across the cell membrane or its rapid degradation by phosphodiesterases (Sandberg et al. 1991). We believe that potentiation was not mediated by the extracellular effects of cpt-cAMP because cAMP (500 μM), which poorly diffuses across the cell membrane (Sandberg et al. 1991), had no effect on contractions. Potentiation of contractions by cpt-cAMP was enhanced and made more consistent by co-application with IBMX. Simultaneous application of a lower concentration of cpt-cAMP (100 μM) with IBMX (100 μM) increased both B3- and B38-evoked contractions to a degree similar to the higher concentration of cpt-cAMP alone. Surprisingly, contractions evoked by stimulating B3 were potentiated more than those of B38 (Figs. 7 and 8). The latency and the relaxation time constant were also reduced to a similar degree for both neurons (Figs. 7 and 8). The potentiation caused by cpt-cAMP/IBMX application was not due to activation of extracellular purinergic receptors because the application of cAMP and adenosine (each at 100 μM) had no effect on contractions. Thus the short-term effects of cpt-cAMP/IBMX, although not identical, were qualitatively similar to the short-term effects of 5-HT and SCP. They selectively facilitated B38-evoked EJPs and increased the relaxation rate of contractions for both neurons. Although cpt-cAMP/IBMX potentiated contractions and reduced the latency for both neurons, the effects on B3-evoked contractions were larger than predicted based on the actions of 5-HT and SCP in this preparation. These differences could be due to some nonspecific effects of cpt-cAMP (see DISCUSSION).

In summary, there is good evidence that activation of the cAMP pathway can modulate EJPs and contractions in buccal muscle I3a. The short-term effects of 5-HT and SCP were mimicked well by procedures that increased cAMP levels or by cAMP analogues. Taken together with the observation that 5-HT and SCP (at 1 μM) potently elevate the cAMP concentration in the muscle, these results suggest that 5-HT and SCP modulate EJPs and contractions, at least in part, via the cAMP second-messenger pathway.

FIG. 9. Effects of phorbol esters on EJPs recorded extracellularly in I3a muscle with a perfusion electrode. A: 4β-phorbol 12,13 diacetate (0.3 μM; β-PDA) facilitated EJPs evoked by B3 or B38 to a similar degree. B: 4α-phorbol 12,13 diacetate (3 μM; α-PDA), a phorbol isomer that does not activate protein kinase C (PKC), had no effect on EJPs even though it was used at a 10-fold higher concentration, suggesting that the facilitation was caused by the activation PKC. EJPs were evoked by alternately stimulating B3 or B38 (interburst interval of 50 s). Bursts were at 16 Hz, and the burst durations were adjusted so the control compound EJPs were similar in amplitude. Recordings for A and B are from different experiments. Pooled results are presented in Fig. 10.

FIG. 10. Summary of the effects of phorbol esters on extracellularly recorded EJPs. Two different active derivatives of phorbol, 4β-phorbol 12,13 diacetate (β-PDA; n = 3 for 0.3 and 3 μM) and 12-deoxyphorbol 13-isobuturate (DPIB; n = 3) facilitated EJPs evoked by B3 and B38 to a similar degree. An inactive isomer of phorbol, 4α-phorbol 12,13 diacetate (α-PDA; n = 3), had no effect on EJPs evoked by either neuron. The mean amplitude of the 3rd EJPs in the bursts were used because the 1st EJP was often too small to measure reliably (e.g., Fig. 9). Increases were calculated using the EJP amplitude before the application of the test agent as the control.
Activation of the PKC pathway facilitates EJPs, but is not selective for B38

Some of the effects of 5-HT appear to be mediated by a second-messenger system other than cAMP pathway because low concentrations of 5-HT (≤0.1 μM) effectively modulate EJPs and contractions (Fox and Lloyd 1998), whereas they have little effect on cAMP in the muscle. Because the activation of both PKA and PKC by 5-HT has been implicated in the facilitation of central sensory neuron synapses in *Aplysia* (Byrne and Kandel 1996; Ghirardi et al. 1992; Sugita et al. 1992, 1997a), we examined whether the activation of PKC facilitates EJPs. Indeed, 4β-phorbol 12,13 diacetate (β-PDA), used at concentrations necessary to reliably activate PKC-mediated processes in intact ganglia (3 μM) (Sugita et al. 1992, 1994b), dramatically facilitated EJPs evoked by both neurons to a similar degree (Figs. 9 and 10). This differs from 5-HT and SCP, which selectively facilitated B38-evoked EJPs. Lower concentrations of β-PDA (0.3 μM) were also effective at facilitating EJPs evoked by the two neurons (Fig. 10). This facilitation appears to be due to activation of PKC because a phorbol isomer that does not activate PKC, 4α-phorbol 12,13 diacetate (α-PDA; 3 μM), did not affect EJPs evoked by either neuron (Fig. 10). A second phorbol derivative that is more selective in activating PKC, 12-deoxyphorbol 13-isobuterate (DPIB; 0.3 μM), also facilitated EJPs evoked by both neurons (Fig. 10). Thus activation of PKC appears capable of facilitating EJPs, but was not selective for B38.

We also looked at the effects of kinase inhibitors on EJPs. The broad spectrum kinase inhibitor H7 (Hidaka et al. 1984) had variable effects but clearly facilitated EJPs in some preparations (by as much as 367% at 100 μM and 471% at 500 μM). Chelerythrine, a relatively specific PKC inhibitor (Herbert et al. 1990), also facilitated EJPs (increased 29 ± 3% at 30 μM and 111 ± 10% at 100 μM; n = 2 for each). We did not further pursue the use of these inhibitors because they caused facilitation themselves.

Activation of the PKC pathway potentiates contractions, but does not increase relaxation rate

Next we examined the effects of phorbol on the properties of contractions evoked by B3 and B38. β-PDA (3 μM) dramatically changed the dynamics of contractions in that short bursts that were barely above threshold for evoking a contraction before β-PDA application produced near maximal contractions that relaxed extremely slowly after its application. Therefore it was necessary to use lower concentrations to study its effects on contractions. We found that β-PDA, at 0.03 and 0.3 μM, dramatically increased contraction amplitude and reduced the latency for both neurons (Figs. 11 and 12). However, unlike 5-HT and SCP, phorbol did not increase the relaxation rate of the contracts. Indeed, the relaxation rate actually decreased during the 0.03 and 0.3 μM β-PDA applications for both neurons (Fig. 12). We believe that these effects were caused by the activation of PKC because α-PDA (3 μM), the inactive phorbol isomer, had no significant effects on the amplitude, latency, or relaxation rate of contractions evoked by either neuron (Figs. 11 and 12).

Many adenylyl cyclases interact with and are regulated by other second-messenger systems including the diacylglycerol (DAG)/PKC pathway (Cooper et al. 1995; Pieroni et al. 1993). Indeed, activation of PKC with phorbol increased the level of cAMP in *Aplysia* sensory neurons (Sugita et al. 1997b). Be-
cause phorbol can activate the cAMP pathway, we tested whether it increased the concentration of cAMP in I3a. β-PDA does not appear to act through the cAMP pathway because it only slightly increased the level of cAMP by 13 ± 9% at 0.3 μM (n = 4; P > 0.05) and 24 ± 12% at 3 μM (n = 4; P > 0.05). Thus facilitation of EJPs by phorbol differs from the actions of 5-HT, SCP, and activators of the cAMP pathway because phorbol facilitated the EJPs and reduced the contraction latency of both motor neurons to a similar degree. The nonselective facilitation of EJPs and the nonselective reduction in latency caused by phorbol supports our hypothesis that selective facilitation of B38-evoked EJPs leads to the selective reduction in latency produced by 5-HT, SCP, and agents that activate the cAMP pathway. Modulation by phorbol, without a concurrent increase in the cAMP levels in the muscle, suggests that there are mechanisms other than the cAMP pathway that can modulate EJPs and contractions.

**DISCUSSION**

The effects of SCP appear to be mediated by the activation of the cAMP pathway. SCP, at concentrations as low as 0.01 μM, increased the level of cAMP in I3a (Lotshaw and Lloyd 1990), and activation of the cAMP pathway, with agents that bypass the SCP receptor and directly activate either adenylyl cyclase or PKA, mimicked the short-term effects of SCP. Activation of the cAMP pathway also mimicked the short-term effects of 1 μM 5-HT; however, it is unlikely that the effects of lower concentrations of 5-HT (<0.1 μM) are mediated by this pathway because, at these concentrations, 5-HT has little effect on the cAMP levels. Because phorbol dramatically facilitated EJPs and potentiated contractions, it is possible that low concentrations of 5-HT activate the PKC pathway.

Many of the effects of 5-HT or SCP are mediated postsynaptically in the I3a muscle fibers. In previous studies, 5-HT and SCP were shown to increase the relaxation rate of contractions, potentiate contractions evoked by the bolus application of glutamate to isolated bundles of muscle fibers, and modulate some step(s) in excitation-contraction coupling because they potentiate B3-evoked contractions even when the B3-evoked EJPs were essentially unchanged (Church et al. 1993; Fox and Lloyd 1997; Lotshaw and Lloyd 1990). In the present study we have shown that 1 μM 5-HT or SCP elevate cAMP levels in the I3a neuromuscular preparation over 250-fold above control values. This preparation contains both muscle fibers and the axons and terminals of neurons that innervate them. Considering the magnitude of the increase in the cAMP levels, it must occur primarily in muscle fibers because they represent nearly the entire tissue volume. It is unlikely that an increase in cAMP exclusively in the axons and terminals of neurons could account for the measured increase in cAMP because of their small volume. It is still possible that cAMP levels increased in the axons and terminals of neurons; however, in this preparation, the concentration of cAMP in neuronal terminals cannot be measured independently. Observations made in another buccal muscle in *Aplysia* (termed I5 or ARC) support the conclusion that most of the increase in cAMP levels is postsynaptic. 5-HT and SCP also potently increase the level of cAMP in I5, and an increase in cAMP was also observed in dissociated muscle fibers (Brezina et al. 1994a,b; Lloyd et al. 1984; Weiss et al. 1979; Whim and Lloyd 1989). In addition, activation of 5-HT and SCP also leads to the phosphorylation of a contractile protein that is believed to mediate the increase in relaxation rates (Probst et al. 1994).
Two different procedures were used to bypass the 5-HT and SCP receptors and stimulate the cAMP pathway, activation of adenylyl cyclase by forskolin and application of cAMP analogues. Both procedures yielded similar results. Individually, each of the agents used in this study may have effects unrelated to their actions on the cAMP pathway. Forskolin activates adenylyl cyclase but has also been shown to affect both voltage-gated and ligand-gated currents in a cAMP-independent manner (Baxter and Byrne 1990b; Brezina et al. 1994a,b; Laurenza et al. 1989). We used dideoxy-forskolin, which has many of the cAMP-independent effects of forskolin but does not activate adenylyl cyclase as a control and found it had little effect. Dideoxy-forskolin (100 μM) does significantly reduce voltage-gated currents in another buccal muscle (Brezina et al. 1994a,b); however, we used lower concentrations and found that they had little effect on EJPs and contractions in I3a. In addition, two hydrophilic derivatives of forskolin that activate adenylyl cyclase but have fewer nonspecific effects were also found to mimic forskolin. cpt-cAMP also has effects unrelated to its actions on the cAMP pathway. It has been shown to inhibit cGMP-specific phosphodiesterases (Connolly et al. 1992), directly activate PKG (Sandberg et al. 1991), and affect both voltage- and ligand-gated channels in a cAMP-independent manner (Brezina et al. 1994a,b; Lambert and Harrison 1990). Some of these PKA-independent effects appear to be associated with the p-chlorophenylthio moiety because cpt-cGMP had similar effects, whereas cAMP, 8-bromo-cAMP, or 8-bromo-cGMP do not (Sugita et al. 1994a). Although cpt-cAMP has many nonspecific effects, we believe that its effects on EJPs and contractions were mediated by the cAMP pathway for several reasons. 1) BiMPS, a different membrane-permeable cAMP analogue that does not activate PKG and does not contain the p-chlorophenylthio moiety (Sandberg et al. 1991), also selectively facilitated B38 evoked EJPs. 2) cpt-cGMP is much less effective at facilitating EJPs than cpt-cAMP. 3) IBMX and cPT-cAMP do not act extracellularly at purinergic receptors. Two native purinergic agonists, adenosine and ATP, had no effect on EJPs even though they were used at the same concentration as IBMX and cPT-cAMP and are structurally similar to them. When the results are viewed collectively, they provide good evidence in support of a prominent role for the cAMP pathway in mediating many of the actions of 5-HT or SCP.

Cyclic nucleotides gated channels have been found in a variety of sensory and nonsensory tissues including vertebrate muscle and invertebrate neurons (Biel et al. 1994, 1999; Kehoe 1990; Price and Goldberg 1993; Sudlow et al. 1993; Zufall et al. 1997). Therefore it is possible that cAMP modulates EJPs and contractions by directly gating ion channels. We could not determine whether cAMP acted directly on channels, if it acted through PKA, or a combination of both mechanisms. However, we believe that PKA has an important role in the modulation of I3a contractions based partially on results obtained in another buccal muscle (15). In both muscles, 5-HT and SCP increase the levels of cAMP, increase contraction amplitude, and increase the relaxation rates of contractions (Fox and Lloyd 1997; Kupfermann 1997; Lotshaw and Lloyd 1990; Weiss et al. 1992). In I5, SCP application leads to the activation of PKA, and application of either SCP or 5-HT causes the phosphorylation of a contractile protein termed twitchin (Hooper et al. 1994; Probst et al. 1994). The degree and time course of the phosphorylation of twitchin correlates well with the increase in the relaxation rates (Probst et al. 1994). By contrast, the role of PKA in the facilitation of EJPs is uncertain because the locus of facilitation has not been identified in I3, and EJPs in I5 are only slightly facilitated by 5-HT and SCP making it a poor model for facilitation (Fox and Lloyd 1997; Lloyd et al. 1984; Weiss et al. 1978).

Second-messenger systems other than the cAMP pathway may be involved in the plasticity at I3a neuromuscular synapses. Phorbol isomers that activate PKC modulated EJPs and contractions evoked by both B3 and B38. However, the effects of phorbol were different from those of 5-HT or SCP because phorbol facilitated EJPs and reduced the latency of contractions equally for both motor neurons. Phorbol also did not increase the relaxation rate of contractions. The differences between the actions of phorbol and 5-HT do not preclude PKC as a mediator of the effects of 5-HT. It is possible that B3 synapses do not express 5-HT receptors linked to this pathway or that phorbol activates PKC isoforms that are not normally activated by 5-HT. For example, sensory neurons in Aplysia contain both Ca-dependent and Ca-independent isoforms of PKC, and 5-HT activates only the Ca-dependent isoform of PKC, but phorbol activates both isoforms (Kruger et al. 1991; Sossin et al. 1993; Sossin and Schwartz 1992). Our results also suggest that 5-HT could act through second-messenger pathways other than the cAMP pathway. First, 0.1 μM 5-HT and SCP have large modulatory effects on I3a (Fox and Lloyd 1998, Lotshaw and Lloyd 1990), but 5-HT does not significantly elevate the level of cAMP, whereas 0.1 μM SCP causes a large increase in cAMP levels. In addition, activation of the PKC pathway facilitated EJPs and potentiated contractions without elevating cAMP levels. Both of these second-messenger pathways probably participate in the modulation of feeding by the MCCs because the maximal short-term effects of MCC stimulation were similar to those of 0.1–0.3 μM 5-HT (Fox and Lloyd 1998); however, our data suggest that the cAMP-independent pathway is more important at physiological frequencies. Of course, the possibility remains that 5-HT might function solely through cAMP if, for example, the increase in cAMP caused by low concentrations of 5-HT was highly compartmentalized. In many ways, these results are reminiscent of the facilitation observed in the central ganglia of Aplysia. PKA and PKC are thought to be involved in mediating the 5-HT–induced facilitation of excitatory postsynaptic potentials (EPSPs) between sensory and motor neurons (Byrne and Kandel 1996). The two pathways were once thought to be activated serially with activation of the PKA leading to PKC activation (Goldsmith and Abrams 1991). Results from other work suggest that both pathways are activated in parallel (Braha et al. 1993; Ghirardi et al. 1992; Sacktor and Schwartz 1990). More recent evidence suggests that both pathways function in parallel but that there is considerable cross-talk between them. Indeed, activation of PKC with phorbol attenuates subsequent responses to 5-HT and raises cAMP concentrations in sensory neurons (Sugita et al. 1997b). Activation of PKC has also been shown to increase adenylyl cyclase activity and increase the cAMP concentration in other systems (Cooper et al. 1995; Pieroni et al. 1993). However, this does not appear to be the case in the I3a neuromuscular preparation because phorbol had little effect on cAMP levels. Furthermore, the effects of phorbol on EJPs were different from the effects of activators of the cAMP pathway. Phorbol equally facilitated EJPs evoked by B3
and B38, whereas 5-HT, SCP, forskolin, and cAMP analogues selectively facilitated B38-evoked EJPs.

Modulation of EJPs and contractions by both the cAMP and PKC pathways has been observed in two other well-studied invertebrate neuromuscular preparations. In lobster, 5-HT facilitates EJPs, increases the tone of muscle, and increases the level of cAMP in both motor neurons and muscle fibers (Goy and Kravitz 1989; Goy et al. 1984; Hempel et al. 1996). Activation of the cAMP pathway with forskolin, IBMX, or 8-bromo-cAMP only partially mimicked the actions of 5-HT. These pharmacological agents had no effect on muscle tone and did not facilitate EJPs to the same degree as 5-HT, suggesting that a component of the facilitation was cAMP-independent (Goy and Kravitz 1989).

Similar results have been obtained from the crayfish neuromuscular junction. The facilitation induced by 5-HT in the crayfish has been divided into two phases: early and late. Although the early phase of facilitation is mimicked by activators of the PKC pathway, the late phase facilitation requires the activation of both the cAMP and PKC pathways (Dixon and Atwood 1989a–c). In addition, the cAMP pathway also modulates vertebrate muscle preparations. Activation of the cAMP pathway modulates electrical and contractile properties of the heart and skeletal muscles (McDonald et al. 1994; Osterrieder et al. 1982; Rodger and Bowman 1983; Tsien 1977).

In conclusion, we provided additional evidence that pharmacological agents that activate the cAMP pathway mimicked most of the effects of 5-HT or SCP. However, it seems likely that some effects of 5-HT are also mediated through another second-messenger pathway because low concentrations of 5-HT modulate EJPs and contractions but do not increase cAMP. It is possible that lower concentrations of 5-HT function through receptors linked to the PKC pathway because phorbol modulated EJPs and contractions without increasing the levels of cAMP. Therefore more than one second-messenger system appears to modulate synaptic efficacy in the I3a neuromuscular system.

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