Modulation of a transient K+ current in the pleural sensory neurons of Aplysia by serotonin and cAMP: Implications for spike broadening

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ABSTRACT To study the contribution of cAMP to the spike broadening produced by serotonin (5-HT) in the pleural sensory neurons of the tail withdrawal reflex, we utilized two phosphodiesterase-resistant cAMP analogs: the Sρ diastereomer of cyclic adenosine 3',5'-monophosphothioate (Sρ-cAMP[S]), which activates protein kinase A, and the antagonist Rρ diastereomer of cyclic adenosine 3',5'-monophosphothioate (Rρ-cAMP[S]), which is a competitive inhibitor of kinase A. When the cAMP agonist Sρ-cAMP[S] was injected into the sensory neurons, it caused spike broadening comparable to that induced by 5-HT. In turn, the cAMP antagonist Rρ-cAMP[S] blocked ~50% of the 5-HT-induced spike broadening. We next examined the K+ currents that are modulated by 5-HT and determined how these currents are affected by cAMP. Confirming Baxter and Byrne (1989) J. Neurophysiol. 62, 665–679, we found that 5-HT modulated two currents, an Sτ-type K+ current (IKS) as well as a transient and voltage-dependent K+ current (IKV). Rρ-cAMP[S] blocked the reduction by 5-HT of the early phase of IKV in parallel with, and to the same degree (60%), as this inhibitor blocked the IKS and spike broadening. These results support the idea that in the pleural sensory neurons cAMP mediates a significant part of the spike broadening that accompanies short-term facilitation produced by 5-HT and that cAMP can produce spike broadening by modulating both IKV and IKS.

Short-term presynaptic facilitation of the connections between the siphon sensory neurons and gill motor neurons in the abdominal ganglion of Aplysia is associated with spike broadening in the siphon sensory neuron (1). Klein et al. (2) found that connective stimulation, which activates serotonergic cells, serotonin (5-HT), and cAMP, a second-messenger pathway activated by connective stimulation and 5-HT, all reduce a specific background K+ current, the Sτ-type K+ current (IKS), which they suggested was responsible for the 5-HT-induced spike broadening (see also refs. 3 and 4). Later, Hochner et al. (6) found that broadening was both necessary and sufficient for short-term facilitation of transmitter release in nondepressed synapses (see also ref. 7). In addition to being modulated in the short term, IKS is also modulated with long-term facilitation lasting one or more days (8).

In the pleural sensory neurons Scholtz and Byrne (9) have similarly found that 5-HT modulates IKS with long-term facilitation, but Baxter and Byrne (10) have found recently that with short-term facilitation 5-HT modulates, in addition to IKS, a change in kinetics of another K+ current, which they characterized as a delayed rectifier, voltage-dependent K+ current (IKV). They found that 5-HT causes an inward movement of the current at the beginning of the voltage step followed by a later outward movement. Because the inward and outward movements of the currents showed similar voltage dependence and sensitivities to tetraethylammonium and 4-aminoypyridine, Baxter and Byrne suggested that IKV is a single current and that 5-HT modulates it by slowing both the kinetics of activation and inactivation. Bath application of cAMP analogs did not modulate this current and produced only a modest spike broadening (10, 11). Therefore, they concluded that 5-HT produced spike broadening by cAMP-independent modulation of IKV. These findings seemed at variance with those obtained by Ghirardi et al. (12) in pleural sensory and motor cells in culture and by Goldsmith and Abrams (13), where inhibitors of protein kinase A blocked spike broadening and short-term facilitation by 5-HT.

The availability of more effective analogs, such as the Sρ diastereomer of cyclic adenosine 3',5'-monophosphothioate (Sρ-cAMP[S]), and of new specific inhibitors of protein kinase A, such as the Rρ diastereomer of cyclic adenosine 3',5'-monophosphothioate (Rρ-cAMP[S]) (13), has encouraged us to reexamine the cAMP dependence of the two types of K+ currents and of the spike broadening in the pleural sensory neurons of intact ganglia. Confirming Baxter and Byrne (10), we found that 5-HT modulated in the short-term not only IKS but also a second, transient IKV. However, we find that the reduction of IKV by 5-HT is blocked by Rρ-cAMP[S] in parallel with and to the same degree (60%) that this inhibitor blocks the modulation of IKS. Moreover, when injected into the sensory neurons, the cAMP agonist Sρ-cAMP[S] caused spike broadening comparable to that induced by 5-HT, and Rρ-cAMP[S], an antagonist of protein kinase A, blocked ~50% of the 5-HT-induced spike broadening, paralleling the blockade of the two K+ currents.

These results suggest that cAMP contributes importantly to spike broadening in the pleural sensory neurons and are consistent with the finding that in nondepressed synapses, where spike broadening is the main mechanism for facilitation (6), short-term facilitation produced by one 5-min pulse of 5-HT is blocked completely by inhibitors of protein kinase A (12). However, we have found that the effect on spike broadening of continuous injection of Sρ-cAMP[S] reaches its peak within 5–10 min and then declines slowly over the next 10–20 min. By contrast, the effect on spike broadening of continuous 5-HT application can be maintained for periods >30 min. In agreement with Ghirardi et al. (12) and Sugita et al. (14), these experiments point to the possibility that other slowly activated and cAMP-independent processes may contribute to spike broadening that emerges after many minutes of continuous exposure to 5-HT.

MATERIALS AND METHODS

Clusters of sensory neuron somata were isolated from the pleural ganglia of Aplysia californica weighing 150–350 g.

Abbreviations: 5-HT, serotonin; Rρ- and Sρ-cAMP[S], cyclic adenosine 3',5'-monophosphothioate, Rρ and Sρ diastereomers, respectively; IKS and IKV, S-type and voltage-dependent K+ currents, respectively.

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The clusters were pinned on the Sylgard (Dow-Corning) floor of a chamber containing 1.25 ml of solution. The physiological solution was prepared from artificial seawater (Instant Ocean; Mentor, OH) to which 10 mM Hepes, pH 7.6, was added. Voltage-clamp experiments were done in solution containing 160 $\mu$M tetrodotoxin and 2 mM tetraethylammonium. The experiments were done at room temperature (22–24°C). Drugs were added and mixed directly into the bath.

For current-clamp experiments, sensory neurons were penetrated with single electrodes that, in bridge configuration, served for both current injection and voltage recording (Axoclamp-2A; Axon Instruments, Burlingame, CA). Membrane potential was kept at $-45$ mV by passing direct current. Single spikes were generated by current pulses of 5-msec duration. Spike durations were determined by measuring the time from peak of the spike to repolarization of the spike to 25% of its amplitude. Cell membrane excitability was measured by the average number of spikes elicited by 500-msec pulses at two current intensities, one that generated one spike and the other with twice that intensity.

The conventional two-electrode voltage-clamp technique was used. In both voltage- and current-clamp experiments the microelectrodes were filled with 0.5 M KCl (in a few experiments 0.5 M potassium acetate was used) and beveled to 8–12 MΩ. The cAMP agonist Sp-cAMP[S] and the antagonist Rp-cAMP[S] (BioLog, La Jolla, CA) were introduced intracellularly via the recording or stimulating electrode. Loading the cells with drugs was achieved by passive diffusion or injecting negative current. Stimulation pulses were repeated at a slow rate of one per min. Voltage and current responses were recorded on a tape recorder (Hewlett-Packard; 3964A) and later analyzed and displayed with a Prowler laboratory computer (Norland, Port Atkinson, WI).

RESULTS

**cAMP Produces Substantial Spike Broadening in the Tail Sensory Neurons of the Pleural Ganglion.** cAMP analogs vary greatly in their effectiveness, perhaps due to variations in their ability to penetrate the cell membrane (15). To overcome these difficulties, as well as to eliminate the consequence that bath application might have on other cells, we intracellularly injected Sp-cAMP[S], an analog resistant to phosphodiesterase. To obtain an initial 10- to 20-min control period, we filled the tip of the microelectrode with 1–2 μl of KCl (0.5 M) and only then added 1–3 μl of Sp-cAMP[S] (20–50 μM) in KCl (0.5 M). This made it possible to follow both excitability and spike duration for $\approx 20$ min before cAMP started to diffuse out of the pipette and exert its effect. We could facilitate this process by injecting a negative current.

Fig. 1 shows the effects of cAMP on spike duration and excitability as a function of the time after penetration of a sensory neuron with an electrode containing Sp-cAMP[S]. Spike duration and number of spikes generated by a current pulse of 1 nA and a duration of 500 msec were stable during the control period. After 20 min, cell excitability began to increase, as reflected by an increase in the number of spikes generated by the $\approx$ current pulses (Fig. 1B2). At 28 min (Fig. 1A2 and B3) excitability and spike duration reached their maximal values (seven-spike increase in excitability and a 27% increase in duration). As with 5-HT, the spike broadening produced by cAMP is evident as a delay in a second, slower, phase of spike repolarization (Figs. 1A2 and 2A1).

On average, Sp-cAMP[S] induced an increase in spike duration of 21.6% (Fig. 1C). This degree of broadening can account for almost the entire broadening detected after a 3-min exposure (Fig. 1A2) and 4.6% of the duration reached at 20 min (24.9% ± 5.7 SEM remains at 7%). Sp-cAMP[S] also induced an effect on excitability that matched that of 5-HT (Fig. 1C). Although the effect on excitability of Sp-cAMP[S] remained at this level for the rest of the record-
broadening of protein kinase A, we used the specific kinase A inhibitor Rp-cAMP[S]. In the experiments outlined in Fig. 2A, the sensory neuron was impaled with electrodes containing 0.5 M KCl and action potentials were generated by a current pulse of 5-msec duration. Adding 20 μM 5-HT resulted in typical spike broadening (Fig. 2A1) and anti-accommodation (Fig. 2A3). A sensory neuron in the second pleural ganglion was penetrated with microelectrodes containing, in addition to 0.5 M KCl, 80 mM Rp-cAMP[S]. This cAMP derivative competes with cAMP but does not activate kinase A (13). About 20 min after impaling this second cell and injecting Rp-cAMP[S] with negative current (the same protocol used in the control cell, Fig. 2A), we measured the response 3 min after application of 20 μM 5-HT. Now, the level of spike broadening was greatly reduced (Fig. 2B1), and the anti-accommodation effect of 5-HT was substantially blocked (Fig. 2B2 and B3).

The pooled data are summarized in Fig. 2C. In control experiments 5-HT caused a 19.8% ± 1.8 SEM increase in spike duration, whereas after Rp-cAMP[S] injection the average broadening was 11.3% ± 1.8 SEM, which is 57% of control. Rp-cAMP[S] was more effective in blocking the 5-HT-induced excitability change (43% ± 16.2 SEM as compared with 483%

± 25 SEM in control). These results suggest that (i) excitability is a more sensitive measure of blockade in cAMP-dependent currents than is spike broadening, and (ii) the blockade by Rp-cAMP[S] of the current modulation responsible for spike broadening is incomplete. As we shall show below, at least part of the incompleteness of the blockage of spike broadening is due to an incomplete blockade of the currents modulated by cAMP. Alternatively, an additional second-messenger system may be recruited that does not contribute to excitability but contributes selectively to spike broadening.

Voltage Clamping Reveals that 5-HT Modulates a Transient K⁺ Current in the Pleural Sensory Neurons. To examine which of the currents are modulated by 5-HT and how these currents are affected by cAMP, we next used voltage-clamp analysis. Confirming Baxter and Byrne, we found that 5-HT modulates two K⁺ currents in the pleural sensory neurons, IKₜ (2, 16) and IKᵥ (10). We distinguished IKᵥ from IKₜ by using two different voltage-command protocols. In both cases we first stepped the membrane potential from a holding potential of −50 mV to −70 mV for 10 sec before applying a 200-msec depolarizing step to one of two test potentials. To examine IKₜ alone, we stepped from −70 mV to −20 mV. To examine IKᵥ and IKₜ together, we stepped from −70 to +30 mV. To eliminate possible contribution of IKᵥ, we carried out these experiments in 2 mM tetraethylammonium, and 160 μM tetrodotoxin was used to block Na⁺ current.

As described by Baxter and Byrne (10), steps from −70 mV to +30 mV activated a transient outward current, which activated rapidly and inactivated slowly (Fig. 3A1). This current is blocked by both 4-aminopyridine (2 mM) and tetraethylammonium (100 mM) (data not shown). 5-HT caused an inward movement, or reduction, of the outward current at the beginning of the pulse, followed by an increase in outward current later in the pulse. This behavior can be characterized by a crossover of the current, at −15 msec, when the control current is compared to the current after exposure to 5-HT. Subtracting the current in the presence of 5-HT from the control current in the absence of 5-HT gives the net total current modulated by 5-HT (Fig. 3A1). Examination of this current indicates that 5-HT can only be effective in causing spike broadening during the first few msecs of a depolarizing pulse because it is only during this period that 5-HT induces an inward movement of the current. The later increase in the outward current can only decrease spike duration, but it is so late as not to be relevant for the duration of a single spike.

With steps from −70 mV to −20 mV (Fig. 3A2), 5-HT caused net reduction of outward current, with the maximal effect at the end of the pulse. The modulated current shown in the subtracted trace is slowly activated with no apparent inactivation. This behavior is typical of IKₜ.

To determine to what degree these several currents are modulated by cAMP, we measured the effects of the kinase A inhibitor, Rp-cAMP[S], on 5-HT modulation of IKₜ and IKᵥ (Fig. 3B). As expected from earlier work showing that IKₜ is modulated by cAMP, Rp-cAMP[S] blocked modulation of this current, as revealed by the voltage step from −70 to −20 mV (Fig. 3B2). The effect of Rp-cAMP[S] on current response to a voltage step from −70 to +30 mV is more complex (Fig. 3B1). Here, Rp-cAMP[S] completely blocks the inward movement of the early current that can contribute to spike broadening, much as it blocks IKₜ. By contrast, Rp-cAMP[S] did not appear to affect the increase in the delayed outward current, which cannot contribute to broadening.

5-HT caused maximal reduction in outward current at 5 msec (Fig. 4A). This inward movement of the current is equivalent to a 13% reduction in the average outward current (22.2 nA). In the presence of Rp-cAMP[S] (Fig. 4C), 5-HT causes only a 5.1% reduction (8.7 nA) of the total outward current and corresponds to 61% inhibition of the 5-HT modulation of the transient phase of IKᵥ. Comparing Fig. 4A

![Figure 2](image-url)
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**Fig. 3.** Voltage-clamp experiments reveal that \( R_p \)-cAMP[\( S \)] blocks the 5-HT modulation of both \( I_{kv} \) (A1 and B1) and a transient outward current \( I_{kv} \) (A2 and B2) and a transient outward current \( I_{kv} \) (A1 and B1). \( I_{kv} \) was activated by stepping the membrane potential from \(-70 \) mV to \(+30 \) mV for 200 msec (A1 and B1). \( I_{kv} \) was activated by voltage steps from \(-70 \) mV to \(-20 \) mV for the same period of time. The upper recordings depict superposition of the two current traces before and 3 min after application of 5-HT (40 \( \mu \)M). The lower trace is the subtraction of the 5-HT current from the control current and represents the total current modulated by 5-HT. Subtracted currents and currents for voltage steps to \(-20 \) mV were digitally smoothed. In B the sensory cell was loaded with \( R_p \)-cAMP[\( S \)]. Note that \( R_p \)-cAMP[\( S \)] blocked the inward movement of the currents both for voltage steps to \(+30 \) mV (compare A1 to B1) and to \(-20 \) mV (compare A2 to B2).

**DISCUSSION**

In examining the short-term effects of 5-HT on the tail sensory neurons of the pleural ganglia, Baxter and Byrne (10) found that 5-HT modulates not only \( I_{ks} \) described by Klein et al. (2) but also modulates \( I_{kv} \). Our results confirm Baxter and Byrne (10) in showing that 5-HT modulates \( I_{kv} \). However, we find, in addition, that the reduction in the early phase of this outward current is modulated by cAMP. Because cAMP modulates both types of outward current reduced by 5-HT (the early phase of \( I_{kv} \) as well as of \( I_{ks} \)), one would predict that cAMP could simulate the broadening produced by 5-HT. Indeed, both we and Goldsmith and Abrams (17) have found that cAMP produces broadening comparable to 5-HT and that inhibitors of kinase A or of adenyl cyclase (18) reduce substantially the 5-HT-induced spike broadening. However, the cAMP injection experiments also point to a potentially unusual finding. Although \( S_p \)-cAMP[\( S \)] can induce large spike broadening similar to that of 5-HT, its maximal effect persists only for \( 5 \) to \( 10 \) min. Therefore, its effect on spike duration begins to decay slowly over the next \( 10 \) to \( 20 \) min, even though the excitability and, therefore, presumably the level of cAMP, is maintained. At these later times, \( S_p \)-cAMP[\( S \)] does not fully obscure the 5-HT effect on spike broadening. These results may indicate that after \( 10 \) to \( 20 \) min of continuous application of 5-HT, cAMP may contribute less to spike broadening than it does during the first 5 to 10 min (see also ref. 13).

Why \( R_p \)-cAMP[\( S \)] is more powerful in blocking the antiaccommodation effect of 5-HT than \( S_p \)-cAMP[\( S \)] is in blocking spike broadening is unclear. This difference may suggest that cAMP is more important in the modulation of excitability and that spike broadening requires, in addition to cAMP, yet another second-messenger system, even at early stages. However, a more reasonable explanation is that \( R_p \)-cAMP[\( S \)] blockade is incomplete. \( R_p \)-cAMP[\( S \)] blocks only 66% of \( I_{ks} \); yet this current is fully dependent on cAMP. The incomplete blockade of the inward modulation of \( I_{kv} \) by \( R_p \)-cAMP[\( S \)], therefore, is consistent with and parallel to the incomplete block of \( I_{ks} \) and suggests that the reason \( R_p \)-cAMP[\( S \)] only blocks \( \approx 50 \)% of the 5-HT-induced spike broadening may be due to the fact that it does not fully block the effects of the rise in cAMP produced by 5-HT. In fact, the affinity of \( R_p \)-cAMP[\( S \)] to kinase A (\( K_i \) \( \approx 10 \) \( \mu \)M) is 10-fold lower than cAMP (13), and therefore a relatively high concentration of \( R_p \)-cAMP[\( S \)] needs to be reached to achieve a complete block. Indeed, in dissociated culture we have found that \( R_p \)-cAMP[\( S \)] blocks 70% of spike broadening in the pleural sensory neurons (12). Moreover, Goldsmith and Abrams (17) find that the Walsh inhibitor peptide, a noncompetitive and perhaps more effective inhibitor of protein kinase A than \( R_p \)-cAMP[\( S \)], blocks spike broadening by 75%, in the ganglion. It therefore seems more likely that the differential effect of \( R_p \)-cAMP[\( S \)] results from a nonlinear relationship between current modulation and the excitable properties of the cell, so that the effects on excitability are more profound than those on spike broadening (17).

Our results, in turn, raise new questions. Is \( I_{kv} \) a single current related to the delayed rectifier \( I_{kv} \) described by Baxter and Byrne, or a composite of two different currents? Which components of the current contribute to spike broadening? The kinetics of the current and the finding that \( R_p \)-cAMP[\( S \)] reduces the modulation by 5-HT of the early inward movement but not the late outward movement suggests that \( I_{kv} \) may consist of two currents. The early and transient component appears similar to the A-type current, \( I_{k dep} \), described by Furukawa et al. (19). This component is modulated by cAMP, and it is only this component of the current that has the direction (inward movement) and early time course (0–20 ms) to contribute to the duration of the action potential. The second, slower component is similar to a delayed rectifier. This component is not modulated by cAMP and is too late to contribute to spike broadening. Alternatively, \( I_{kv} \) may be a single complex current, and cAMP may simply modulate its activation kinetics without affecting its inactivation.

Based on these findings we propose the following scheme for 5-HT-induced spike broadening. 5-HT causes rapid elevation in free cAMP level (ref. 20; unpublished data), which leads to closure of \( I_{ks} \) and reduction in the transient phase of \( I_{kv} \). The modulation of these two currents by cAMP contributes to the spike broadening that develops rapidly after 5-HT application and induces short-term facilitation of transmitter release in nondepressed synapses. This suggestion is consistent with the findings of Braha et al. (21) and Ghirardi et al. (12) that in nondepressed synapses, when cAMP is increased by the main mechanism for facilitation (6), blockade of protein kinase A blocks completely short-term facilitation produced by one 5-min pulse of 5-HT, whereas blockade of protein kinase C has no effect on either facilitation or spike broadening at this early
time point. By contrast, the late slowly developed spike broadening may involve an additional second-messenger system. As suggested by Ghirardi et al. (12) and Sugita et al. (14), the protein kinase C system might become relatively more important when the sensory neurons are exposed to exogenous 5-HT for long periods of time. According to this view, the action of protein kinase C may be recruited under two circumstances: (i) for mobilization of transmitter when the synapse become depressed (5, 21); and (ii) for broadening when 5-HT is present continuously for many minutes (7, 14).