

Enhancement of synaptic transmission by cyclic AMP modulation of presynaptic I_h channels

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Presynaptic activation of adenylyl cyclase and subsequent generation of cAMP represent an important mechanism in the modulation of synaptic transmission. In many cases, short- to medium-term modulation of synaptic strength by cAMP is due to activation of protein kinase A and subsequent covalent modification of presynaptic ion channels or synaptic proteins. Here we show that presynaptic cAMP generation via serotonin receptor activation directly modulated hyperpolarization-activated cation channels (I_h channels) in axons. This modulation of I_h produced an increase in synaptic strength that could not be explained solely by depolarization of the presynaptic membrane. These studies identify a mechanism by which cAMP and I_h regulate synaptic plasticity.

Serotonin is an important neuromodulator of synaptic transmission in many phyla^{1–7}. In crustaceans, serotonin acts as a neurohormone; on binding to a G_s -coupled receptor⁸, it strongly enhances neuromuscular transmission by increasing the number of quanta released per action potential^{9–11}. Studies on both the inhibitor (GABAergic) and excitor (glutamatergic) nerve innervating the muscle have implicated two distinct processes: serotonin increases the absolute number of vesicles available for transmitter release¹², and serotonin increases the kinetics of release from the inhibitor nerve¹³.

Serotonin alters neither resting presynaptic $[Ca^{2+}]_i$ nor calcium influx during an action potential in crayfish nerve terminals^{13,14}. Its actions seem to be mediated by at least two second-messenger systems, one involving phospholipase C (PLC) and another involving adenylyl cyclase^{8,15}. Serotonin enhancement of transmission is blocked by presynaptic injection of a selective PLC inhibitor⁸. The downstream messenger and its target that mediate this enhancement are uncertain, but this pathway may involve cAMP. Compounds increasing $[cAMP]_i$ can mimic and potentiate serotonin action^{15,16}, and serotonin increases cAMP levels when added to lobster neuromuscular preparations, although a pre- or postsynaptic locus for cAMP production was undetermined¹⁷. In addition, presynaptic injection of an adenylyl cyclase inhibitor reduces serotonin enhancement¹⁵.

Here we show that cAMP generation is an important component of serotonin enhancement of synaptic transmission, and furthermore, that the target for cAMP is presynaptically located I_h channels.

RESULTS

Serotonin-induced enhancement involves cAMP

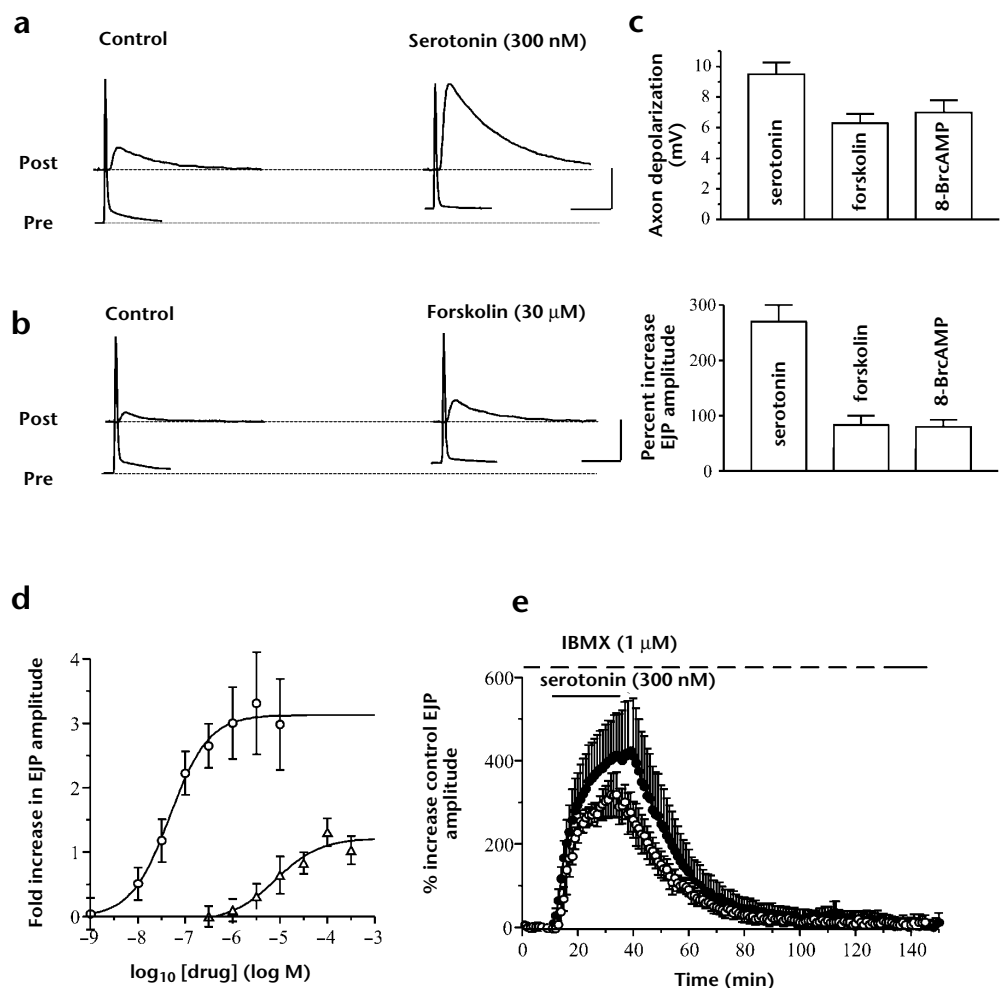
We recorded excitatory junction potentials (EJPs) from crayfish muscle cells at the neuromuscular junction while stimulating innervating glutamatergic axons. Superfusion of serotonin (300 nM, 25 min) resulted in a maximal increase of 310% in EJP

amplitude (Fig. 1a) with an EC_{50} of 60 ± 19 nM and a Hill coefficient of 1.14 ± 0.22 ($n = 4$; Fig. 1d). Simultaneous intracellular recording of axon membrane potential revealed an associated depolarization of the resting potential by ~ 10 mV (Fig. 1a and c) and a slight reduction in action potential amplitude and the area beneath its voltage trace, consistent with previous reports^{8,15}. Forskolin, an activator of adenylyl cyclase, partially mimicked the effects of serotonin (Fig. 1b and c), maximally increasing EJP amplitude by 120% (EC_{50} , 19 ± 10 μ M; Hill slope, 1.09 ± 0.26 ; $n = 3$), and depolarizing the axon membrane by ~ 7 mV. Amplitude of the action potential and area beneath its trace were also slightly reduced. The membrane-permeable cAMP analog 8-Br-cAMP (300 μ M) mimicked the effect of forskolin, enhancing EJP amplitude by $80 \pm 12\%$ ($n = 6$).

We next sought to determine the role of endogenous cAMP generation in serotonin-induced synaptic enhancement. The phosphodiesterase inhibitor IBMX prevents breakdown of cAMP, so we would expect it to potentiate serotonin enhancement if cAMP were generated after serotonin receptor activation. Application of a maximal concentration of serotonin (300 nM) increased EJP amplitude by $304 \pm 46\%$. After washout (2 h) to allow EJP amplitude to return to pre-drug levels, subsequent incubation in a low concentration of IBMX (1 μ M) increased EJP amplitude by $30 \pm 19\%$. When this EJP amplitude was taken as the new baseline, application of serotonin in the continued presence of IBMX potentiated EJP enhancement ($457 \pm 122\%$ increase in EJP amplitude, $n = 5$, $p < 0.1$, Fig. 1e). A second application of serotonin in the absence of IBMX produced no increase in EJP amplitude over that seen during the first application ($p > 0.1$, $n = 4$).

As IBMX is also a nonselective adenosine-receptor antagonist, we investigated whether the observed potentiation might be due to inhibition of adenosine receptors rather than inhibition of phosphodiesterase. At a concentration 100-fold higher than that used for IBMX, application of the nonselective adenosine receptor antagonist, 8-(p-sulphophenyl)-theophylline (8-PST, 100 μ M; $n = 5$), similarly increased basal EJP amplitude by $43 \pm 16\%$

Fig. 1. Serotonin and forskolin enhance synaptic transmission. (a, b) Intracellular recording of axonal action potentials (APs, Pre) and muscle excitatory junction potentials (EJPs; Post) before (control) and after application of serotonin (300 nM, 25 min; a) and forskolin (30 μ M, 25 min; b). Each trace is the average of all EJPs/APs recorded for 1 min at 2-Hz stimulation. Scale bars, 25 ms and 1 mV (Post) or 30 mV (Pre). (c) Bar charts summarizing the axonal depolarization (top) and enhancement of EJP amplitude (bottom) induced by serotonin (300 nM), forskolin (30 μ M) or 8-Br cAMP (300 μ M); $n = 4$ –12. (d) Cumulative concentration–response curves for serotonin (\circ) and forskolin (Δ). The lower maximal increase in EJP amplitude with forskolin than with serotonin demonstrates that adenylyl cyclase activation cannot entirely account for serotonin-induced enhancement. Each point represents the mean \pm s.e. of four experiments. (e) Time course of serotonin (300 nM)-induced enhancement of EJP amplitude (\circ). After EJPs returned to control amplitudes following washout of serotonin, each preparation was incubated for 25 min with a low concentration of the phosphodiesterase inhibitor IBMX (1 μ M). IBMX alone resulted in a small enhancement of EJP amplitude (normalized to control in graph). A second serotonin application was potentiated in the presence of IBMX ($n = 5$, \bullet). This effect might be explained by increased cAMP generation over control levels in response to serotonin application.



(IBMX response, $30 \pm 19\%$, $n = 5$). However, in contrast to IBMX, 8-PST did not potentiate the serotonin (300 nM) response; the increase in EJP amplitude induced by serotonin in the presence of 8-PST was not different from that elicited by serotonin in the absence of 8-PST ($3 \pm 11\%$ difference; $p > 0.05$, $n = 5$). Therefore, IBMX potentiation of the serotonin-induced enhancement seems to result from phosphodiesterase inhibition, although the effect of IBMX alone on EJP amplitude may be due to a nonspecific action, or even to inhibition of adenosine receptors.

Serotonin and forskolin act presynaptically

The locus of action of both serotonin and forskolin (and hence cAMP generation) was examined using analysis of miniature EJPs (mEJPs). In Normal Van Harrevald's solution containing TTX (1 μ M), the mean frequency of mEJPs recorded over a 30-minute period was 0.31 ± 0.1 Hz with a mean amplitude of 254 ± 36 μ V ($n = 12$). After incubation for 20 minutes in either serotonin (1 μ M) or forskolin (30 μ M), the same muscle fiber was recorded for 30 minutes in the presence of the drug. In 5 of 6 paired experiments, serotonin induced a significant increase in frequency ($p < 0.05$, Kolmogorov-Smirnov test) but not amplitude of mEJPs (Fig. 2a and b), with a mean increase in frequency of $64 \pm 11\%$

(Fig. 2b; $p < 0.05$, Student's t -test). Forskolin significantly increased frequency in 3 of 6 experiments (Kolmogorov-Smirnov test, $p < 0.05$) with a mean increase in frequency of $42 \pm 33\%$ and no increase in amplitude of mEJPs ($p > 0.1$, Student's t -test, Fig. 2b). Consistent with serotonin's enhancement of the size of the vesicle pool¹², this finding supports the assumption that the locus of action of both serotonin and forskolin is presynaptic.

PKA is not involved

The downstream effects of cAMP at the crayfish neuromuscular junction have previously been attributed to activation of PKA¹⁵. We tested the effects of two membrane-permeable inhibitors of PKA on the enhancement induced by serotonin and 8-Br-cAMP. A submaximal concentration of serotonin (100 nM, 25 min) elicited an increase in EJP amplitude of $144 \pm 24\%$. In the presence of 30 μ M H-7, which should fully inhibit PKA, PKC and PKG ($K_i = 3$ –6 μ M^{18,19}), a second application of serotonin resulted in an amplitude increase no different from the control response ($148 \pm 33\%$, $n = 4$, $p > 0.05$). H-7 was similarly ineffective against the increase induced by 8-Br-cAMP (8-Br-cAMP alone, $74 \pm 23\%$; 8-Br-cAMP plus H-7, $95 \pm 38\%$; $n = 3$; $p > 0.05$). However, H-7 (30 μ M) prevents an unrelated

presynaptic phorbol ester-induced potentiation of transmission at the crayfish neuromuscular junction (V.B. and R.S.Z., unpublished observation), demonstrating that this compound permeates the axon sufficiently to inhibit PKC.

In addition to H-7, we tested the highly specific, cell-permeable PKA inhibitor *Rp*-8-Br-cAMPS. Incubation with *Rp*-8-Br-cAMPS (300 μ M) alone caused a $40 \pm 13\%$ increase in EJP amplitude, suggesting that this cAMP analog may mimic cAMP itself. When serotonin was also added, EJP amplitude increased by $292 \pm 68\%$ of pre-drug amplitude, not significantly different from the paired response with serotonin alone ($324 \pm 125\%$, $n = 3$; Fig. 3a). Similar effects of *Rp*-8-Br-cAMPS were found against 8-Br-cAMP-induced increases in EJP amplitude (Fig. 3b). Because *Rp*-8-Br-cAMPS is a cAMP analog, and it increased EJP amplitude significantly, these results suggest that the target of cAMP may be a cyclic nucleotide-binding effector other than PKA.

Presynaptic I_h channels are modulated by cAMP

Depolarization of the axon by forskolin, 8-Br-cAMP and serotonin (Fig. 1a–c) suggests that presynaptic I_h channels may be targets for cAMP. Indeed, modulation of I_h channels by the direct binding of cyclic nucleotides (including *Rp*-8-Br-cAMPS²⁰) and subsequent

shifts of the activation curve to more depolarized potentials^{20–25} would explain depolarization on addition of cAMP analogs^{8,15}.

The presence of I_h channels in the crayfish excitor axon was investigated by intracellular injection of hyperpolarizing current pulses (500 ms, -5 to -50 nA) into the axon. Upon hyperpolarization of the membrane beyond resting potential, I_h should become activated, typically producing a 'depolarization sag' back toward the resting potential, whereas termination of the pulse results in a transient depolarizing overshoot of the original resting potential (after-depolarization potential; ADP)^{25,26}. ADPs were produced by injecting current pulses of different amplitudes (Fig. 4). With large current injections, the ADP was of sufficient amplitude to initiate firing of action potentials, as demonstrated for I_h channels in many neurons²⁵. When the I_h blocker Cs^+ (1 mM) was applied to the preparation, resting membrane potential hyperpolarized by approximately 4 mV, and ADP amplitude was significantly reduced (Fig. 4a). Injection of between -5 and -50 nA of current elicited ADPs (Fig. 4b; $n = 6$) that were blocked by Cs^+ ($n = 3$) or the specific I_h blocker ZD7288 (ref. 27; $n = 3$). These data not only demonstrate the presence of I_h channels in excitor axons, but also suggest that these channels contribute to the axon's resting potential. However, it should be noted that,

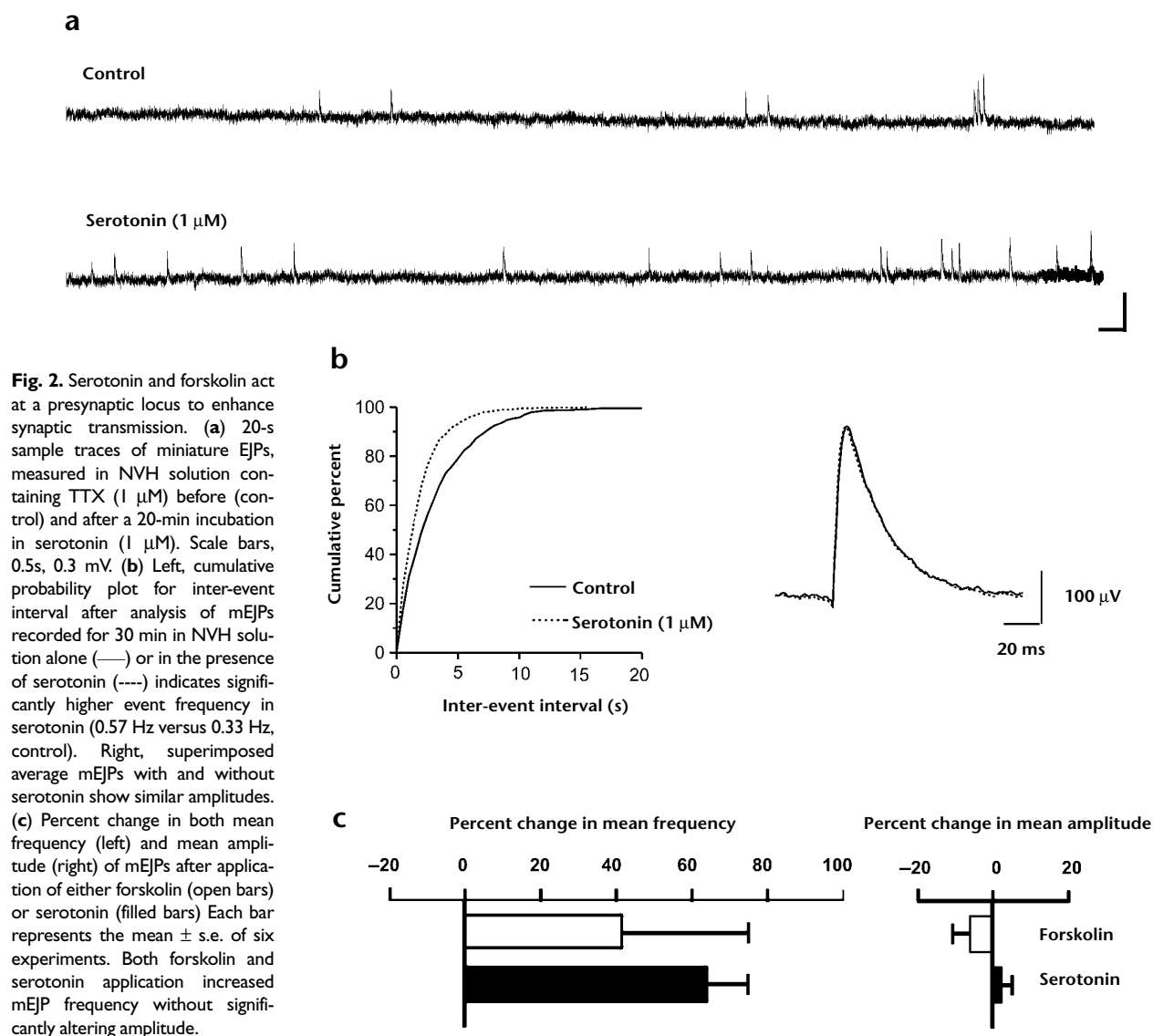
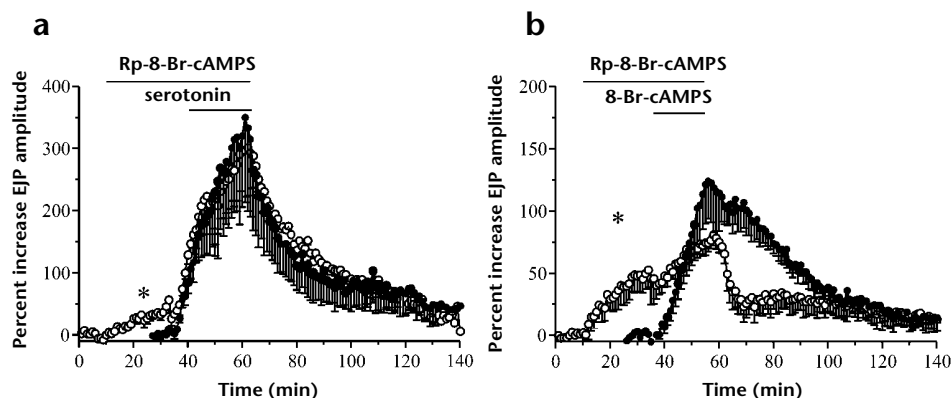


Fig. 3. Rp-8Br-cAMPS elevates EJP amplitude and does not significantly block serotonin enhancement of transmission. (a) Time course of the increase of EJP amplitude in response to serotonin (100 nM; ●). After serotonin was washed out and EJP amplitude reached a steady baseline, preparations were incubated with the membrane-permeable specific PKA inhibitor Rp-8Br-cAMPS (300 μ M). Surprisingly, this incubation enhanced EJP amplitude (○,*) by approximately 50% over control. Serotonin-induced enhancement was affected little by the PKA inhibitor (○).

(b) Time course of the increase of EJP amplitude by the membrane-permeable cAMP analog 8-Br-cAMP (●, 300 μ M). After washout of 8-Br-cAMP, EJP amplitude reached a steady baseline, and preparations were incubated with Rp-8Br-cAMPS (300 μ M). Again, this incubation increased EJP amplitude $48 \pm 4\%$ over control (○,*) Addition of 8-Br-cAMP together with Rp-8Br-cAMPS resulted in a slightly smaller increase in EJP amplitude ($78 \pm 7\%$; ○) than 8-Br-cAMP applied alone ($120 \pm 30\%$; ●).



whereas ADPs elicited by current injection up to and including -30 nA could be blocked effectively, ADPs elicited by larger current injections were not completely blocked by either Cs^+ or ZD7288 (Fig. 4a and b), suggesting that ADPs evoked by injection of more than -30 nA may involve conductances other than I_h channels.

To determine whether I_h channels were modulated by cAMP, we attempted to block the serotonin- and forskolin-induced depolarization of the axon (Fig. 1a and b) using Cs^+ and ZD7288. Application of serotonin (300 nM) resulted in a maximum axon depolarization of 9.5 ± 0.75 mV from a mean resting membrane potential of -71 ± 1 mV ($n = 12$; Fig. 5a) within 16 minutes of serotonin application. Addition of Cs^+ (3 μ M–3 mM) reversed this depolarization in a concentration-dependent manner (IC_{50} , 260 ± 20 μ M; Hill coefficient, 1.26 ± 0.05 ; $n = 5$, Fig. 5b). A slight broadening of the action potential and an increase in area beneath the voltage trace was observed in Cs^+ , consistent with additional block of a potassium conductance. However, addition of the potassium-channel blocker Ba^{2+} (1 mM) to the nerve reproduced the

effects on the action potential seen with Cs^+ , but did not affect serotonin-induced depolarization (Fig. 5a). ZD7288 (100 nM–100 μ M; 25 min) potently reduced serotonin-induced depolarization of the axon, with an IC_{50} similar to that for its action on I_h channels^{27,28} (IC_{50} , 6 ± 2 μ M; Hill coefficient, 1.19 ± 0.01 ; $n = 4$, Fig. 5b).

Serotonin seemed to alter I_h activity through cAMP generation, as application of forskolin (30 μ M) also produced axon depolarization of 6.3 ± 0.6 mV from a mean resting potential of -71 ± 1.4 mV ($n = 8$, Fig. 5c and d). The significantly smaller size of the depolarization with forskolin than with serotonin (~ 10 mV) probably resulted from the barely submaximal concentration of forskolin used (Fig. 1d). Incubation with Cs^+ (1 mM, $n = 4$) or ZD7288 (30 μ M, $n = 4$) abolished the depolarization induced by a second application of forskolin (Fig. 5c and d). In summary, we demonstrated that cAMP generated in the axon acts on I_h , resulting in depolarization with a time course paralleling that of synaptic enhancement.

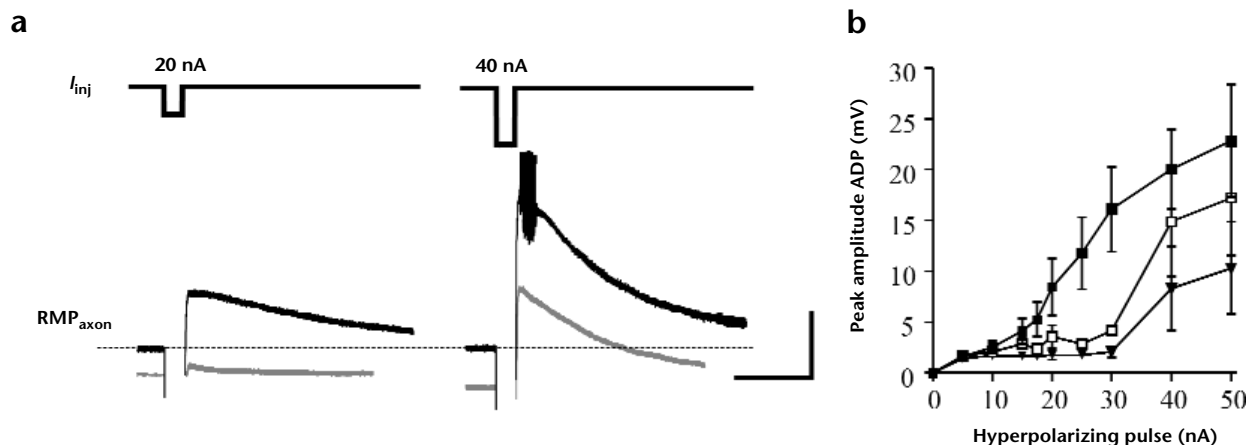


Fig. 4. I_h channels are involved in maintenance of resting membrane potential, and I_h activation results in anomalous membrane rectification in excitatory axons. (a) Injection of axons with hyperpolarizing current (500 ms) resulted in after-depolarizing potentials (ADPs; traces in black). Resting membrane potential of the axon (dashed line) was -66 mV. Membrane responses during the pulse were truncated. With large hyperpolarizing pulses (typically ≥ 40 nA), the ADP was sufficient to reach threshold for action potential generation (APs truncated in trace). Scale bar, 2 s, 10 mV. In the same axon, resting membrane potential became relatively hyperpolarized in the I_h channel blocker Cs^+ (1 mM; compare gray traces with dashed line), and ADP amplitudes in response to hyperpolarizing pulses were reduced (gray traces). (b) Plots of peak ADP amplitude versus size of injected hyperpolarizing current in controls (■, $n = 6$), with Cs^{2+} (□, 1 mM, $n = 3$) or with ZD7288 (▼, 30 μ M, $n = 3$).

Modulation of I_h by cAMP results in synaptic enhancement

To investigate the involvement of I_h modulation in synaptic enhancement, we studied the increase of EJP amplitude by serotonin, forskolin and 8-Br-cAMP in the absence and presence of ZD7288 (30 μ M) or Cs^+ (1 mM), with Ba^{2+} (1 mM) as a control.

In the absence of any other drug, serotonin (100 nM) caused a 2.9 ± 0.3 -fold increase in EJP amplitude (control response; $n = 13$). Two hours later, recordings from the same muscle cells incubated in Cs^+ (1 mM) 10 minutes before and during a second serotonin application showed a $43 \pm 7\%$ reduction compared with control responses to serotonin (1.8 ± 0.2 -fold increase in EJP amplitude; $p < 0.05$, $n = 6$), whereas application of 1 mM Ba^{2+} before and during a third serotonin application insignificantly increased EJP amplitude $17 \pm 13\%$ over controls, consistent with the slight widening of the action potential we observed ($n = 6$; Fig. 6a and b). A 25-minute application of ZD7288 before and during serotonin application was as effective as Cs^+ in reducing synaptic enhancement, decreasing the response to serotonin by $42 \pm 13\%$ from the control ($n = 7$, $p < 0.05$; Fig. 6a). Neither Cs^+ nor ZD7288 applied on its own reduced basal EJP amplitude ($n = 4$ each).

Both forskolin's (30 μ M) and 8-Br-cAMP's (300 μ M) enhancement of EJP amplitude (1.4 ± 0.2 -fold increase, $n = 9$ and 0.8 ± 0.1 -fold increase, $n = 6$, respectively) were markedly

blocked in the presence of ZD7288 (30 μ M), which decreased synaptic enhancement by $67 \pm 6\%$ (Fig. 6c) and $70 \pm 18\%$ (Fig. 6d) relative to the respective control responses. Enhancement of EJP amplitude by 8-Br-cAMP was blocked by Cs^+ equally well ($82 \pm 2\%$ reduction of the control response, $n = 3$). However, Cs^+ was less effective in blocking forskolin enhancement, reducing the response to only $31 \pm 15\%$ of the control response ($n = 3$). Although the reason for this is not obvious, Cs^+ blockade of a potassium conductance in these cells may have induced a depolarization that counteracted the effect of blocking I_h to a greater extent than observed in other experiments.

It is conceivable that the reduction of the serotonin and forskolin response by ZD7288 and Cs^+ ions may have been a result only of the membrane hyperpolarization produced by these agents, rather than by a specific block of an I_h conductance. We felt this was unlikely, as hyperpolarizing the axon by ~ 5 mV by manipulating extracellular potassium has no effect on either serotonin-induced axonal depolarization or serotonin-induced synaptic enhancement^{14,29}. Nevertheless, we eliminated hyperpolarization of the axon in the presence of ZD7288 (30 μ M) by slightly elevating extracellular potassium concentration to 6.75–7.0 mM (Fig. 7a). Even with the offset of hyperpolarization afforded by increased $[\text{K}^+]_e$,

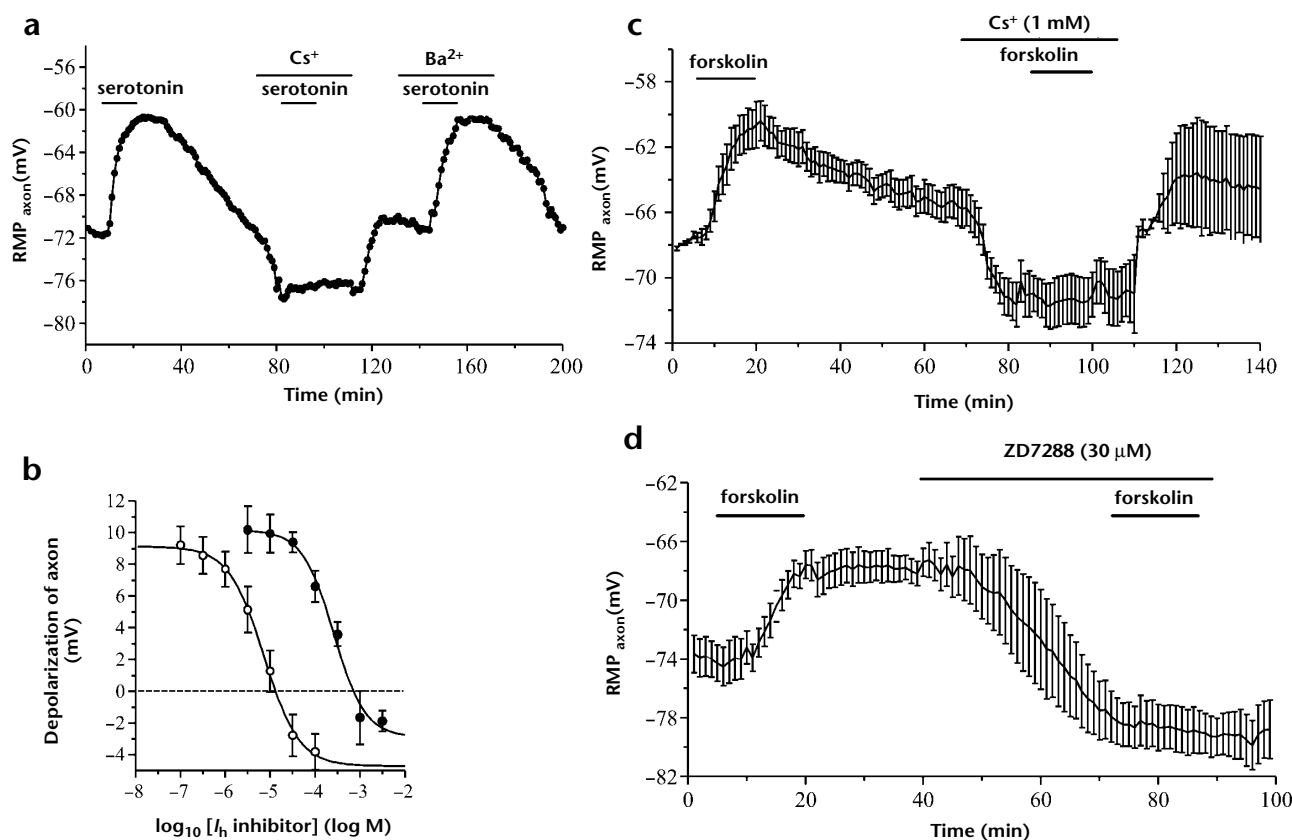


Fig. 5. I_h modulation by cAMP generation results in axon depolarization. (a) Application of serotonin (300 nM) to axons resulted in an approximately 10-mV membrane depolarization ($n = 5$, \bullet). After washout of serotonin, membrane depolarization slowly reversed to pre-serotonin levels. Application of Cs^+ (1 mM) to the axon resulted in a rapid membrane hyperpolarization of approximately 5 mV. Subsequent application of serotonin in the presence of Cs^+ was ineffective in eliciting a membrane depolarization. After washout of Cs^+ , incubation with Ba^{2+} (1 mM) affected neither resting membrane potential nor subsequent depolarization in response to serotonin ($n = 4$; typical s.e., ± 1.5 mV). (b) Cumulative concentration-inhibition curves for Cs^+ (\bullet ; $n = 3$ separate experiments) and the irreversible I_h blocker ZD7288 (\circ ; $n = 4$) against axon depolarization induced by serotonin (300 nM). Each point represents the mean \pm s.e. (c, d) Forskolin (30 μ M) mimics serotonin at the axon, suggesting that cAMP generation is responsible for increased I_h activation and resulting depolarization. The effects of forskolin can be blocked by either Cs^{2+} (c; 1 mM) or ZD7288 (d; 30 μ M).

forskolin-induced synaptic enhancement after ZD7288 treatment was reduced to the same extent as without elevating $[K^+]_e$ ($68 \pm 23\%$ reduction, $n = 3$, Fig. 7b).

In summary, pooled results from the above experiments show that I_h activation by cAMP was responsible for $62 \pm 7\%$ ($n = 18$) of the cAMP-induced synaptic enhancement, and activation of I_h via cAMP generation accounted for $43 \pm 6\%$ ($n = 13$) of the serotonin-induced enhancement of synaptic strength.

Axonal hyperpolarization enhances synaptic transmission

Our results thus far implicated cAMP modulation of I_h channels as a vital component of the increase in synaptic strength observed on addition of either serotonin or forskolin. We extended this observation to see whether voltage-gated activation of I_h channels *per se* was sufficient to increase synaptic transmission at the crayfish neuromuscular junction. First, the fluorescent dye Fura-2 (17 mM in 200 mM KCl) was iontophoresed (-10 nA, 10 min) into the axon via a microelectrode. Diffusion of the dye along the length of the axon allowed visualization of small tertiary branches and boutons innervating individual muscle fibers. Using a new intracellular electrode (3 M KCl), we reimpaled the axon within

several hundred microns of boutons innervating a muscle fiber, which was also impaled with a microelectrode. We then simultaneously recorded both evoked action potentials (2 Hz) in the excitor nerve and subsequent EJPs in the muscle (Fig. 8a). After 10 minutes, stimulation was stopped and the axon hyperpolarized by injection of -30 nA for 1 minute via the intracellular electrode, a protocol that elicits a large ADP, but is not sufficient to induce spontaneous firing of action potentials (Fig. 4). The increased time of current injection used in this experiment (1 min versus 0.5 s in Fig. 4) did not significantly affect the amplitude of the ADP (13 ± 6 mV after 1-min and 16 ± 4 mV after 0.5-s current injection) or its sensitivity to ZD7288 ($79 \pm 21\%$ block and $87 \pm 18\%$ block, respectively; $n = 3$), suggesting that this prolonged current protocol elicited an ADP indicative mainly of I_h channel activation. During axon hyperpolarization, we often noted an increase in the number of spontaneous EJPs (Fig. 8a) which was not observed previously using methods that polarize large axonal branches³⁰. If no increase in spontaneous events was detected during hyperpolarization, it was assumed that the hyperpolarizing electrode was too far from boutons to elicit a response in the recorded muscle fiber, and the experiment was discounted (4 of

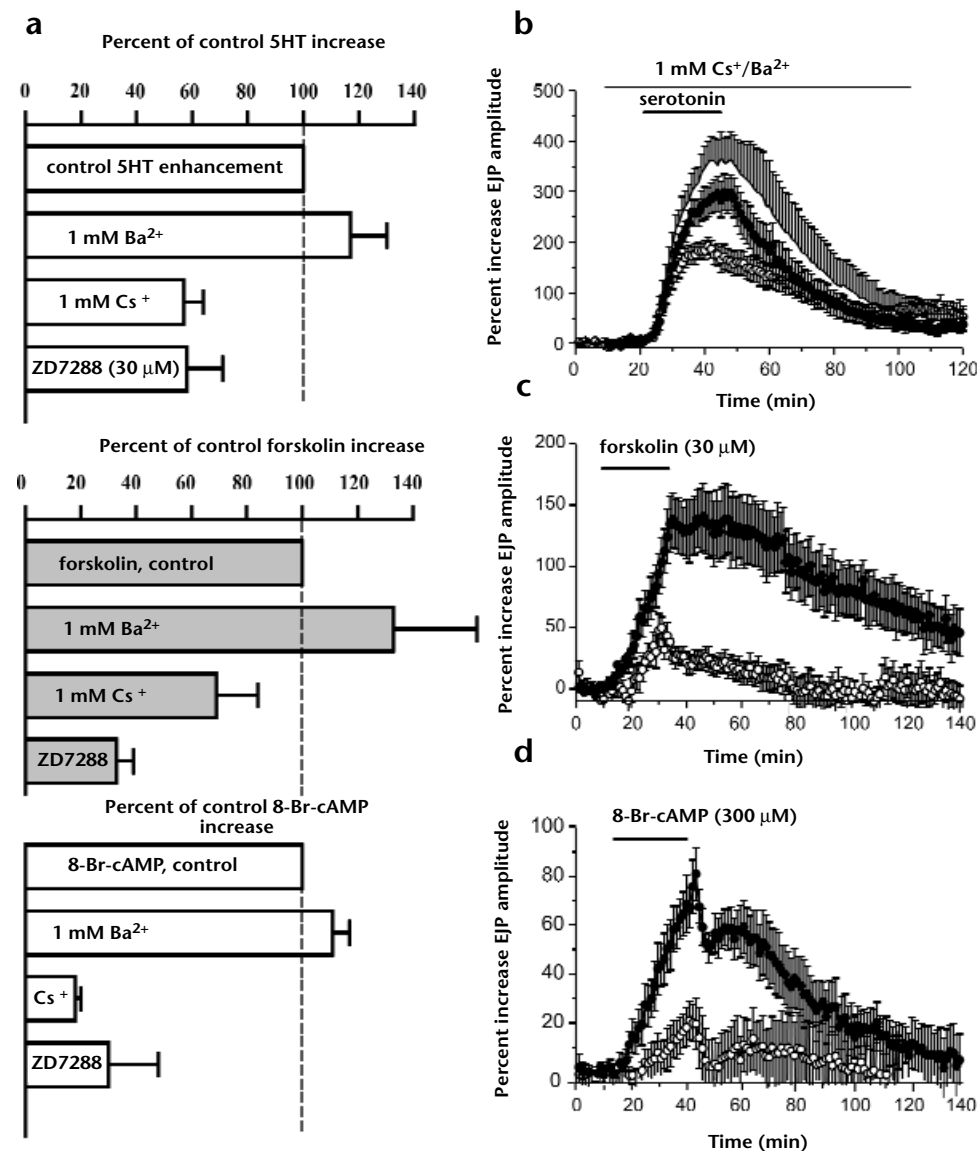


Fig. 6. Inhibition of I_h channels resulted in a reduction of synaptic enhancement by serotonin, forskolin and 8-Br-cAMP. (a) Effects of Ba²⁺ (1 mM) and the I_h blockers Cs⁺ (1 mM) or ZD7288 (30 μM) on EJP amplitude enhancement by serotonin (100 nM; upper), forskolin (30 μM; middle, gray bars) or 8-Br cAMP (300 μM; lower). To allow visual comparison of the percent inhibition of synaptic enhancement by either Cs⁺ or ZD7288, enhancements with serotonin, forskolin or 8-Br-cAMP alone (control enhancement) were normalized to 100%. (b–d) Data from which bar charts were generated. (b) Time course of the percent increase in EJP amplitude with serotonin alone (100 nM; ●) or after concurrent incubation with Cs⁺ (1 mM; ○) or Ba²⁺ (1 mM, —). (c, d) Percent increase in EJP amplitude during application of forskolin (c; ●) or 8-Br-cAMP (d; ●) alone or after a 30-min pre-incubation with ZD7288 (30 μM, ○). Each point represents the mean \pm s.e. of 4–7 different experiments.

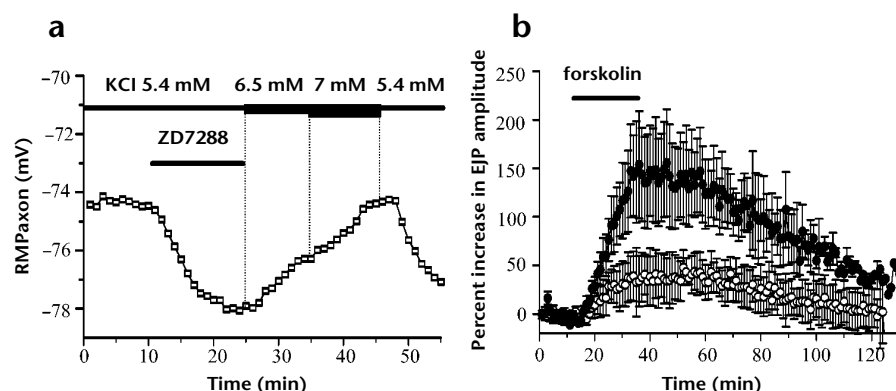


Fig. 7. Reversal of the ZD7288-mediated axonal hyperpolarization does not affect ZD7288 block of forskolin-induced synaptic enhancement. (a) The axon was impaled, and ZD7288 (30 μM) was applied after determining the postsynaptic control response to forskolin (b, ●). Resulting presynaptic membrane hyperpolarization was then offset by stepwise increases in extracellular potassium to return the axon to its original resting potential. The recovery of hyperpolarization when the $[K^+]_o$ was returned to normal (5.4 mM) demonstrates the irreversibility of I_h block by ZD7288. (b) After determining the postsynaptic control response to forskolin (●), ZD7288 was applied and the resulting hyperpolarization of the axon membrane potential offset back to the original resting potential (see a) before recording the response to forskolin from a different muscle fiber in the same preparation (○, $n = 3$). ZD7288 still reduced forskolin-induced synaptic enhancement under these conditions.

sistent with the notion that EJP enhancement is a result of hyperpolarization *per se*. Indeed, if hyperpolarization independent of I_h activation mediated the enhancement of EJP amplitude, one would expect a greater increase after ZD7288 application, where blockade of I_h conductance would increase the hyperpolarization induced by current injection. We therefore conclude that a relatively brief I_h channel activation, independent of cAMP modulation, is also capable of eliciting a more prolonged synaptic enhancement.

DISCUSSION

Neuronal I_h channels subserve fundamental physiological functions such as contribution to membrane potential^{31–34}, integration of synaptic input to neurons³⁵ and the rhythmicity of various brain regions^{36–39}. Binding of cAMP to I_h results in a concentration-dependent shift of the

12). After axon hyperpolarization, action potentials and subsequent EJPs were again evoked at two Hz. EJP amplitude significantly increased following hyperpolarization, increasing an average of $54 \pm 11\%$ by 10–20 minutes after current injection (Fig. 8a and b; $p < 0.05$, $n = 8$).

In separate experiments, ZD7288 (30 μM) was applied for 30 minutes before and during the -30 nA hyperpolarization of the axon (Fig. 8b). Again, individual experiments were abandoned if spontaneous release was not seen during the hyperpolarizing pulse, although this was rare (1 of 7) in the presence of ZD7288. The increase in spontaneous release observed during hyperpolarization with ZD7288 probably indicated that hyperpolarizing current pulses applied during I_h block resulted in greater changes in potential compared with those in control trials. EJP amplitude sometimes increased slightly (average $11 \pm 8\%$) and transiently between 1 and 5 minutes after the termination of the pulse, correlating positively with the level of spontaneous activity during the hyperpolarization, although this was not quantified further. However, ZD7288 completely prevented the enhancement of EJP amplitude between 10 and 20 minutes after current injection ($2 \pm 5\%$ reduction from basal amplitude, $n = 6$, significantly different from the control trials; $p < 0.001$, Student's unpaired t -test).

It should be noted that, although this experiment is unavoidably compromised by the probable difference in axon membrane potential change elicited by the current pulse with or without I_h channel block, the prevention of any increase by ZD7288 is incon-

activation curve to more depolarized voltages through a phosphorylation-independent allosteric interaction with the channel^{40,41}. Thus receptors both positively and negatively coupled to adenylyl cyclase can shift I_h activation by up to ± 20 mV²⁵, and β -adrenoceptors²³, serotonin receptors^{23,42,43}, A_1 adenosine receptors⁴⁴ and μ -opioid receptors²¹ regulate I_h in this way. The chan-

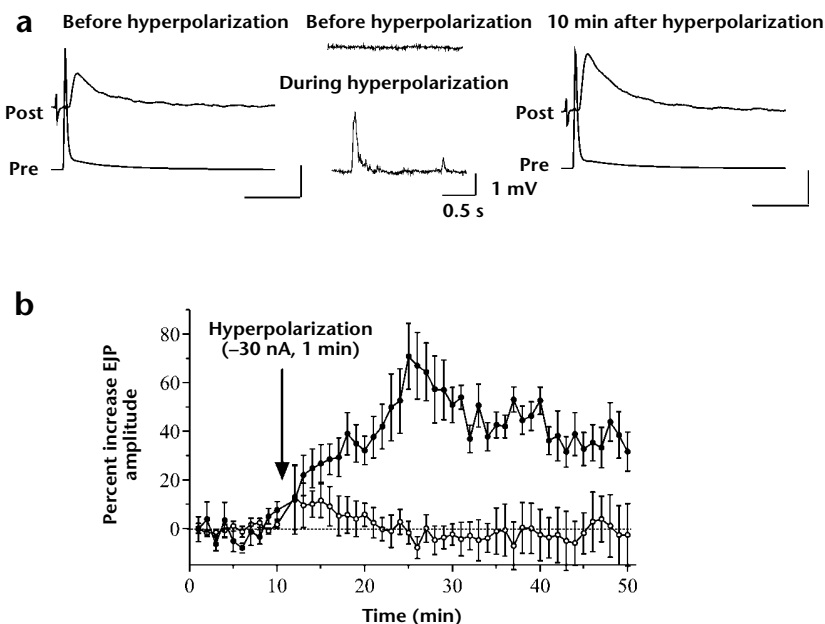


Fig. 8. Brief activation of I_h channels by hyperpolarization is sufficient to induce long-lasting enhancement of EJP amplitude. (a) Intracellular recording of axonal action potentials (APs; pre) and muscle excitatory junction potentials (EJPs; post) before (control) and 10 minutes after hyperpolarizing current injection (1 min, -30 nA) into the axon to activate I_h channels. Each trace is the average of all EJPs/APs recorded for 1 min at 2-Hz stimulation. Scale bars, 25 ms and 1 mV (post) or 25 mV (pre). Spontaneous EJPs increase upon hyperpolarization (middle panel). (b) Time course of EJP amplitude before, during and after hyperpolarization (-30 nA, 1 min) to activate presynaptic I_h channels with ZD7288 (○; 30 μM; $n = 6$) or without ZD7288 (●; $n = 8$).

nels we describe were similar to other reported I_h channels based on their activation by hyperpolarization, the contribution made to axonal resting potential, their increased activation by cAMP and their selective pharmacological block by both Cs^+ and ZD7288. The I_h current is a mixed cation current (Na^+/K^+)²⁵, and under physiological conditions, current is carried predominantly by the movement of sodium ions. In the crayfish axon, this also seems to be the case, as serotonin-induced depolarization of membrane potential was prevented when external sodium was replaced with choline¹⁵.

The mechanism by which serotonin-induced activation of I_h channels in the axon results in an increase in the total number of vesicles available for release¹² remains elusive. It is clear from previous studies that the amplitude of depolarization *per se* elicited by serotonin or forskolin is insufficient to account for the enhancement of neurotransmission, as depolarizing the axon by the same amount using either high $[\text{K}^+]$ ^{14,29} or current injection through intracellular recording electrodes⁴⁵ could not mimic the increase in synaptic strength. In addition, the observation that EJP amplitude remains elevated for up to 20 minutes after a relatively brief (1 minute) axonal hyperpolarization would suggest that I_h activation is required only for initiating a downstream process resulting in synaptic enhancement. Synaptic enhancement after axon hyperpolarization is observed not only in crayfish³⁰ but also in vertebrate junctions⁴⁶. Before knowledge of I_h channels, either a prolongation of the action potential³⁰ or a hyperpolarization-dependent mobilization of vesicles was assumed responsible^{30,47}. As we did not stimulate the nerve and record EJPs during hyperpolarization, and action potentials were unaltered after hyperpolarization, the former hypothesis can be rejected. However, active I_h -dependent mobilization of vesicles from non-releasable to releasable pools represents an attractive possibility, and the role of microtubule and actin transport pathways in this cAMP-induced enhancement are being investigated.

I_h clearly can be included with other presynaptic voltage- and ligand-gated ion channels modulated by neurotransmitters to inhibit or facilitate transmission. The many reports of I_h channels in vertebrate and invertebrate axons^{42,47–49} suggest physiological importance of cAMP-induced I_h modulation in regulating synaptic strength in a variety of organisms.

METHODS

Crayfish (*Procambarus clarkii*, 2–3 inches) were obtained from Niles Biological (Sacramento, California). Preparation of the innervated dactyl opener muscle of the first walking leg is described¹⁴. Autotomized legs were continuously superfused by a gravity-fed perfusion system with Normal Van Harrevald's solution, containing 195 mM NaCl, 13.5 mM CaCl_2 , 5.4 mM KCl, 2.6 mM MgCl_2 and 10 mM Na-HEPES at pH 7.4 and 14–17°C. Serotonin, H-7, 8-(phenylsulfonyl)-theophylline and 8-Br-cAMP were obtained from Sigma; Fura-2 from Molecular Probes (Eugene, Oregon); and ZD7288 from Tocris Cookson (Ballwin, Missouri). All other drugs were from Calbiochem (La Jolla, California). All paired control experiments contained final concentrations of DMSO equal to those used for drug delivery.

Electrophysiology. Sharp electrodes were used to impale both proximal muscle fibers (electrode resistance, 12–25 M Ω) and/or either primary or secondary branches of the excitor nerve axon (beveled electrode resistance, 25–45 M Ω). Excitor nerves were stimulated (2 Hz) during recording using a suction electrode containing the excitor axon freed from the meropodite segment of the leg. Signals were amplified (Neuroprobe 1600 Amplifier, A-M systems Carlsborg, Washington), filtered at 2 kHz, and digitized at 5 kHz and the average of all EJPs elicited each minute was saved to computer using pClamp7 software (Axon Instruments Foster

City, California). EJP amplitudes were measured off-line (Clampfit 6.05, Axon Instruments). For miniature EJP recording, beveled sharp electrodes (resistance 5–10 M Ω) were used to continuously acquire recordings subsequently filtered at 1 kHz, digitized at 2.5 kHz and analyzed off-line using Minianalysis Program version 4.0.1 (Synaptosoft, Leonia, New Jersey).

Data presentation and statistical analysis. As control EJP amplitudes were extremely variable from fiber to fiber, results were expressed as percent change from control EJP amplitude, taken as the average EJP amplitude over ten minutes of continuous recording in the absence of drug. Data are plotted as mean \pm s.e. percent change from this control level. When effects of different drugs were tested within a single muscle fiber, results were analyzed by Student's paired *t*-test; the Kolmogorov-Smirnov test was used to compare differences in cumulative probability for analysis of mEJPs. Significance was assumed if *p* < 0.05, unless otherwise stated.

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