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Calcium Influx Through HCN Channels Does Not Contribute to cAMP-Enhanced Transmission

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Zhong, Ning, Vahri Beaumont, and Robert S. Zucker. Calcium influx through HCN channels does not contribute to cAMP-enhanced transmission. *J Neurophysiol* 92: 644–647, 2004. First published March 10, 2004; 10.1152/jn.00112.2004. Serotonin is a native neuromodulator of synaptic transmission at glutamatergic neuromuscular junctions of crayfish limb muscles. During times of stress, serotonin binds to presynaptic receptors, which activate adenylyl cyclase to elevate presynaptic levels of cAMP. cAMP binds to two presynaptic target proteins, hyperpolarization and cyclic nucleotide-activated (HCN) ion channels and an exchange protein activated by cAMP (Epac), and activation of these effectors results in enhancement of transmitter release to action potentials. cAMP elevation also results in a small preterminal rise in $[Ca^{2+}]_i$, which we show here to result from Ca^{2+} influx through the presynaptic HCN channels opened by cAMP. Little or no Ca^{2+} influx occurs through voltage-dependent Ca^{2+} channels, despite the small presynaptic depolarization caused by current through the HCN channels. Loading terminals with BAPTA delays the rise in preterminal $[Ca^{2+}]_i$ without affecting the enhancement of transmission to cAMP elevation. This dissociation of the dynamics of the $[Ca^{2+}]_i$ rise and synaptic enhancement, plus the small magnitude and location of $[Ca^{2+}]_i$ elevation distant from release sites, seems to preclude any direct role for this $[Ca^{2+}]_i$ elevation in cAMP-dependent enhancement of transmission.

INTRODUCTION

Increased stress and states of arousal are accompanied by increases in muscle tension (Asterita 1985). In crayfish, this is caused, at least in part, by an increase in circulating levels of serotonin, which acts to enhance transmitter release to motor neuron action potentials at glutamatergic neuromuscular junctions onto tonic limb muscles (Glusman and Kravitz 1982). Recent studies of the mechanism of serotonin action reveal that at least part of its action is mediated by an elevation of presynaptic levels of cAMP, which in turn operates on two protein targets: hyperpolarization and cyclic nucleotide-activated (HCN) channels (Beaumont and Zucker 2000) and exchange protein activated by cAMP (Epac) (N. Zhong and R. S. Zucker, unpublished observations). Activation of both targets is required for full enhancement of transmission, but it is not yet clear how the activation of either of these targets enhances transmitter release.

Previous work (Delaney et al. 1991) showed that serotonin could cause a modest elevation of presynaptic $[Ca^{2+}]_i$ level, although the measured rises were somewhat haphazard and confined to preterminal regions of the motor neuron endings. Very rarely was serotonin seen to elevate $[Ca^{2+}]_i$ in presynaptic boutons, but in some axonal branches, $[Ca^{2+}]_i$ rose by 30–120 nM from a typical resting level of 165 nM. Presynaptic EGTA injection sufficient to reduce the build-up of $[Ca^{2+}]_i$ during repetitive stimulation had no effect on serotonin enhancement of transmission, but the effects on the modest and sporadic $[Ca^{2+}]_i$ rises in serotonin were not monitored.

A recent report (Yu et al. 2004) indicates that cloned human HCN4 channels admit a very small amount of Ca^{2+}, about 0.6% of the net inward current, when expressed in HEK293 cells, and that under certain conditions, this Ca^{2+} entry can enhance transmitter release to subsequent action potentials. This suggested the possibility that the small and sporadic rises in $[Ca^{2+}]_i$ accompanying exposure to serotonin might reflect a Ca^{2+} influx through presynaptic HCN channels and that Ca^{2+} might act locally near channel mouths, perhaps at undetectable levels in presynaptic boutons, to sensitize synaptic transmission. We have therefore collected some previously obtained but unpublished results and performed some additional experiments to test this possibility more directly.

METHODS

Small (2–2.5 in) crayfish (*Procambarus clarkii*) were procured from Niles Biological (Sacramento, CA). First walking legs were isolated by autotomy and mounted for electrophysiology and $[Ca^{2+}]_i$ measurement as described previously (Delaney et al. 1991; Zhong et al. 2001). The opener muscle was exposed, and the exciter motor neuron was stimulated with a suction electrode in the leg nerve in the meropodite. Preparations were bathed in ~1 ml of a medium containing (in mM) 195 NaCl, 13.5 $CaCl_2$, 5.4 KCl, 2.6 $MgCl_2$, and 10 Na-HEPES (pH 7.4) at 15–17°C. Drugs were applied by switching the perfusion solution (1–2 ml/min) to one containing the drug or drugs shown. Drugs used included serotonin (Sigma Chemical, St. Louis, MO), forskolin (ED Biosciences, Pasadena, CA), ZD 7288 (Tocris Cookson, Ballwin, MO), ω-agatoxin IVA (Calbiochem, La Jolla, CA), and BAPTA-AM (Molecular Probes, Eugene, OR). Nerves were loaded with BAPTA by incubating for 30 min in 50 μM BAPTA-AM and washing for 30 min in normal solution to permit full hydrolysis of BAPTA-AM. Forskolin and BAPTA-AM were prepared as stock solutions in DMSO and dissolved before use in a final solution containing 0.1% or less DMSO. Control experiments showed that synaptic transmission was unaffected by this concentration of DMSO. Excitatory junctional potentials (EJPs) were recorded from proximal muscle fibers using sharp microelectrodes (15–25 MΩ) filled with 3 M KCl. The motor nerve was stimulated at 2 Hz; intracellular signals were filtered at 2 kHz and digitized at 5 kHz for storage on a personal computer using pClamp7 (Axon Instruments, Union City, CA).

Presynaptic $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive ratiometric fluorescent dye fura-2 (Teflabs, Austin, TX). Fura-2 was iontophoresed from a microelectrode (containing 17 mM in 200 mM

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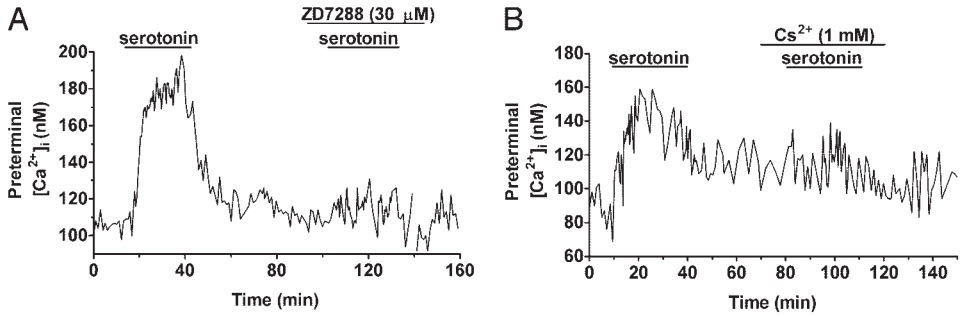

FIG. 1. Serotonin (300 nM) elevates $[Ca^{2+}]_i$ in preterminal motor neuron branches. This elevation was blocked by the hyperpolarization and cyclic nucleotide-activated (HCN) channel inhibitors ZD 7288 (*A*) and Cs^+ (*B*).

KCl) that penetrated the primary or a secondary motor nerve branch within 200 μm of the preterminal branches and boutons that were imaged. Injecting –10 nA current for 10–15 min produced a final fura-2 concentration in imaged processes of about 150 nM, estimated as described previously (Mulkey and Zucker 1992). The apparatus used for processing fluorescence images obtained using an intensified CCD camera (Quantex, Sunnyvale, CA), and procedures and calibrations used for $[Ca^{2+}]_i$ determination were as described before (Zhong et al. 2001). Electrophysiological and $[Ca^{2+}]_i$ measurements are presented as mean ± SE, and two-tailed Student's *t*-tests were used to assess statistical significance.

RESULTS

Our first set of experiments is directed toward identifying the origin of the rise in presynaptic $[Ca^{2+}]_i$ on exposure to a concentration of serotonin (300 nM) that enhances synaptic transmission at neuromuscular junctions of the crayfish dactyl opener muscle (Beaumont and Zucker 2000). First, we confirmed that detectable $[Ca^{2+}]_i$ elevations are confined to preterminal axonal branches, as reported previously (Delaney et al. 1991). In 16 of 19 experiments, we observed modest $[Ca^{2+}]_i$ elevations (41 ± 10 nM) in scattered presynaptic axonal regions. $[Ca^{2+}]_i$ usually rose to a plateau level in 5–10 min; examples of effects of serotonin are shown in Fig. 1. In the other three preparations, we detected no changes (<10 nM) in presynaptic $[Ca^{2+}]_i$. We never observed a change in $[Ca^{2+}]_i$ from the resting level in presynaptic boutons. Resting $[Ca^{2+}]_i$ level ranged between 50 and 170 nM in these experiments.

Since serotonin acts mainly by elevating presynaptic cAMP activity (Beaumont and Zucker 2000), we checked for effects of the adenylyl cyclase activator forskolin (30 μM) on presynaptic $[Ca^{2+}]_i$. In six experiments, forskolin increased $[Ca^{2+}]_i$ by 38 ± 19 nM, which was very similar to the average effect of serotonin (41 μM). As with serotonin, $[Ca^{2+}]_i$ elevations were only observed in preterminal axonal branches of 5–15 μm diam. In two additional control experiments, we saw no effects of serotonin on $[Ca^{2+}]_i$ when the external Ca^{2+} was removed and substituted with 0.5 mM EGTA. Thus the rise in preterminal $[Ca^{2+}]_i$ appears to be due to an influx from outside through channels opened by cAMP elevated by forskolin or serotonin.

To test whether Ca^{2+} entered through HCN channels, we used the HCN channel inhibitor ZD 7288 to block channel openings by cAMP. We used ZD 7288 at 30 μM, or six times its half-maximal concentration for blocking HCN channels in crayfish (Beaumont and Zucker 2000), a dose that should produce an 83% block of HCN channels (assuming a single and totally effective binding site). In three experiments, 30 μM ZD 7288 reduced the effect of serotonin from 59 ± 18 nM (without the inhibitor) to 12 ± 2 nM ($P = 0.05$, 2-tailed paired Student's *t*-test), which is an 80% reduction. Figure 1*A* shows results of one such experiment. Similarly, in three experiments, 1 mM Cs^+ reduced the serotonin-induced increase from 42 ± 15 to 11 ± 12 nM without Cs^+ ($P < 0.05$); a typical result is shown in Fig. 1*B*. At four times the half-maximal dose in blocking HCN channels (Beaumont and Zucker 2000), 1 mM Cs^+ should produce an 80% block, which may be compared with the observed 74% reduction in $[Ca^{2+}]_i$ elevation. Control experiments confirm that when preparations are successively exposed to serotonin or forskolin with a wash in between, both exposures increase $[Ca^{2+}]_i$ (see Fig. 2*B*).

The previous results suggested that the rise in $[Ca^{2+}]_i$ induced by serotonin results from influx through HCN channels. However, another potential source of Ca^{2+} influx into nerve terminals is through P-type Ca^{2+} channels, which are the only type present in these motor nerve terminals (Araque et al. 1994; Wright et al. 1996). We tested for influx through these channels by measuring the $[Ca^{2+}]_i$ rise induced by 300 nM serotonin in the presence of 300 nM ω-agatoxin IVA. In four experiments, the $[Ca^{2+}]_i$ rise in preterminal axons was 32 ± 21 nM, which was not significantly different from the effect of serotonin without a P-channel blocker. Similarly, 30 μM forskolin elevated preterminal $[Ca^{2+}]_i$ by 30 ± 15 nM in the presence of 300 nM ω-agatoxin IVA, which was not significantly different from the effect of forskolin without the P-channel blocker. We confirmed that 300 nM ω-agatoxin IVA completely blocks postsynaptic responses to motor nerve stimulation, as well as the typical rise in $[Ca^{2+}]_i$ transients observed with no blocker present (Delaney et al. 1991). Thus it appears that the preterminal $[Ca^{2+}]_i$ rise induced by cAMP is mainly, and probably entirely, due to influx through HCN channels.

Even though the preterminal $[Ca^{2+}]_i$ rise is quite modest and is only detectable in a presynaptic compartment distinct and distant from presynaptic boutons where transmitter is secreted, it seemed possible that a tiny, unmeasurable Ca^{2+} influx through HCN channels at presynaptic boutons could mediate the HCN channel–dependent action of cAMP on synaptic transmission. Although presynaptic EGTA injection had been found to be without effect on serotonin's enhancement of transmission, EGTA binds Ca^{2+} quite slowly, only within about 10 ms at 200 nM $[Ca^{2+}]_i$, and so might not be able to capture Ca^{2+} ions acting very close to their point of entry before they bind to an effector target. To test this possibility, we loaded nerve terminals with the Ca^{2+} chelator BAPTA, which binds Ca^{2+} ions about 100 times faster than EGTA (Neher 1986), and compared effects of 30 μM forskolin on

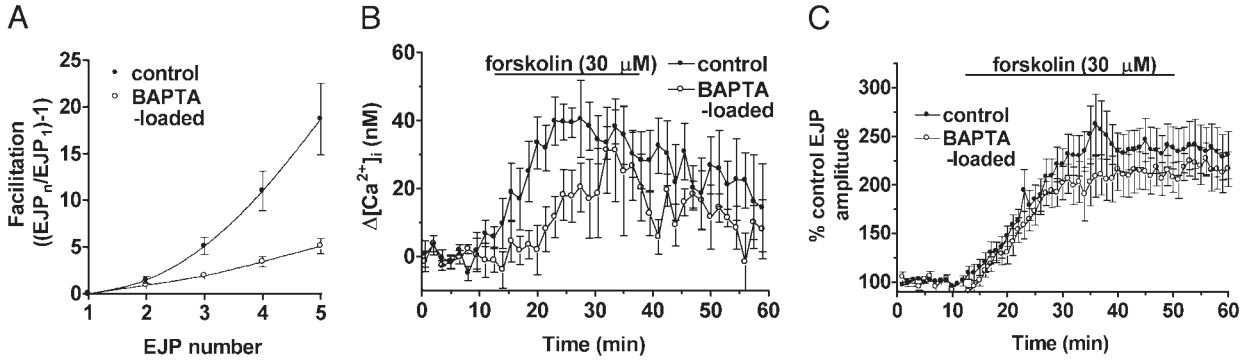

FIG. 2. Effects of loading motor nerve terminals with the Ca^{2+} chelator BAPTA. *A*: facilitation of transmitter release [measured as fractional increase of the *n*th excitatory junctional potential (EJP) divided by the 1st EJP for a 5-pulse 100-Hz train] was reduced by BAPTA. *B*: forskolin (30 μM) elevated preterminal $[Ca^{2+}]_i$, and BAPTA retarded this elevation. *C*: forskolin enhanced synaptic transmission, while BAPTA was without effect on this enhancement. *A–C*: averaged responses from the same preparations.

transmission and presynaptic $[Ca^{2+}]_i$ levels before and after BAPTA loading.

In eight experiments, after loading nerve terminals with BAPTA, baseline synaptic transmission was reduced (by 42 ± 9%). Synaptic facilitation to a brief train of action potentials was also substantially diminished (by 59 ± 13%, see Fig. 2*A*). This result is expected, since the addition of exogenous Ca^{2+} buffer capacity should slow the accumulation of residual Ca^{2+} responsible for facilitation (Zucker and Regehr 2002). Treating a presynaptic bouton as a single well mixed compartment, residual calcium Ca(t) depends on the Ca^{2+} influx rate $I(t)$, pump rate at the surface P, and the Ca^{2+} buffer ratio β, and is described by the equation

$$\frac{d\mathrm{Ca}(t)}{dt}V = -\frac{PA\mathrm{Ca}(t)}{\beta} + \frac{I(t)A}{\beta}$$

where $\mathrm{Ca} = [Ca^{2+}]_i$, V = bouton volume, A = bouton surface area, and $P = P_{max}/(\mathrm{Ca} + K_p) \approx P_{max}/K_p$ if $\mathrm{Ca} \ll K_p$, with P_{ma} and K_p the maximum pump rate and its Ca^{2+} affinity, respectively, and the buffer ratio (the ratio of bound plus free $[Ca^{2+}]_i$ to free $[Ca^{2+}]_i$) depends on total concentrations of each of i buffers ($B_{tot,i}$) and their Ca^{2+}-dissociation constants ($K_{D,i}$)

$$\beta = 1 + \sum_i \frac{B_{tot,i}}{\mathrm{Ca} + K_{D,i}} \approx 1 + \sum_i \frac{B_{tot,i}}{K_{D,i}} \text{ if } \mathrm{Ca} \ll K_{D,i}$$

Thus the addition of an exogenous Ca^{2+} buffer increases β and prolongs the time constant of $[Ca^{2+}]_i$ equilibration [$\tau = \beta V/PA$ for small Ca(t)] and retards the accumulation of facilitation. Similarly, BAPTA loading should retard the rise in $[Ca^{2+}]_i$ due to an influx of Ca^{2+} [$I(t)$] induced by serotonin or forskolin, and this effect was also observed (Fig. 2*B*). The steady-state level of $[Ca^{2+}]_i$ equals I/P and is unaffected by BAPTA because it is independent of β.

The interesting result is the effect of BAPTA loading on the enhancement of transmission by forskolin. There was no significant change in either the amplitude or the time course of the cAMP-dependent enhancement of transmitter release. Without BAPTA loading, the rise in $[Ca^{2+}]_i$ in preterminal axons reached a peak of about 40 nM at 25 min after forskolin addition, with a half-rise time of 5.8 ± 0.68 min. This is somewhat before the enhancement of transmission reached its peak (at ~35 min), with a half-rise time of 11.1 ± 0.54 min. After BAPTA loading, the peak rise in $[Ca^{2+}]_i$ (to ~30 nM) was delayed until 35 min, with the half-rise time now occurring at 13.6 ± 0.90 min, while the enhancement of release was not delayed at all, with its half-rise time still at 11.1 ± 0.65 min. Thus without BAPTA, the half-rise time of the $[Ca^{2+}]_i$ elevation to forskolin preceded the half-rise time of the effect on transmission, while after BAPTA loading the half-rise time of the $[Ca^{2+}]_i$ elevation often followed the half-rise time of the effect on transmission. The differential effects of BAPTA on the time courses of $[Ca^{2+}]_i$ change and enhancement of transmission are difficult to reconcile with the rise in $[Ca^{2+}]_i$ serving as a direct mediator of the enhanced transmission.

DISCUSSION

In these experiments, we have probed the origin of the cAMP-mediated $[Ca^{2+}]_i$ rise caused by serotonin and sought evidence that it might contribute to cAMP-dependent enhancement of transmitter release. We have made several findings. *1*) The $[Ca^{2+}]_i$ elevation is quite modest and occurs only in preterminal axonal branches; no detectable $[Ca^{2+}]_i$ elevation occurs in presynaptic boutons or varicosities where transmitter is released (Cooper et al. 1995). It is difficult to imagine how this $[Ca^{2+}]_i$ rise, which is too small and in the wrong place, can directly enhance synaptic transmission. This confirms previous observations (Delaney et al. 1991). *2*) Most, if not all, of the $[Ca^{2+}]_i$ elevation is due to entry of $[Ca^{2+}]_i$ through HCN channels opened by cAMP. Little, if any, enters through the presynaptic P-type voltage-dependent Ca^{2+} channels that admit the Ca^{2+} responsible for triggering transmitter release to action potentials. This confirms more directly the previous inference (Delaney et al. 1991; Dixon and Atwood 1985; Wojtowicz and Atwood 1984) that the small (~4–10 mV) depolarization of presynaptic terminals caused by the cAMP activation of HCN channels (Beaumont and Zucker 2000) cannot enhance transmission by admitting Ca^{2+} through voltage-dependent channels. Since the cAMP-dependent $[Ca^{2+}]_i$ elevation requires external Ca^{2+}, it does not arise from Ca^{2+} released from internal stores independent of Ca^{2+} entry. *3*) Increasing the cytoplasmic Ca^{2+} buffering power reduces evoked transmission, slows the growth of synaptic facilitation, and slows the rise in preterminal $[Ca^{2+}]_i$ so that it follows slightly, instead of precedes slightly, the cAMP-dependent enhancement in synaptic transmission. It is difficult to imagine how this $[Ca^{2+}]_i$ rise can be responsible for enhancing release,

which under these circumstances precedes it (as measured by the time to reach half-maximum). *4*) Presynaptic BAPTA had no effect at all on the cAMP-dependent enhancement of transmission. If this enhancement were due to the local action of occult, undetectable Ca^{2+} influx into nerve terminals, BAPTA should have captured these Ca^{2+} ions before they could act, and blunted the enhancement of transmission. Our results would appear to exclude this possibility.

In conclusion, the enhancement of transmitter release is mediated by activation of HCN channels and Epac (Beaumont and Zucker 2000; N. Zhong, and R. S. Zucker, unpublished observations), operating on the transmitter release machinery, or perhaps the supply of vesicles available for release (Wang and Zucker 1998), apparently without any direct involvement of Ca^{2+} ions in the process of enhancement.

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DISCLOSURES

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