Presynaptic Effectors Contributing to cAMP-Induced Synaptic Potentiation in *Drosophila*

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Received 16 May 2005; accepted 25 August 2005

ABSTRACT: **cAMP analogs and activation of adenylyl cyclase by forskolin strongly potentiate synaptic transmission at the *Drosophila* neuromuscular junction. These effects are generally attributed to activation of cAMP-dependent protein kinase. Recent reports on crustacean and mammalian synapses have implicated other cAMP-dependent effectors in synaptic potentiation. *Drosophila* neuromuscular junctions were tested for effects of two known cAMP-dependent effectors: hyperpolarization-activated, cyclic nucleotide-regulated channels (HCNCs) and guanine nucleotide exchange protein activated by cAMP (Epac). Forskolin-induced enhancement of synaptic transmission was drastically reduced by a blocker of HCNCs, but not completely eliminated. A specific agonist for Epac modestly enhanced synaptic potentials. This agonist also stabilized their amplitudes in the presence of a blocker of HCNCs. The observations implicate HCNCs and Epac in cAMP-dependent potentiation that does not require cAMP-dependent protein kinase, indicating that additional previously unexplored factors contribute to synaptic plasticity in *Drosophila*. Genetic and molecular techniques available for *Drosophila* can be used to define the underlying molecular basis for cAMP-dependent synaptic potentiation.** J Neurobiol 66: 273–280, 2006

***Keywords:* *Drosophila melanogaster*; cyclic 3′,5′-adeosine monophosphate; hyperpolarization-activated, cyclic nucleotide-regulated channels; guanine nucleotide exchange factor; forskolin; neuromuscular junction; synaptic transmission**

INTRODUCTION

The initial discovery of several *Drosophila* learning mutants that have deficiencies in neuronal cAMP production or removal (Byers et al., 1981; Davis and Kiger, 1981; Dudai and Zvi, 1984; Livingstone et al., 1984) has stimulated many subsequent studies on cAMP's effects on synaptic transmission in *Drosophila*, mostly on the larval neuromuscular junction (NMJ), which is easily accessible for optical and electrophysiological studies. Mutants with deficiencies in cAMP metabolism exhibit abnormalities in transmitter release and short-term synaptic plasticity (Zhong and Wu, 1991; Renger et al., 2000), and formation of the NMJ (Budnik et al., 1990; Cheung et al., 1999). Striking effects on evoked (Kuromi and Kidokoro, 2000) and spontaneous (Yoshihara et al., 1999, 2000) transmitter release promoted by membrane-permeant cAMP analogs at the *Drosophila* larval NMJ have reinforced the view that cAMP acutely regulates presynaptic processes governing exocytosis of synaptic vesicles. In addition, long-term effects are thought to ensue from cAMP's action on transcription of synaptic proteins via cAMP-dependent transcription factors (Davis et al., 1996), as in other neural systems (Dash

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Contract grant sponsor: Canadian Institutes of Health Research (CIHR) (H.L.A.).

Contract grant sponsor: National Science Foundation (R.S.Z.).

Published online 2 December 2005 in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/neu.20218

et al., 1990; Bourtchuladze et al., 1994). All of the previous studies on neuromuscular transmission in *Drosophila* have either assumed or produced evidence to support the interpretation that cAMP exerts both its acute and its long-term effects through activation of its best-known local effector, protein kinase A (PKA).

Recent studies on several other neural systems, in particular crustacean NMJs (Beaumont and Zucker, 2000) and the mammalian giant brain-stem synapse, the calyx of Held (Kaneko and Takahashi, 2004), have provided evidence that cAMP effectors other than PKA are important regulators of transmitter release. At crustacean NMJs, several studies on cAMP-enhanced transmission have supported a key role for hyperpolarization-activated, cyclic nucleotide-regulated channels (HCNCs). When these channels are blocked pharmacologically or otherwise interfered with, synaptic enhancement attributable to an increase in cAMP is severely attenuated (Beaumont and Zucker, 2000; Beaumont et al., 2002). At the crustacean NMJ (Zhong and Zucker, 2005) and at the calyx of Held (Kaneko and Takahashi, 2004), an additional effector, exchange protein activated by cAMP (Epac), has been implicated by the action of a specific agonist for Epac that does not activate PKA. Synaptic potentiation produced by cAMP at the calyx of Held may be entirely due to activation of Epac (Kaneko and Takahashi, 2004).

In view of the increasing number of studies of synaptic transmission utilizing *Drosophila*, in which conditional manipulations of specific proteins can be more readily achieved and assayed for their physiological effects than in most other organisms, it is important to establish whether these recently implicated cAMP-dependent effectors contribute to synaptic enhancement in *Drosophila*. If this were the case, the prevailing view that cAMP-dependent enhancement is entirely due to PKA would have to be revised. In addition, the genetic and molecular tools available for *Drosophila* could be employed to decipher the subcellular pathways involved in the potentiation.

The present study is an initial exploration of this hypothesis using available pharmacological reagents. The positive results obtained open the way for more detailed investigations with genetic mutants, conditional molecular expression, and other tools available for *Drosophila*.

METHODS

Fly Stocks

Drosophila melanogaster Canton-special (CS) strain was maintained on standard cornmeal-agar medium with dry yeast (Fleischmann's) in bottles housed in an incubator at 22 ± 1°C. Fly stocks were changed weekly to ensure a non-crowded population. Wandering third-instar larvae were selected for experiments.

Preparation

Larvae were dissected in chilled Schneider's insect medium (Sigma, St. Louis, MO) in a magnetic strip well glued to a glass base, as described previously (Atwood et al., 1993). Use of Schneider's medium suppresses unwanted contractions of body wall muscles during dissection. Dissection exposed the larval body wall muscles from which recordings were made in segments A3 and A4, using HL3 solution as the physiological recording solution (Stewart et al., 1994). HL3 solution used in all experiments contains (in m*M*): 70 NaCl, 5 KCl, 20 $MgCl_2$, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 5 N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid (BES). In these experiments, the concentration of $CaCl_2$ in HL3 was 0.5 m*M*. This concentration, which is less than that found normally in hemolymph (Stewart et al., 1994), was selected to avoid contractions after application of potentiating drugs and to allow better observations of the percentage increase or decrease of the excitatory junction potential (EJP). The final HL3 recording solution was adjusted drop-wise to a pH of 7.2 using 0.5 *M* NaOH.

Reagents

Both the HCNC blocker ZD7288 and the adenylyl cyclase activator forskolin (FKL) were obtained from Tocris (Ellisville, MO). FKL stock solution was dissolved in dimethyl sulphoxide (DMSO; Sigma); the final concentration of DMSO did not exceed 0.1% in the preparation chamber. The Epac agonist 8-CPT-2-Me-cAMP (8-CPT) was obtained from BioLog and is highly membrane permeant as indicated by the lipophilicity of cAMP agonists (Krass et al., 1997). The Epac antagonist Brefeldin A (BFA) was purchased from Calbiochem. All drugs were delivered by superfusion in HL3 solution over the preparation.

Electrophysiology

Synaptic transmission efficacy at the NMJ was assayed by intracellular whole-cell recording of evoked EJPs. Microelectrodes with resistances of 60–80 MΩ (Stoelting, borosilicate glass 1.5 mm OD/0.84 mm ID with omega dot fiber) filled with 3 *M* KCl were used to impale larval ventral longitudinal muscle 6 in segment 4. Voltage signals were amplified with an Axoclamp-2A amplifier (Axon Instruments, Foster City, CA). The amplifier was calibrated and the recording scale was adjusted accordingly. Evoked signals were filtered at 5 kHz, and digitized at 10 kHz directly to computer with Maclab data acquisition system and Scope software (ADInstruments, Castle Hill, NSW, Australia).

The evoked EJP was produced by sucking the segmental nerve into a fire-polished stimulating electrode with 11–12 μm ID, filled with HL3 solution. Stimulus pulses,

0.03 ms in duration, were delivered at 1 Hz. The stimulating voltage was adjusted to recruit both axons innervating muscle 6 (Kurdyak et al., 1994), producing a compound EJP in the muscle fiber. The compound EJP includes contributions of both Type 1b and Type 1s boutons (Atwood et al., 1993) on muscle 6; in the present study, we did not attempt to determine whether the reagents that we used affected the two types differentially.

Statistical Analysis

MS Excel was used in data analysis; results were presented as the percent change from the initial baseline EJP amplitude, taken as the average EJP amplitude acquired from 100 sweeps continuously recorded at 1 Hz. Data are plotted as the mean ± SD percentage change from baseline level using Sigma Plot. Statistical analysis of results noted in all the figures was performed using Student's *t* test.

RESULTS

Contribution of HCNCs

The *Drosophila* NMJ exhibits robust enhancement of neurosecretion in response to application of membrane-permeant cAMP analogs (Kuromi and Kidokoro, 2000) or the adenylyl cyclase activator, FKL (Yoshihara et al., 2000). In our experiments, application of 30 μM forskolin reliably increased the compound EJP amplitude by about 150%, while the vehicle used to dissolve FKL (DMSO, 0.1%) produced no effect by itself [Fig. 1(A)]. Effects of FKL were predominantly presynaptic, because mean amplitude of spontaneously occurring quantal events remained close to 1 mV before and after drug application. This has been reported in more detail by Yoshihara et al. (2000).

The FKL-induced enhancement was drastically attenuated by pharmacological agents that block HCNCs [Fig. 1(B)]. The blocking agent ZD7288 was bath-applied after washout of FKL, and by itself caused no change in the compound EJP monitored at a low frequency of stimulation. Subsequent reapplication of FKL elicited only a small enhancement of the EJP. Most of the blocking effect of ZD7288 was apparent at a concentration of 30 μM, because doubling the concentration to 60 μM elicited very little further reduction in EJP amplitude [Fig. 1(B)]. These results are in agreement with an extensive series of experiments at the crayfish NMJ, in which an important role for HCNCs has been established (Beaumont and Zucker, 2000).

Evidence for Epac's Contribution

A membrane-permeant analog of cAMP, 8-CPT, is known to activate Epac and not PKA (Enserink et al., 2002). This compound, applied by itself, increased the EJP by about 50% of initial value (Fig. 2). An antagonist of other guanine nucleotide exchange proteins, BFA (Morinaga et al., 1996), applied at a concentration of 100 μM, blocked the EJP enhancement produced by 8-CPT (Fig. 2). No significant changes in amplitude of spontaneously occurring quantal events were observed. These results are consistent with findings at the crayfish NMJ (Zhong and Zucker, 2005).

In summary, the HCNC antagonist ZD7288 greatly attenuates the FKL-induced enhancement of

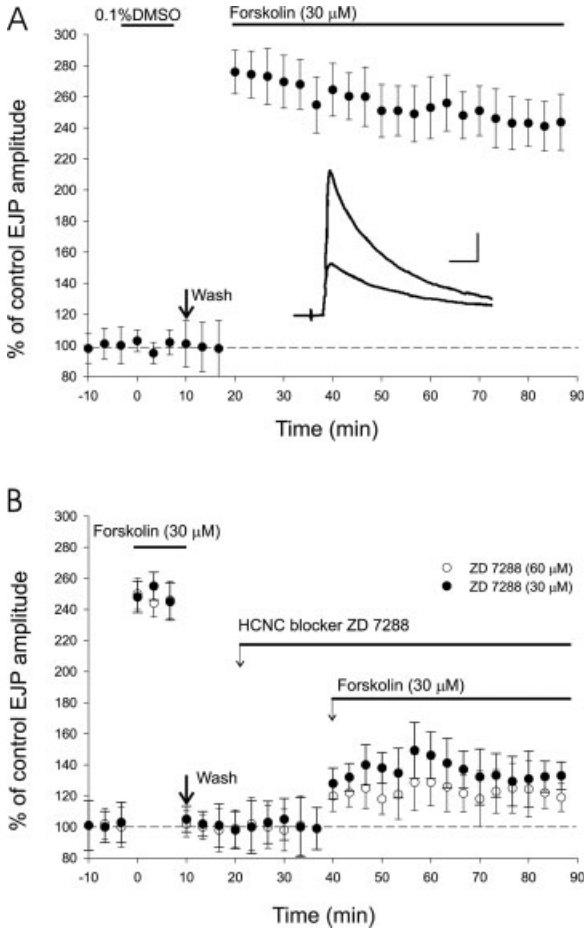

Figure 1 Effects of HCNC block on synaptic enhancement by cAMP. (A) Compound EJP amplitude, recorded over time, was enhanced by adding forskolin (30 μM), an activator of adenylyl cyclase. DMSO (0.1%) was used to dissolve forskolin and was tested separately; no effect on the EJP occurred. Forskolin increased EJP amplitude by more than 150% ($n = 10$). The inset illustrates a representative recording of the compound EJP before and after application of forskolin (scale: 10 mV, 40 ms). (B) HCNC blocker, ZD7288, had no effect on the unpotentiated EJP amplitude but attenuated the forskolin-induced EJP enhancement (30 μM, filled circles, $n = 5$; 60 μM, open circles, $n = 5$).

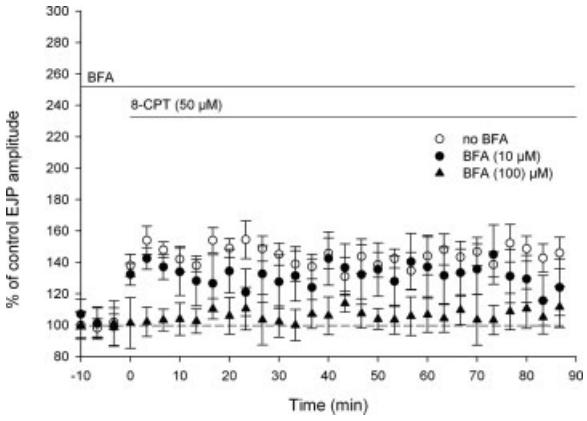

Figure 2 Evidence for participation of Epac in synaptic enhancement by cAMP. In the absence of any other drug, the specific agonist for Epac, 8-CPT (50 μM), caused a 40% increase in EJP amplitude (open circles, $n = 5$). This enhancement was not significantly reduced by 10 μM Brefeldin A (BFA, filled circles, $n = 5$), but was totally blocked by 100 μM BFA (triangles, $n = 5$).

the EJP, and a specific Epac agonist produces a modest enhancement of the EJP, which is blocked by the Epac antagonist BFA (Fig. 3).

Previous studies have reported increased dispersion of release and lengthening of the time course of quantal events in mutants with abnormal cAMP concentration (Renger et al., 2000), and shortening of the time course of quantal events after addition of FKL in a mutant embryonic preparation (Yoshihara et al., 2000). The possible contribution of such effects was checked by measuring the rise time and the time of decay to half-amplitude of signal-averaged EJPs from representative experiments of Figure 3. There was no difference in rise time of EJPs before and after drug treatments. Mean values were: controls, 9.8 ms (five experiments); FKL, 9.4 ms (five experiments); 8-CPT, 8.2 ms (three experiments); ZD7288 in combination with FKL [Fig. 1(B)], 9.4 ms (three experiments). However, drug treatments increased the time for decay to half-amplitude by 13–55%. Mean values and range of values for the same sets of experiments were: controls, 44.6 ms (39.8–48.6 ms); FKL, 70.0 ms (69.2–71.4 ms); 8-CPT, 52.0 ms (49.7–56.3 ms); ZD7288 in combination with FKL, 69.0 ms (67.4–70.7 ms). The lengthening of decay time suggests that there could be less synchronized release of quanta and/or lengthening of quantal events after drug treatment; because no changes in rise time were observed, the latter effect seems more likely. However, such effects, if present, cannot account for the much larger changes in amplitude of EJPs induced by drug treatments, and thus were not analyzed further in the present study.

Maintenance of EJP Enhancement

Previous experiments on the crayfish NMJ showed that synaptic enhancement induced by FKL could not be reduced by subsequent application of ZD7288, indicating that HCNCs are required only for induction of cAMP-dependent synaptic enhancement, but not its maintenance (Beaumont et al., 2002). At the *Drosophila* NMJ, however, we found that FKL enhancement had to be in place for 20–30 min before it became resistant to the HCNC blocker. When ZD7288 was applied 10 or 20 min after FKL, the enhanced EJP slowly declined. When ZD7288 was applied 30 minutes after FKL, the enhanced EJP retained its amplitude (Fig. 4).

This result is consistent with the previous experiments on the crayfish NMJ (Zhong and Zucker, 2005), because in the crayfish experiments ZD7288 was applied 30 min after FKL, and the effects of earlier applications were not examined.

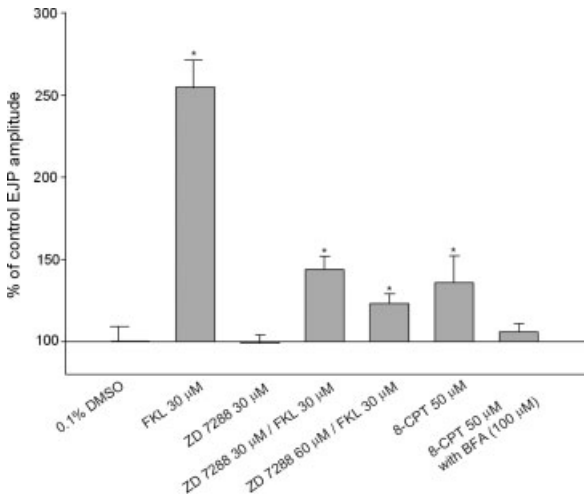

Figure 3 Summary of experiments testing participation of Epac and HCNC in forskolin-induced EJP enhancement. In the absence of other drugs, neither 0.1% DMSO nor ZD7288 (30 μM) produced any effect on the basal EJP amplitude ($p = 0.38$, 0.54 respectively). Forskolin (30 μM) enhanced the EJP to approximately 2½ times its initial amplitude ($p < 0.001$). ZD7288 (30 μM) reduced this enhancement by approximately 2/3 ($p < 0.05$); additional reduction occurred at 60 μM ZD7288. The specific Epac agonist 8-CPT (30 μM) increased the EJP amplitude to 136 ± 6% of initial value ($p < 0.05$); this enhancement was blocked by BFA (100 μM) ($p = 0.2$). Absolute amplitudes (mean ± SD) of EJPs were: for 0.1% DMSO, 21.37 ± 0.88 mV; for 30 μM FKL, 53.43 ± 6.68 mV; for 30 μM ZD7288, 19.53 ± 0.58 mV; for 30 μM ZD7288 and 30 μM FKL, 33.6 ± 3.96 mV; for 60 μM ZD7288 and 30 μM FKL, 25.77 ± 3.88 mV; for 50 μM 8-CPT, 28.49 ± 6.55 mV; for 50 μM 8-CPT and 100 μM BFA, 22.20 ± 0.65 mV.

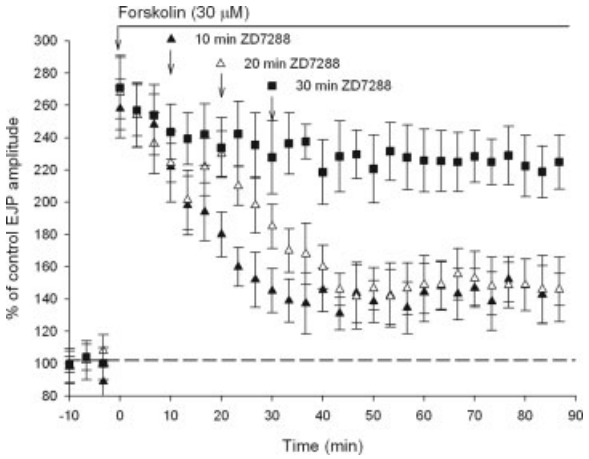

Figure 4 Effects of HCNC block on maintenance of EJP enhancement. Enhancement of transmission by forskolin (30 μM) was not maintained when ZD7288 was added 10 min (▲, $n = 3$) or 20 min (△, $n = 3$) after forskolin. When ZD7288 was added 30 min after forskolin (■, $n = 5$), enhancement of transmission was maintained.

Stabilization of EJP Amplitude by Epac Agonist

Application of the Epac agonist 8-CPT stabilized the FKL-enhanced EJP and prevented further decline associated with early application of ZD7288. Thus, when 8-CPT and ZD7288 were applied together 10 min or 20 min after the EJP had been potentiated by FKL, there was no appreciable decline in the EJP (Fig. 5). As at the crayfish NMJ (Zhong and Zucker, 2005), Epac appears capable of maintaining FKL-enhanced transmission when HCNCs are blocked.

When FKL was replaced after 10 min by ZD7288 and 8-CPT, elevated responses were maintained for about 20 min thereafter, then began to decline slowly toward baseline [Fig. 6(A)]. The amplitude of the response leveled off at a value similar to that attained after application of 8-CPT alone (Fig. 2). When FKL was present alone for 20 min, and then replaced by ZD7288 and 8-CPT [Fig. 6(B)], the potentiated EJP persisted for the duration of the experiment (at least 80 min). These observations indicated that FKL enhancement must remain in place for a period of 20–30 min before enhanced synaptic transmission becomes resistant to the effects of the HCNC blocker.

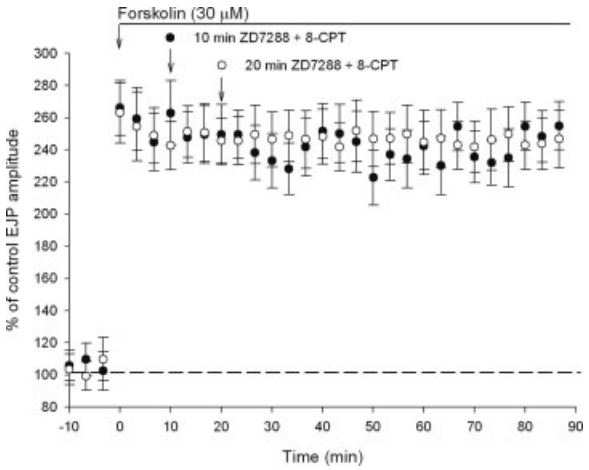

Figure 5 Stabilization of EJP amplitude by Epac agonist. Forskolin-induced enhancement of EJP amplitude was maintained when 8-CPT (50 μM) and ZD7288 (30 μM) were applied together 10 min (●, $n = 3$) or 20 min (○, $n = 5$) after forskolin-induced enhancement of EJPs.

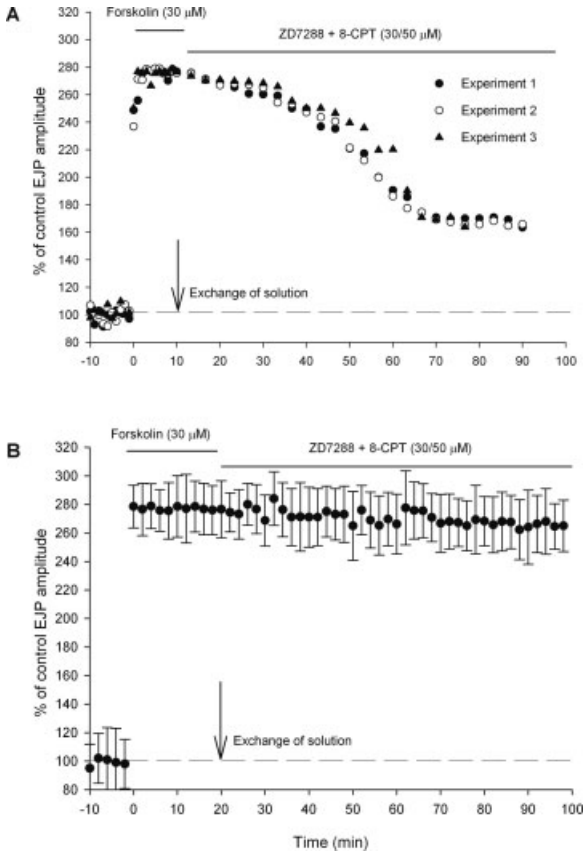

Figure 6 Persistence of EJP enhancement after removal of forskolin. (A) Removal of forskolin after addition of ZD7288 (30 μM) and 8-CPT (50 μM) resulted in a gradually declining EJP amplitude. The EJP amplitude stabilized at approximately 160% of the initial baseline amplitude. Results of three separate experiments are shown. (B) Removal of forskolin after 20 min coupled with block of HCNC and activation of Epac by 8-CPT resulted in maintained enhancement of EJP amplitude ($n = 5$).

DISCUSSION

Modulation of synaptic transmission is thought to play an important role in adaptive mechanisms within the nervous system and in memory formation (Kandel and Schwartz, 1982). Both pre- and postsynaptic processes are involved, and several second-messenger pathways participate (Nguyen et al., 1995; Salin et al., 1996; Carroll et al., 1998; Tzounopoulos et al., 1998). Previous studies conducted over many years in a range of organisms, especially in vertebrates, mollusks, and arthropods, have implicated cAMP-regulated mechanisms in both short- and long-term modification of synaptic strength. A major message of this previous work is that elevation of cAMP can act through several pathways in the presynaptic neuron to cause increased release of neurotransmitter, manifested as both short- and long-term increases in synaptic strength (Kennedy et al., 1992; Bailey et al., 1996; Byrne and Kandel, 1996; Salin et al., 1996). Most previous studies of the role of cAMP in short-term synaptic enhancement have implicated cAMP-activated PKA as an essential step. It is generally thought that PKA phosphorylates presynaptic ion channels (Kandel and Schwartz, 1982) or target proteins involved in mobilization and/or release of synaptic vesicles (Trudeau et al., 1998) to achieve its short-term enhancing effects. For example, studies on mammalian synapses formed in culture have provided evidence that PKA has a direct action on the secretory machinery, modulating its sensitivity to intracellular Ca^{2+} and thereby regulating vesicular exocytosis (Trudeau et al., 1996, 1998).

Long-lasting synaptic changes induced by cAMP elevation in the presynaptic neuron require protein synthesis and are associated with structural changes in several well-studied examples (Bailey and Kandel, 1993; Davis et al., 1996). The present experiments address short-term synaptic modulation and a possible transition to a longer-lasting, less malleable modification.

The well-established and widely accepted role for PKA in modulating transmitter release does not rule out other cAMP-dependent mechanisms. Recent evidence from both mammalian (Kaneko and Takahashi, 2004) and crustacean (Beaumont and Zucker, 2000; Zhong and Zucker, 2004) synapses has indicated that two other mechanisms responding to cAMP are coupled to presynaptic regulation of neurotransmission. First, hyperpolarization-activated nonselective cation channels (variously termed I_h channels, HCN channels, or HCNCs in recent literature) appear to be linked to the presynaptic vesicular release machinery, possibly through cytoskeletal elements (Siegelbaum, 2000). These channels are regulated by cyclic nucleotides acting on intracellular binding sites. Second, a major role for cAMP-activated guanine exchange factors (exchange proteins activated by cAMP) Epac 1 and 2, which can activate the monomeric G protein Rap (Kopperud et al., 2003) and bind to certain isoforms of the vesicle protein Rim (Ozaki et al., 2000), has been demonstrated in both crustacean (Zhong and Zucker, 2004) and mammalian (Kaneko and Takahashi, 2004) synapses.

In the present study, we have provided initial evidence for both of these cAMP responsive systems in *Drosophila* synapses. Many previous studies in *Drosophila* have supported a role for cAMP-dependent mechanisms in learning and memory (Byers et al., 1981), experience-dependent synaptic strengthening (Budnik et al., 1990; Davis et al., 1996; Cheung et al., 1999), the movement of vesicles between reserve and readily releasable pools (Kuromi and Kidokoro, 2000; Renger et al., 2000), and the regulation of spontaneous neurotransmitter release (Yoshihara et al., 2000). In both *Drosophila* and in crayfish, effects of FKL and other agents that promote effects of cAMP are predominantly presynaptic, because quantal size is not affected, while frequency of spontaneous quantal emission is increased (Yoshihara et al., 2000; Beaumont and Zucker, 2000). Our observations confirmed a lack of effect on mEJP amplitude. Lengthening of EJPs after FKL treatment was detected, indicating a possible postsynaptic effect, but the small effects observed cannot account for the large changes in EJP amplitude. While it has generally been assumed that all of these processes are dependent on PKA, no previous study has tested the alternatives. Here we have employed pharmacological tests for the involvement of HCNCs and Epac, and found that FKL-induced enhancement of synaptic transmission can be altered by these agents. The results are similar to those of previous more extensive experiments carried out on the crayfish NMJ (Zhong and Zucker, 2004). Thus at present, it appears likely that the mechanisms governing cAMP-dependent potentiation of transmitter release and its transition to a long-lasting form may be shared among arthropods, and possibly also with other phyla. This being the case, it would be feasible to use the *Drosophila* model, with its associated genetic tools, to investigate further the details of the cAMP-dependent mechanisms of presynaptic regulation of transmitter release.

An interesting feature of the present experiments is the possible transition from a temporary, readily reversible cAMP-dependent enhancement to a long-lasting, less readily reversible enhancement. When FKL-induced potentiation of the EJP is challenged by

application of the HCNC blocker ZD7288 10–20 min after its initiation, it is slowly reversible; thereafter, it becomes resistant to this challenge. Apparently, transition to a more durable enhancement requires at least 20 min of elevated cAMP. Participation of Epac in the formation of the long-lasting phase is indicated by the prevention of ZD7288's effect during the period in which it can reverse the potentiation. This ''consolidation time'' for HCNC activation, after which further HCNC activation is unnecessary so long as Epac is activated, is reminiscent of the process at crayfish NMJs called ''temporal synaptic tagging'' (Beaumont et al. 2002). In those junctions, sustained presynaptic activity induces a long-term facilitation of transmitter release by a process that also involves HCNC activation. Following the induction protocol, which requires 10 min of stimulation and a further 10 min for tagging to be ''set'' (Zhong and Zucker, 2004), cAMP-dependent enhancement of release still occurs, but is now resistant to block of HCNCs .

We thank Marianne Hegström-Wojtowicz for technical assistance.

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