Distinct Roles of CaMKII and PKA in Regulation of Firing Patterns and K⁺ Currents in Drosophila Neurons

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Received 11 April 2000; accepted in final form 19 December 2000

Yao, Wei-Dong and Chun-Fang Wu. Distinct roles of CaMKII and PKA in regulation of firing patterns and K⁺ currents in Drosophila neurons. J Neurophysiol 85: 1384–1394, 2001. The Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and the cAMP-dependent protein kinase A (PKA) cascades have been implicated in neural mechanisms underly- ing learning and memory as supported by mutational analyses of the two enzymes in Drosophila. While there is mounting evidence for their roles in synaptic plasticity, less attention has been directed toward their regulation of neuronal membrane excitability and spike information coding. Here we report genetic and pharmacological analyses of the roles of PKA and CaMKII in the firing patterns and underlying K⁺ currents in cultured Drosophila central neurons. Genetic perturbation of the catalytic subunit of PKA (DCO) did not alter the action potential duration but disrupted the frequency coding of spike-train responses to constant current injection in a subpopulation of neurons. In contrast, selective inhibition of CaMKII by the expression of an inhibitory peptide in ala transformants prolonged the spike duration but did not affect the spike frequency coding. Enhanced membrane excitability, indicated by spontaneous bursts of spikes, was observed in CaMKII-inhibited but not in PKA-diminished neurons. In wild-type neurons, the spike train firing patterns were highly reproducible under consistent stimulus conditions. However, disruption of either of these kinase pathways led to variable firing patterns in response to identical current stimuli delivered at a low frequency. Such variability in spike duration and frequency coding may impose problems for precision in signal processing in these protein kinase learning mutants. Pharmacological analyses of mutations that affect specific K⁺ channel subunits demonstrated distinct effects of PKA and CaMKII in modulation of the kinetics and amplitude of different K⁺ currents. The results suggest that PKA modulates Shaker A-type currents, whereas CaMKII modulates Shal-A type currents plus delayed rectifier Shab currents. Thus differential regulation of K⁺ channels may influence the signal handling capability of neurons. This study provides support for the notion that, in addition to synaptic mechanisms, modulations in spike activity patterns may represent an important mechanism for learning and memory that should be explored more fully.

INTRODUCTION

The Ca²⁺/calmodulin-dependent protein kinase II (CaMKII) and cAMP-dependent protein kinase A (PKA) cascades play critical roles in learning and memory behaviors in both vertebrates (Byrne and Kandel 1996; Chen and Tonegawa 1997) and invertebrates (Davis 1996; Tully and Quinn 1989). CaMKII and PKA are necessary for spatial memory performance in mice (Huang et al. 1995; Mayford et al. 1996; Silva et al. 1992a). In Drosophila, inhibition of CaMKII by genetic transformation alters both nonassociative and associative conditioning (Griffith et al. 1993) and disruptions of cAMP pathways by mutations that alter the dance (a phosphodiesterase gene), rutabaga (an adenyl cyclase gene), or PKA gene impair learning and memory in a variety of behavioral paradigms (Aceves-Pina and Quinn 1979; Drain et al. 1991; Dudai et al. 1976; Duerr and Quinn 1982; Goodwin et al. 1997; Heisenberg et al. 1985; Li et al. 1995; Quinn et al. 1974; Siegel and Hall 1979; Spatz et al. 1974; Wustmann et al. 1996).

The cellular basis of CaMKII- and PKA-mediated behavioral plasticity has been intensively studied. The major focus has been modifications in synaptic strength and nerve terminal sprouting (Bailey and Kandel 1993; Bliss and Collingridge 1993), especially in the context of hippocampal long-term potentiation in mammals (Huang et al. 1995; Malenka et al. 1989; Malinow et al. 1989). In Drosophila, inhibition of CaMKII and mutations of dnc and rut genes alter synaptic transmission and nerve terminal arborization at larval neuromuscular junctions (Wang et al. 1994; Zhong and Wu 1991; Zhong et al. 1992).

Modulation of intrinsic membrane properties of neurons can profoundly change network dynamics and performance (Gilbert 1989; Harris-Warrick and Marder 1991) and may represent another important cellular mechanism for behavioral plasticity, one that has received less attention in learning and memory studies. To more fully understand the cellular basis of learning and memory, it will be important to determine the roles of PKA and CaMKII in regulation of intrinsic membrane properties in neurons. Although dnc and rut mutations have been shown to affect central neuron firing patterns (Zhao and Wu 1997), it is not known to what degree this effect is mediated by PKA, a downstream component of the cAMP pathway. It is also not known how the electrical activities of central neurons are regulated by CaMKII or how this regulation compares with modulation by PKA. Some useful insights into the cellular operations that subserve learning and memory processes may be gained from such studies given the apparently similar deficiencies in synaptic and behavioral plasticity caused by gene knockouts of these two kinases in mammalian systems (Huang et al. 1995; Mayford et al. 1996; Silva et al. 1992a,b).

Inhibition of CaMKII causes limbic epilepsy in mice (Butler et al. 1995) and enhanced nerve firing in Drosophila (Griffith et al. 1993).
et al. 1994), suggesting a possible role for CaMKII in the regulation of ion channels. In *Drosophila*, voltage-dependent K⁺ currents are altered by dnc and rut (Delgado et al. 1998; Zhao and Wu 1997; Zhong and Wu 1997; Levin et al. 1992; Wu 1997; Byers et al. 1981; Livingston et al. 1984), raising the possibility that K⁺ channels may be downstream targets of PKA regulation. Correspondingly, central neurons of these memory mutants show aberrant spike frequency coding (Zhao and Wu 1997). Furthermore K⁺ channel mutants display abnormal habituation in a defined neural circuit, similar to altered habituation found in *dnc* and *rut* flies (Engel and Wu 1996, 1998). These findings suggest a close link among K⁺ channel activity, neuronal spike patterns, and behavioral plasticity.

The *Drosophila* “giant” neuron culture system has facilitated patch-clamp characterization of neuronal spike activity and the underlying ionic currents (Renger et al. 1999; Saito and Wu 1991; Wu et al. 1990; Yao and Wu 1999a,b; Zhao et al. 1995). Two transgenic fly strains, *ala1* and *ala2*, carry a heat-shock inducible mini-gene for a specific peptide inhibitor of CaMKII (Griffith et al. 1993). *DCO-K* is an EMS-induced mutation that inactivates a catalytic subunit of PKA, causing dramatically reduced PKA activity (Li et al. 1995). These *Drosophila* strains, together with several mutants and transfectants of K⁺ channel subunits, allowed us to study how PKA and CaMKII regulate neuronal firing patterns and how such effects may be mediated by modulations of identified K⁺ channels.

**METHODS**

**Fly stocks**

Canton S (CS) was the wild-type control strain used in all experiments. *Sh¹M* and *eag¹* were both from the collection of Dr. S. Benzer at Cal Tech. *Sh¹M* is a null allele of *Sh* (Zhao et al. 1995). *Shab¹* was generated in Dr. S. Singh’s Lab at SUNY Buffalo. Three lines of ShD transformants were generated by inserting a P-element vector containing a ShD cDNA fused to a hsp-70 promoter into the first, second, or third chromosome in the *Sh¹M* genetic background. No basal expression of ShD channels is observed at room temperature (Zhao et al. 1995). *DCO-K¹*, a cold-sensitive allele of the *DCO* gene that encodes the major catalytic subunit (RI) of PKA, is lethal at 18°C but viable at 25°C (Li et al. 1995). The transgenic CaMKII inhibitor strains *ala1* and *ala2* were generated with P-element insertions into the first and second chromosome, respectively (Griffith et al. 1993). Expression of the inhibitory peptide is under control of the heat shock promoter hsp-70. The *ala* transformants show a low basal level of expression of the inhibitory peptide even without heat-shock treatment (Griffith et al. 1993). All fly stocks were maintained on standard *Drosophila* media at 20–22°C except for *DCO-K¹*, which was kept at 25°C. All strains used were homozygous for the corresponding mutations.

**Cell culture and electrophysiology**

The procedure for culturing *Drosophila* “giant” neurons has been described previously (Saito and Wu 1991). Briefly, embryos at the gastrulation stage were gently dissociated in modified Schneider medium (GIBCO, Grand Island, NJ) containing 200 ng/ml Insulin (Sigma, St. Louis, MO), 20% fetal bovine serum (FBS), 50 µg/ml streptomycin, and 50 U/ml penicillin. Cells were washed in the above medium and resuspended in medium containing 2 µg/mg Cytochalasin B (Sigma), and then plated on glass coverslips. Cultures were maintained in humidified chambers at room temperature (20–25°C) for 2–5 days prior to recordings.

Standard whole cell patch-clamp recording has been described previously (Saito and Wu 1991). Recording bath solutions (Jan and Jan 1976) contained (in mM) 128 NaCl, 2 KCl, 4 MgCl₂, 1.8 CaCl₂, and 35.5 sucrose, buffered with 5 HEPES at pH 7.1. Patch pipettes were filled with solution containing (in mM) 144 KCl, 1 MgCl₂, 0.5 CaCl₂, and 5 EGTA, buffered with 10 HEPES, pH 7.1. K⁺ channels were isolated by adding TTX (0.2 µM) and Cd²⁺ (0.2 mM) to the bath solution. PKA inhibitor Rp-cAMPS and CaMKII inhibitors KN-62 and KN-93 were from Calbiochem (La Jolla, CA). Recordings were obtained at room temperature from isolated neurons with a patch clamp amplifier (Axopatch 1B, Axon Instruments, Foster City, CA). Data acquisition and analysis were carried out using pCLAMP and Axograph software (Axon Instruments).

**Heat-shock protocol**

Induction of transgene expression in ShD, *ala¹*, and *ala2* cultures was accomplished by placing cell cultures in a 38.5°C incubator for 45 min. The heat-shock temperature and duration were established in previous studies to be sufficient for induction of either peptide (Griffith et al. 1993; Wang et al. 1994; Zhao et al. 1995). Detectable expression of the CaMKII inhibitory peptide in *ala* lines, but not ShD channels, was significantly induced by the cellular stress imposed by the current cell culture procedures. *ala* phenotypes with or without heat shock did not differ, and thus results from heat-shock-treated and non–heat-shocked neurons were pooled in all experiments.

**RESULTS**

**Alterations of excitability and firing patterns in *ala* and *DCO* neurons**

Whole cell current-clamp experiments were performed on the soma of isolated giant neurons to minimize complications introduced by synaptic connectivity. On injection of depolarizing currents, three types of voltage responses were observed in both wild-type and mutant neurons: all-or-none, graded, and nonregenerative (Saito and Wu 1991). Among these, the all-or-none type action potentials have been best characterized (Zhao and Wu 1997) and will be emphasized here. In wild-type cells with all-or-none action potentials, firing patterns can be functionally classified into adaptive, tonic, and delayed categories based on their instantaneous spike frequency and first spike latency (Fig. 1), following the criteria described previously (Zhao and Wu 1997). Specifically, the delay to first spike, and the intervals between the first two spikes (*fₚ₀₀*) and the last two spikes (*fₚ₉₉*) in the spike train were used to distinguish three different firing patterns. “Delayed” showed a delayed onset (>100 ms) of the first spike. “Adaptive” displayed decreasing firing frequency within a spike train and normally conformed to the criteria of latency <100 ms and *fₚ₀₀*< *fₚ₉₉* < 0.7. “Tonic” showed firing patterns with latency <100 ms but *fₚ₀₀*< *fₚ₉₉* > 0.7, hence the firing frequency in these neurons was relatively constant.

It should be noted that such categorization was based on spike trains during the first run of a current-clamp protocol immediately after a whole cell configuration was established. This stimulation protocol consisted of a series of 400-ms current steps ranging from −30 to 300 pA. The spike train in response to a current pulse at the strength of 40 pA above threshold was taken for categorizing the firing patterns. Firing
patterns classified this way represented a condition closer to the "native" excitability states of the neuron.

Because both CaMKII and PKA cascades are implicated in mechanisms subserving learning and memory, we sought to discern how the two systems modulate distinct aspects of neuronal firing patterns. *ala*1 and *ala*2 neurons, in which CaMKII activity is inhibited by a specific inhibitory peptide, typically displayed prolonged action potential waveforms (Figs. 1 and 2) and abnormal spontaneous activity (Fig. 3) with the following characteristics. First, the spike duration, which was determined as the width at the inflection point during an action potential take-off in a spike train, was substantially prolonged in mutant neurons ("Abnormal"). Note the fluctuations in firing rate in *DC0*4 neurons and action potential prolongation in *ala* neurons which maintained the regularity in firing rate.

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### TABLE 1. Firing properties of wild-type and mutant neurons

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Incidence</th>
<th>Mean Frequency</th>
<th>Amplitude</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (44)</td>
<td>18</td>
<td>19 ± 1.7</td>
<td>31.7 ± 4.7</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>Adaptive</td>
<td>68</td>
<td>16.5 ± 1.5</td>
<td>49.9 ± 1.9</td>
<td>4.0 ± 0.4</td>
</tr>
<tr>
<td>Tonic</td>
<td>14</td>
<td>18.3 ± 3.4</td>
<td>31.6 ± 5.1</td>
<td>4.2 ± 0.4</td>
</tr>
<tr>
<td>Delayed</td>
<td>20</td>
<td>21.6 ± 1.9</td>
<td>39.6 ± 2.6</td>
<td>5.7 ± 0.6*</td>
</tr>
<tr>
<td><em>ala</em>1 (25)</td>
<td>64</td>
<td>18.0 ± 2.2</td>
<td>42.7 ± 3.3*</td>
<td>6.0 ± 1.1*</td>
</tr>
<tr>
<td>Adaptive</td>
<td>16</td>
<td>13.2 ± 1.5</td>
<td>39.5 ± 3.8</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>Tonic</td>
<td>20</td>
<td>13.3 ± 2.3*</td>
<td>39.2 ± 5.8</td>
<td>6.0 ± 0.8*</td>
</tr>
<tr>
<td>Delayed</td>
<td>60</td>
<td>24.5 ± 2.0*</td>
<td>41.4 ± 4.2*</td>
<td>5.8 ± 0.9*</td>
</tr>
<tr>
<td><em>ala</em>2 (28)</td>
<td>22</td>
<td>15.0 ± 2.6</td>
<td>45.8 ± 10.0</td>
<td>5.7 ± 1.4</td>
</tr>
<tr>
<td>Adaptive</td>
<td>18</td>
<td>36.3 ± 6.0*</td>
<td>17.1 ± 0.8*</td>
<td>3.7 ± 0.1</td>
</tr>
<tr>
<td>Tonic</td>
<td>50</td>
<td>28.2 ± 4.1*</td>
<td>43.9 ± 4.7</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Delayed</td>
<td>17</td>
<td>17.8</td>
<td>16.5</td>
<td>3.5</td>
</tr>
</tbody>
</table>

Data are means ± SE except for *DC0* delayed type, which had few cells. Total number of cells firing regular all-or-none action potentials categorized for each genotype is indicated in parentheses. Mean frequency is determined based on the number of spikes elicited by a 400-ms depolarization current step at a strength of 40 pA above threshold. Amplitude is measured as the voltage difference between the peak and the valley of the first action potential in a spike train. Duration is measured at the inflexion point during take-off of the first spike. *P* < 0.05, Student’s *t*-tests.
CNS (Yao and Wu 1999a). The percentage of these putative pace-making cells in ala (3–5%) was similar to that of wild-type culture (2%; Fig. 3). The incidence of adaptive, tonic, and delayed firing as well as the spike amplitude and the mean spike frequency in response to suprathreshold stimulation were also summarized in Table 1.

Both abnormal spontaneous activity and aberrant spike frequency coding have been demonstrated in neurons with dnc and rut mutations (Zhao and Wu 1997), both of which disrupt cAMP metabolism and cause learning and memory deficiencies (Aceves-Pina et al. 1983; Byers et al. 1981; Dudai et al. 1976; Livingstone et al. 1984). Although the majority of the cAMP effects in eukaryotes may be mediated through activation of PKA, intracellular cAMP has also been shown to interact directly with ion channels in some cell types (Delgado et al. 1991; Dhallan et al. 1990). Thus to establish the functional roles of PKA in mediating the phenotypes of the memory mutants dnc and rut, it is necessary to employ mutants such as DCOX4 in which direct perturbation of the kinase activity occurs.

We found that very few DCOX4 neurons displayed sporadic bursting activities with a percentage not statistically different from that of wild type (Fig. 3). This indicates that the increased spontaneous activity in dnc and rut neurons (Zhao and Wu 1997) may be mediated by mechanisms other than PKA modulation. The resting membrane potential, distribution of firing patterns, and action potential duration are not affected in DCOX4 (Table 1 and data not shown). The firing frequency was significantly increased in adaptive and tonic DCOX4 neurons. The action potential amplitude was significantly decreased in adaptive but slightly reduced in tonic neurons in DCOX4. We noticed that the most remarkable mutant phenotype of firing patterns in DCOX4, however, is the erratic firing rate during current injection (Figs. 1 and 4). These findings show that abnormality in firing frequency coding can occur without altered action potential duration (Figs. 1 and 2). With respect to this particular phenotype, the DCOX4 results were in general consistent with the dnc and rut study (Zhao and Wu 1997), suggesting that defective frequency coding caused by abnormal cAMP levels may be mediated by PKA.

In contrast, frequency coding of all-or-none spike trains in ala neurons in response to current injections appeared normal, and the instantaneous firing rates in tonic and adaptive categories were indistinguishable between ala and wild type (Figs. 1 and 4). It is remarkable that the spike frequency coding remained intact despite the fact that the action potential duration in some ala neurons is significantly prolonged compared with wild-type neurons (Figs. 1, 2, and 4 and Table 1). These results indicate that perturbations of CaMKII and PKA activities exert influences on distinct neuronal firing parameters. CaMKII appears to play a major role in defining the width of individual action potentials and maintaining their waveform during sustained activity. PKA, on the other hand, is more important to the regularity of spike frequency coding.

Altered stability of spike frequency coding in ala and DCO neurons

Responses to individual current pulses can reveal erratic patterns of spike trains in mutant neurons, such as ala1, ala2, and DCOX4. However, a single-pulse paradigm cannot determine the stability of the spike patterns generated by a given neuron. Such endogenous stability of firing patterns is important to ensure a reliable and consistent input-output relationship during signal processing by the neuron. We examined this problem by using a revised paradigm involving repetitive stimulation at a low frequency. When neurons were stimulated by step current injections (80–120 pA) at 0.5 Hz or lower to minimize the interaction between successive pulses, wild-type neurons displayed highly reproducible firing patterns in response to these identical repeated stimuli (Fig. 5A). The firing pattern, in particular the number of spikes in each spike train, varied to a much greater extent in ala and DCOX4 neurons. To quantify the variation in the number of spikes evoked by a fixed current pulse for each genotype, the coefficient of variance (CV = SD/Mean) was determined for 20 repeated stimuli and presented in relation to the mean spike number for each cell (Fig. 5B). A markedly increased CV was seen in a large proportion of ala1, ala2, and DCOX4 neurons compared with wild type, indicating significant intrinsic instability in the mutant neurons. Although our frequency analysis of different firing patterns indicates that mutant neurons do not fire fewer action potentials in general (Table 1), the variability among responses to identical stimuli was most evident for neurons that generated fewer spikes (Fig. 5B). Thus both CaMKII and PKA play crucial roles in maintaining the stability of neuronal firing patterns, which may be important for the proper performance of neural circuits during information processing.
of the double-exponential approximation for Type 1 currents was significantly reduced compared with WT neurons (Fig. 6). The specific effect of \( \text{ala} \) mutations on the fast decay time constant is consistent with previous pharmacological studies that showed accelerated decay of \( K^+ \) currents after CaMKII inhibition (Peretz et al. 1999; Yao and Wu 1999b). However, the slow inactivation time constant (\( \tau_s \), not shown) in Type 1 currents and the decay rate (\( \tau \)) of Type 2 currents did not significantly differ among genotypes (Fig. 6). These results suggest that CaMKII may differentially regulate certain channels with distinct kinetic properties in \( Drosophila \) neurons.

To examine how the amplitudes of \( I_K \) and \( I_{\text{al}} \) in the two current types were affected in \( \text{ala} \) and \( DC0^{X4} \) neurons, we used a prepulse paradigm to separate the early, inactivating currents (\( I_\lambda \)) and the delayed, noninactivating currents (\( I_K \); Fig. 7A). We found that both \( \text{ala} \) and \( DC0^{X4} \) mutations affected neurons displaying Type 1 currents. In these cells, current density of \( I_\lambda \) was reduced significantly in both \( \text{ala} \) and \( DC0^{X4} \) neurons (Fig. 7B). When the current-voltage relations (\( I-V \) curves) are normalized for the three genotypes (Fig. 7C), it is apparent that the reductions in current amplitude were independent of membrane potentials. The current density of \( I_K \) was also reduced in \( \text{ala} \) and \( DC0^{X4} \) neurons. However, there was an apparent voltage dependence of this effect with more severe reduction at lower

\[ \text{Altered } K^+ \text{ currents in } \text{ala} \text{ and } DC0^{X4} \text{ neurons} \]

Voltage-activated \( K^+ \) currents regulate membrane repolarization and hence excitability and firing patterns in neurons (Hille 1992; Rudy 1988). The altered neuronal firing patterns and aberrant spontaneous hyperexcitability observed in \( \text{ala} \) and \( DC0^{X4} \) neurons suggested that CaMKII and PKA affect \( K^+ \) current mechanisms. In the giant neuron culture system, various voltage-activated \( K^+ \) currents and the effects of mutations of identified \( K^+ \) channel subunits have been well characterized (Yao and Wu 1999a; Zhao et al. 1995). Abnormality in kinetics and amplitude of different current components may be readily determined. Some of the striking mutant phenotypes observed in current clamp experiments might be correlated to changes in \( K^+ \) current properties that could be revealed in voltage-clamp records.

Voltage-activated \( K^+ \) currents were examined in saline containing TTX and \( Ca^{2+} \) to eliminate inward \( Na^+ \) and \( Ca^{2+} \) currents as well as outward \( Ca^{2+} \)-activated \( K^+ \) currents (Saito and Wu 1991). Two types of \( K^+ \) currents with different inactivation kinetics can be seen in wild-type neurons (Fig. 6A) (cf. Delgado et al. 1998). The decay of Type 1 currents could be fitted by a two-exponential process with fast [\( \tau_1 = 68.3 \pm 4.1 \) (SE) ms] and slow (\( \tau_2 = 1,133 \pm 221 \) ms) components. Type 1 currents were encountered in the majority of giant neurons (~60%, \( n = 59 \)). Type 2 currents, found in the remaining cells (~40%), inactivated with a single-exponential time course (\( \tau = 1,066 \pm 485 \) ms). Both 4-aminopyridine (4-AP)- and TEA-sensitive components were observed in the two types of currents. In general, Type 1 currents contained a greater 4-AP-sensitive, fast-inactivating component, and Type 2 currents were more sensitive to TEA (data not shown).

We found similar fractions of neurons in wild-type and mutant cultures expressing Type 1 versus Type 2 currents [wild type (WT): 73 vs. 27%; \( \text{ala1} \): 75 vs. 25%; \( \text{ala2} \): 73 vs. 27%; \( DC0^{X4} \): 62 vs. 38%, no significant differences under 1-sample binomial tests]. In \( \text{ala1} \) and \( \text{ala2} \) but not in \( DC0^{X4} \) neurons, \( \tau_1 \)
whereas affects both the kinetics and amplitude of Type 1 currents, \( I \) gives current with a prepulse (\( A_2 \)) from the one without (\( A_1 \)). Further current- and voltage-clamp correlation analysis in the membrane potentials (especially in \( ala \) neurons) (Fig. 7C), which is also reflected in the significantly shifted half-activation voltage in \( ala \) and \( DCO^{2-64} \) (Fig. 7D). The amplitudes of \( I_A \) and \( I_K \) components of Type 2 currents were not significantly different between WT and \( ala \) neurons [WT: \( I_A = 13.17 \pm 1.29 \), \( I_K = 19.85 \pm 0.77 \) (\( n = 3 \)); \( ala \): \( I_A = 13.59 \pm 4.32 \), \( I_K = 17.31 \pm 2.39 \) (\( n = 3 \)); \( P > 0.05 \), t-tests; consistent results were also obtained from nonseparated \( I_A \) and \( I_K \) (data not shown)]. All currents were recorded at +20 mV. In contrast, the \( DCO^{2-64} \) mutation did not affect \( I_A \) but decreased \( I_K \) components of Type 2 current significantly [\( DCO^{2-64} \): \( I_A = 19.02 \pm 2.78 \), \( I_K = 9.01 \pm 2.09 \) (\( n = 5 \)); \( P < 0.01 \)]. Taken together, \( ala \) affects both the kinetics and amplitude of Type 1 currents, whereas \( DCO^{2-64} \) selectively affects current amplitude but in both Type 1 and Type 2 currents. Such distinct effects on current density may contribute to the differences in the regulation of excitability and firing patterns by CaMKII and PKA. Further current- and voltage-clamp correlation analysis in the same cell may reveal how neurons of different firing patterns express varying portions of CaMKII- and PKA-sensitive \( K^+ \) current components.

**Genetic dissection of CaMKII and PKA modulation on \( K^+ \) currents**

Voltage-gated \( K^+ \) channel \( \alpha \) subunits in *Drosophila* neurons, encoded by the identified genes \( Sh, Shal, Shab, \) and \( Shaw \), generate \( K^+ \) currents with distinct kinetics and voltage dependence when expressed in heterologous systems (Butler et al. 1989; Iverson and Rudy 1990; Timpe et al. 1988). Visible point mutations of \( Sh \) (Wu and Ganetzky 1992) and \( Shab \) (Singh and Singh 1999) provide powerful tools to dissect the downstream effectors of PKA and CaMKII for modulating \( K^+ \) currents. Highly selective pharmacological agents that inhibit CaMKII and PKA are also available to enhance the genetic approach.

KN-62 and -93 are specific inhibitors of CaMKII (Sumi et al. 1991; Tokumitsu et al. 1990) while Rp-cAMPS (adenosine 3',5'-cyclic monophosphorothioate, Rp-Isomer; 50 \( \mu M \)) selectively inhibits PKA activity. When applied to the bath solution, CaMKII inhibitors KN-62 (10 \( \mu M \)) and KN-93 (1 \( \mu M \)) and PKA inhibitor Rp-cAMPS (50 \( \mu M \)) all reduced both the peak and sustained \( K^+ \) currents in WT neurons (Fig. 8).

*Sh* mutations alter A-type currents in muscle (Salkoff and Wyman 1981; Wu and Haugland 1985) and in neurons (Baker and Salkoff 1990). The null mutation \( Sh^M \) deletes all \( Sh \) subunits in neurons and could be used to test whether \( Sh \) subunits are a target for CaMKII or PKA modulation. As shown in Figs. 9 and 10, the non-Sh currents that remain in \( Sh^M \) neurons, including both the A-type and the sustained components, were significantly affected by KN-93 in both amplitude and kinetics to a degree similar to those of WT neurons. In contrast, the non-Sh currents were apparently resistant to modulation by Rp-cAMPS (Figs. 9B and 10B).

FIG. 7. Reduced \( I_A \) and \( I_K \) of Type 1 \( K^+ \) currents in \( ala \) and \( DCO^{2-64} \) neurons. \( A \): the voltage protocol used to separate \( I_A \) from \( I_K \). A 1-s preconditioning pulse to +10 mV from a holding potential of +80 mV was used to inactivate \( I_A \) while leaving \( I_K \) intact (\( A_2 \)). Subtraction of the voltage-activated current with a prepulse (\( A_2 \)) from the one without (\( A_1 \)) gives \( I_A (A_1 - A_2) \). \( B \): \( I-V \) relation of \( I_A \) and \( I_K \). \( C \): relative reduction of \( I_A \) and \( I_K \) in \( ala \) and \( DCO \) neurons. The \( I_A \) and \( I_K \) for mutants are normalized to the corresponding values for WT, which are shown as a horizontal line at 1.0. \( D \): activation \( V_{m1/2} \) and slopes for \( I_A \) and \( I_K \). The membrane conductance (determined by the formula \( G = B(V - V_x) \), where \( l \) is the current density in pA/pF, and \( V_r \) the reversal potential (−75 mV) was fit to the Boltzmann relationship \( G = G_0/[1 + \exp((V_m1/2 - V)/V_{shpd})] \), where \( G_0 \), \( V_{m1/2} \) and \( V_{shpd} \) are the maximum conductance, half-activation voltage (mV), and slope (mV/e-fold), respectively.

FIG. 8. Modulation of \( K^+ \) currents by CaMKII and PKA inhibitors in WT neurons. CaMKII antagonists KN-93 and -62 suppressed both the transient and sustained \( K^+ \) currents and accelerated the decay process of the transient component. The PKA inhibitor Rp-cAMPS reduced the transient and the sustained \( K^+ \) currents without affecting the current decay kinetics. \( K^+ \) currents were elicited by depolarization steps (1 s) from a holding potential of −80 mV to between −60 and +60 mV in 20-mV increments. The final drug concentrations in saline: KN-93, 1 \( \mu M \); KN-62, 10 \( \mu M \); Rp-cAMPS, 50 \( \mu M \).
To directly assay the modulation of an identified Shα subunit by CaMKII kinase and PKA, we used ShD transgenic strains in which a copy of ShD cDNA was inserted into the first, second, or third chromosome in the ShM host background to express ShD channels in neurons lacking endogenous Sh channels. The expressed ShD current, induced by heat shock (see METHODS), exhibited fast inactivation kinetics and extremely slow recovery from inactivation (Zhao et al. 1995). These distinct kinetic properties allow the isolation of this current from the native K1 currents by a twin pulse paradigm (Renger et al. 1999; Zhao et al. 1995). As shown in Figs. 9 and 10, ShD current was inhibited by Rp-cAMPS but not by KN-93. Taken together, the results support the idea that the Sh product is a target for PKA, but not for CaMKII, modulation.

The Shab gene has been proposed to be responsible for the major slowly inactivating delayed rectifier K+ currents in Drosophila neurons (Tsunoda and Salkoff 1995b) and muscle cells (Singh and Singh 1999). We found that a point mutation, Shab1 (Singh and Singh 1999), eliminated most of the steady-state currents in giant neurons (Fig. 9). This mutation allowed us to analyze further how CaMKII and PKA modulate the different K+ currents in neurons. Figures 9 and 10 show that in Shab-deficient neurons, the transient A current (more prominent due to the removal of the delayed rectifier) was still significantly modified by KN-93 and Rp-cAMPS. However, these two drugs did not significantly affect the small sustained non-Shab components remaining in Shab-deficient neurons.

The A current in Drosophila neurons is thought to be primarily composed of Shal currents (Tsunoda and Salkoff 1995a) with a smaller contribution from Sh (Baker and Salkoff 1991). The sustained current consists of a number of components including the delayed rectifiers Shab (Tsunoda and Salkoff 1995a) and the steady-state components of A-type Sh and Shal currents. Currents conferred by Shab subunits have also been implicated as part of the sustained current (Tsunoda and Salkoff 1995a). The suppression of A-type currents in Shab1 neurons is consistent with the idea that CaMKII and PKA modulate Shal and Sh channels, respectively (see preceding text). In contrast, the lack of significant effects of CaMKII inhibitors on the non-Shab sustained current in Shab1 neurons suggests that modulation of the Shab current may be responsible for part of the reduction in sustained currents of WT neurons by those drugs.

In summary, a working scheme about PKA and CaMKII modulation of K+ channels can be proposed based on the genetic and pharmacological data: modulation of A currents is mediated by PKA on Sh channels and CaMKII on Shal channels (Fig. 11), whereas modulation of delayed rectifier K+ currents by CaMKII is mediated primarily by Shab. Future work on Shaw mutants, when they become available, should significantly improve our understanding of how different Shα subunits can modulate various K+ currents in Drosophila neurons.

**FIG. 9.** Modulation of K+ currents by CaMKII and PKA inhibitors in different K+ channel mutants and ShD transformants. A: modulatory effects of KN-93 on K+ currents recorded from ShM and Shab1 mutants and on heat-shock (HS)-induced ShD currents. The HS-induced slow-recovering ShD currents were extracted by a protocol described previously (Renger et al. 1999; Zhao et al. 1995). B: modulatory effects of Rp-cAMPS on K+ currents in ShM, Shab, and ShD transformant cells. Voltage-activated K+ currents were elicited by depolarizing the neurons to +40 mV (1 s) from a holding potential of −80 mV. The final concentrations of KN-93 and Rp-cAMPS in saline were 1 and 50 μM, respectively.

To directly assay the modulation of an identified Shα subunit by CaMKII kinase and PKA, we used ShD transgenic strains in which a copy of ShD cDNA was inserted into the first, second, or third chromosome in the ShM host background to express ShD channels in neurons lacking endogenous Sh channels. The expressed ShD current, induced by heat shock (see METHODS), exhibited fast inactivation kinetics and extremely slow recovery from inactivation (Zhao et al. 1995). These distinct kinetic properties allow the isolation of this current from the native K+ currents by a twin pulse paradigm (Renger et al. 1999; Zhao et al. 1995). As shown in Figs. 9 and 10, ShD current was inhibited by Rp-cAMPS but not by KN-93. Taken together, the results support the idea that the Sh product is a target for PKA, but not for CaMKII, modulation.

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provide further evidence concerning the genetic basis of CaMKII and PKA modulation of K⁺ currents.

**DISCUSSION**

In this study, we report that CaMKII and PKA modulate different aspects of neuronal firing properties, resulting at least in part from differential targeting of voltage-gated K⁺ channels by the two enzymes. Our results establish roles for the two Ca²⁺/CaM-activated pathways in neuronal spike shaping and firing patterning. These properties provide another regulatory mechanism, in addition to modulation of synaptic efficacy, which may contribute to behavioral modifications.

**Differential regulation of neuronal function by PKA and CaMKII**

Neuronal firing patterns carry highly structured temporal information. Firing rate and action potential shape are two major parameters that dictate the timing and amount of neurotransmitter release. Neuronal electrical activity can be highly plastic, being affected by both short- and long-term modulatory mechanisms (Getting 1989; Marder et al. 1996; McCormick 1992; Turrigiano et al. 1994, 1995). Such functional plasticity has important implications not only for behavioral modification but also for activity-dependent refinement of proper neural circuits during development (Budnick et al. 1990; Cline 1991; Hubel and Wiesel 1970). Given the critical roles of PKA and CaM kinase in behavioral plasticity, it is not surprising that at the cellular level, both enzymes regulate neuronal firing properties. Comparisons of how the two signaling pathways modulate neuronal membrane properties can provide insights into the cellular mechanisms underlying behavioral modifications (Fig. 11).

The activity-dependent accumulation of Ca²⁺ could activate both PKA and CaM kinase activity through a Ca²⁺/calmodulin-dependent mechanism (Fig. 11B). Direct binding of Ca²⁺/CaM is required for CaMKII activation while Ca²⁺/CaM-dependent activation of adenylyl cyclase stimulates cAMP synthesis, which in turn activates PKA. **DCO**²⁺ neurons in which the PKA catalytic subunit is defective showed aberrant frequency coding, unstable firing patterns, enhanced firing frequency, and decreased spike amplitude, but no changes in action potential width (Figs. 1, 2, and 4, Table 1). These phenotypes are in general consistent with the defects found in dnc and rut neurons (Zhao and Wu 1997) which have a defective cAMP phosphodiesterase and adenylyl cyclase, respectively. The exception is that dnc and rut neurons display spontaneous activity not seen in **DCO**²⁺, which raises the possibility of regulation mediated by direct cAMP binding to ion channels (Delgado et al. 1991; Dhallan et al. 1990; Nakamura and Gold 1987) or via non-**DCO**²⁺-dependent PKA pathways. In comparison, neurons of ala1 and ala2 mutants, which express a CaMKII inhibitory peptide (Griffith et al. 1993), exhibited unstable firing patterns in repeated stimulation trials, but no fluctuations in spike frequency coding during single current injections. Furthermore the action potential waveform was prolonged in ala neurons. Because CaMKII is highly abundant at the synapse (Kennedy 1997; Nairn et al. 1985), its regulation of action potential duration could influence the dynamics of Ca²⁺ influx and thus the amount of transmitter release. Indeed, enhancement of synaptic currents and irregularity of release patterns has been reported for the larval neuromuscular junction of Drosophila ala transmutants (Wang et al. 1994).

The preceding observations suggest that different second-messenger systems may regulate distinct aspects of neuronal firing properties. This can enhance the capacity of the nervous system to fulfill specific functional requirements for a variety of behavioral tasks. Based on previous observations, cAMP and CaM kinase pathways are known to exert different effects on nerve terminal growth and synaptic transmission at the larval neuromuscular junction (Wang et al. 1994; Zhong and Wu 1991; Zhong et al. 1992), which demonstrates that differential regulation of different aspects of neuronal function by the two signaling cascades can occur within the same cell. It will be interesting to further investigate firing patterns between these mutants in neurons associated with identified neural circuits underlying distinct behaviors.

**Correlation of K⁺ current modulation with firing patterns**

The firing properties of Drosophila “giant” neurons are shaped by a plethora of ionic currents, including voltage-activated outward K⁺ currents, inward Ca²⁺ and Na⁺ currents, and Ca²⁺-activated outward K⁺ currents (Saito and Wu 1993). Iₖ has been shown in this preparation to modulate the onset, the duration, and the frequency of action potentials whereas I₅ primarily contributes to the repolarization of the action potential, determining its duration (Saito and Wu 1993; Yao and Wu 1999a; Zhao and Wu 1997). The distinct patterns of adaptive,
tonic, and delayed firing reflect differences in expression of different K⁺ currents (Zhao and Wu 1997). Consistent with this notion, our preliminary results suggest that Type 1 currents can support all three types of firing patterns while Type 2 neurons only fire adaptive spikes (unpublished observations).

Although a complete explanation of mutant firing patterns will require knowledge of both inward and outward currents, some insights can be obtained by an initial analysis of K⁺ currents. For example, kinase inhibitors and K⁺ channel blockers can be used to explore the contributions of pharmacologically and kinetically distinct K⁺ current components. Specifically, 4-AP has been shown to increase the firing frequency and shorten the latency to the onset of spikes, which is particularly striking for delayed type neurons, whereas TEA broadens the duration of action potentials without changing the firing rate, which is most obvious for adaptive type neurons (Zhao and Wu 1997). Current- and voltage-clamp correlation studies from the same cells demonstrated that K⁺ currents in adaptive cells have a large TEA-sensitive component and delayed cells a large 4-AP-sensitive component (Zhao and Wu 1997). In our preliminary experiments we found that KN-93 prolonged action potential duration especially in adaptive cells and Rp-cAMPS greatly reduced the latency and turned adaptive cells into fast-spiking cells (Yao and Wu, Peng and Wu, unpublished observations). These observations are consistent with the hypothesis that PKA has a major effect on Sh currents and CaM kinase affects non-Sh currents including Shab Iₖ. However, the kinase inhibitors did not completely mimic the modulation of either Iₐ or Iₖ channel blockers (data not shown), suggesting that kinase inhibitors may affect firing patterns in a more complicated manner. For example, Iₛₕ and Iₑₐₔ are known to affect the initiation and duration of action potentials and may be modulated by protein kinases. A more complete understanding of the ionic basis of firing patterns would require voltage-and current-clamp correlation analysis in the same cells of each firing pattern and should include not only K⁺ currents but also other ionic currents.

Distinct regulation of K⁺ channel subunits by different second-messenger systems

It has been established that a variety of voltage-gated K⁺ channels in Drosophila neurons are composed of a combination of pore-forming α subunits, including Sh, Shal, Shab, and Shaw, and auxiliary β subunits such as hyperkinetic (Yao and Wu 1999a). Based on the genetic and pharmacological analyses presented above, a scheme of K⁺ channel modulation by PKA and CaMKII can be proposed (Fig. 11B).

Our data show that KN-93 affected Iₐ in WT neurons and non-Sh transient currents in ShM mutant neurons to a similar degree, suggesting that Shal, but not Sh, is modulated by CaMKII. This was supported by the fact that ShD current is resistant to KN-93 treatment. Since Sh RNA undergoes extensive alternative splicing (Kamb et al. 1988; Pongs et al. 1988; Schwarz et al. 1988), further tests of whether other Sh products are modulated by CaMKII may be performed in future genetic and pharmacological studies. On the other hand, our results show that Rp-cAMPS modulated ShD currents in ShD transformant neurons but not the non-Sh currents in ShM mutants, which is consistent with previous findings: the Sh polypeptide contains PKA phosphorylation sites (Schwarz et al. 1988) responsible for modulation of inactivation of Sh channels expressed in Xenopus oocytes (Drain et al. 1994), and in Drosophila muscle, the transient Sh current is modified by dnc mutations and by acute application of cAMP analogs (Zhong and Wu 1993). Taken together, these observations demonstrate that Sh channels are a target of PKA but probably not of CaMKII.

The sustained K⁺ current consists of multiple components as discussed in RESULTS, but its major component, the delayed rectifier current, is thought to be mediated by the Shab subunit (Fig. 9) (cf. Singh and Singh 1999; Tsunoda and Salkoff 1995b). The fact that the sustained currents in both WT and ShM neurons were substantially suppressed, following KN-93 treatments, to a level comparable to the steady-state currents in Shab₁ neurons (see preceding text) (cf. Perez et al. 1998; Singh and Singh 1999; Yao and Wu 1999b) strongly suggests that the delayed rectifier Shab channel mediates the majority of CaMKII effects on sustained K⁺ currents.

The differential effects of CaMKII and PKA on distinct K⁺ channel subunits discussed in the preceding text may contribute to the action potential phenotypes observed in ala and DC0⁻³d neurons. While the modulation of Sh currents by PKA appears to be important for generating regular firing patterns for reliable frequency coding, modulation of non-Sh currents by CaMKII may contribute to the control of action potential repolarization and duration. Both PKA and CaMKII, however, may regulate the stability of firing patterns through possible effects on multiple channel types (see preceding text), which may be critical for reliability and precision in signal processing in the nervous system.

In conclusion, PKA and CaMKII target different K⁺ channel subunits to confer differential regulation of neuronal excitability and spike patterning. The two second-messenger systems may modulate separate parameters of neuronal function, including nerve terminal outgrowth (Wang et al. 1994; Zhong et al. 1992), synaptic function and plasticity (Wang et al. 1994; Zhong and Wu 1991), and neuronal firing patterns (Zhao and Wu 1997; this study). These parameters might be conditioned differentially during nervous system activity and thus may underlie specific behavioral modifications by different stimuli in different model systems.

We thank Dr. Jeff Engel for comments on the manuscript. This work was supported by National Institutes of Health grants to C.-F. Wu.

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