Role of Calcium-Calmodulin–Dependent Protein Kinase II in Modulation of Sensorimotor Synapses in Aplysia

KEIKO NAKANISHI,1 FAN ZHANG,1 DOUGLAS A. BAXTER,1 ARNOLD ESKIN,2 AND JOHN H. BYRNE1
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Nakanishi, Keiko, Fan Zhang, Douglas A. Baxter, Arnold Eskin, and John H. Byrne. Role of calcium-calmodulin–dependent protein kinase II in modulation of sensorimotor synapses in Aplysia. J. Neurophysiol. 78: 409–416, 1997. The Ca2+-calmodulin–dependent protein kinase II (CaMKII) inhibitor, 1-[(N, O-bis(5-isooquinolinesulfonfyl)–N-methyl-L-tyrosyl)–4-phenylpiperazine] (KN-62), was used to investigate the role of CaMKII in synaptic transmission and serotonin (5-HT)-induced facilitation in Aplysia. Application of KN-62 (10 µM) by itself increased the duration of action potentials greater than 30 ms. Correlation analysis revealed that the effects of KN-62 had no effect on excitability of isolated sensory neuron soma but did not block spike broadening produced by 5-HT. KN-62 had no effect on excitability of isolated sensory neuron soma nor did it block 5-HT–induced enhancement of excitability. These results indicate that the attenuation of short-term facilitation by KN-62 is not due to modulation of the membrane currents contributing to 5-HT–induced spike broadening or enhancement of excitability. Rather, these data are consistent with the hypothesis that CaMKII contributes to the regulation of sensorimotor connections and that it has a role in spike-duration–independent processes contributing to short-term facilitation.

INTRODUCTION

Plasticity of the connections between sensory neurons and their follower motor neurons in Aplysia has been used extensively as a model system to study the cellular and molecular mechanisms of simple forms of learning such as sensitization (for recent reviews see Byrne and Kandel 1996; Byrne et al. 1993; Kandel et al. 1987). The synaptic plasticity associated with sensitization is induced, at least in part, by serotonin (5-HT), acting via at least two kinases, adenosine 3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase [protein kinase A (PKA)] (Bacskai et al. 1993; Bernier et al. 1982; Greenberg et al. 1987; Occor and Byrne 1985) and Ca2+-phospholipid-dependent protein kinase [protein kinase C (PKC)] (Braha et al. 1990; Sacktor and Schwartz 1990; Sossin and Schwartz 1992; Sugita et al. 1992).

A third protein kinase, Ca2+-calmodulin–dependent protein kinase II (CaMKII), also may be involved. For example, 5-HT induces the translocation of a subunit of Ca2+-calmodulin–dependent kinases from the membrane–cytoskeleton complex to the cytoplasm (Saitoh and Schwartz 1983, 1985). In addition, the level of mRNA for calmodulin (CaM) is increased by treatment with 5-HT (Eskin et al. 1993; Zhang et al. 1995; Zwartjes et al. 1991). The electrophysiological effects of CaMKII have not been examined, however. To investigate the role of CaMKII in the efficacy and modulation of sensorimotor connections, we examined the effects of a specific inhibitor of CaMKII, 1-[(N, O-bis(5-isooquinolinesulfonfyl)–N-methyl-L-tyrosyl)–4-phenylpiperazine] (KN-62) (Tokumitsu et al. 1990), on transmission and 5-HT–induced facilitation of sensorimotor synapses in pleural–pedal ganglia. We also examined the effect of KN-62 on the excitability and spike duration in sensory neurons to determine whether the effects of KN-62 on the synaptic efficacy could be attributed to actions on one or more of the membrane currents.

METHODS

Aplysia californica were obtained from Alacrity Marine Biological Specimens (Redondo Beach, CA) and Marine Specimens Unlimited (Pacific Palisades, CA). Animals (70–300 g) were maintained in aquaria containing aerated artificial seawater (ASW; Instant Ocean, Aquarium Systems, Mentor, OH) at ~15°C. Before dissection, animals were weighed and anesthetized by injection of a volume of isotonic MgCl2 equal to one-half of their body volume. Pleural–pedal ganglia were removed and placed in a Sylgard (Dow Corning, Midland, MI)–lined recording chamber (volume 0.5 ml) containing a 50:50 solution of isotonic MgCl2:ASW. The connective tissue overlying the ganglion was removed surgically. The 50:50 solution then was exchanged ~100 times with ASW that was buffered with 10 mM Tris (pH 7.4).

Standard electrophysiological techniques were used for intracellular recordings from sensory and motor neurons. Recordings were conducted at 15 ± 1°C. Only those neurons having resting membrane potentials greater than −35 mV were used in these studies.

After finding a connection between a sensory and a motor neuron, testing occurred once every 5 min and consisted of eliciting a single action potential in the sensory neuron with a 30-ms, suprathreshold depolarizing pulse and recording the monosynaptic excitatory postsynaptic potential (EPSP) produced in the motor neuron (Zhang et al. 1994). During tests, motor neurons were hyperpolarized by ~30 mV to prevent the EPSP from triggering an action potential. Measurements of input resistance of the motor neuron (while the cell was hyperpolarized) were made before each test by intracellularly injecting 1-, 1- or 2-nA hyperpolarizing constant current pulses. After three baseline trials, ganglia were exposed to KN-62 (10 or 1 µM) or control vehicle (N-1-[N-methyl-p-(5-isooquinolinesulfonyl) benzyl]-2-(4-phenylpiperazine ethyl)-5-isooquinolinesulfonamide) (KN-04); an inactive form of KN-62, 15 min before the application of 15 µM 5-HT (Fig. 1). The amplitudes of EPSPs were normalized to the baseline EPSP, which was defined as the average of three EPSPs (0, 5, and 10 min) recorded immediately before the application of KN-62 or the control vehicles.

The experiments using CaM inhibitors [calmidazolium, 25 µM and trifluoperazine (TFP), 50 µM] were similar to those using
Measurements of spike duration and excitability

Clusters of sensory neuron somata were isolated surgically from pleural ganglia and were pinned to the floor of a recording chamber containing ASW (Sugita et al. 1992, 1994), and the preparation was maintained at 15 ± 1°C. Two-electrode current-clamp techniques (Sugita et al. 1992, 1994) were used. The membrane potential of the sensory neuron was monitored and was adjusted via baseline (time = 10 min), experimental or control solution was applied to bath; 15 min after treatment with KN-62 or control, 15 μM serotonin (5-HT) subsequently was applied to bath. Value of each trial was normalized to mean of 3 baseline trials preceding treatment.

KN-62 except an interstimulus interval (ISI) of 1 min was used. This ISI, which leads to more pronounced synaptic depression, was selected to maximize the possibility of observing a selective effect of CaM on depressed synapses. The amplitude of EPSPs were normalized to the baseline EPSP, which was defined as the average of three baseline trials (0, 1, and 2 min).

Results

Kn-62 increased the amplitude of EPSPs and attenuated 5-HT–induced facilitation

To examine the role of CaMKII in sensorimotor synaptic transmission in *Aplysia*, single action potentials were elicited repeatedly in sensory neurons before and after the application of 10 μM KN-62. Control experiments were identical except that KN-04 was used instead of KN-62. The role of CaMKII in facilitation was tested by comparing the effects of 5-HT in the presence of KN-62 with the effects of 5-HT in controls (Fig. 1). Figure 2A illustrates a typical result. In the control experiments, repeated stimulation (ISI = 5 min) of the sensory neuron led to synaptic depression. Application of 5-HT led to synaptic facilitation (Fig. 2A). Figure 2A illustrates the effects of KN-62. Application of KN-62 by itself increased the amplitude of EPSP. In addition, KN-62 attenuated the facilitation produced by 5-HT. Average data are presented in Fig. 2A, and further analyses of the data are illustrated in Fig. 2, B and C. The mean amplitude of the three control EPSPs during the pre-5-HT period was 80.9 ± 3.9% of the baseline (mean ± S.E., n = 23; Fig. 2B). In contrast, the mean amplitude of the three KN-62 EPSPs during the pre-5-HT period was 108.8 ± 4.6% of the baseline (n = 12). The KN-62–induced increase in the amplitudes of EPSPs was statistically significant (∫/ = 4.39, two tailed P < 0.001). In the post-5-HT period, the mean amplitude of the control EPSPs was 159.9 ± 12.6% of the pre-5-HT period, whereas the mean amplitude of the KN-62 EPSPs was 91.7 ± 7.0% of the pre-5-HT period (Fig. 2C). This difference was statistically significant (∫/ = 3.74, two tailed P < 0.007). These results suggested that CaMKII may have a dual role in regulating transmitter release and 5-HT–induced facilitation.

The effects of KN-62 did not appear to be due to differences in the initial state of the synaptic connections or a generalized effect on the motor neuron. The average amplitudes of the three baseline EPSPs (∫/., 0, 5, and 10 min) were 7.1 ± 0.9 mV in the control group and 7.3 ± 0.5 mV in the KN-62 group (∫/ = 0.16). The input resistance of the motor neurons in baseline period was 10.7 ± 0.4 MΩ in the control group and 11.1 ± 1.2 MΩ in the KN-62 group (∫/ = 0.74).

We also examined the effects of a lower concentration of KN-62 (1 μM). The lower concentration of KN-62 produced an increase in the pre-5-HT EPSPs and attenuated 5-HT–induced facilitation. However, these effects were not statistically significant, indicating that concentrations of KN-62 > 1 μM were required to significantly affect the sensorimotor synapses in *Aplysia*.

Because 5-HT increases the level of mRNA for CaM (Eskin et al. 1993; Zhang et al. 1995; Zwartjes et al. 1991), we also examined the effects of inhibitors of CaM (25 μM calmidazolium and 50 μM TFP) on the amplitudes of EPSPs. Calmidazolium and TFP had effects on the pre-5-HT EPSPs in the inhibitor and the control groups. The values for each group were obtained by normalizing the average of the three trials in the post-5-HT period to the average value of the three trials in the pre-5-HT period (Fig. 1). A P value of < 0.05 (two-tailed) was considered significant.
and 5-HT–induced facilitation similar to, but weaker than, KN-62. Calmidazolium and TFP increased the amplitude of the pre-5-HT EPSP (control, 78.2 ± 9.6%, n = 6 vs. calmidazolium, 86.8 ± 4.1%, n = 7; and control, 85.2 ± 14.7%, n = 5 vs. TFP, 109.5 ± 20.4%, n = 4). These effects were not statistically significant, however. In addition, both inhibitors appeared to attenuate 5-HT–induced facilitation (control 292.5 ± 86.0% vs. calmidazolium 147.0 ± 11.6% and control 216.0 ± 65.0% vs. TFP 98.3 ± 9.4%), but again the effects were not statistically significant.

KN-62 did not affect the excitability of sensory neurons nor did it affect 5-HT–induced enhancement of excitability

The effects of KN-62 on synaptic efficacy and on 5-HT–induced facilitation may be due to an action of KN-62 on membrane currents in sensory neurons. To investigate this possibility, we examined the effects of KN-62 on excitability and spike broadening in isolated somata of sensory neurons. KN-62 (10 μM) by itself had no effect on the excitability of sensory neurons (Fig. 3, A1 and A2). Figure 3B illustrates the mean changes in excitability (control, 113.7 ± 4.9% versus KN-62, 127.5 ± 7.6%). These effects were not statistically significant (t17 = 1.55). After application of 5-HT, there was an increase in excitability in both the control and KN-62 groups. Figure 3C illustrates the mean changes in excitability produced by 5-HT (5-HT, 322.3 ± 62.5% versus 5-HT + KN-62, 248.2 ± 21.5%). There was also no statistically significant difference between these two groups (t17 = 1.07). These results indicate that the attenuation of 5-HT–induced synaptic facilitation by KN-62 (Fig. 2C) was probably not due to block of the membrane current(s) that contribute to the regulation of excitability. The 5-HT–induced enhancement of excitability in *Aplysia* is believed to be due to PKA-mediated closure of S-K+ channels (Baxter and Byrne 1990a; Klein et al. 1982; Pollock et al. 1985; Siegelbaum et al. 1982; Sugita et al. 1994). Application of KN-62 (10 μM) led
FIG. 3. KN-62 (10 μM) did not change excitability of sensory neuron nor did it affect 5-HT-induced enhancement of excitability. A1: typical results illustrating excitability of a sensory neuron in control saline (baseline), after application of KN-62 and after application of 5-HT to bath, which still contained KN-62. A2: summary data from 10 control (N-(1-[N-methyl-p-(5-isouquinolinesulfonyl) benzyl]-2-(4-phenylpiperazine)ethyl]-5-isouquinolinesulfonamide) (KN-04) and 9 KN-62 experiments. B: summary data for control and KN-62 groups during pre-5-HT period. C: 5-HT-induced enhancement of excitability in presence (KN-62 + 5-HT) and absence (control + 5-HT) of KN-62. There was no significant difference between KN-62 and control groups in B and C.

<table>
<thead>
<tr>
<th>A1</th>
<th>Baseline</th>
<th>KN-62</th>
<th>5-HT</th>
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<tbody>
<tr>
<td></td>
<td><img src="image1" alt="Baseline" /></td>
<td><img src="image2" alt="KN-62" /></td>
<td><img src="image3" alt="5-HT" /></td>
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</tbody>
</table>

**DISCUSSION**

An increasing body of evidence indicates that CaMKII is an important kinase involved in synaptic plasticity in both invertebrate and vertebrates (see Byrne et al. 1993; Lisman 1994 for reviews; see also Chapman et al. 1995; Malenka et al. 1989; Malinow et al. 1989; Silva et al. 1992; Wang et al. 1994). The present finding that a CaMKII inhibitor, KN-62, attenuated the facilitation produced by 5-HT supports the hypothesis that CaMKII is involved in synaptic plasticity, in general, and in heterosynaptic facilitation of the *Aplysia* sensorimotor synapse in particular. Thus in addition to the previously described roles of PKA and PKC in facilitation, CaMKII also may be involved.

**Specificity of KN-62**

The evidence for the involvement of CaMKII in synaptic transmission and its plasticity is based on the use of
KN-62, which is considered a specific inhibitor of CaMKII. KN-62 does not affect PKA, PKC, myosin light chain kinase, and Ca\(^{2+}\)/CaM-dependent phosphodiesterase in rat, even at a concentration of 100 \(\mu\)M (Ishikawa et al. 1990). It is not known whether KN-62 affects Ca\(^{2+}\)/CaM-dependent adenyl cyclase, however. Nor have biochemical studies been performed to examine its specificity in Aplysia. Indirect evidence indicates that KN-62 does not affect PKA and PKC in Aplysia, however. For example, the 5-HT–induced increases in excitability of the sensory neurons are known to be predominately mediated by PKA (Baxter and Byrne 1990a; Klein et al. 1986). An activator of PKC can increase excitability (Sugita et al. 1992), but this effect appears due, at least in part, to a PKC-induced activation of cAMP levels (S. Sugita and J. H. Byrne, unpublished observation). In addition, the 5-HT–induced increases in spike duration are mediated mainly by the combined actions of PKA and PKC (Baxter and Byrne 1990a; Braha et al. 1993; Castellucci et al. 1982; Sugita et al. 1992). These actions also are not affected by KN-62. Although additional studies are needed, the present results raise the intriguing possibility that CaMKII plays a dual role in regulating synaptic efficacy and 5-HT–induced short-term facilitation in Aplysia.

**Interrelationships among kinases**

CaMKII, PKA, and PKC have at least one common action in that they all seem to participate in 5-HT–induced short-term facilitation of the sensorimotor connections (Table 1). Two mechanisms are believed to contribute to the facilitation. One mechanism for facilitation is broadening of the sensory neuron spike, whereas a second one is via spike-duration–independent (SDI) processes (see Byrne and Kandel 1996 for review). PKA and PKC seem to engage both spike-duration dependent and SDI processes whereas CaMKII does not. Although KN-62 increased the spike duration slightly, CaMKII does not appear to be necessary for 5-HT–induced spike broadening. CaMKII also does not appear to be necessary for the enhancement of excitability produced by 5-HT. These results suggest that CaMKII may play a role exclusively in SDI processes.

We do not know how CaMKII is activated in response to 5-HT. One possibility is that a receptor to 5-HT is linked to adenylyl cyclase, however. Nor have biochemical studies been performed to examine its specificity in Aplysia. Indirect evidence indicates that KN-62 does not affect PKA and PKC in Aplysia, however. For example, the 5-HT–induced increases in excitability of the sensory neurons are known to be predominately mediated by PKA (Baxter and Byrne 1990a; Klein et al. 1986). An activator of PKC can increase excitability (Sugita et al. 1992), but this effect appears due, at least in part, to a PKC-induced activation of cAMP levels (S. Sugita and J. H. Byrne, unpublished observation). In addition, the 5-HT–induced increases in spike duration are mediated mainly by the combined actions of PKA and PKC (Baxter and Byrne 1990a; Braha et al. 1993; Castellucci et al. 1982; Sugita et al. 1992). These actions also are not affected by KN-62. Although additional studies are needed, the present results raise the intriguing possibility that CaMKII plays a dual role in regulating synaptic efficacy and 5-HT–induced short-term facilitation in Aplysia.

**Table 1. Involvement of second messenger systems in short-term effects of 5-HT**

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<th>Second Messenger System</th>
<th>EPSP</th>
<th>Spike Duration</th>
<th>Excitability</th>
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<tr>
<td>cAMP/PKA</td>
<td>Yes(^a)</td>
<td>Yes(^b)</td>
<td>Yes(^c)</td>
</tr>
<tr>
<td>DAG/PKC</td>
<td>Yes(^d)</td>
<td>Yes(^e)</td>
<td>No(^f)</td>
</tr>
<tr>
<td>CaMKII</td>
<td>Yes(^g)</td>
<td>No(^h)</td>
<td>No(^i)</td>
</tr>
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5-HT, serotonin; EPSP, excitatory postsynaptic potential; cAMP, adenosine 3',5'-cyclic monophosphate; PKA, protein kinase A; DAG, diacylglycerol; PKC, protein kinase C; CaMKII, Ca\(^{2+}\)/calmodulin-dependent protein kinase II. \(^a\) Brunelli et al. 1976; Castellucci et al. 1980; Braha et al. 1990; Klein 1993. \(^b\) Baxter and Byrne 1990a; Castellucci et al. 1982. \(^c\) Baxter and Byrne 1990a; Klein et al. 1986. \(^d\) Braha et al. 1990; Sugita et al. 1992. \(^e\) Sugita et al. 1992, but Braha et al. 1993. \(^f\) Braha et al. 1993, but Sugita et al. 1992. \(^g\) Present study.
at this locus would broaden the spike and enhance synaptic transmission. After treatment with 5-HT, CaMK is released from the membrane-cytoskeleton complex to the cytoplasm (Saitoh and Schwartz 1983, 1985). The free CaMK in the cytoplasm may have a facilitatory effect on synaptic transmission similar to that in the giant synapse of squid (Llinas et al. 1985). A block of CaMK at this locus would inhibit 5-HT-induced facilitation. An alternative hypothesis is that the complex effects of CaM and CaMKII inhibitors arise from cross-talk among the multiple second messenger systems implicated in the plasticity at the sensorimotor synapse (e.g., Byrne et al. 1993). These interactions may occur at multiple levels, ranging from the receptors to substrate proteins.

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