Neuron, Vol. 5, 789–796, December, 1990, Copyright © 1990 by Cell Press

Cyclic AMP–Dependent Protein Kinase Decreases $GABA_A$ Receptor Current in Mouse Spinal Neurons

Nada M. Porter,* Roy E. Twyman,* Michael D. Uhler,†‡ and Robert L. Macdonald*§

*Departments of Neurology
†Department of Biological Chemistry
‡Mental Health Research Institute
§Department of Physiology
University of Michigan Medical Center
Ann Arbor, Michigan 48104

Summary

GABA, the major inhibitory neurotransmitter in the mammalian brain, binds to $GABA_A$ receptors, which form chloride ion channels. The predicted structure of the $GABA_A$ receptor places a consensus phosphorylation site for cAMP-dependent protein kinase (PKA) on an intracellular domain of the channel. Phosphorylation by various protein kinases has been shown to alter the activity of certain ligand- and voltage-gated ion channels. We have examined the role of phosphorylation by the catalytic subunit of PKA in the regulation of $GABA_A$ receptor channel function using whole-cell and excised outside-out patch-clamp techniques. Inclusion of the catalytic subunit of PKA in the recording pipettes significantly reduced GABA-evoked whole-cell and single-channel chloride currents. Both heat inactivation of PKA and addition of the specific protein kinase inhibitor peptide prevented the reduction of GABA-evoked currents by PKA. Neither mean channel open time nor channel conductance was affected by PKA. The reduction in GABA receptor current by PKA was primarily due to a reduction in channel opening frequency.

Introduction

The mammalian γ-aminobutyric acid type A ($GABA_A$) receptor is a chloride ion channel that opens to at least four different conductance levels (Bormann et al., 1987). The 27 pS conductance level accounts for approximately 90% of the current carried through the channel and thus has been referred to as the main conductance state (Hamill et al., 1983; Macdonald et al., 1989). The exact structure of the $GABA_A$ receptor has not yet been determined; however, cDNA cloning of the CNS receptor from several mammalian species has shown that it may contain multiple α, β, γ, and δ subunits (Schofield et al., 1987; Khrestchatisky et al., 1989; Lolait et al., 1989; Pritchett et al., 1989; Shivers et al., 1989; Ymer et al., 1989; Lüddens et al., 1990).

Several recent reports have suggested a role for cAMP-dependent protein kinase (PKA) in the modulation of the $GABA_A$ receptor. First, the cloning and sequencing of the receptor have shown that the β subunit contains a consensus sequence for phosphorylation by PKA (Schofield et al., 1987). The proposed structure of the receptor places this sequence on an intracellular domain of the β subunit, consistent with a location at which PKA could act. Second, cAMP analogs and the adenylate cyclase activator forskolin decreased GABA-gated chloride flux in synaptosomes (Heuschneider and Schwartz, 1989; Jalilian Tehrani et al., 1989). In electrophysiological studies, these compounds reduced the peak current evoked by GABA and increased the rate of decay of the current in cultured cortical (Jalilian Tehrani et al., 1989) and hippocampal neurons (Harrison and Lambert, 1989a, 1989b). Finally, the direct phosphorylation by PKA of the β subunit isolated from porcine (Kirkness et al., 1989) and rat (Browning et al., 1990) brain has been demonstrated.

Phosphorylation of the nicotinic acetylcholine receptor (nAChR), another ligand-gated ion channel, has been well characterized and shown to play a major role in the regulation of this receptor (Miles and Huganir, 1988). Specifically, phosphorylation of the nAChR by a variety of protein kinases increases the rate of desensitization of this receptor. In view of the important role of phosphorylation on the nAChR, we undertook the following electrophysiological studies to determine the direct effects of PKA on the $GABA_A$ receptor.

Results

PKA Reduced GABA-Evoked Whole-Cell Currents in Cultured Mouse Spinal Neurons

In whole-cell recordings, application of GABA (5 μM) to the soma of a spinal cord neuron reproducibly evoked an inward chloride current and an associated increase in membrane conductance (Figure 1a). After the initial increase in current and membrane conductance, the GABA-evoked response typically declined over time. All neurons tested were sensitive to GABA ($n = 19$). In cells in which PKA (50 μg/ml) was included in the internal pipette solution, the peak current was significantly reduced by 60% (Table 1). The effect of PKA was detected upon the first application of GABA to neurons. This was within 1–2 min of giga-seal formation and patch rupture for the whole-cell recording mode. In contrast to the control group in which all cells tested were sensitive to GABA, no GABA-evoked current was detected in 14 of 35 PKA cells tested. An example of a neuron in which the GABA-evoked inward current and increase in membrane conductance were completely blocked by PKA is shown in Figure 1b. In the presence of a lower concentration of PKA (20 μg/ml), the peak current evoked by GABA was reduced by 43%, suggesting a concentration-dependent effect of PKA (Table 1). In 4 of 18 neurons tested, no GABA-evoked current was detected at this lower concentration of PKA.

To eliminate the possibility that PKA was blocking GABA current nonspecifically by clogging the record-

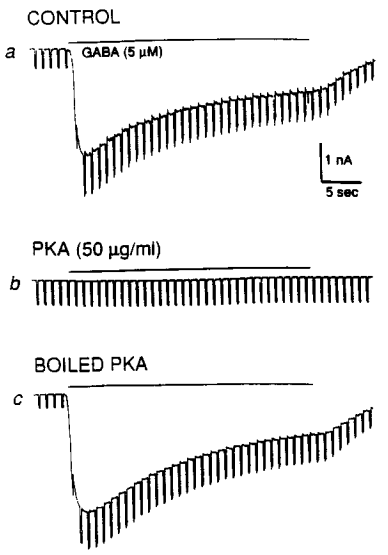

Figure 1. Effect of PKA on Whole-Cell GABA Responses in Mouse Spinal Cord Neurons

GABA (5 μM; overbar) was applied by pressure ejection for 30 s. Recording pipettes contained 2 mM Mg-ATP. (a) In a control cell, application of GABA evoked an inward chloride current (downward deflection) and an associated increase in membrane conductance. (b) An example of a cell in which PKA (50 μg/ml) was added to the internal pipette solution. PKA completely blocked the GABA-evoked current and increase in membrane conductance. (c) A cell in which PKA was heat-inactivated before being added to the internal pipette solution. The GABA-evoked increase in current and membrane conductance were not blocked in this instance. Neurons were voltage clamped at −75 mV, and membrane conductance was determined by applying constant short duration (200 ms) hyperpolarizing voltage commands. Calibration bars apply throughout.

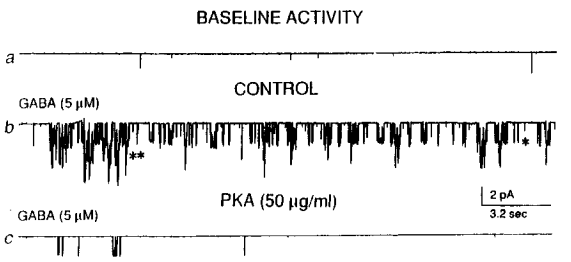

Figure 2. Effect of PKA on GABA-Evoked Single-Channel Chloride Currents in Mouse Spinal Cord Neurons

Recordings were from excised outside-out membrane patches voltage clamped at −75 mV. Recording pipettes contained 2 mM Mg-ATP. (a) In the absence of GABA, rare chloride channel openings (downward deflections) were observed. (b) In control patches, application of GABA (5 μM) produced an increase in chloride channel opening. Channels opened to a main conductance state of 27 pS (double asterisks) and to a less frequent subconductance state of 19 pS (single asterisk). Multiple channels in a patch may open simultaneously to the main conductance state. (c) In a patch with PKA in the internal pipette solution, GABA-activated opening of chloride channels was greatly reduced. Calibration bars apply throughout.

ing pipette, 150 mM KCl was applied to all PKA cells following application of GABA. KCl evoked an inward current and an increase in membrane conductance, suggesting that the observed reduction in GABA-evoked current in the presence of PKA was not due to technical difficulties of recording. We next tested the specificity of PKA by including heat-inactivated catalytic subunit in the internal pipette solution. The GABA-evoked current and increase in membrane conductance were not reduced in these cells (Figure 1c; Table 1). We further examined the issue of nonspecificity of the kinase effect by substituting an equivalent amount of bovine serum albumin (50 μg/ml) for PKA in the internal pipette solution. The peak GABA-evoked current in neurons injected with bovine serum albumin was 1725 ± 290 pA (n = 6; $P > 0.05$) and was not reduced when compared with control neurons (see Table 1). These results suggested that PKA may reduce GABA-evoked currents in spinal cord neurons by a specific mechanism of action.

Table 1. Effect of PKA on the Peak Amplitude of the Whole-Cell Current Evoked by 5 μM GABA

Treatment	Peak Current (pA)	n
Control	1947 ± 225	19
PKA (50 μg/ml)	794 ± 186[a]	35
PKA (20 μg/ml)	1102 ± 240[b]	18
PKA (heat-inactivated)	1541 ± 277	8

Values represent the mean ± SEM; n is the number of cells tested.
[a] $P < 0.01$ with respect to control by ANOVA and Dunnett's test.
[b] $P < 0.05$.

PKA Reduced GABA-Evoked Single-Channel Currents

The effects of PKA (50 μg/ml) on single-channel $GABA_A$ receptor currents also were examined in excised outside-out membrane patches. In the absence of GABA, only occasional, brief spontaneous openings were observed (Figure 2a). In control patches (approximately 80% tested positive for GABA sensitivity), application of GABA evoked bursting inward chloride currents with two predominant conductance states (Figure 2b). The chord conductances were 27 pS and 19 pS and represented approximately 93% and 7% of the total open time, respectively. Multiple single-channel recordings were summed to produce an average ensemble current per application of GABA. The average current per patch as calculated from all conductance states was 0.89 pA (n = 9 patches; 43,707 openings) in the presence of 5 μM GABA. In patches in which PKA was included in the internal pipette solution, very few openings were observed (Figure 2c) and the average GABA-evoked chloride current was reduced by 97% to 0.03 pA (n = 7 patches; 1,296 openings). A reduction in average current can result from a decrease in channel conductance, channel opening frequency, channel open time, or a combination of these factors. To determine the mechanism by which PKA reduced the total GABA-evoked current, we examined each of these parameters.

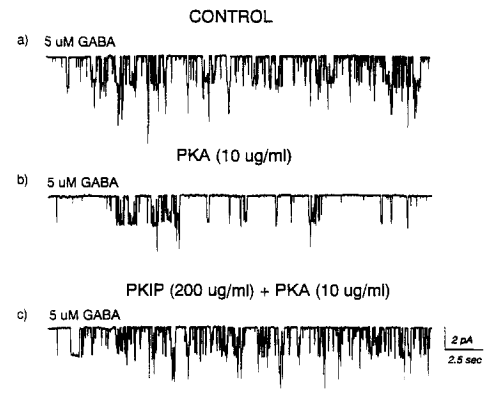

Figure 3. Effect of PKA and PKIP on GABA-Evoked Single-Channel Currents

Recordings were from excised, outside-out membrane patches voltage clamped at −75 mV. Recording pipettes contained 2 mM Mg-ATP. (a) In control patches (n = 17 patches), application of 5 μM GABA evoked chloride channel opening. (b) In patches in which a lower concentration of PKA (10 μg/ml; n = 16) was included in the internal pipette solution, GABA-activated opening of channels was reduced. (c) In patches in which both PKA (10 μg/ml) and PKIP (200 μg/ml; n = 13) were included in the internal medium, no reduction in GABA-evoked channel openings was observed. Recordings from patches shown in (b) and (c) were obtained from the same cell. Calibration bars apply throughout.

The chord conductance of the main state channel was unaffected by exposure to PKA (27.5 pS for control versus 27.9 pS for kinase-exposed patches), suggesting that the decrease in total current observed was not due to a change in channel conductance. Since the 27 pS channel was the dominant conductance state, kinetic analysis was performed only on this state. The frequency of channel opening in control patches in the presence of GABA was 24.8 ± 9.4 openings per s (mean ± SEM). In patches exposed to PKA, GABA-evoked channel openings were significantly reduced to 1.3 ± 0.6 openings per s ($P < 0.025$, Mann-Whitney U-test). To obtain an accurate estimate of the mean open time, only patches that had few simultaneous channel openings were analyzed (Colquhoun and Hawkes, 1983). In those patches, the mean channel open time of the 27 pS conductance state in the presence of GABA in control patches was 5.0 ms (11,898 openings) and 5.9 ms (1,296 openings) in PKA-exposed patches. Although the mean open time in PKA patches was slightly increased, this shift was not significant when examined on a per patch basis.

PKIP Prevented PKA Reduction of GABA-Evoked Single-Channel Currents

We also determined the effects of a lower concentration of PKA (10 μg/ml), which more closely approximated physiological levels of this enzyme (Hofmann et al., 1977). In addition, we examined the ability of the specific PKA inhibitor PKIP (Cheng et al., 1986) to prevent the effect of PKA on $GABA_A$ receptor currents. Figure 3a shows a typical response of a patch to application of 5 μM GABA. Bursting inward chloride currents were evoked, and the total average ensemble current evoked through all conductance states in this set of experiments was 0.39 pA (n = 17 patches). In patches in which the lower concentration of PKA was included in the pipette solution, fewer openings were once again observed (Figure 3b) and the average GABA-evoked current was reduced by 74% to 0.10 pA (n = 16 patches). GABA-evoked current was not reduced, however, when the specific PKA inhibitor PKIP was included in the pipette solution with PKA (Figure 3c). The average GABA-evoked current in patches exposed to PKA + PKIP was 0.32 pA (n = 13 patches).

Approximately 80% of control and PKA + PKIP patches were sensitive to GABA. However, only 55% of the patches treated with PKA alone showed typical responses to GABA; the remainder displayed either trace openings or no response. As in the previous experiment using a higher concentration of PKA, the chord conductance of the main state was unaffected by either PKA or PKIP. Table 2 shows the $GABA_A$ receptor 27 pS main conductance state open and closed properties. The mean channel open time was unaffected by treatment with either PKA or the combination of PKA + PKIP when examined on a per patch basis. The channel opening frequency was 15.9 ± 2.6 openings per s in control patches and 18.3 ± 3.1 openings per s in patches exposed to PKA + PKIP. The opening frequency was significantly reduced in PKA patches to 5.7 ± 1.6 openings per s ($P < 0.01$ with respect to control and PKIP-treated patches by ANOVA and Newman-Keuls' test). Consistent with this reduction in channel opening frequency in PKA-treated patches, an increase in the mean closed time of the main conductance state also was observed (Table 2). The results from the single-channel studies indicate that the primary mechanism by which PKA reduces $GABA_A$ receptor current is by reducing channel opening frequency.

The Distributions of Open Times Were Best Fitted by Multiple Exponential Functions and Were Unaltered by PKA or PKIP

Since the mean or average open and closed times may

Table 2. $GABA_A$ Receptor Main Conductance Open and Closed Properties

	Control	PKA	PKA + PKIP
Mean open time (ms)	5.9	4.8	4.7
Mean closed time (ms)	118.0	196.1	93.3
Percentage open	8.3	3.3	7.9
Average current (pA)	0.17	0.07	0.17
Number of openings	47,131	16,896	26,307
Number of patches	17	16	13

Mean open and closed times, percentage open, average current, and number of openings were derived from detected openings (see Experimental Procedures) and represent values only for the 27 pS main conductance state. Mean closed time refers to the mean closed duration between 27 pS main conductance openings. PKA (10 μg/ml) and PKIP (200 μg/ml) were included in the internal recording pipette solution.

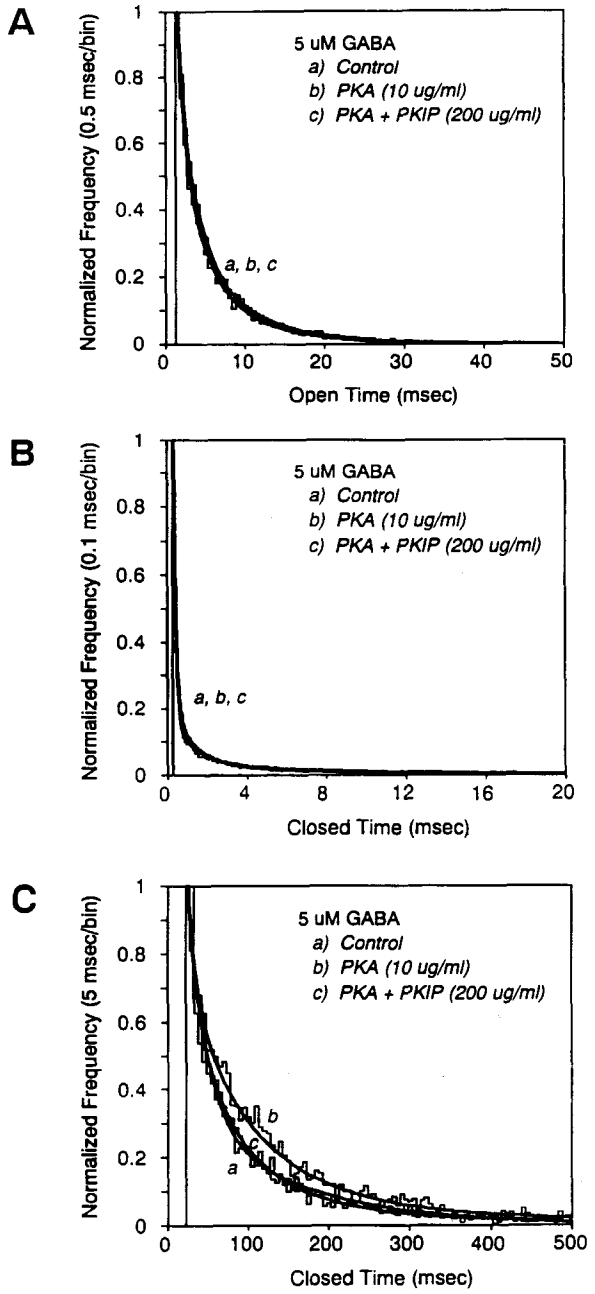

Figure 4. Frequency Distributions of Openings and Closings Evoked by 5 μM GABA in the Presence of PKA or PKA + PKIP

Distributions were normalized and overlayed to display relative frequency distribution. Histograms were fitted with three exponential functions, and the curves were drawn according to the fits (see Results). (A) Openings were put into bins of 0.5 ms and plotted from bins 3–100. GABA open time frequency histograms were unaltered by either PKA (10 μg/ml) or PKA (10 μg/ml) + PKIP (200 μg/ml). (B) Short closures were put into bins of 0.1 ms and plotted from bins 3–200. GABA short closed time frequency distributions were unaltered by either PKA or PKA + PKIP. (C) Long closures were put into bins of 5 ms and plotted from bins 5–100. In the presence of PKA, the GABA long closed time distribution was increased. No difference in long closures between the control and PKA + PKIP groups was observed.

not accurately describe the kinetic behavior of the channel, frequency distributions for channel openings and closings were examined. Open and closed time frequency histograms for the previous experiment are shown in Figure 4. In all cases, open time frequency distributions were best fitted by three exponential functions called components 1, 2, and 3. These three components corresponded to the shortest, intermediate, and longest open time constants, respectively, and were not different across all treatments (Figure 4A). For all treatments, the component 1 time constant ranged from 0.6 to 0.8 ms, with a mean of 0.7 ± 0.1 ms (mean ± SD). The component 2 time constant ranged from 2.8 to 3.4 ms, with a mean of 3.1 ± 0.3 ms, and the component 3 time constant ranged from 8.0 to 8.9 ms, with a mean of 8.6 ± 0.5 ms.

PKA Altered Long, But Not Short, Closed Time Frequency Distributions

Since closed times covered a very wide range, closed time distributions were plotted with two different bin magnitudes (Figures 4B and 4C). The distribution of short closed times ranged from 0.3 to 20 ms in bins of 0.1 ms. The distribution of long closed times ranged from 5 to 500 ms in bins of 5 ms. In all cases, closed time frequency distributions were best fitted by three exponential functions. Short closed times were unaltered by treatment with either PKA or the combination of PKA + PKIP (Figure 4B). The mean of the short closed time constants for all three treatments was as follows: component 1, 0.1 ± 0.01 ms; component 2, 1.1 ± 0.1 ms; and component 3, 10.0 ± 0.4 ms.

In contrast to the results obtained with the short closed time distributions, the long closed time distribution for PKA was shifted to the right (Figure 4C). This increase in the long closed time distribution was due to an increase in the two longest time constants. No difference in the shortest time constant between the control, PKA, and PKA + PKIP groups was observed, and it ranged from 5.0 to 6.7 ms, with a mean of 5.9 ± 0.9 ms. The means for the two longer time constants for the control and PKA + PKIP groups were 26.9 ± 0.1 ms and 118.6 ± 6.5 ms, respectively. In the presence of PKA, the intermediate and long time constants were 50.6 and 161.4 ms. This increase in the long closed time distribution in the presence of PKA is consistent with a decrease in channel opening frequency.

Discussion

PKA Reduced $GABA_A$ Receptor Current Possibly by Phosphorylation of the Channel Protein

The present report directly demonstrates that phosphorylation of $GABA_A$ receptors alters channel function. Previous studies have indirectly inferred an action of PKA on $GABA_A$ receptor function with agents that may act as second messengers, such as cAMP analogs or forskolin (Heuschneider and Schwartz,

1989; Jalilian Tehrani et al., 1989). It is noteworthy that several recent studies suggested that in some cases the effects of these agents may be independent of an action on PKA, thus leading to a potential misinterpretation of results (Hoshi et al., 1988; Wagoner and Pallotta, 1988; Heuschneider and Schwartz, 1989; Lambert and Harrison, 1989, Soc. Neurosci., abstract). Whether the effects of PKA on GABA-evoked current are exerted via phosphorylation of the receptor/channel directly or an associated protein that controls channel activity remains to be determined. However, the former mechanism appears to be more likely, since direct phosphorylation of the β subunit by PKA has recently been demonstrated in purified preparations of receptor isolated from mammalian brain (Kirkness et al., 1989; Browning et al., 1990). The present study also extends to the central nervous system the observation made in the peripheral nervous system that phosphorylation regulates ligand-gated ion channels. Studies examining the effects of phosphorylation on the nAChR have been done primarily on skeletal muscle or on preparations of receptor isolated from the electric organ of fish (Miles and Huganir, 1988).

Heterogeneity of $GABA_A$ Receptors

PKA completely blocked $GABA_A$ receptor current in approximately 40% of the neurons dialyzed with PKA, whereas in the remaining cells the current was reduced by 32%. This heterogeneity was observed also in patches. As in previous studies (Macdonald et al., 1989), approximately 80% of the patches obtained were GABA sensitive; however, in the presence of PKA only 55% of the patches obtained were sensitive to GABA. It is possible that the heterogeneity of the response to GABA in cells exposed to PKA may reflect heterogeneity of the β subunit. Multiple β subunits have been isolated by both protein chemistry (Browning et al., 1990) and cDNA cloning techniques (Lolait et al., 1989; Ymer et al., 1989). Moreover, the different β subunits appear to be substrates for different protein kinases. In purified preparations of the $GABA_A$ receptor, a higher molecular weight β polypeptide was preferentially phosphorylated by PKA, whereas a β peptide of lower molecular weight was phosphorylated by protein kinase C (Browning et al., 1990). Interestingly, no direct phosphorylation of either β peptide by calcium/calmodulin-dependent kinase II was observed. Alternatively, the amount of GABA-evoked current in the presence of PKA may be directly related to the degree of phosphorylation of the receptor, as is observed for the nAChR (Hopfield et al., 1988). The nAChR contains multiple phosphorylation sites, and the amount of desensitization of the receptor is dependent on the stoichiometry of the phosphorylation. A similar case may exist for the $GABA_A$ receptor, since the proposed structure of the native receptor includes two β subunits (Mamalaki et al., 1987; Schofield et al., 1987) and thus two sites at which PKA may act.

Mechanism of Reduction of $GABA_A$ Receptor Currents by PKA

To evaluate the effects of PKA on the microscopic kinetics of the $GABA_A$ receptor, frequency distributions of channel open times were examined. The open time frequency distributions were best fitted with at least three exponential functions, suggesting the presence of three open states with different mean durations. This finding is consistent with a kinetic model of the $GABA_A$ receptor shown to have a minimum of three open states and two binding sites (Macdonald et al., 1989; Twyman et al., 1990). Since the open time frequency distribution (Figure 4A), mean open time, and open time constants were not significantly altered, PKA did not affect the $GABA_A$ receptor closing kinetics or the rate of entry into the three open states. These results suggest that when receptor activation by GABA occurs in the presence of PKA, gating proceeds in a normal fashion.

Further characterization of channel kinetics can be obtained by examining closed time frequency distributions. Ion channels appear to open and close in bursts when openings occur in rapid succession. The closings within a burst of openings are short and usually reflect the activity of a single, fully bound receptor/channel (Colquhoun and Hawkes, 1983; Macdonald et al., 1989). Closed time frequency distributions were best fitted with multiple exponential functions, suggesting the presence of multiple closed states. The distribution of short closings was unaltered in PKA and indicated that the short closings of single receptor/channels were not affected (Figure 4B). However, long closings, which generally result from partially bound and unbound receptors, were prolonged in the presence of PKA (Figure 4C) and reflected the observed decrease in opening frequency. These results indicate that PKA could have reduced $GABA_A$ receptor current by producing a desensitized state of the bound receptor, by reducing the rate of transition from the unbound to the single-agonist-bound state of the receptor, or by increasing the rate of transition from the single-agonist-bound to the unbound state. Either of these actions would produce a decrease in channel opening frequency without altering channel gating properties.

Regulation of the $GABA_A$ Receptor by Phosphorylation

It should be noted that previous studies have demonstrated that ATP prevents rundown of GABA-evoked whole-cell currents (Stelzer et al., 1988; Gyenes et al., 1988). In the single-channel studies presented here, the addition of ATP to the internal recording medium did not appear to maintain GABA-evoked channel openings or affect channel kinetics (unpublished data). It is possible that ATP may maintain GABA-evoked whole-cell currents by a mechanism independent of PKA. More recently, Chen et al. (1990) have demonstrated that a phosphorylation process main-

tained GABA-evoked whole-cell currents in hippocampal neurons and that a calcium/calmodulin-dependent phosphatase accelerated the rundown of the current. It is possible that the maintenance of GABA-evoked currents by an ATP-dependent process is via an indirect mechanism or is due to an interaction with other reported $GABA_A$ receptor subunits or subunit variants. The γ2 subunit contains a consensus sequence for potential phosphorylation by tyrosine kinases (Pritchett et al., 1989), and a kinase activity of unknown identity is associated with the α subunit (Sweetnam et al., 1988). Protein kinase C also may be involved in the modulation of the $GABA_A$ receptor. A β subunit variant is preferentially phosphorylated by protein kinase C (Browning et al., 1990), and activators of protein kinase C have been shown both to reduce (Sigel and Baur, 1988; Stelzer et al., 1988; Moran and Dascal, 1989) and to have no effect on GABA-evoked whole-cell currents (Harrison and Lambert, 1989a). Additional studies examining the direct effects of protein kinase C on GABA currents are necessary to clarify these results. Nonetheless, the results from these studies indicate that the regulation of $GABA_A$ receptor activity by phosphorylation may be quite complex.

Functional Importance of PKA Phosphorylation of $GABA_A$ Receptors

It is unclear from the study presented here whether PKA actually blocks the activation of the $GABA_A$ receptor channel or whether it promotes a fast desensitization. Some analogy may be drawn between the present results and the well-documented studies demonstrating desensitization of the nAChR following phosphorylation (Huganir et al., 1986; Hopfield et al., 1988). The $GABA_A$ receptor and the nAChR share similar membrane topology and some sequence similarity (Schofield et al., 1987), and thus it is tempting to speculate that the physiological effects of PKA may be the same in both systems. The consequences of any alteration in the GABAergic system in the brain are likely to be important. In view of GABA's role as the major inhibitory neurotransmitter in the brain, any reduction in receptor function, as demonstrated in the study presented here, may lead to increased neuronal excitability.

Experimental Procedures

Cell Culture

Primary cell cultures were established from spinal cords of 12- to 14-day-old murine fetuses as previously described (Ransom et al., 1977). Briefly, cells were mechanically dissociated in medium consisting of 80% minimum essential medium (MEM; GIBCO, Grand Island, NY), 10% fetal calf serum, 10% heat-inactivated horse serum, glucose (5.5 g/liter), and $NaHCO_3$ (1.5 g/liter). The final suspension was plated on collagen-coated plates and incubated (5% CO_2, 95% air, 37°C) for 3 days prior to the first medium change. On day 5 the medium was replaced with medium lacking the fetal calf serum (MEM/H) and containing 5-fluoro-2'-deoxyuridine (20 μg/ml final concentration), to prevent the proliferation of nonneuronal cells. The medium was replaced with MEM/H after 2 days, and all subsequent changes were made twice a week. Cells were maintained for 2–5 weeks before being used in experiments.

Solutions

Prior to recording, the growth medium of a culture was exchanged for 2 ml of extracellular bath solution that contained the following: 142 mM NaCl, 8.1 mM KCl, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 10 mM glucose, and 10 mM Na-HEPES (pH 7.4). For whole-cell recordings, tetrodotoxin (1 μM) was added to the external solution to eliminate spontaneous action potentials. The internal micropipette solution contained 153 mM KCl, 1 mM $MgCl_2$, 2 mM Mg-ATP, 5 mM EGTA, and 10 mM K-HEPES (pH 7.3). This combination of external and internal solutions resulted in an equilibrium potential of 0 mV for chloride and −75 mV for potassium. GABA was diluted from a frozen 10 mM stock with external bath solution to the desired concentration and applied by a pressure ejection micropipette (10–20 μm tip diameter; 0.8–1.0 psi) positioned next to the cell or patch. All recordings were performed at room temperature (22°C–24°C).

PKA Isolation and Determination of Activity

The catalytic subunit of PKA was isolated and purified from bovine heart as previously described (Reimann and Beham, 1983). The catalytic subunit was then diluted to either 10, 20, or 50 μg/ml in internal recording medium just prior to the experiment. To prevent possible breakdown of PKA, dithiothreitol (5 mM) was added to the internal recording solution when the lowest concentration of the catalytic subunit was used. Since PKA interfered with giga-seal formation, recording pipette tips were prefilled with a small amount of control internal solution and then backfilled with internal solution containing PKA. In the single-channel studies, PKIP (200 μg/ml; Sigma, St. Louis, MO) was included with PKA in the internal recording medium. The solutions were preincubated at 37°C for 3–5 min prior to use to ensure activation of the inhibitor peptide. Heat inactivation of PKA was carried out by boiling for 5–10 min. The activity of PKA in internal recording medium was determined by a protein kinase assay using the synthetic peptide kemptide as substrate (Uhler and McKnight, 1987). Inactivation of PKA following either boiling or treatment with PKIP was determined in the same manner.

Current Recording

Recording micropipettes (microhematocrit capillary tubes; Fisher Scientific, Pittsburgh, PA) were pulled in three stages using a model P-87 Flaming Brown Micropipette Puller (Sutter Instruments, San Francisco, CA). Whole-cell and patch-pipette resistances were approximately 5 and 10 MΩ, respectively.

Whole-cell and excised outside-out patch-clamp recordings were obtained as previously described (Hamill et al., 1981) using a model L/M EPC-7 amplifier (List Medical Instruments, Darmstadt, FDR). Whole-cell current responses were low-pass-filtered (3 dB at 1 kHz, 8-pole Butterworth; A. P. Circuit Corporation) and simultaneously recorded on a chart recorder (Gould, Cleveland, OH) and stored on a digital video cassette recording system (Sony SL-2700 modified to 0–20 kHz) via a digital audio processor (Sony PCM-501ES, 14-bit, 44 kHz). Single-channel data currents were low-pass-filtered (3 dB at 10 kHz, 8-pole Bessel filter) and stored on the VCR system.

For whole-cell and patch recordings, membranes were voltage clamped at −75 mV. Membrane conductance was determined by applying constant short duration (200 ms) hyperpolarizing voltage commands to neurons during the whole-cell recording mode.

Single-Channel Current Analysis

The single-channel data were played back from the VCR system and digitized (20 kHz sampling rate) for computer analysis with a low-pass (3 dB at 2 kHz) 8-pole Bessel filter interposed. Current amplitudes and durations were determined by a locally written computer program (Macdonald et al., 1989). Channel openings were detected using the 50% threshold crossing method and were accepted as valid events if their durations were greater than

twice the system dead time (system dead time = 70 μs). Openings were constrained to be greater than twice the rise time of the low-pass filter (rise time = 130 μs) to obtain an accurate estimation of channel amplitude (Colquhoun and Sigworth, 1983). As a result of undetected openings or closings, the measured times for events will generally be longer than the "true" open and closed times (Colquhoun and Sigworth, 1983). In the text, the terms "open" and "closed times" refer to measured times not corrected for unobserved transitions (McManus et al., 1987).

Durations of channel openings and closings from all patches were pooled and collated into frequency histograms. Only closed times between main conductance state openings were analyzed. Open time frequency histograms were fitted starting from at least twice the system dead time. A locally modified, nonlinear curve-fitting program was used to fit histograms to a sum of n exponential functions (Macdonald et al., 1989). Estimates of time constants were determined using the method of maximum likelihood estimation and approximated a 95% confidence interval. The number of significant exponential functions necessary to fit the frequency distributions was determined by chi-square analysis.

Acknowledgments

We thank Dr. Carl J. Rogers for writing the single-channel detection program and for computer hardware and software implementation. We also thank Ms. Nancy E. Ciliax for maintaining cultures and for illustrations. This work was supported by an individual NRSA postdoctoral fellowship to N. M. P. (NS08216), by an American Academy of Neurology and Neuropharmacology Fellowship and a CIDA award (NS01266) to R. E. T., by USPHS grants to M. D. U. (GM38788) and R. L. M. (NS19613), and by a grant from the Lucille P. Markey Charitable Trust.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 USC Section 1734 solely to indicate this fact.

Received July 17, 1990; revised September 7, 1990.

References

Bormann, J., Hamill, O. P., and Sakmann, B. (1987). Mechanism of anion permeation through channels gated by glycine and γ-aminobutyric acid in mouse cultured spinal neurones. J. Physiol. *385*, 243–286.

Browning, M. D., Bureau, M., Dudek, E. M., and Olsen, R. W. (1990). Protein kinase C and cAMP-dependent protein kinase phosphorylate the β subunit of the purified γ-aminobutyric acid A receptor. Proc. Natl. Acad. Sci. USA *87*, 1315–1318.

Bruning, J. L., and Kintz, B. L. (1977). Computational Handbook of Statistics (Glenview, Illinois: Scott, Foresman and Co.).

Chen, Q. X., Stelzer, A., Kay, A. R., and Wong, R. K. S. (1990). $GABA_A$ receptor function is regulated by phosphorylation in acutely dissociated guinea-pig hippocampal neurons. J. Physiol. *420*, 207–221.

Cheng, H.-C., Kemp, B. E., Pearson, R. B., Smith, A. J., Misconi, L., Van Patten, S. M., and Walsh, D. A. (1986). A potent synthetic peptide inhibitor of the cAMP-dependent protein kinase. J. Biol. Chem. *261*, 989–992.

Colquhoun, D., and Hawkes, A. G. (1983). The principles of the stochastic interpretation of ion-channel mechanisms. In Single-Channel Recordings, B. Sakmann and E. Neher, eds. (New York: Plenum Publishing Corp.), pp. 135–175.

Colquhoun, D., and Sigworth, F. J. (1983). Fitting and statistical analysis of single-channel records. In Single-Channel Recordings, B. Sakmann and E. Neher, eds. (New York: Plenum Publishing Corp.), pp. 191–263.

Gyenes, M., Farrant, M., and Farb, D. H. (1988). "Run-down" of γ-aminobutyric $acid_A$ receptor function during whole-cell recording: a possible role for phosphorylation. Mol. Pharmacol. *34*, 719–723.

Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981). Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflügers Arch. *391*, 85–100.

Hamill, O. P., Bormann, J., and Sakmann, B. (1983). Activation of multiple-conductance state chloride channels in spinal neurones by glycine and GABA. Nature *305*, 805–808.

Harrison, N. L., and Lambert, N. A. (1989a). The effects of protein kinase activators on $GABA_A$ receptor function in cultured rat hippocampal neurones. J. Physiol. *412*, 17P.

Harrison, N. L., and Lambert, N. A. (1989b). Modification of $GABA_A$ receptor function by an analog of cyclic AMP. Neurosci. Lett. *105*, 137–142.

Heuschneider, G., and Schwartz, R. D. (1989). cAMP and forskolin decrease γ-aminobutyric acid-gated chloride flux in rat brain synaptoneurosomes. Proc. Natl. Acad. Sci. USA *86*, 2938–2942.

Hofmann, F., Bechtel, P. J., and Krebs, E. B. (1977). Concentrations of cyclic AMP-dependent protein kinase subunits in various tissues. J. Biol. Chem. *252*, 1441–1447.

Hopfield, J. F., Tank, D. W., Greengard, P., and Huganir, R. L. (1988). Functional modulation of the nicotinic acetylcholine receptor by tyrosine phosphorylation. Nature *336*, 677–680.

Hoshi, T., Garber, S. S., and Aldrich, R. W. (1988). Effect of forskolin on voltage-gated K^+ channels is independent of adenylate cyclase activation. Science *240*, 1652–1655.

Huganir, R. L., Delcour, A. H., Greengard, P., and Hess, G. P. (1986). Phosphorylation of the nicotinic acetylcholine receptor regulates its rate of desensitization. Nature *321*, 774–776.

Jalilian Tehrani, M. H., Hablitz, J. J., and Barnes, E. M. (1989). cAMP increases the rate of $GABA_A$ receptor desensitization in chick cortical neurons. Synapse *4*, 126–131.

Khrestchatisky, M., MacLennan, A. J., Chiang, M.-Y., Xu, W., Jackson, M. B., Brecha, N., Sternini, C., Olsen, R. W., and Tobin, A. J. (1989). A novel α subunit in rat brain $GABA_A$ receptors. Neuron *3*, 745–753.

Kirkness, E. F., Bovenkerk, C. F., Ueda, T., and Turner, A. J. (1989). Phosphorylation of γ-aminobutyrate (GABA)/benzodiazepine receptors by cyclic AMP-dependent protein kinase. Biochem. J. *259*, 613–616.

Lolait, S. J., O'Carroll, A.-M., Kusano, K., Muller, J. M., Brownstein, M. J., and Mahan, L. C. (1989). Cloning and expression of a novel rat $GABA_A$ receptor. FEBS Lett. *246*, 145–148.

Lüddens, H., Pritchett, D. B., Köhler, M., Killisch, I., Keinänen, K., Monyer, H., Sprengel, R., and Seeburg, P. H. (1990). Cerebellar $GABA_A$ receptor selective for a behavioural alcohol antagonist. Nature *346*, 648–651.

Macdonald, R. L., Rogers, C. J., and Twyman, R. E. (1989). Kinetic properties of the $GABA_A$ receptor main conductance state of mouse spinal cord neurones in culture. J. Physiol. *410*, 479–499.

Mamalaki, C., Stephenson, F. A., and Barnard, E. A. (1987). The $GABA_A$/benzodiazepine receptor is a heterotetramer of homologous α and β subunits. EMBO J. *6*, 561–565.

McManus, O. B., Blatz, A. L., and Magleby, K. L. (1987). Sampling, log binning, fitting, and plotting durations of open and shut intervals from single channels and the effects of noise. Pflügers Arch. *410*, 530–553.

Miles, K., and Huganir, R. L. (1988). Regulation of nicotinic acetylcholine receptors by protein phosphorylation. Mol. Neurobiol. *2*, 91–124.

Moran, O., and Dascal, N. (1989). Protein kinase C modulates neurotransmitter responses in Xenopus oocytes injected with rat brain. Mol. Brain Res. *5*, 193–202.

Pritchett, D. B., Sontheimer, H., Shivers, B. D., Ymer, S., Kettenmann, H., Schofield, P. R., and Seeburg, P. H. (1989). Importance

of a novel $GABA_A$ receptor subunit for benzodiazepine pharmacology. Nature *338*, 582–585.

Ransom, B. R., Neale, E., Henkart, M., Bullock, P. N., and Nelson, P. G. (1977). Mouse spinal cord in cell culture. I. Morphology and intrinsic neuronal electrophysiologic properties. J. Neurophysiol. *40*, 1132–1150.

Reimann, E. M., and Beham, R. A. (1983). Catalytic subunit of cAMP-dependent protein kinase. Meth. Enzymol. *99*, 51–55.

Schofield, P. R., Darlison, M. G., Fujita, N., Burt, D. R., Stephenson, F. A., Rodriguez, H., Rhee, L. M., Ramachandran, J., Reale, V., Glencorse, T. A., Seeburg, P. H., and Barnard, E. A. (1987). Sequence and functional expression of the $GABA_A$ receptor shows a ligand gated receptor super-family. Nature *328*, 221–227.

Shivers, B. D., Killisch, I., Sprengel, R., Sontheimer, H., Köhler, M., Schofield, P. R., and Seeburg, P. H. (1989). Two novel $GABA_A$ receptor subunits exist in distinct neuronal subpopulations. Neuron *3*, 327–337.

Sigel, E., and Baur, R. (1988). Activation of protein kinase C differentially modulates neuronal Na^+, Ca^{2+}, and γ-aminobutyrate type A channels. Proc. Natl. Acad. Sci. USA *85*, 6192–6196.

Stelzer, A., Kay, A. R., and Wong, R. K.S. (1988). $GABA_A$ – receptor function in hippocampal cells is maintained by phosphorylation factors. Science *241*, 339–341.

Sweetnam, P. M., Lloyd, J., Gallombardo, P., Malisan, R. T., Gallagher, D. W., Tallman, J. F., and Nestler, E. J. (1988). Phosphorylation of the $GABA_A$/benzodiazepine receptor alpha subunit by a receptor associated protein kinase. J. Neurochem. *51*, 1274–1284.

Twyman, R. E., Rogers, C. J., and Macdonald, R. L. (1990). Intraburst kinetic properties of the $GABA_A$ receptor main conductance state of mouse spinal cord neurones in culture. J. Physiol. *423*, 193–220.

Uhler, M. D., and McKnight, G. S. (1987). Expression of cDNAs for two isoforms of the catalytic subunit of cAMP-dependent protein kinase. J. Biol. Chem. *262*, 15202–15207.

Wagoner, P. K., and Pallotta, B. S. (1988). Modulation of acetylcholine receptor desensitization by forskolin is independent of cAMP. Science *240*, 1655–1657.

Ymer, S., Schofield, P. R., Draguhn, A., Werner, P., Köhler, M., and Seeburg, P. H. (1989). $GABA_A$ receptor β subunit heterogeneity: functional expression of cloned cDNAs. EMBO J. *8*, 1665–1670.