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Protein Kinase C Enhances Recombinant Bovine α1β1γ2L $GABA_A$ Receptor Whole-Cell Currents Expressed in L929 Fibroblasts

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Summary

The β1 and γ2L subunits of the γ-aminobutyric acid type A receptor (GABAR) contain phosphorylation sites for PKC. To determine the effect of PKC on GABAR function, whole-cell recordings were obtained from mouse fibroblasts expressing recombinant α1β1γ2L receptors, and catalytically active PKC (PKM) was applied via the recording pipette. The first experiment was a population study. Intracellular application of PKM increased GABAR currents, and the enhancement was antagonized by coapplication of the PKC inhibitory peptide. No acceleration or deceleration of GABAR desensitization was observed. The second experiment was a reimpalement study in which paired recordings were made successively from individual cells. Enhancement of GABAR currents by PKM was again obtained. PKM increased GABAR currents at high (>10 μM) but not at low (<10 μM) GABA concentrations, resulting in increases in both EC_{50} and maximal GABAR current. Thus, PKC phosphorylation enhanced recombinant α1β1γ2L GABAR current by increasing maximal current without increasing the affinity of GABA for the GABARs.

Introduction

γ-Aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the vertebrate brain (Olsen and Venter, 1986). The $GABA_A$ receptor (GABAR) is a hetero-oligomeric receptor-channel complex consisting of different subunit families (α, β, γ, δ, and ρ), and multiple subtypes of each GABAR subunit family have been cloned (α1-6, β1-4, γ1-3, δ1, and ρ1-2; Olsen and Tobin, 1990; Burt and Kamatchi, 1991; Macdonald and Olsen, 1994). The GABAR forms a chloride ion channel, and a variety of clinically important agents, including anxiolytic, anticonvulsant, hypnotic, anesthetic, and muscle relaxant drugs, have extracellular binding sites on the GABAR and can allosterically modulate GABAR function (Macdonald and Olsen, 1994).

Neuronal membrane currents evoked by voltage or neurotransmitters can be modulated by intracellular second messengers and regulatory proteins (Moran and Dascal, 1989). However, little is known about the intracellular regulation of GABARs. Protein phosphorylation is an important posttranslational mechanism through which cellular proteins and intracellular events are modified (Hunter and Cooper, 1985). Previous studies have provided evidence for a role of phosphorylation in the regulation of the function of various K^+ and Ca^{2+} channels (Browning et al., 1985; Levitan, 1985; Dascal et al., 1986) as well as the nicotinic acetylcholine receptor (nAChR; Huganir et al., 1986; Downing and Role, 1987; Miles and Huganir, 1988; Swope et al., 1992). Based on the structural homology between the nAChR and the GABAR (Schofield et al., 1987), it might be predicted that phosphorylation would also play an important role in regulating GABAR function.

Recently, it has been shown that an unidentified protein kinase associated with a partially purified preparation of GABAR could phosphorylate the receptor in vitro (Sweetnam et al., 1988). Moreover, it has been demonstrated that highly purified GABAR proteins could be phosphorylated by exogenously added cyclic AMP-dependent protein kinase (PKA; Kirkness et al., 1989; Browning et al., 1990; Moss et al., 1992b) or by Ca^{2+}/phospholipid-dependent protein kinase (PKC; Browning et al., 1990). These findings suggest that GABARs might be regulated by protein phosphorylation.

Functionally, PKA has been shown to reduce GABAR currents in mouse spinal neurons (Porter et al., 1990) and to decrease GABAR-mediated chloride flux in mouse microsacs (Leidenheimer et al., 1991). Phorbol esters and diacylglycerol analogs that activate PKC have been demonstrated to reduce recombinant GABAR currents expressed in Xenopus laevis oocytes (Sigel and Baur, 1988; Moran and Dascal, 1989; Sigel et al., 1991; Kellenberger et al., 1992; Leidenheimer et al., 1992; Krishek et al., 1994) and to decrease the $^{36}Cl^-$ uptake in mouse cerebellar membrane vesicles (Leidenheimer et al., 1992). In addition, GABAR currents recorded from human embryonic kidney cells cotransfected with murine α1, β1, and γ2L GABAR subunit cDNAs together with the rat α isoform of PKC were reduced by phorbol esters (Krishek et al., 1994).

Serines 327 and 343 of the γ2L subunit as well as serine 409 of the β1 subunit are consensus sites for phosphorylation by PKC and are located in the major intracellular loop of the GABAR between the proposed third and fourth transmembrane domains (Whiting et al., 1990; Moss et al., 1992a). Furthermore, purified fusion proteins of the major intracellular domain of β1 and γ2L subunits produced in Escherichia coli have been shown to be phosphoylated rapidly by PKC, and site-directed mutagenesis of these specific serine residues eliminates PKC phosphorylation (Moss et al., 1992a). Recently, Krishek et al. (1994) reported that site-directed mutagenesis of these serine residues differentially reduced the effects of phorbol esters on GABAR currents expressed in human embry-

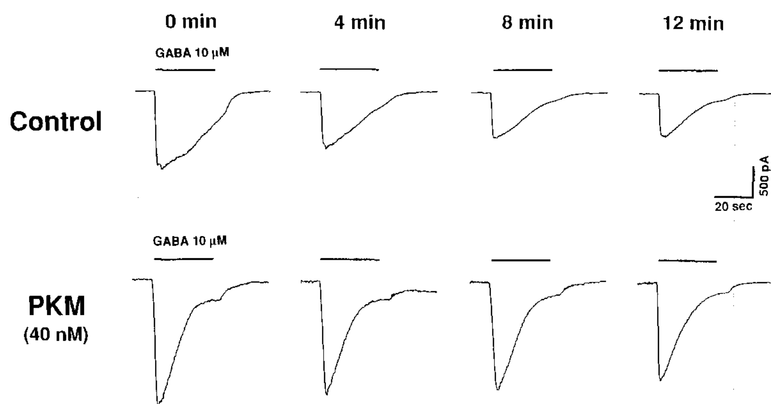

Figure 1. PKM Enhanced Recombinant α1β1γ2L GABAR Whole-Cell Currents in Transfected L929 Cells

To both the control cell (upper panel) and PKM-treated cell (lower panel), GABA (10 μM) was applied four times by pressure ejection for 30 s each time at 4 min intervals. GABA was applied in the same way for all the subsequent figures, except Figure 7. In a control cell, application of GABA resulted in an inward chloride current, which declined (ran down) with repetitive GABA ejection over time (upper panel). In another cell that was treated with PKM (40 nM) intracellularly, the amplitudes of evoked currents were larger than control responses. This cell also showed less run-down of peak whole-cell currents. Recordings were made from cells 48 hr after transfection. For this and all subsequent experiments, cells were held at −75 mV.

onic kidney cells and Xenopus oocytes. The functional significance of phosphorylation of GABARs by direct PKC treatment, however, remains uncertain.

To determine whether acute PKC phosphorylation alters GABAR function, we transiently expressed bovine α1, β1, and γ2L GABAR subunits in mouse L929 fibroblasts and recorded GABAR whole-cell currents from transfected cells. We compared GABAR currents obtained with control pipette solution with those obtained with constitutively active PKC (PKM; Huang and Huang, 1986) in the recording pipette (Lin, et al., 1992, 1993, Soc. Neurosci., abstracts).

Results

PKM Enhanced Recombinant Bovine α1β1γ2L GABAR Whole-Cell Currents

Transient transfection of bovine α1, β1, and γ2L subunit cDNAs into L929 cells resulted in expression of functional GABARs. GABA evoked inward currents in transfected L929 cells when the cells were voltage clamped to −75 mV. The reversal potential of the GABAR current was 0 mV, since symmetrical recording and bath chloride solutions were used. The effect of PKM on GABAR function was examined by comparing peak currents evoked from control and PKM-treated cells. GABA (10 μM) was applied for 30 s, 0, 4, 8, or 12 min after obtaining the whole-cell recording. Repetitive application of GABA resulted in a gradual decrease of GABAR whole-cell currents (i.e., current run-down) during the 12 min recording period (Figure 1, upper panel; Figure 2, open circles). In the presence of intracellular PKM (40 nM), the amplitude of peak GABAR whole-cell currents increased (Figure 1, lower panel; Figure 2, closed circles). In control cells, the mean amplitude of the initial peak whole-cell current at 0 min was 968 ± 192 pA, and the current declined to 546 ± 101 pA at 12 min (mean ± SEM; n = 22; Figure 2, open circles). In contrast, with PKM in the recording pipette, the mean amplitude of the initial peak current at 0 min was larger (1264 ± 212 pA), and though the PKM-treated current gradually decreased to 945 ± 166 pA at 12 min (n = 24; Figure 2, closed circles), it was still larger than the current at 12 min from control cells. The increase of mean peak whole-cell current amplitude by PKM treatment was not significant at the first GABA application but became significant for subsequent applications (4, 8, and 12 min time points; Figure 2, closed versus open circles). On the other hand, the rate of GABAR run-down was not affected by PKM. In control cells, GABAR currents ran down to 71% ± 7% of the initial value in 12 min. With 40 nM PKM present in the recording pipette, GABAR currents declined to 81% ± 7% of the initial value in 12 min. The difference was not significant.

The PKC-Inhibitory Peptide Suppressed Enhancement of Recombinant Bovine α1β1γ2L GABAR Currents by PKM

In six cells, the specificity of PKC phosphorylation enhancement of GABAR currents was studied. The specific PKC-inhibitory peptide (PKC-I; 4 μM) was included in the intracellular recording solution with PKM (40 nM). PKC-I antagonized the enhancement of GABAR currents by PKM (Figure 2, open squares versus closed circles). In control cells, GABAR currents declined from 968 ± 192 pA to 546 ± 101 pA in 12 min (n = 22; Figure 2, open circles). In cells treated with PKC-I plus PKM, peak GABAR currents declined from 805 ± 175 pA to 395 ± 120 pA in 12 min (n = 6; Figure 2, open squares). The peak whole-cell currents recorded from these two groups of cells were not statistically different from each other. Interestingly, the mean peak whole-cell GABAR currents in cells treated with PKM plus PKC-I were smaller than the currents from control cells at each corresponding time point, although the difference was not significant (Figure 2).

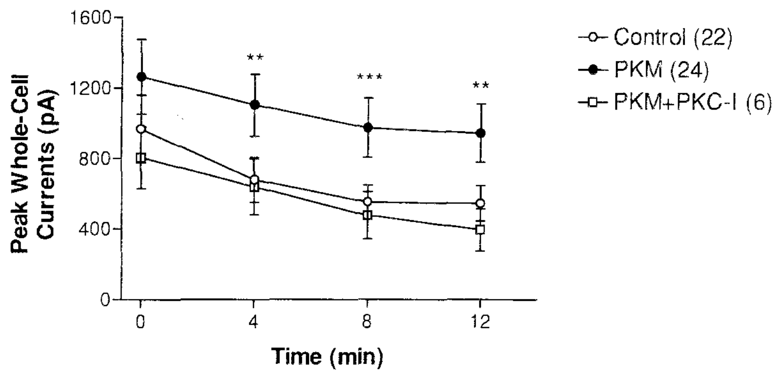

Figure 2. PKM Increased Recombinant α1β1γ2L GABAR Peak Whole-Cell Currents, and the Enhancement Was Antagonized by PKC-I

Magnitudes of peak whole-cell currents evoked by 10 μM GABA from transfected L929 cells were enhanced by PKM treatment. Data are expressed as mean ± SEM. The enhancement of peak whole-cell currents evoked in the PKM-treated cells (closed circles; n = 24) was not significant at the first GABA application, but for subsequent applications, GABAR currents were increased significantly (the 4, 8 and 12 min time points) when compared with the GABAR currents from control cells (open circles; n = 22). On the other hand, PKC-I treatment reversed the enhancement of GABAR peak whole-cell currents produced by PKM. PKC-I (4 μM) was applied together with PKM (40 nM) through recording pipettes. GABAR currents obtained from cells treated with PKC-I plus PKM (open squares; n = 6) were not different from currents obtained from control cells (open circles; n = 22). The asterisks represent significant differences in mean peak currents between the control cells and the PKM-treated cells (two asterisks, $p < .05$; three asterisks, $p < .025$).

Acute Desensitization Was Not Altered by PKM

Acute desensitization of GABAR currents was determined by calculating the percentage of desensitization (%D) of currents evoked by a 5 s application of 100 μM GABA. The fast application multipuffer system was used to obtain a more accurate estimate of the desensitization rate. Comparison of the percentage of desensitization between control (n = 25) and PKM-treated (n = 12) cells showed that the acute desensitization was not affected by 40 nM PKM treatment. For control and PKM-treated cells, GABAR currents desensitized to 20% ± 4.0% and 12% ± 2.8% of peak current magnitudes, which corresponded to desensitization rates of 16%/s and 17.6%/s, respectively. The differences in desensitization magnitudes and rates were not significant (independent Student's t test).

Successive Recordings from the Same Cell with Control and PKM-Containing Intracellular Solutions Provided Direct Evidence That PKM Enhanced Recombinant Bovine α1β1γ2L GABAR Currents

Although intracellular PKM treatment enhanced GABAR currents in the population study (Figure 2), we were concerned about the variability of whole-cell current magnitudes obtained from transfected L929 cells. The variability of current amplitudes could be a problem, since control and PKM-treated currents were obtained from different cells. To achieve a more reliable control for PKM treatment, we performed a "reimpalement" study on individual cells. Recordings were obtained sequentially from individual transfected cells with control internal solution-filled pipettes followed by PKM-containing pipettes or control solution-filled pipettes. Whole-cell currents obtained sequentially from the same cell were then compared.

We determined whether the reimpalement method was feasible by performing control recordings sequentially on individual cells. For recordings either with control solution-containing or with PKM-containing pipettes, GABA (10 μM) was applied four times for 30 s at 4 min intervals over a 12 min period as described for the population study. A whole-cell recording was obtained from a transfected L929 cell with a control intracellular recording solution-filled pipette, and GABA was applied to the cell to evoke GABAR currents. After the initial recording, the pipette was removed, and the cell was allowed to recover for at least 30 min. Then, another recording (reimpalement recording) was made with a control internal solution-filled pipette. Seven cells were studied using this protocol (control/control cells).

To examine the effect of PKM on GABAR whole-cell currents from individual cells, initial control recordings were made with control intracellular recording solution-filled pipettes, and reimpalement recordings were obtained from the same cell with PKM-containing, internal solution-filled pipettes after a recovery period of at least 30 min. Five cells were investigated in this way (control/PKM cells).

Current tracings obtained from a control/control cell are illustrated in Figure 3. Currents ran down in the initial control recording over 12 min (Figure 3, upper panel; Figure 4, open circles). The magnitudes of control currents obtained during the reimpalement recording using another control internal solution-filled pipette (Figure 3, lower panel; Figure 4, closed circles) were smaller when compared with the currents obtained during the initial control recording of this cell.

In contrast, currents obtained during the reimpalement recording of the control/PKM cells were increased in magnitude. Figure 5 shows current tracings obtained from a control/PKM cell, with the control (initial) currents illustrated in the upper panel and the PKM-treated (reimpalement) currents illustrated in the lower panel. Reimpalement of this cell with a PKM-containing pipette resulted in an increase of GABAR currents (Figure 5, lower panel; Figure 6, closed circles) from control level (Figure 5, upper panel; Figure

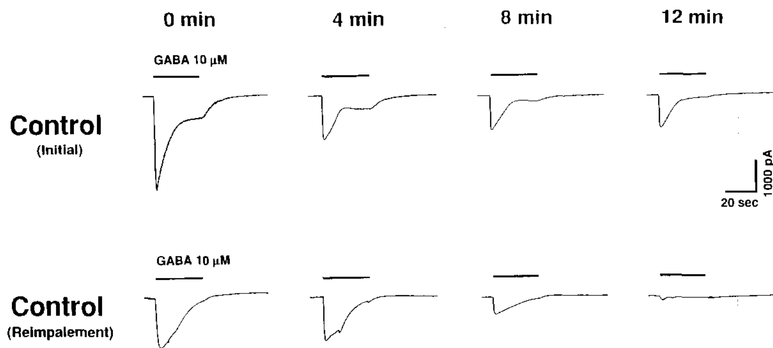

Figure 3. Recombinant α1β1γ2L GABAR Peak Whole-Cell Currents Were Not Increased by Reimpalement with a Control Solution-Filled Recording Pipette

Current tracings obtained from a control/control transfected L929 cell are illustrated. Two sequential recordings of GABAR currents were performed on this cell. The initial recording was made by a control intracellular recording solution-filled pipette, and GABA (10 μM) was applied four times at the indicated time points. After a recovery period of 30 min, the same cell was reimpaled and recorded from with another control pipette, and GABAR currents were evoked with four GABA applications at corresponding time points. Tracings in the upper panel show GABAR whole-cell currents recorded during the control recordings (initial), and tracings in the lower panel show currents obtained during the reimpalement control recordings (reimpalement). In this cell, GABAR currents declined in the initial recording as well as in the reimpalement control recording. The currents recorded during the reimpalement control recordings were smaller than the currents obtained during the initial control recording at each corresponding time point. In the absence of PKM, the reimpalement recording made on a single cell with a control solution-filled pipette did not enhance GABAR currents.

6, open circles) at each corresponding time point of GABA application. The currents recorded with the PKM-containing pipette were larger at each time point when compared with the corresponding control currents.

The currents obtained from these experiments were compared to determine whether the control/PKM cell currents were enhanced by PKM treatment when compared with their same-cell initial controls and with the control/control currents. The reimpalement approach allowed same-cell paired comparisons of current amplitudes (i.e., paired control versus control, or control versus PKM currents recorded from the same cell) to be made. Control currents obtained during the initial whole-cell recording (Figure 4, open circles) and control currents obtained during the reimpalement whole-cell recording (Figure 4, closed circles) were compared. While mean currents are shown in Figure 4, comparisons were made by performing paired t tests on control and reimpalement currents from individual control/control cells (n = 7). To normalize currents, the first current of the initial recording was taken as 100%. All subsequent currents were normalized to this current by expressing them as a percentage of this current. Asterisks in Figure 4 represent significant differences in normalized (in %) current amplitudes obtained during the initial and the reimpalement recordings from individual cells. Compared with the corresponding normalized initial recording control currents, there was a slight but significant change in the reimpalement control at the 4 and 12 min time points (Figure 4, asterisks). However, at 0 and 8 min, two sets of normalized control currents obtained from individual cells were not different. In

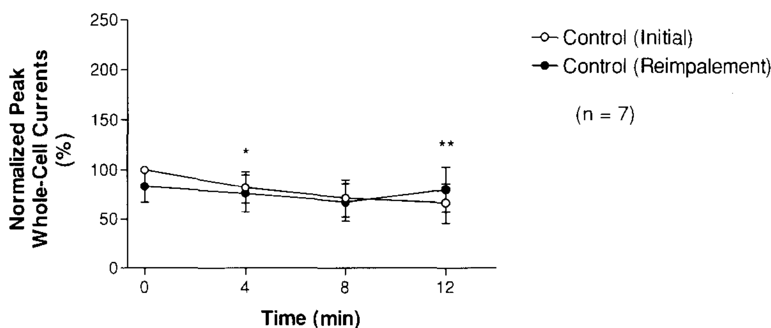

Figure 4. Recombinant α1β1γ2L GABAR Peak Whole-Cell Currents Were Not Increased by Reimpalement with Control Solution-Filled Pipettes

Normalized mean peak whole-cell currents (%) obtained from individual control/control transfected L929 cells are shown (mean ± SEM; n = 7). Two sequential recordings of GABAR currents were obtained from each cell. The initial recording was made using a control intracellular recording solution-filled pipette. After a recovery period of at least 30 min, the same cell was reimpaled, and another recording was obtained with another control solution-filled pipette. GABA was applied four times at the indicated time points for both the initial and the reimpalement control recordings, and the evoked GABAR currents were compared. Open circles represent mean GABAR peak whole-cell currents obtained during the initial control recording, and the closed circles represent mean currents obtained during the reimpalement control recording. All evoked currents were normalized to the first response evoked at the 0 min time point during the initial recording (100%) from each cell. Comparisons were then made by performing paired t tests on each pair of control currents obtained at corresponding time points from the same cells (n = 7). Normalized GABAR currents declined during the initial recording as well as during the reimpalement control recording. The currents obtained at the corresponding time points during the reimpalement control recording (closed circles) were reduced slightly (n = 7) when compared with the currents obtained during the initial control recording (open circles) from the same cells. The only exception was that the reimpalement currents evoked by GABA at 12 min were increased slightly. The asterisks represent significant differences in currents obtained during the initial and the reimpalement control recordings of individual cells (one asterisk, $p < .10$; two asterisks, $p < .05$).

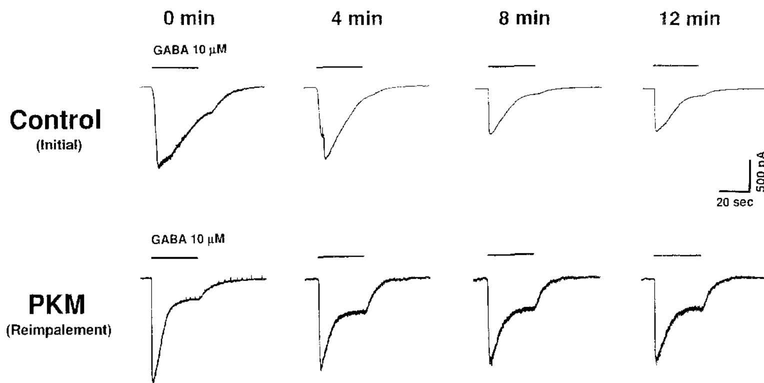

Figure 5. PKM Reimpalement Increased Recombinant α1β1γ2L GABAR Peak Whole-Cell Currents in a Transfected L929 Cell

Current tracings obtained from a control/PKM cell are illustrated. Two sequential recordings of GABAR currents were performed on this cell. The initial recording was made by a control intracellular recording solution-filled pipette. After a recovery period of 30 min, a whole-cell recording was obtained with a PKM-containing, internal solution-filled pipette. GABA (10 μM) was applied four times at the indicated time points. Tracings in the upper panel show GABAR whole-cell currents obtained during the control recordings (initial), and tracings in the lower panel show currents obtained during the PKM-treated recordings (reimpalement). In this cell, the amplitudes of GABAR currents, obtained during the reimpalement recording (PKM-treated), were greater than the currents obtained during the initial recording (control) at all corresponding time points.

general, these data confirmed that reimpalement of the same cell with a control solution-filled pipette did not result in an increase in current amplitude (Figure 4).

Paired comparisons were also performed on currents obtained during successive recordings from individual control/PKM cells (n = 5). In Figure 6, asterisks again represent significant differences between current amplitudes obtained during the initial and the reimpalement recordings. All currents are shown as average values pooled from five cells. To normalize currents, the first current of the initial recording was again taken as 100%, and all subsequent currents were normalized to this current by expressing them as a percentage of this current. The average currents obtained during PKM reimpalement recordings (Figure 6, closed circles) were larger than those obtained during initial control recordings (Figure 6, open circles). While mean currents are shown in Figure 6, comparisons were made by performing paired t tests on initial control and reimpalement PKM-treated currents from individual control/PKM cells (n = 5). The enhancement was significant at each time point (paired t test; n = 5; Figure 6, asterisks).

Properties of Recombinant Bovine α1β1γ2L GABARs Expressed in Mouse Fibroblast L929 Cells Were Altered by PKM Treatment

By performing another reimpalement study on five cells, GABAR whole-cell currents were shown to increase in a concentration-dependent manner, with GABA concentration ranging from 0.1 to 300 μM under both control (Figure 7, open circles) and PKM (40 nM)-treated (closed circles) conditions. A multipuffer fast application system instead of the puffer pressure-ejection system was used to obtain better application and washout of drugs. The control and PKM-treated currents obtained from each cell were normalized to the maximal control current (taken as 100%). The normalized currents obtained from a total of five cells were then pooled and averaged at each GABA concentration to form the two normalized concentration–

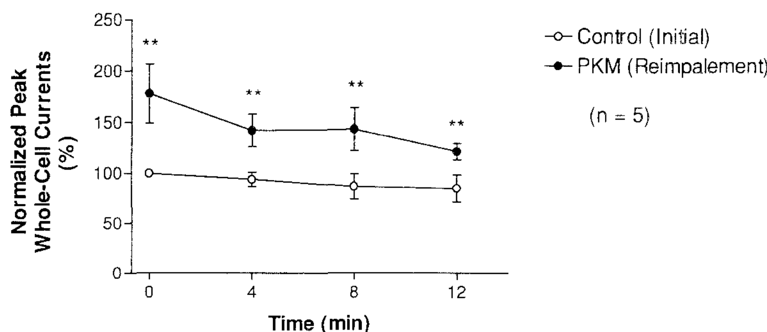

Figure 6. PKM Reimpalement Increased Recombinant α1β1γ2L GABAR Peak Whole-Cell Currents in Individual Transfected L929 Cells

Normalized mean peak whole-cell currents (%) obtained from individual control/PKM cells are shown (mean ± SEM; n = 5). Recordings were obtained from each cell sequentially with two different solution-filled pipettes. The initial recording was made using a control intracellular recording solution-filled pipette. After recovery, the same cell was reimpaled with a PKM-containing pipette. GABA (10 μM) was applied four times at the indicated time points for both the initial and the reimpalement recordings. Open circles represent mean GABAR peak whole-cell currents recorded during the initial control recording, and closed circles represent mean currents obtained during the PKM-treated reimpalement recording. All peak whole-cell currents were normalized to the first control current obtained at 0 min during the initial recording (100%) from the same cell. Comparisons were made by performing paired t tests on each pair of the control and PKM-treated currents from the same individual cells. GABAR currents declined in the initial control recording as well as in the reimpalement recording. However, PKM (40 nM) treatment significantly increased the normalized peak GABAR whole-cell currents at each time point when compared with the control currents from the same cells. The asterisks represent the significant differences in currents obtained during the initial and the reimpalement recordings for individual cells ($p < .05$).

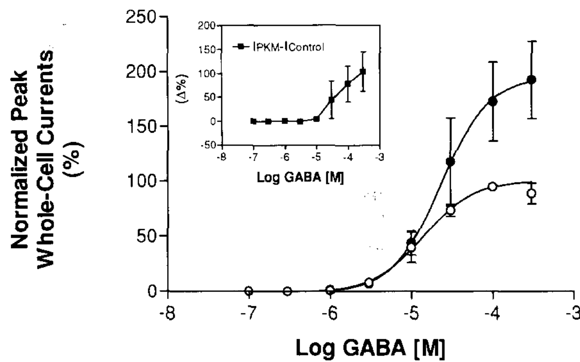

Figure 7. PKM Increased the Efficacy but Decreased the Potency of GABA on Recombinant α1β1γ2L GABARs Expressed in Transfected L929 Cells

When GABA was applied to recombinant α1β1γ2L GABARs expressed in transfected L929 cells in a reimpalement study (control/PKM cells; n = 5), the GABA-evoked whole-cell currents increased in a concentration-dependent manner during both the initial control and the PKM-treated reimpalement recordings. Open circles represent the average peak whole-cell currents obtained during the initial control recording, and closed circles represent the average amplitudes of peak whole-cell currents obtained during the PKM-treated reimpalement recording. All currents were normalized to the maximal current (100%) obtained during the initial control recordings of each individual cell. Concentrations of GABA used ranged from 0.1 μM to 300 μM, and GABA was applied by a multipuffer fast application system at 5 s durations. The curves were fit to the logistic equation by nonlinear regression. The Hill coefficient was 1.4, and the EC_{50} was 14 μM for cells in control condition; these measurements were 1.5 and 23 μM, respectively, for the same group of cells in the PKM-treated condition. The maximal response and EC_{50} values obtained from each cell under control and PKM-treated conditions were compared by using paired Student's t test and showed significant increases ($p < .025$ for both the maximal response and EC_{50}). These data indicated that PKM decreased the GABA potency but increased the efficacy.

response curves shown in Figure 7. The curves were fit to the logistic equation by nonlinear regression. The Hill coefficient and EC_{50} were 1.4 and 14 μM, respectively, for control cells and 1.5 and 23 μM for the same group of cells that were treated with PKM. In addition to increasing EC_{50}, PKM increased the maximal GABAR current responses to 195% of control. Paired comparisons of EC_{50} and V_{max} values between control and PKM-treated concentration–response curves obtained from each individual cell revealed that the increases of EC_{50} and the maximal response were both statistically significant ($p < .025$ for both, paired Student's t test). The differences between the normalized currents (Δ%) obtained during control and PKM-treated recordings from each individual cell at different GABA concentrations are shown in the inset of Figure 7 (n = 5). The enhancement of whole-cell currents by PKM became significant as GABA concentration increased. At GABA concentrations lower than 10 μM, PKM produced no effect on whole-cell GABAR currents.

Discussion

PKC Enhanced the Magnitude but Did Not Alter the Rate of Run-Down of Recombinant α1β1γ2L GABAR Currents

Direct intracellular PKM application resulted in an increase in GABAR peak whole-cell currents throughout a 12 min recording period. However, the rate of GABAR current run-down was not altered by PKM treatment. Variable rates of current run-down were observed among both control and PKM-treated cells. When the GABAR currents were normalized to the initial current recorded and averaged, the rates of run-down were not different between control and PKM-treated cells. The data suggest that it is the magnitude of currents, but not their rate of run-down, that was affected by PKC phosphorylation. Current run-down was a phenomenon most likely related to the experimental protocol used. In previous studies, it has been shown that ATP prevents run-down of GABAR whole-cell currents (Gyenes et al., 1988; Stelzer et al., 1988), and it has been suggested that the function of the GABAR is maintained by a phosphorylation process of the receptor, or some closely associated regulatory protein, and is destabilized by a Ca^{2+}-dependent dephosphorylation process (Chen et al., 1990). In the present study, however, intracellular addition of PKM and Mg^{2+}-ATP (PKM-treated cells) failed to change the run-down rates (data not shown). The persistence of the run-down in the presence of PKM plus Mg^{2+}-ATP might be attributed to a gradual loss of diffusible regulatory molecules from the recorded cell or to a dephosphorylation process. It could also be possible that other phosphorylation processes other than PKC phosphorylation are important to maintain GABAR function. The stabilization of currents toward a higher current level than the control in PKM-treated cells may be due to a persistent increase of GABAR function by PKC phosphorylation.

PKC Phosphorylation Enhanced Recombinant GABAR Currents

PKC-I is a synthetic PKC-inhibitory peptide (19–31) that is identical to the intrinsic pseudosubstrate sequence of PKC and functions as a negative control for PKC assays. Data from cells treated with PKC-I plus PKM suggest that PKC enhances GABAR currents by specific phosphorylation rather than a nonspecific interaction between PKC and target proteins. The enhancement of GABAR currents observed following successive recordings from the same cell in the reimpalement study was consistent with the data obtained in the population study. The amplitudes of GABAR whole-cell currents obtained from different cells varied. The advantage of the successive recording (reimpalement) method employed here was to provide a

more reliable paired comparison, although the latter was technically difficult to perform. Results from both studies provide evidence supporting the conclusion that PKC phosphorylation up-regulates rather than down-regulates GABAR function.

Nevertheless, whether the effects of PKC on GABAR current are exerted directly by phosphorylation of the receptor–channel protein complex, or indirectly, via phosphorylation of an associated protein that controls channel activity, remains to be determined. The former mechanism appears to be more likely, because in vitro studies have shown that GABAR subunits could be phosphorylated by exogenous PKA (Kirkness et al., 1989; Browning et al., 1990) or by PKC (Browning et al., 1990). It has been identified that serine 409 in the GABAR β1 subunit and serines 327 and 343 in the γ2L subunit are high affinity substrates for PKC in vitro (Moss et al., 1992a). Site-directed mutagenesis experiments are currently in progress to create mutant bovine β1 and γ2L subunit cDNAs with all known PKC phosphorylation sites removed. Future study of PKC on these mutants would determine whether PKC phosphorylation regulates GABAR function by direct or indirect action on receptor proteins.

PKC Phosphorylation Increased the Apparent Efficacy but Decreased the Potency of GABA at the GABAR

The increase of EC_{50} and maximal GABA response by PKM treatment suggests that PKC phosphorylation alters at least two different GABAR properties. The increased maximal responses to GABA (efficacy) accounted for the enhancement of GABAR current magnitude to 10 μM GABA by PKM. However, the increased EC_{50} indicated that PKC phosphorylation also decreased GABA potency. Interestingly, the effect of PKC phosphorylation was GABA concentration-dependent. PKM did not enhance GABAR currents at GABA concentrations lower than 10 μM. However, when the GABA concentration was increased (from 10 to 300 μM), enhancement of GABAR responses by PKM was produced.

PKC Phosphorylation Had No Effect on the Rate of GABAR Desensitization

Similar to the report by Leidenheimer et al. (1992), but employing different preparation and measurement methods, the data in the present study show that the rate of acute desensitization of GABAR current was not affected by PKC phosphorylation. The increase in GABAR currents by PKC phosphorylation resulted from an increase of GABA efficacy. However, the change of the GABA concentration–response relation by PKM treatment (Figure 7) does not rule out an effect of PKC phosphorylation on GABARs in other desensitized states. The enhancement of GABAR currents could be due to phosphorylation-mediated reversal of a nonconducting desensitized state. Resolution of this issue will require a study using very rapid GABA application techniques.

Possibility of Differential Phosphorylation of Native GABARs

It is unclear whether the total number or the specific position of amino acid residues of the PKC phosphorylation sites on GABAR subunits determines the extent of up-regulation of the GABAR function produced by PKC. The structure and distribution of GABARs in the central nervous system are heterogeneous (Vicini, 1991). Therefore, depending on the presence of available phosphorylation sites, different subunits might have varying potential for being phosphorylated. As a result, the presence of differential regulation by PKC phosphorylation of GABARs in different brain areas or, more accurately, of GABARs with different subunit composition, is likely.

Comparison of the Present Study with Previous Studies of PKC Phosphorylation of GABARs

Several laboratories have reported that 12-myristate 13-acetate or other active phorbol esters inhibited GABAR currents expressed in Xenopus oocytes (Sigel and Baur, 1988; Moran and Dascal, 1989; Sigel et al., 1991; Kellenberger et al., 1992; Leidenheimer et al., 1992; Krishek et al., 1994) and kidney cells (Krishek et al., 1994). It has also been shown that phorbol esters had no effect on GABARs in cultured neurons (Malenka et al., 1987; Harrison and Lambert, 1989, J. Physiol., abstract; Harrison and Lambert, 1989; Ticku and Mehta, 1990).

PKC phosphorylation increased the rate of the fast phase of desensitization of native nAChRs (Huganir and Greengard, 1990), another member of the ligand-gated receptor–channel superfamily. However, neither the fast nor the slow phase of desensitization of recombinant α1β1γ2L GABARs was altered by PKC activation (Leidenheimer et al., 1992).

In contrast to the above studies, we demonstrated enhancement of GABAR currents by PKC phosphorylation, suggesting that PKC up-regulates GABAR function. However, this finding is different from previous studies (Sigel and Baur, 1988; Stelzer et al., 1988; Harrison and Lambert, 1989, J. Physiol., abstract; Harrison and Lambert, 1989; Moran and Dascal, 1989; Sigel et al., 1991; Kellenberger et al., 1992; Leidenheimer et al., 1992; Krishek et al., 1994) in which PKC activator phorbol esters instead of direct PKM treatment were used to produce PKC phosphorylation. The differences in these results could be due to the following: different experimental protocols to achieve PKC phosphorylation; different basal level of endogenous PKC phosphorylation of a given GABAR population before more PKC was activated or before PKM was dialyzed intracellularly; different basal level of phosphorylation of receptor proteins by other protein kinases; different subunit composition of receptors studied or different cells used. An attenuating effect of phorbol esters on cellular PKC levels has been reported (Wolf et al., 1984; Rodriguez-Pena and Rozengurt, 1984; Woodgett and Hunter, 1987), and this should be taken into account when interpreting data obtained with phorbol esters.

Possible Mechanisms of PKC Phosphorylation to Regulate GABARs

Protein phosphorylation is one of the most important and effective ways to regulate protein activity (Nestler and Greengard, 1984). Protein phosphorylation has been correlated with modulation of receptor activity by either enhancing or terminating the action of the ligand (Hemmings et al., 1989). Alternatively, it has been proposed that protein phosphorylation may act as an intracellular ligand with the phosphorylation sites on target proteins envisioned as cytoplasmic ligand binding sites (Ferrer-Montiel et al., 1991).

The GABAR is not G protein coupled, and therefore, GABARs cannot activate the PKC phosphorylation cascade to modulate receptor function. The up-regulation of GABAR function produced by PKC phosphorylation could be generated indirectly, similar to the regulation of nAChRs by the neuropeptide calcitonin gene-related peptide (CGRP; Huganir and Greengard, 1990). CGRP is a cotransmitter with ACh at the neuromuscular junction and is known to regulate cAMP levels in muscle cells (Miles et al., 1989). It was suggested that CGRP stimulates phosphorylation by activation of PKA and thus serves as a physiological regulator of nAChR phosphorylation and desensitization at the neuromuscular junction (Mulle et al., 1988; Miles et al., 1989). Efforts to study actions of the cotransmitters with GABA in the brain are crucial for establishing the physiological mechanisms for PKC phosphorylation of GABARs.

Conclusions

In the present study, direct intracellular application of PKM increased recombinant α1β1γ2L GABAR currents transiently expressed in transfected mouse L929 fibroblast cells. The enhancement was antagonized by PKC-I. A similar enhancement of GABAR current by PKC was observed in a single-cell reimpalement study, in which whole-cell recordings were obtained successively from the same cell. From the same cell, the initial recording was made with a control intracellular solution-filled pipette, and the reimpalement recording was performed using either a PKM-containing, internal solution-filled pipette or a control pipette. No comparable increase of GABAR currents was obtained when the subsequent recordings were made with a control pipette instead of a PKM-containing pipette. PKM treatment increased the efficacy but decreased the potency of GABA on recombinant α1β1γ2L GABARs. In contrast, neither acute desensitization, or the rate of current run-down was affected by PKM treatment.

In the brain, GABARs might be subject to differential phosphorylation from a variety of protein kinases, depending on the structural specificity and stoichiometry of protein kinase phosphorylation sites in specific subunits and on the activity of protein kinases. In this study, we used direct application of PKM instead of incubation with PKC activators, which excluded possible nonspecific effects produced by PKC activators. The consistency of observations made from experiments with application of both PKM and PKC-I and with application of PKM alone further supported the conclusion that enhancement of α1β1γ2L GABAR currents by PKC was due to specific PKC phosphorylation that increased the efficacy of GABA activation of GABARs. Together, these data suggest that PKC phosphorylation of GABAR up-regulates its function.

Experimental Procedures

Plasmid Construction of GABAR Subunit cDNAs

Full-length cDNAs encoding the bovine α1, β1, and γ2L subunits of GABARs were kindly provided by Dr. E. Barnard (Schofield et al., 1987; Shingai et al., 1991). Expression plasmids were constructed as described previously (Angelotti et al., 1993). The cDNAs were released from the original plasmids with appropriate restriction enzymes (BRL). These cDNAs contained the complete open reading frames and about 10–100 bp of the 5′ and 3′ untranslated sequences and were then subcloned into the BglII site of the mammalian expression vector pCMVNeo (Huggenvik et al., 1991) to form pCMVbα1, pCMVbβ1, and pCMVbγ2L. A marker gene, *LacZ*, encoding for β-galactosidase was also subcloned into expression vector pCMVNeo to form pCMVβGal (Angelotti et al., 1993).

Cell Line and Transient Transfection

A mouse fibroblast cell line L929 (from ATCC) was used in this study. Cells were grown in Dulbecco's modified Eagle medium (DMEM; BRL) with 10% horse serum (BRL) and 100 IU/ml each of penicillin and streptomycin (BRL) at 37°C in 5% CO_2/95% air. Cells were split with 0.5% trypsin/0.2% EDTA (BRL) 1 day before transfection and were plated into a 60 mm Corning dish. On the next day, cells were cotransfected with pCMVbα1, pCMVbβ1, pCMVbγ2L, and pCMVβGal by using a modified calcium phosphate precipitation method (Chen and Okayama, 1987). The total amount of DNA per 60 mm dish for each transfection was 16 μg in 500 μl of transfection buffer. After 4–5 hr, the cells were shocked with a 15% glycerol/PBS solution. On the day after transfection, cells were again trypsinized and treated with 75 μl of DNase I twice for 5 min each at 37°C. After DNase I (BMB) digestion, the cells were collected by centrifugation at 400 × g (or 2000 rpm) for 10 min and then plated to Mecanex-gridded 35 mm dishes or standard 35 mm Corning dishes as described previously (Angelotti et al., 1993). Electrophysiological analysis was carried out 48–72 hr after transfection.

Identification of Transfected Cells with FDG Fluoroscopy

To detect expression of the marker gene (*LacZ*) product β-D-galactosidase in individual cells, fluorescein digalactoside (FDG; Molecular Probes) staining was performed on living L929 cells immediately before electrophysiological recordings. By this staining, positively transfected cells were identified as described previously (Angelotti et al., 1993). Parallel staining with 5-bromo-4-chloro-3-indoyl β-D-galactoside (X-gal; BRL) on fixed cells was also performed to determine the transfection efficiency (Sanes et al., 1986).

Solutions

After FDG fluoroscopy and prior to electrophysiological recording, the dish was rinsed several times and finally bathed in 2 ml of extracellular bath solution, which had the following composition: 142 mM NaCl, 8 mM KCl, 1 mM $CaCl_2$, 6 mM $MgCl_2$, 10 mM D-glucose, 10 mM HEPES (pH to 7.4). The intrapipette recording solution used consisted of 153 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES, 5 mM EGTA, and 2 mM Mg^{2+}-ATP (pH to 7.3). All chemicals were obtained from Sigma unless otherwise stated. A difference of 15–20 mOsm between external and internal solution was kept. The combination of external and internal solutions resulted in an equilibrium potential of 0 mV for Cl^- and −75 mV for K^+.

Drug Application

The GABA used to evoke chloride current was diluted from frozen 10 mM stocks with extracellular bath solution to desired concentrations. A concentration of 10 μM was used for repetitive application by pressure ejection (1.0–1.5 psi) through a micropipette (5–10 μm tip diameter) for most of the present study. The tip of the micropipette was positioned next to the cell recorded during application. Each GABA application lasted for 30 s and was repeated at 4 min intervals. On the other hand, to determine the concentration–response relation and acute desensitization rates of GABAR, a multipuffer fast application system was used (see below). In the latter case, GABA was applied for 5 s by stopping a suction pump via a valve driver (General Valve Corp., NJ) and was terminated/washed away by reopening the valve. The tip diameter of drug puffers was about 60 μm, and the tip was positioned approximately 50 μm from the recorded cell.

Electrophysiological Recording

Whole-cell patch-clamp recording (Hamill et al., 1981) was carried out to record GABAR currents in transfected L929 cells. Currents were monitored with a List L/M EPC-7 amplifier and filtered with a Frequency Devices 902LPF low-pass 8 pole Bessel filter (3 dB, 1 kHz). Filtered signals were then simultaneously recorded to a Gould polygraph chart recorder as well as to a Sony SL-2700 VCR via a Sony PCM-501ES digital audio processor (14 bit, 44 kHz). All cells were voltage clamped at −75 mV during the recordings.

Population Study and Reimpalement Study

The recording experiments were performed in two different ways. One was the population study, in which the data from control and PKM-treated groups were collected separately from different cells, and the comparisons were made between the difference of mean values from both groups. Another study was a same-cell reimpalement study, in which each pair of the control and PKM-treated GABAR currents was obtained from the same cell sequentially. In the reimpalement study, the initial recording was performed with a control intracellular solution-filled pipette, and within 12 min, four GABA applications were given to evoke currents. After the initial recording, the pipette was removed gently, and the cell was allowed to recover for at least 30 min. The reimpalement recording was carried out on the same cell afterwards with either a PKM-containing pipette (for control/PKM cell) or a control internal solution-filled pipette (for control/control cell). Another four repetitive GABA applications were applied during the reimpalement recording to evoke GABAR currents, and these currents were compared with the responses in the initial recording from the same cell. Since the amplitude of GABAR whole-cell currents from different cells varied, the advantage of the same-cell reimpalement study was to provide a more reliable paired comparison. Two different reimpalement manipulations used in this study (control/control and control/PKM) were designed to allow us to test the feasibility of the reimpalement technique and to determine the role of PKC on GABAR functions.

Preparation of PKM and PKC-I

The PKC was isolated and purified from bovine brain as previously described (Woodgett and Hunter, 1987; Browning et al., 1990). Removal of Ca^{2+}/phospholipid dependency of the PKC was achieved by trypsin treatment. After trypsin treatment, PKM was repurified by soybean trypsin inhibitor affinity column chromatography and was concentrated and dialyzed against a buffer with the following composition: 2 mM TEA, 5 mM EGTA, 6 mM $MgCl_2$, 140 mM KCl, 10 mM HEPES, 1 mM $CaCl_2$ (pH to 7.5 with KOH). PKM was stored at −70°C and diluted to 40 nM with internal recording solution just before use.

PKC-I was synthesized and has a sequence identical to amino acids 19–31 of PKC. This sequence functions as an endogenous auto-inhibitor of PKC activity. After synthesis, PKC-I was lyophilized and stored at room temperature or at −70°C. A final concentration of 4 μM of PKC-I was made freshly before use with internal recording solution.

Acute Application of PKM and PKC-I

Both PKM and PKC-I were dissolved in internal recording solutions and dialyzed into cells through recording pipettes after rupture of cell membranes. In our experiments, working solution of PKC-I (4 μM) was always prepared and applied together with PKM (40 nM) and served as a negative control. To allow enough time for PKM or PKM plus PKC-I to enter into the cell, application of GABA usually started about 1 min after rupture of cell membranes.

Analysis of Desensitization

The percentage of desensitization (%D) generated by a single 5 s GABA application was calculated according to the following formula:

$$\%D = [(I_{GABApeak} - I_{GABAend})/I_{GABApeak}] \times 100\%,$$

where $I_{GABApeak}$ is the peak current amplitude evoked by 100 μM GABA and $I_{GABAend}$ is the remaining current at the end of a 5 s GABA application. The calculation of percentage of desensitization was used as an approximate estimate of the acute desensitization rate. For analysis of acute desensitization rates, 100 μM GABA was applied by a multipuffer fast application system to either control cells ($n = 25$) or PKM (40 nM)-treated cells ($n = 12$).

Obtaining and Fitting of Concentration–Response Curves

Concentration–response curves were also obtained using the multipuffer system. GABA concentrations ranged from 0.1 to 300 μM. Currents evoked by GABA under control or PKM-treated conditions were obtained from the individual cells ($n = 5$) using a reimpalement method. Control recordings were performed first with a control intrapipette solution–filled recording pipette, and PKM-treated recordings were performed on the same cells after at least a 30 min recovery period with a PKM (40 nM)-containing pipette. For comparing the effects of PKM on EC_{50} and maximal GABA-evoked current, all currents were normalized to the maximal control current (as 100%) within each cell. The curves were fit to the logistic equation by nonlinear regression.

Analysis and Statistics

In both the population study and the successive recording study, normalized and absolute peak whole-cell currents were collected. GABAR currents recorded with PKM treatment were compared with either the regular control or the PKM plus PKC-I control. The statistics used were independent t test for the population study data, and paired as well as independent t test for the same-cell reimpalement study data (Bolton, 1990).

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