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Activation of Protein Kinase C Selectively Inhibits the γ-Aminobutyric Acid$_A$ Receptor: Role of Desensitization

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SUMMARY

The effects of protein kinase C (PKC) activators on γ-aminobutyric acid$_A$ (GABA$_A$) receptor function were studied by two-electrode voltage-clamp in *Xenopus* oocytes expressing brain mRNA or subunit cDNAs and in isolated mouse brain cerebellar membrane vesicles (microsacs), using $^{36}Cl^-$ uptake. Both oocytes and microsacs showed transient (desensitizing) and sustained (nondesensitizing) GABA$_A$ receptor responses. In oocytes expressing brain mRNA, the PKC activator phorbol myristoyl acetate (PMA), but not the inactive analog phorbol 12-monomyristate, inhibited both transient and sustained GABA-gated chloride currents. The inhibition by PMA was concentration dependent, with an EC$_{50}$ of approximately 5 nM, and resulted in a decrease in the efficacy, but not the potency, of GABA. Additionally, PMA inhibited GABA-gated chloride currents in oocytes expressing $\alpha_1\beta_1\gamma_{2L}$ subunit cDNAs. The effect of PMA on recombinant receptors was significantly antagonized by PKC inhibitory peptide (PKCI). In the microsac preparation, the PKC activators (−)-7-octylindolactam V and PMA inhibited the sustained phase of $^{36}Cl^-$ flux without altering the transient phase. The action of PMA was blocked by kinase inhibitors and by depletion of Mg-ATP and was mimicked by protein phosphatase inhibitors. These results demonstrate that activation of PKC inhibits GABA$_A$ receptor function, and the results from the microsac experiments suggest that PKC-dependent phosphorylation preferentially inactivates a nondesensitized form or state of the receptor.

GABA is the major inhibitory neurotransmitter in mammalian brain, and its actions are mediated by GABA$_A$ and GABA$_B$ receptor subtypes. The GABA$_A$ receptor is a heterooligomeric ligand-gated chloride channel consisting of α, β, γ, and δ subunits. Multiple isoforms of each subunit have been cloned and sequenced, leading, at least theoretically, to a vast number of possible subunit combinations and receptor subtypes (1). Several GABA$_A$ receptor subunits have been shown to be phosphorylated *in vitro*, providing the possibility of posttranslational regulation of GABA$_A$ receptor function (2).

There is evidence that Ca^{2+}-phospholipid-dependent protein kinase (PKC) phosphorylates at least one β subunit as well as the γ_{2L} subunit of the receptor (3, 4), suggesting that activation of PKC could alter GABA$_A$ receptor function. Indeed, *Xenopus* oocytes injected with either brain mRNA or GABA$_A$ receptor subunit cDNAs express GABA-gated chloride currents that are potently inhibited by phorbol ester activators of PKC (5–7). In contrast, phorbol esters have not been found to inhibit GABA$_A$ receptor function in neurons. For example, Ticku and Mehta (8) have shown that 1 μM phorbol dibutyrate does not alter GABA$_A$ receptor-mediated $^{36}Cl^-$ uptake in cultured spinal cord neurons. In CA$_1$ hippocampal pyramidal cells, phorbol dibutyrate increases GABA$_A$ receptor-mediated inhibitory postsynaptic potentials; however, this effect is thought to be due to an enhanced synaptic release of GABA and not to PKC modulation of the GABA$_A$ receptor protein itself (9).

GABA$_A$ receptors display complex kinetics of activation, multiple conductance states, and several phases of desensitization (10–13). It is possible that PKC may alter one of these distinct processes, and this kinetic selectivity could underlie the discrepancies in the literature between oocytes and neurons. In the present study, we assessed the effects of phorbol esters on the rapid (transient) phase and the slow (sustained) phase of GABA-activated chloride flux, using both *Xenopus* oocytes expressing brain mRNA or GABA$_A$ receptor subunit cDNAs and brain membrane vesicles. Additionally, we investigated whether the effects of phorbol esters could be blocked by PKC inhibitors and whether protein phosphatases would mimic the actions of phorbol esters. These experiments indicate that GABA$_A$ receptors are inhibited by PKC and suggest that, for

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ABBREVIATIONS: GABA, γ-aminobutyric acid; DMSO, dimethylsulfoxide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; PKC, protein kinase C; PKCI, protein kinase C inhibitor peptide; PMA, phorbol 12-myristate 13-acetate; PMM, phorbol 12-mono-myristate; Oct. V, (−)-7-octylindolactam V.

GABA$_A$ receptors *in situ*, PKC selectively modulates a particular receptor state or desensitization phase.

Materials and Methods

Reagents

Adult (60–90-day-old) male ICR and BALB/c mice were obtained from Harlan Sprague Dawley (Indianapolis, IN), and adult female *Xenopus laevis* frogs were purchased from Xenopus I (Ann Arbor, MI). Fast Track mRNA isolation kits were purchased from Invitrogen (San Diego, CA). Drugs and chemicals used and their respective sources were as follows: muscimol and GABA, Research Biochemicals Incorporated (Natick, MA); collagenase type IA, picrotoxin, PMA, PMM, staurosporine, sphingosine, and PKCI, Sigma Chemical Company (St. Louis, MO); okadaic acid, calyculin A, and Oct. V, LC Services Corporation (Woburn, MA); and $^{36}Cl^-$ (specific activity, 6.5 mCi/g of Cl), ICN (Irvine, CA). All other reagents used were analytical grade and were purchased from Sigma or Fisher Scientific (Pittsburgh, PA).

Oocyte Experiments

Preparation of brain mRNA and cDNAs. For mRNA isolations, polyadenylated mRNA was extracted from BALB/c mouse whole brain, using a Fast Track isolation kit, as previously described (14). In brief, mouse brains were removed after decapitation and frozen quickly in liquid nitrogen. The frozen tissue was ground to a fine powder with a mortar and pestle. It was then incubated for 2 hr in lysis buffer at 47°. Oligo(dT) was added to bind mRNA, and the samples were centrifuged and washed several times. The mRNA was then isolated by column elution. Each preparation was purified further by phenol/chloroform extraction. Samples were precipitated with ethanol and stored at −20° until used. When needed, aliquots were microcentrifuged and the pellet was dried and resuspended in sterile, diethyl pyrocarbonate-treated water for injection. Sequences of cDNAs encoding human GABA$_A$ receptor α_1, β_1, and γ_{2L} subunits have been reported previously by Seeburg and colleagues (15). The cloning of the subunit cDNAs used here will be described elsewhere.[1] cDNAs were subcloned into pCDM8 vector (Invitrogen) for expression studies. The cDNAs were then suspended in diethyl pyrocarbonate-treated water, aliquoted, and stored at −20° until injection.

Oocyte preparation and microinjection. Oocytes were prepared as previously described (14). Stage V and VI oocytes were isolated, and the theca and epithelial cell layer were dissected away manually. The underlying follicular cell layer was removed by a 10-min treatment in Sigma type IA collagenase (0.5 mg/ml) dissolved in modified Barth's saline [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, adjusted to pH 7.5]. For mRNA expression, a 50-nl mRNA solution (150 ng/50 nl) was injected into the oocyte cytoplasm, using a 10-μl glass micropipette (20–30-μm tip diameter) attached to a Drummond microdispenser (Drummond Scientific, Broomall, PA). For expression of cDNAs, a 30-nl mixture of $\alpha_1\beta_1\gamma_{2L}$ cDNAs (1.5 ng/30 nl) was injected into the nucleus of the oocyte by the "blind" method described by Colman (16). Injected oocytes were then incubated in modified Barth's saline supplemented with 2 mM sodium pyruvate, 100 units/ml penicillin, 100 mg/ml streptomycin, 50 mg/ml gentamycin, and 0.5 mM theophylline. Recording of oocytes expressing cDNAs and mRNAs began 1 and 3 days after injection, respectively.

Electrophysiological recording. The cells were impaled in the animal pole hemisphere with two microelectrodes (1–20 MΩ) containing 3 M KCl and were voltage-clamped at −90 mV with an Axoclamp IIA amplifier (Axon Instruments, Inc., Burlingame, CA). Only those cells with resting membrane potentials of at least −60 mV were used for data collection. For experiments showing both transient and sustained currents, GABA (30 μM) applications were 5 min in duration. The effects of PMA and PMM on these currents were determined 10 and 25 min after phorbol application. For the remaining experiments, GABA (30 μM) was applied for 30 sec and, thus, only the transient current was measured. PKCI was microinjected (300 ng in 30 nl) into the oocytes 1 hr before PMA application, and GABA-gated chloride currents were measured 40 min after PMA treatment. For GABA concentration-response experiments, all concentrations of GABA were applied to an oocyte both before and after PMA treatment. In all experiments, phorbol esters were prepared in a solution containing 0.1% DMSO and were bath applied for 3 min. Drug concentrations were calculated by assuming complete equilibrium. The *n* for the oocyte experiments refers to the number of oocytes tested. Several different donors were used.

Microsac Experiments

Membrane preparation. The procedure for preparation of membrane vesicles (microsacs) was described by Harris and Allan (17). ICR mice were sacrificed by decapitation, and the cerebellum or cerebral cortex was dissected and retained. For each preparation, four to 10 cerebella or one to three cortices were pooled. The final pellet was suspended in assay buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM D-glucose, 1 mM $CaCl_2$, 10 mM HEPES, pH 7.5), yielding a preparation containing 4–5 mg of protein/ml of suspension. To prevent phosphorylation from occurring in some experiments (18), the final microsac pellet was lysed in a solution of 6 mM tricine and 1 mM magnesium acetate (pH 7.5), and microsacs were then resealed with a calcium- and magnesium-free assay buffer containing 1 mM EDTA. For experiments with the lysed/resealed microsacs, measurement of $^{36}Cl^-$ uptake was performed in calcium- and magnesium-free, EDTA-containing assay buffer.

$^{36}Cl^-$ uptake. This procedure was described by Hahner *et al.* (19). Microsacs (0.2 ml) were incubated in a 34° water bath for 5 min. Uptake was then initiated by the addition and immediate vortexing of 0.2 ml of a solution maintained at 34°, containing $^{36}Cl^-$ (2 μCi/ml of assay buffer) with or without 3 μM muscimol. Thirty seconds (unless indicated otherwise) after addition of $^{36}Cl^-$, influx was terminated by addition of 4 ml of ice-cold assay buffer, containing 0.1 mM picrotoxin, and rapid filtration under vacuum (15 cm Hg) onto a premoistened GB100R filter (MicroFiltration Systems, Dublin, CA), using a Hoefer manifold (Hoefer Scientific, San Francisco, CA). These filters were washed, with the manifold towers removed, with an additional 12 ml of ice-cold assay buffer containing 0.1 mM picrotoxin. The amount of $^{36}Cl^-$ bound to the filters in the absence of membranes (no-tissue blank) was subtracted from all values. For desensitization experiments, membranes (0.2 ml) were preincubated with or without 3 μM muscimol for 5–30 sec before a 3-sec uptake was initiated by addition of 0.2 ml of $^{36}Cl^-$ solution containing 0.2 mM muscimol (final concentration, 0.1 mM). Uptake was stopped at the 3-sec time point by the quench/filtration/wash procedure described above.

Muscimol-dependent uptake (i.e., influx through GABA$_A$ receptor-activated Cl^- channels) was defined as the amount of $^{36}Cl^-$ taken up while muscimol was present in the medium (total uptake) minus the amount of $^{36}Cl^-$ taken up when muscimol was not present (basal or muscimol independent). To evaluate effects of drugs on the muscimol-dependent uptake, basal uptake was also measured in the presence of these drugs, and these values were used to determine the muscimol-dependent uptake for that drug. Muscimol was dissolved in water and all other drugs were dissolved in DMSO; the final concentration of DMSO in the assay was 0.1% and was tested as a vehicle control for all experiments with these agents. Thus, all drugs were tested for effects on muscimol-independent (basal) uptake of $^{36}Cl^-$, as well as for effects on uptake of $^{36}Cl^-$ in the presence of muscimol.

Statistics

An analysis of variance for repeated measures was used to compare time-course data and concentration-response curves. For experiments involving only two comparisons, a Student's *t* test was used.

[1] P. Whiting. Cloning of human cDNAs coding for GABA$_A$ receptor subunits, manuscript in preparation.

Results

Function of $GABA_A$ receptors expressed in *Xenopus* oocytes. Injection of mouse brain mRNA in oocytes resulted in expression of $GABA_A$ receptors that displayed a large transient response to GABA, as well as a smaller sustained response (Fig. 1). Both the sustained and transient currents were markedly inhibited by PMA (25 nM), and this inhibition developed slowly, reaching a maximum (complete inhibition in the case of the sustained current) about 25 min after application of PMA (Fig. 2). In contrast to PMA, the inactive phorbol ester PMM did not affect either the transient or sustained GABA currents.

Because the transient current was much larger and more accurately measured, we performed subsequent experiments on this current. PMA (5–25 nM) produced a concentration-dependent inhibition of the GABA response, with an EC_{50} of approximately 5 nM (Fig. 3). To investigate whether the inhibition of $GABA_A$ receptor function was due to a decrease in the efficacy or shift in the potency of GABA, GABA concentration-response experiments were performed (Fig. 4). The effect of PMA on GABA-gated chloride currents was due to a robust decrease in the efficacy of GABA. The GABA EC_{50s} values for control and PMA-treated oocytes were approximately 45 and 30 μM, respectively. In addition to testing PMA on oocytes expressing brain mRNA, experiments were conducted on recombinant $GABA_A$ receptors in oocytes coexpressing human $\alpha_1\beta_1\gamma_{2L}$ subunit cDNAs. Similarly, PMA (25 nM) inhibited GABA-gated chloride currents by approximately 80% (Fig. 5).

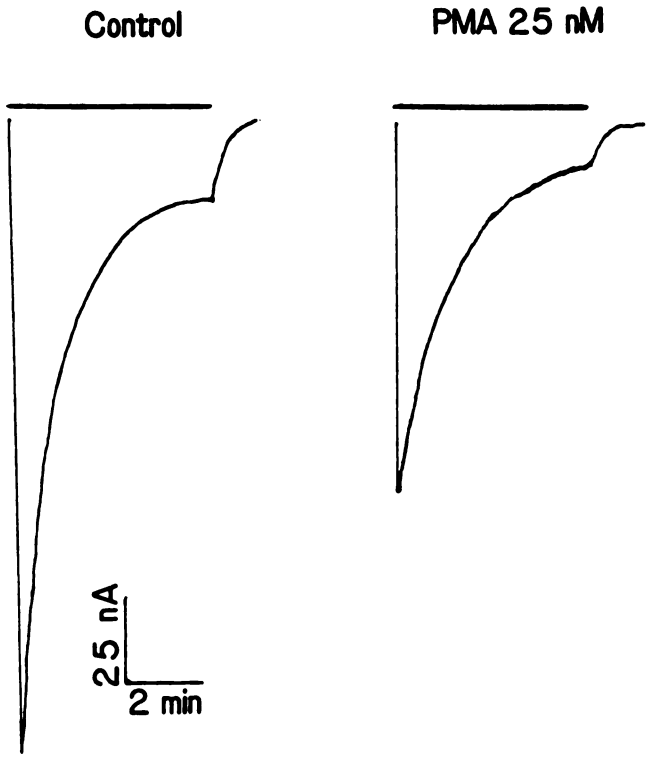

Fig. 1. Effects of PMA on GABA-gated chloride currents in an oocyte injected with brain mRNA. *Abscissa*, time; *ordinate*, current (nA). GABA (30 μM) was applied for 5 min, as indicated by the *horizontal bar at the top of each tracing*, and produced a rapid transient current as well as a sustained current. The *tracing on the left* was obtained prior to PMA treatment. The *tracing on the right* was obtained 10 min after application of PMA (25 nM).

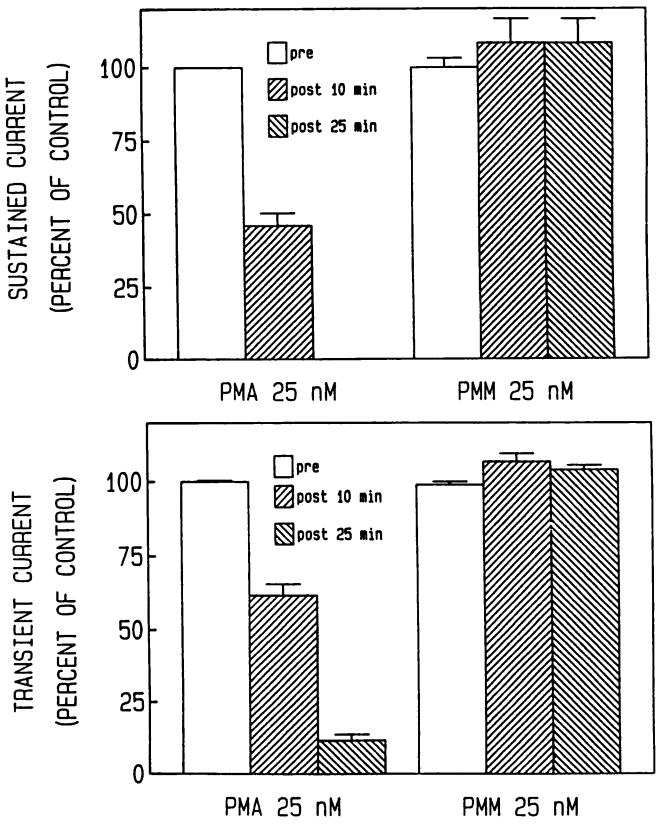

Fig. 2. Effects of PMA and PMM on the GABA-activated transient and sustained currents produced in oocytes expressing brain mRNA. *Ordinates*, currents as a percentage of the control GABA response. *Upper*, data for the sustained current; *lower*, data for the transient current. After determination of control GABA responses, PMA or PMM was applied (25 nM for 3 min), and GABA responses were tested again 10 and 25 min later. Values are mean ± standard error (n = 3–8 different oocytes). No bar is shown for the sustained current 25 min after PMA application because this current was not detectable.

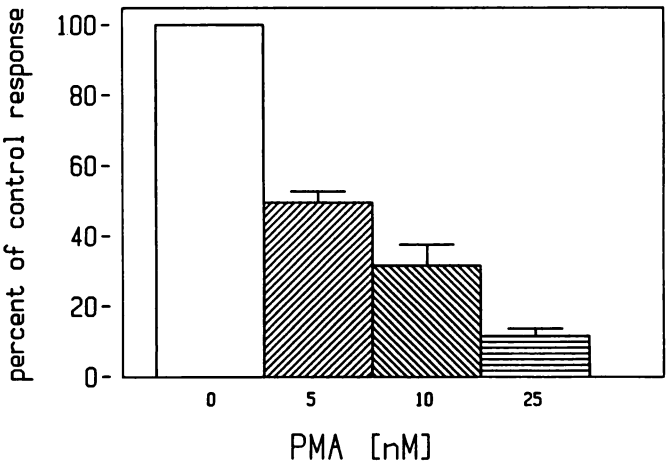

Fig. 3. PMA (5–25 nM) produces a concentration-dependent inhibition of GABA-gated chloride currents in oocytes expressing mouse brain mRNAs. Data are presented as percentage of the GABA (30 μM) control response (GABA responses before PMA treatment). The IC_{50} for this effect is approximately 5 nM (n = 3–5).

Intracellular injection of PKCI before PMA application significantly antagonized the effect of PMA.

Brain membrane $GABA_A$ receptor function. Muscimol-stimulated uptake of $^{36}Cl^-$ by cerebellar microsacs displayed an

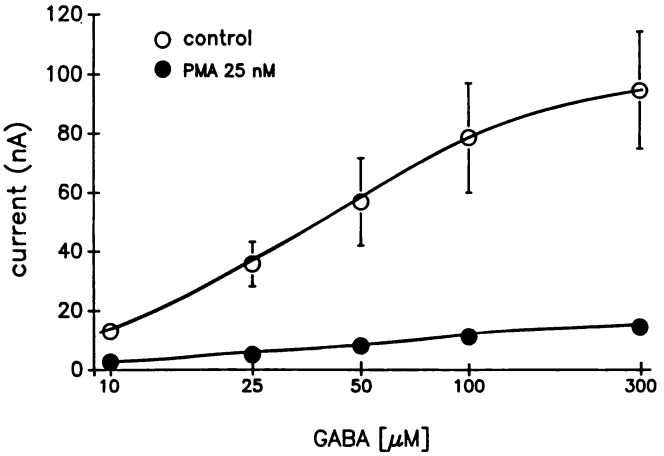

Fig. 4. PMA inhibits GABA-gated chloride currents over a wide range of GABA concentrations. *Abscissa*, GABA response, in current (nA). *Ordinate*, increasing concentrations of GABA. PMA (25 nM) produces a large decrease in the efficacy of the GABA response without altering GABA potency ($n = 5$). The EC_{50} concentrations of GABA in control and PMA-treated oocytes were approximately 45 and 30 μM, respectively.

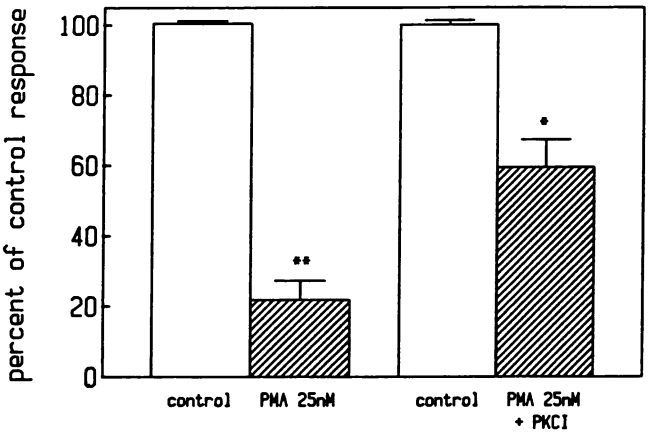

Fig. 5. PMA (25 nM) inhibits GABA-gated chloride currents in oocytes expressing human $\alpha_1\beta_1\gamma_{2L}$ cDNAs. *Abcissa*, percentage of control GABA (30 μM) responses before PMA exposure. *, Significant inhibition of GABA control current, $p < 0.0001$ ($n = 6$ or 7). PKCI partially antagonizes the inhibitory effects of PMA. PMA plus PKCI is significantly different from PMA alone (**, $p < 0.001$, one-tailed, independent t test).

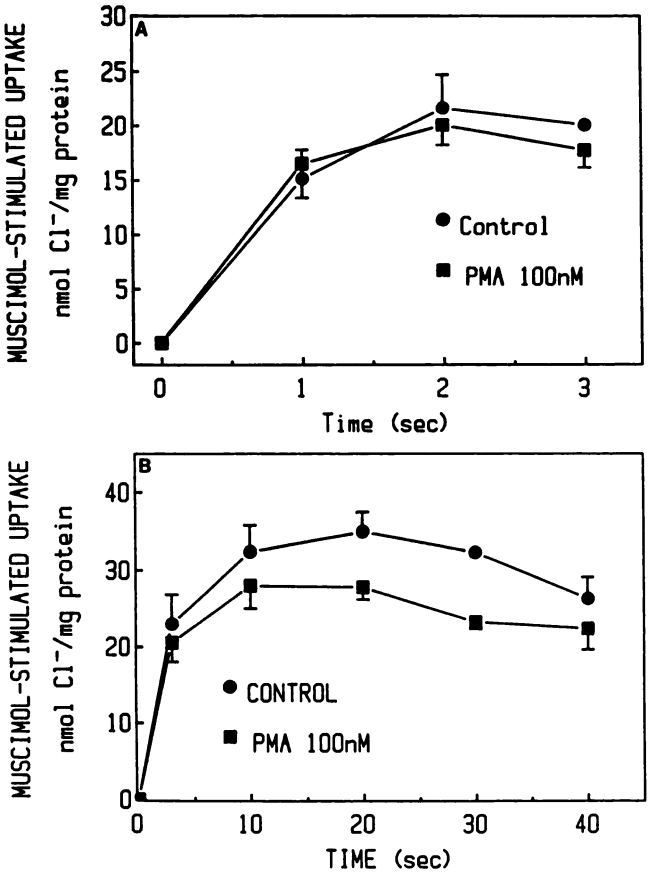

Fig. 6. PMA (100 nM) inhibits the slow phase but not the fast phase of muscimol-stimulated $^{36}Cl^-$ uptake. *Abscissa*, uptake time; *ordinate*, muscimol-stimulated uptake of $^{36}Cl^-$ for each time point. Values are mean ± standard error ($n = 3$). The time course of uptake was clearly biphasic, with much of the rapid phase completed at 1 sec (A). The average rate of uptake was about 15 nmol of Cl^-/mg of protein/sec for the first second and declined to only 4 nmol of Cl^-/mg of protein/sec between 1 and 2 sec. PMA (100 nM) did not affect the rapid phase of muscimol-activated $^{36}Cl^-$ uptake, using flux times of 1, 2, and 3 sec. For the slow uptake phase, the average uptake rate from 3 to 20 sec was 0.8 nmol of $^{36}Cl^-$/mg of protein/sec (B). At the 20- and 30-sec time points, there was a significant effect of PMA [$F = 11(1,20)$, $p < 0.004$]. For several values, the error bars are smaller than the points.

initial rapid phase that was complete by 3 sec and a slower phase that persisted for 20–30 sec (Fig. 6A), in agreement with previous studies (12, 19). PMA (100 nM) did not alter the muscimol-dependent uptake measured with a 1-, 2-, or 3-sec flux period (rapid phase of uptake) (Fig. 6A). For the 3-sec uptake period, a total of 37 membrane preparations were treated with PMA and the uptake was 101 ± 2% of control. In contrast to these results, PMA inhibited muscimol-stimulated chloride uptake during the 30-sec flux period; the muscimol-dependent uptake was 28 ± 3 in control and 18 ± 2 nmol of $^{36}Cl^-$/mg of protein/30 sec in PMA-treated microsacs (mean ± standard error, $n = 23$). The uptake of $^{36}Cl^-$ in the absence of muscimol (basal uptake) was not significantly affected by PMA (112 ± 6 and 110 ± 5 nmol of Cl^-/mg of protein/30 sec for control and PMA-treated microsacs, respectively) Thus, 100 nM PMA inhibited the muscimol-dependent uptake by 36% ($p < 0.01$) but produced no effect on the basal uptake. Similar to cerebellar microsacs, in cortical preparations PMA (30 nM) inhibited the slow phase of muscimol-stimulated $^{36}Cl^-$ uptake by 19 ± 4%, while not altering the fast phase of uptake. Because PMA was more effective at inhibiting chloride flux by cerebellar than cortical microsacs, the cerebellar preparation was used for all subsequent experiments.

The inhibitory action of PMA in the microsacs was concentration dependent, with a maximal effect occurring at 30 nM and a half-maximal effect at approximately 3 nM, which agrees closely with the results from the oocytes (Figs. 3 and 7). The maximal inhibition was about 40%. PMM, an analog of PMA that does not activate PKC, produced no change in $^{36}Cl^-$ uptake at a concentration of 10 nM and only slight inhibition at concentrations of 100–1000 nM (Fig. 7). Similar to PMA, the PKC activator Oct. V (60 and 300 nM) also reduced muscimol-dependent uptake (Table 1). This inhibition was observed at Oct. V concentrations (60–300 nM) that inhibit [^{3}H]PMA binding (IC_{50} of 29 nM) (20).

Protein phosphorylation can be prevented in the microsac preparation by lysing and resealing the vesicles in the absence of Mg^{2+} and presence of EDTA (to deplete Mg-ATP) (18). Under these conditions, PMA (10–100 nM) failed to inhibit

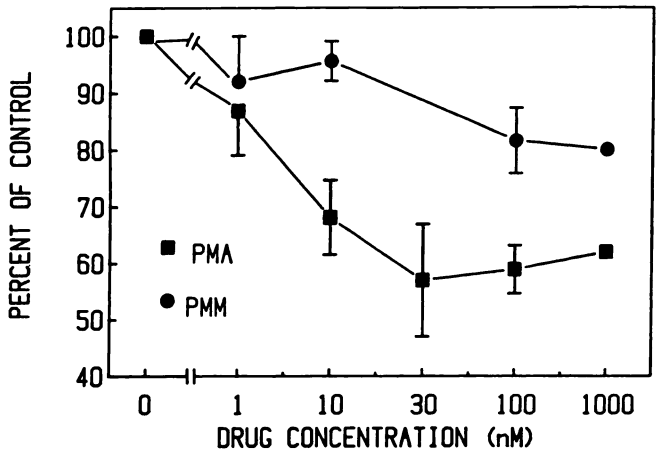

Fig. 7. PMA is a more effective inhibitor of muscimol-stimulated $^{36}Cl^-$ uptake than is PMM. *Abscissa*, drug concentrations, in nM; *ordinate*, muscimol-stimulated uptake of $^{36}Cl^-$, as a percentage of the control (no phorbol) uptake. Uptake time was 30 sec. Values are mean ± standard error (n = 3–8). For several values, the error bars are smaller than the points.

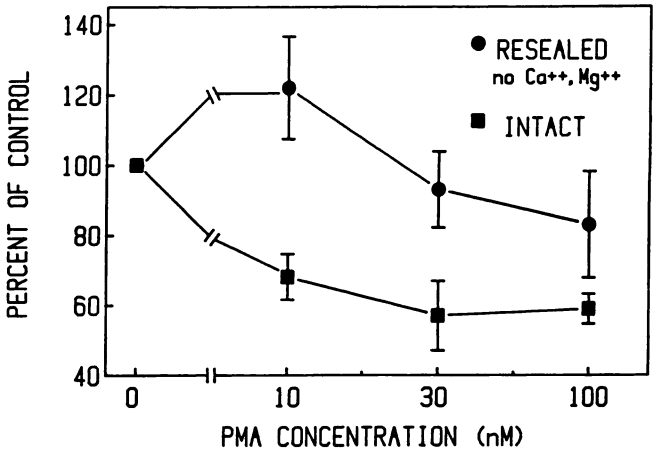

Fig. 8. Effects of PMA are prevented when phosphorylation is prevented by lysing and resealing of the microsacs (see Materials and Methods). *Abscissa*, concentrations of PMA, in nM; *ordinate*, muscimol-stimulated uptake of $^{36}Cl^-$, as a percentage of the control (no PMA) uptake. Uptake time was 30 sec. Values are mean ± standard error (n = 9). PMA was significantly more effective in intact, compared with lysed/resealed, microsacs [F = 31(1,48), $p < 0.001$].

TABLE 1

Effect of phosphorylation modulators on muscimol-stimulated $^{36}Cl^-$ uptake

Values represent percentage of control (no drug) muscimol-dependent uptake, measured with a 30-sec flux period, and are given as mean ± standard error.

Treatment	$^{36}Cl^-$ uptake	n
	% of control	
PKC activators		
PMA, 30 nM	71 ± 5[a]	12
Oct V, 60 nM	86 ± 1[a]	4
300 nM	68 ± 6[b]	7
Phosphatase inhibitors		
Okadaic acid, 100 nM	67 ± 15[c]	7
Calyculin A, 1 nM	52 ± 14[c]	4
10 nM	41 ± 6[b]	3
Kinase inhibitors		
Sphingosine, 3 μM	92 ± 19	4
Staurosporine , 10 nM	92 ± 5	27
Drug combinations		
PMA, 30 nM, + Okadaic acid, 100 nM	76 ± 11[a]	7
PMA, 30 nM, + sphingosine, 3 μM	105 ± 14	6

[a] Significantly different from no-drug control, $p < 0.01$.
[b] Significantly different from no-drug control, $p < 0.001$.
[c] Significantly different from no-drug control, $p < 0.05$.

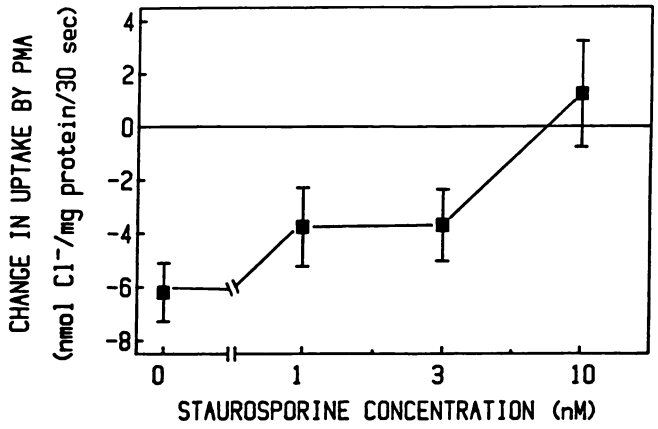

Fig. 9. The protein kinase inhibitor staurosporine antagonizes effects of PMA on muscimol-stimulated $^{36}Cl^-$ uptake. *Abscissa*, concentration of staurosporine, in nM; *ordinate*, change in muscimol-stimulated uptake of $^{36}Cl^-$ produced by PMA (30 nM). Uptake time was 30 sec. Values are mean ± standard error (n = 5). There is a significant effect of staurosporine [F = 3.4(4,23), $p < 0.04$].

muscimol-stimulated $^{36}Cl^-$ uptake significantly (Fig. 8), providing evidence that the PMA effect may be dependent on phosphorylation. Consistent with this finding, the effects of PMA were reduced by the protein kinase inhibitor staurosporine in a concentration-dependent manner, with 10 nM preventing the action of PMA (Fig. 9). The concentration of staurosporine required to inhibit the PMA effect is similar to the potency (IC_{50} = 2.7 nM) with which staurosporine inhibits PKC (21). Staurosporine (10 nM) did not alter the muscimol-independent (basal) uptake of $^{36}Cl^-$ (97% of control, n = 21). Staurosporine itself did not affect the slow phase of muscimol-dependent $^{36}Cl^-$ uptake when tested at concentrations of 1–10 nM; with 10 nM staurosporine the uptake was 92% of control (Table 1). The fast phase of $^{36}Cl^-$ uptake was not affected by 10 nM staurosporine (data not shown). Sphingosine, another kinase inhibitor, also antagonized the PMA effect (Table 1).

The protein phosphatase inhibitor okadaic acid (100 nM) mimicked the action of PMA, by inhibiting the slow phase of muscimol-stimulated $^{36}Cl^-$ flux (Table 1) without altering the fast phase (data not shown). The inhibition produced by okadaic acid was not additive with that of PMA, suggesting a similar mechanism of action for the two compounds. Another protein phosphatase inhibitor, calyculin A (1 and 10 nM), also potently inhibited the slow phase of muscimol-stimulated $^{36}Cl^-$ uptake (Table 1). The concentrations of these compounds required to decrease muscimol-dependent $^{36}Cl^-$ uptake are consistent with the potency of these drugs as phosphatase inhibitors. For example, okadaic acid inhibits rabbit skeletal muscle phosphatase (protein phosphatase 1) with an IC_{50} of 60 nM, and calyculin A inhibits the same enzyme with an IC_{50} of 2 nM (22).

Effects of PMA on the fast and slow phase of muscimol-stimulated $^{36}Cl^-$ flux were also studied in muscimol preincubation experiments (12). With this approach, receptor desensitization is produced by exposure of the membranes to muscimol for 3–30 sec, and $^{36}Cl^-$ uptake is then measured for a 3-

sec period, using a maximally effective concentration of muscimol (100 μM). This allows the extent of receptor desensitization to be calculated (12). Preincubation with muscimol for 3 sec produced about 50% desensitization, and preincubation for 30 sec produced about 70% desensitization (Fig. 10). This is consistent with the time course of $^{36}Cl^-$ uptake shown in Fig. 6, which demonstrates a rapid phase of influx that desensitizes before 3 sec, followed by a slower phase of desensitization. Several distinct phases of GABA-activated $^{36}Cl^-$ flux have been previously demonstrated by the detailed kinetic analysis of Cash and Subbarao (12). It should be noted that our preincubation experiments rule out the possibility that the slowing of $^{36}Cl^-$ uptake observed in the time-course experiment is due to the vesicles rapidly filling (equilibrating) with the external $^{36}Cl^-$ solution, rather than the receptors desensitizing. The predesensitization measurements were all obtained with a 3-sec uptake period, yet they demonstrate the same transient and sustained phases of uptake obtained in the time-course experiments [which is consistent with the results of Cash and Subbarao (12)]. The predesensitization experiments also demonstrate that PMA did not alter the fast phase of desensitization but enhanced the extent of the slow phase desensitization (Fig. 10). At the 30-sec preincubation time, only 20.4 ± 5.3% of the control (no preincubation) uptake remained (Fig. 10). In the presence of PMA, this value was reduced to 10.7 ± 5.3%. The control versus PMA values were significantly different from each other (one-tailed, paired t test, $p < 0.005$). Thus, PMA inhibited this predesensitized response by approximately 60%. These predesensitization experiments confirm the time-course results shown (Fig. 6), which indicate that PMA did not alter the initial phase of muscimol action but inhibited the sustained or desensitized phase. PMA did not produce a statistically significant change in the rate of desensitization (slope of lines in Fig. 10).

A mechanism by which phorbol esters could inhibit muscimol-stimulated $^{36}Cl^-$ uptake is by reducing the uptake of GABA by glial cells, because glial uptake of GABA is reported to be modulated by PKC (23). Such a reduction of uptake would cause an accumulation of the endogenous GABA normally present in the microsac preparation. This would lead to the desensitization of the GABA$_A$ receptor and, therefore, less muscimol-stimulated $^{36}Cl^-$ uptake (although this mechanism would be expected to alter both the rapid and slow phases of uptake). To test this possibility, we measured the uptake of [^{3}H]GABA by microsacs in the presence and absence of PMA. We found that 100 nM PMA did not alter the uptake of GABA by cerebellar microsacs (data not shown), ruling out GABA uptake as a mechanism of action of PMA in our system.

Discussion

In this study, we provide the first comparison of effects of activators/inhibitors of PKC on the function of GABA$_A$ receptors expressed in oocytes with those of brain membrane vesicles. We observed that PMA, but not PMM, inhibited GABA-gated chloride currents in oocytes expressing mouse brain mRNA. The PMA effect was concentration dependent, with an EC_{50} of approximately 5 nM, and GABA concentration-response experiments showed that the effect of PMA was due to a large decrease in the efficacy of GABA, with little change in the GABA EC_{50}. Additionally, PMA was observed to inhibit recombinant receptors expressed from human $\alpha_1\beta_1\gamma_{2L}$ subunits.

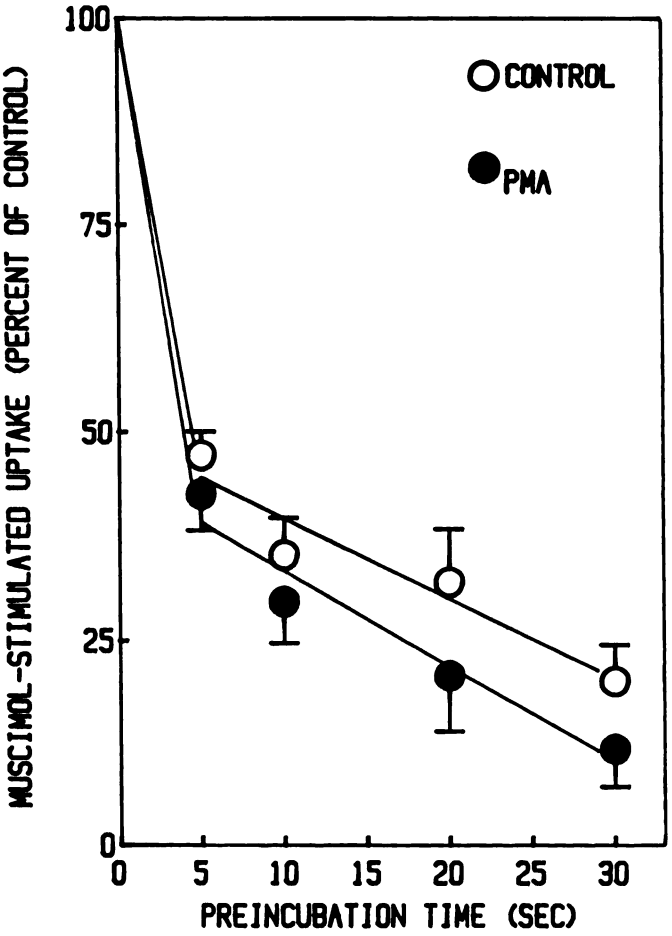

Fig. 10. Effects of PMA (100 nM) on desensitization of muscimol-stimulated $^{36}Cl^-$ uptake. *Abscissa*, desensitization (muscimol preincubation) time, in sec; *ordinate*, muscimol-stimulated uptake of $^{36}Cl^-$, as a percentage of the uptake obtained in the absence of desensitization (preincubation without muscimol). Control uptake (no desensitization) was 28 ± 1 nmol of chloride/mg of protein/3 sec without PMA and 30 ± 2 nmol of chloride/mg of protein/3 sec with PMA. Values for the slope are −0.96 ± 0.1%/sec for control and −1.1 ± 0.1%/sec for PMA (t = 1.7, $p > 0.1$); values for the intercept at 30% of control are 21 ± 5 sec for control and 13 ± 4 sec for PMA (t = 3.7, $p < 0.01$). *Lines* were fit by linear regression. Values are mean ± standard error (n = 6).

In oocytes coexpressing these subunits, PKCI significantly antagonized the PMA effect, providing evidence that the action of PMA is due to activation of PKC.

Our results confirm and extend previous studies of effects of phorbol esters on GABA$_A$ receptors expressed in *Xenopus* oocytes (5–7). Previous studies have shown that nanomolar concentrations of phorbol esters inhibit GABA-gated chloride currents by 60–70% in oocytes expressing chick (5) and rat (6) brain mRNAs and that PMA modulates a variety of recombinant receptor subtypes (7). In contrast to the results obtained in oocytes, two studies in neurons have failed to demonstrate phorbol effects on GABA$_A$ receptors (8, 9).

To address the question of whether PMA inhibits the function of GABA$_A$ receptors in their native membranes, we performed muscimol-stimulated $^{36}Cl^-$ uptake studies on membrane vesicles prepared from mouse cerebellum or cortex. Our results show that activators of PKC, as well as inhibitors of protein phosphatases, reduce muscimol-stimulated $^{36}Cl^-$ uptake by brain membrane vesicles. The action of PMA did not occur

under nonphosphorylating conditions, was blocked by the kinase inhibitors staurosporine and sphingosine, and was not mimicked by PMM (a phorbol ester that does not activate PKC) but was mimicked by the PKC activator Oct. V. Taken together, these findings provide evidence that phosphorylation by PKC can inhibit the function of $GABA_A$ receptors *in situ*.

Interestingly, the modulation of $GABA_A$ receptors in the microsacs was selective for the slow phase of $^{36}Cl^-$ uptake. This was shown by time-course experiments, in which PMA inhibited muscimol action at an uptake time of 30 sec but not at times of 3 sec or less, and by experiments showing that predesensitization of $GABA_A$ receptors was necessary for the inhibitory action of PMA. It was possible that the ability of PMA to inhibit $^{36}Cl^-$ uptake at 30 sec and not at 3 sec could be due to an enhancement of $^{36}Cl^-$ efflux by PMA, which would produce a greater reduction of $^{36}Cl^-$ accumulation at the longer uptake times. If this were the case, PMA would be expected to inhibit not only muscimol-dependent $^{36}Cl^-$ uptake but also muscimol-independent (basal) $^{36}Cl^-$ uptake at the 30-sec uptake time. This, however, did not occur. Furthermore, results from the predesensitization experiments also rule out an effect of PMA on $^{36}Cl^-$ efflux. At the 3-sec $^{36}Cl^-$ uptake time used in the predesensitization experiments, PMA was able to inhibit $GABA_A$ receptor function if, and only if, a large fraction of the receptors were desensitized.

An important question is why effects of PKC activation are observed, in the microsac preparation, only after receptor desensitization. It is possible that PKC may selectively modulate only a subtype of $GABA_A$ receptor (perhaps a nondesensitizing or slowly desensitizing receptor). If PMA did preferentially modulate a receptor subtype, this could account for the difference in PMA modulation between cerebellar and cortical microsacs, because different subunits may be expressed in these regions. However, a differential effect of PKC on receptor subtypes remains to be demonstrated, because a recent study of oocytes expressing recombinant $GABA_A$ receptors shows that a variety of recombinant receptors are affected by PMA (7).

Another possibility is that PKC may alter the desensitization kinetics of $GABA_A$ receptors. In this regard, PKC phosphorylation increases the rate of the fast phase of desensitization of the nicotinic acetylcholine receptor (24), a ligand-gated ion channel homologous to the $GABA_A$ receptor (25). In our predesensitization experiments, however, PMA did not significantly increase the $GABA_A$ receptor desensitization rate for the slow phase of uptake. Although not measured, it is also unlikely that PMA would alter the fast phase desensitization rate, because PMA did not significantly alter muscimol-stimulated $^{36}Cl^-$ uptake at 1–3 sec.

The desensitization kinetics of the $GABA_A$ receptor are quite complex, with transitions occurring between three open and three closed states after prolonged application of GABA (13). Our working hypothesis is that a particular state of the receptor may be selectively affected by PKC (perhaps a nondesensitizing state of the receptor) or that phosphorylation by PKC favors a particular state. Detailed single-channel studies will be required to test this hypothesis.

Another important question is why activation of PKC has not been observed to affect $GABA_A$ receptor function in neurons. It is possible that the neurons tested with PMA thus far did not possess $GABA_A$ receptors that are PKC substrates. This, however, appears unlikely, because, as mentioned above, a variety of recombinant $GABA_A$ receptors respond to PMA (7). Alternatively, the techniques used to study these neurons may not have been optimal for observing the nondesensitizing state or form of the receptor. For example, the nondesensitizing component of $GABA_A$ has been described in frog sensory neurons (10), but PMA was not tested on these neurons.

Although PMA inhibited GABA responses in both microsacs and oocytes, the PMA inhibition was greater in oocytes. This is not an unexpected finding, because the oocyte is an intact cell and would possess physiological levels of PKC/phosphorylation factors (such as Mg-ATP). In contrast to the oocytes, it is likely that the microsacs lose PKC/phosphorylation factors during the isolation/homogenization procedure and, therefore, would display a less robust response to PMA. Another difference between the two preparations is that ion transport studies measure cumulative $^{36}Cl^-$ uptake, whereas electrophysiological recordings measure instantaneous current. In this regard, the cumulative measurement of $^{36}Cl^-$ uptake could attentuate the PMA effect in microsacs, because the effect occurs only at later uptake times. A third difference between the two preparations is that the $GABA_A$ receptor is in its native membrane in the microsacs. It is unknown whether this could affect PKC modulation of the receptor. Yet another factor that could potentially contribute to differences in the PMA effect is subunit composition of the receptor. For example, the microsacs were prepared from cerebellum, whereas the oocytes were injected with whole-brain mRNA. Thus, the microsacs would only possess receptors found in the cerebellum, whereas the oocytes may possess all possible receptor subtypes, including receptors composed of subunits that would not normally associate *in vivo*. Lastly, the method of applying GABA in the two preparations is different. In the oocyte, GABA application is rather slow, because of the large size of the cell and the relatively slow perfusion flow rate used in our studies. In contrast, the addition of muscimol to the microsacs occurs in rapidly mixing membranes. This difference between application methods would alter the kinetic properties of receptor activation and perhaps influence the PMA effect.

Activity of neuronal ion channels other than the $GABA_A$ receptor depends critically on protein phosphorylation, particularly by the PKC family (24, 26). Modulation of rapidly acting ion channels by PKC provides a mechanism for feedback from slower intracellular signaling systems, such as neuromodulator receptors coupled through phospholipase C. Such interactions have been proposed as an underlying mechanism for learning and memory, long term potentiation, and other neuroadaptive phenomena (27). Because of the high activity of PKC in brain and its prevalence as a neuronal regulator, it is likely that PKC may regulate $GABA_A$ receptor function *in vivo*. For example, we found that both baclofen, acting through $GABA_B$ receptors, and adenosine, acting through A_1 receptors, selectively inhibit the slow phase of $GABA_A$ receptor action. This inhibition is prevented by staurosporine (19),[2] suggesting that PKC signaling provides a cross-talk pathway between some types of GTP-binding protein-linked receptors and $GABA_A$ receptors.

What might be the physiological importance of inactivating a nondesensitizing state of the $GABA_A$ receptor? Receptor desensitization appears to be an important mechanism for terminating synaptic actions of GABA during normal synaptic

[2] L. D. Hahner and R. A. Harris, unpublished observations.

transmission (28) and may be critical during brief flurries of GABA release that could occur normally or during seizures (29). The PKC signaling system may provide a mechanism for terminating the action of receptors not completely desensitized by GABA. In addition, the apparent high sensitivity of the nondesensitizing response to GABA agonists (19) suggests that it could be activated by residual synaptic GABA and provide a tonic inhibition that would be reduced by activation of PKC. Resolution of the role of PKC in modulating GABAergic neurotransmission will require the identification of the PKC substrate(s) responsible for inhibiting GABA$_A$ receptor function and detailed kinetic analysis of GABA$_A$ receptors after PKC phosphorylation.

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