

Regulation of Recombinant γ -Aminobutyric Acid (GABA)_A and GABA_C Receptors by Protein Kinase C

NATALIA FILIPPOVA, ANNA SEDELNIKOVA, YINONG ZONG, HENRY FORTINBERRY, and DAVID S. WEISS

Department of Neurobiology, University of Alabama School of Medicine, Birmingham, Alabama

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ABSTRACT

Activation of protein kinase C (PKC) by phorbol 12-myristate 13-acetate induced a continuous decrease in the γ -aminobutyric acid (GABA)-activated current amplitude from recombinant GABA receptors (formed by $\rho 1$ or $\alpha\beta\gamma$ subunits) expressed in *Xenopus* oocytes. This decline was due to internalization of receptors from the plasma membrane as confirmed by a decrease in surface fluorescence with green fluorescence protein-tagged receptors as well as a concomitant decrease in surface [³H]GABA binding. PMA specifically caused internalization of GABA receptors, but not neuronal acetylcholine receptors (α_7 or $\alpha_4\beta_2$), indicating the internalization was not a general, non-specific phenomenon. Mutation of $\rho 1$ PKC phosphorylation

sites, identified by in vitro phosphorylation, did not prevent GABA receptor internalization, nor did coexpression of the $\rho 1$ M3-M4 intracellular loop along with $\rho 1$ GABA receptors. It is likely that PKC-mediated phosphorylation of other proteins, rather than $\rho 1$ itself, was required for the internalization. Both $\rho 1$ and $\alpha\beta\gamma$ receptors did not degrade after phorbol 12-myristate 13-acetate-induced internalization, but returned to the membrane surface within 24 h. These data suggest internalized receptors can exist in an intracellular compartment that can be delivered back to the plasma membrane. Thus, by regulating GABA receptor surface expression, PKC may play a key role in the regulation of GABA-mediated inhibition.

γ -Aminobutyric acid (GABA)-activated chloride channels represent a pharmacologically diverse class of receptors that mediate inhibitory synaptic activity in the central nervous system (CNS). At least two classes of fast inhibitory GABA receptors exist in the CNS: GABA_A receptors composed of α , β , γ , δ , ϵ , π , and θ subunits (Schofield et al., 1987; Lolait et al., 1989; Pritchett et al., 1989; Shivers et al., 1989; Ymer et al., 1989; Hedblom and Kirkness, 1997; Bonnert et al., 1999) and GABA_C receptors presumably composed of ρ subunits (Cutting et al., 1991). Both classes have a Cl[−]-selective pore, but in contrast to GABA_A, GABA_C receptors are insensitive to bicuculline, barbiturates and benzodiazepines (Sivilotti and Nistri, 1989; Polenzani et al., 1991; Johnston, 1996).

The distribution of GABA_A and GABA_C receptors in the CNS is well documented (Richards et al., 1987; Houser et al., 1988; Benke et al., 1991; Zimprich et al., 1991; Wisden et al., 1992; Enz et al., 1996; Koulen et al., 1998), although the mechanism responsible for delivery of receptors to and from the membrane surface is poorly understood. Although GABA_A and GABA_C receptors can be localized in the same neuron, they exist at separate synaptic sites (Koulen et al.,

1998). In addition to surface receptors, a significant cytoplasmic pool exists in cells (Mammen et al., 1997; Wan et al., 1997). Whether these receptors can be dynamically shuttled back and forth between an intracellular pool and the cell surface is not well established.

GABA_A receptors can be potentiated or inhibited by direct phosphorylation via protein kinase C (PKC), PKA, or tyrosine protein kinases (Moss et al., 1992; Moss et al., 1995; Lin et al., 1996; McDonald et al., 1998). The results from these studies, however, have been controversial. Recombinant $\alpha 1\beta 1\gamma 2$ receptors expressed in HEK293 cells and GABA_A (mRNA injection) or recombinant $\alpha 1\beta 1\gamma 2S$ receptors expressed in *Xenopus* oocytes demonstrated a reduction in the amplitude of the GABA-activated current in response to PKC activators (Sigel and Baur, 1988; Kellenberger et al., 1992; Krishek et al., 1994; Chapell et al., 1998). In contrast, introduction of catalytically active PKC into L929 fibroblasts expressing recombinant $\alpha 1\beta 1\gamma 2L$ receptors caused an enhancement of the GABA-mediated current (Lin et al., 1996). Previously, for GABA_C receptors, we provided evidence that internalization is a potential mechanism for phosphorylation-dependent inactivation of $\rho 1$ receptors transfected in HEK cells (Filippova et al., 1999). Both recombinant GABA_A and GABA_C receptors are inactivated in a PKC-dependent manner by phorbol 12-myristate 13-acetate (PMA) in *Xeno-*

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ABBREVIATIONS: GABA, γ -aminobutyric acid; CNS, central nervous system; PKC, protein kinase C; GST, glutathione S-transferase; PMA, phorbol 12-myristate 13-acetate; GFP, visualize green fluorescence protein; nACh, neuronal acetylcholine.

pus oocytes (Sigel and Baur, 1988; Chapell et al., 1998; Kusama et al., 1998).

To gain insight into the mechanism of PKC-dependent modulation of recombinant GABA_A and GABA_C receptors, we used: 1) the two-electrode voltage clamp to measure GABA-activated currents, 2) fluorescence microscopy to visualize green fluorescence protein (GFP)-tagged GABA receptor subunits on the membrane surface, and 3) [³H]GABA binding in intact oocytes to estimate changes in the number of receptors on the cell surface. We found that activation of PKC induced the internalization of GABA receptors and these internalized receptors could, in time, return back to the cell surface. Thus, by regulating GABA receptor surface expression, PKC may play a key role in the regulation of GABA-mediated inhibition.

Materials and Methods

Clones, Constructs, and In Vitro Transcription. The human $\rho 1$ and rat $\alpha 1$, $\beta 2$, $\gamma 2$ subunits were obtained via the polymerase chain reaction as previously described (Amin et al., 1994; Amin and Weiss, 1994), and subcloned into the p-ALTER-1 vector (Promega, Madison, WI). Henceforth, $\alpha 1$, $\beta 2$, and $\gamma 2$ will be referred to as α , β , and γ , respectively. Altered Sites (Promega) was used for the site directed mutagenesis that was verified by cDNA sequencing.

The $\beta 2$ subunit was subcloned into the pEGFP-N1 vector (Clontech Laboratories, Palo Alto, CA) between the *EcoRI* and *BamHI* sites, leaving GFP attached to the carboxy terminus. For in vitro transcription, the β -GFP construct was subcloned into the pcDNA3.1 vector (Promega). The 6HIS construct consisted of the wild-type $\rho 1$ subunit with 10 glycine residues followed by six histidine residues at the C terminus. For expression of the M3-M4 intracellular (IC) loop of the $\rho 1$ receptor, a cDNA fragment encoding residues 347 to 436 was subcloned into the pGEM vector (Promega). For the in vitro phosphorylation assays, the same segment of the M3-M4 IC loop was cloned into pGEX-2T (Pharmacia, Piscataway, NJ), producing a fusion protein with glutathione *S*-transferase (GST) at the amino terminus of the IC loop. The clones for the rat $\alpha 7$, $\alpha 4$, and $\beta 2$ subunits of the neuronal acetylcholine (nACh) receptor were kindly provided by Dr. M Quick (University of Alabama School of Medicine, Birmingham, AL) in the pCMV vector.

cDNAs of each clone were linearized and run-off capped cRNA was transcribed from the linearized cDNAs with standard in vitro transcription procedures. Integrity and yield of the cRNA were verified on a 1% agarose gel.

Xenopus Oocyte Expression. *X. laevis* (Xenopus I, Ann Arbor, MI) were anesthetized by MS-222, and oocytes were surgically removed and placed in a solution that consisted of 85.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 50 U/ml penicillin, and 50 μ g/ml streptomycin, pH 7.5. Oocytes were dispersed in this solution without Ca²⁺, but in the presence of 0.3% Collagenase A (Boehringer-Mannheim, Indianapolis, IN). After isolation, stage 6 oocytes were thoroughly rinsed and maintained at 18°C in the above-mentioned solution plus 1 mM Ca²⁺. Micropipettes for injecting cRNA were pulled on a Sutter P87 horizontal puller. cRNA was diluted 2- to 10-fold with pyrocarbonate-treated water. A total of 0.5 to 5 ng of cRNA was injected into each oocyte.

Voltage Clamp of Oocytes. Two-electrode voltage-clamp procedures were used for current recording 3 or 4 days after cRNA injection. Oocytes were placed on a 300- μ m nylon mesh suspended in a small volume chamber (<100 μ l). The oocyte was perfused continuously with a solution containing 92.5 mM NaCl, 2.5 mM KCl, 5 mM HEPES, 1 mM CaCl₂, 1 mM MgCl₂, and 0.5 mM Na₂PO₄, pH 7.5. The solution was switched to the test solution, which is identical with the perfusion solution plus drug (e.g., GABA, carbachol). In experiments with nACh receptors, atropine (0.5 μ M) was added to block musca-

rinic ACh receptors. All experiments were performed at room temperature.

Recording microelectrodes were pulled on a P87 Sutter horizontal puller and filled with 3 M KCl. The electrode resistances ranged from 1 to 3 M Ω . Standard two-electrode voltage-clamp techniques (GeneClamp 500; Axon Instruments, Foster City, CA) were used to record currents at a holding potential of -70 mV. The current signal was filtered at 10 Hz and recorded on paper with a Gould EasyGraf chart recorder (Gould Instrument Systems Inc., Valley View, OH). At the same time, on-line digitization of the signal at 20 Hz with 12-bit resolution was carried out by using the MacADIOS data acquisition board (GW Instruments, Somerville, MA), Igor software (Wavemetrics, Lake Oswego, OR) in conjunction with a set of macros to drive the GW board (provided by Bob Wytenbach, Cornell University, Ithaca, NY) in a Macintosh (Apple Computer, Cupertino, CA). Dose-response and dose-inhibition relationships were fit with the following Hill equation with a nonlinear least-squares method:

$$I = \frac{I_{\max}}{1 + (EC_{50}/[A])^n} \quad (1)$$

$$I = \frac{I_{\max}}{1 + ([A]/IC_{50})^n} \quad (2)$$

where I is the peak current response at a given concentration of agonist (A), I_{\max} is the maximum current response, EC_{50} is the concentration of the agonist with a half-maximal activation, IC_{50} is the concentration of the agonist with a half-maximal inhibition, and n is the Hill coefficient.

For the PMA experiments, oocytes were incubated in 100 nM PMA for 10 min. Calphostin C (1 μ M) was added in the perfusion solution or injected into the oocytes. The final dimethyl sulfoxide dilution for the oocyte injection was 1000-fold. The injection of oocytes with dimethyl sulfoxide diluted 1000-fold did not modify the GABA-activated currents. The degree of the PMA effect on GABA-activated currents varied between batches of oocytes. For example, the percentage of inactivation in the presence of 100 nM PMA varied between 54 and 98%.

Comparison of the Zn²⁺ sensitivity of the wild-type and 6HIS $\rho 1$ receptors was accomplished by coapplying different concentrations of Zn²⁺ along with 3 μ M GABA. Oocytes expressing the 6HIS construct were exposed to the exoprotease, carboxypeptidase A (Sigma Chemical Co., St. Louis, MO) for ~120 min then thoroughly rinsed before subsequent two-electrode voltage-clamp analysis. All results are presented as mean \pm S.E. Data were compared statistically by the Student's *t* test. Statistical significance was determined at the 5% level.

Fluorescence Analysis. The intensity of the fluorescence from $\alpha\beta$ -GFP γ receptors was analyzed with a Diaphot 300 fluorescence microscope (Nikon, Tokyo, Japan). The GABA-activated current from oocytes injected with $\alpha\beta$ -GFP γ cRNA was tested using two-electrode voltage clamp before examining the fluorescence.

Western Blot Analysis. Noninjected oocytes and oocytes injected with the cRNA coding for the $\rho 1$ IC loop (40 per each condition) were homogenized, centrifuged, and layered on a discontinuous 20% sucrose gradient in the presence of a protease inhibitor cocktail (Sigma Chemical Co.), 1 mM dithiothreitol, and 5 mM MgCl₂ in a Tris-buffered saline. After centrifugation, the top layer (cytosol) and pellet (membrane) fractions were collected. The proteins from the membrane fraction were solubilized by 60 mM urea. The membrane and cytosolic fractions were then boiled in SDS-polyacrylamide gel electrophoresis loading buffer and separated on a 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The immunoblotting procedure included a 2-h incubation with a mouse polyclonal antibody (1:500) raised against the M3-M4 IC loop of the $\rho 1$ receptor followed by a 1-h incubation in an alkaline phosphatase-conjugated

anti-IgG antibody diluted 1:1000. A 14-kDa band from the cytosol fraction was detected as the IC loop protein.

[³H] Binding Assay. To estimate [³H]GABA binding, we used the single-oocyte binding method as previously described (Chang and Weiss, 1999). Briefly, single oocytes injected with $\rho 1$ cRNA were held by suction at the end of a pipette tip. The oocyte was first incubated in [³H]GABA for 60 to 90 s (specific activity = 94 Ci/mmol; Amersham life Sciences, Arlington Heights, IL), then rinsed for 6 s in a 150 ml of ice-cold stirring bath to remove free [³H]GABA from the oocyte surface. Next, the oocyte was held in a 250- μ l incubation solution for 90 s to allow the bound [³H]GABA to dissociate from the receptor. The released counts per minute were then determined in a liquid scintillation counter.

In Vitro Phosphorylation. The GST-IC loop fusion protein was purified from the bacterial cell line Epicurian Coli BL21-Gold(DE3)pLysS (Stratagene, La Jolla, CA) with glutathione-Sepharose (Amersham, Uppsala, Sweden) under nondenaturing conditions. The in vitro phosphorylation assay was carried out with the fusion protein still bound to the Sepharose. The catalytic domain of protein kinase C (Calbiochem, San Diego, CA) was added to the beads in the presence of [γ -³²P]ATP for 25 min. The Sepharose was then rinsed, boiled, and ran on a denaturing 15% polyacrylamide gel and subjected to autoradiography.

Results

PMA-Induced Inactivation of Homomeric $\rho 1$ GABA_C and $\alpha\beta\gamma$ -GABA_A Receptors. The GABA-activated current from homomeric $\rho 1$ receptors expressed in *Xenopus* oocytes was stable during long-term recording (up to 2 h). The activator of PKC, PMA (100 nM; 10-min application) induced a continuous decrease in the amplitude of the GABA-activated current (normalized value was 0.32 ± 0.02 ; $n = 4$ after 55 min of recording) (Fig. 1, A and B). The inactive analog of PMA, α PMA (1 μ M; 10-min application), had no significant effect on the GABA-evoked current (1.03 ± 0.06 ; $n = 4$) after 30 min of recording (Fig. 1, A and B).

A similar experimental paradigm was used to study PKC activation on $\alpha\beta\gamma$ -GABA_A receptors. PMA (100 nM; 10-min treatment) induced a continuous inactivation of recombinant GABA_A receptors expressed in *Xenopus* oocytes (Fig. 2A). The time course of inactivation was similar to the PMA-induced inactivation of $\rho 1$ receptors. After 30 min from the end of PMA treatment, the amplitude of the GABA-activated current was 0.22 ± 0.06 ($n = 5$) from the initial value (Fig. 2C). α PMA (1 μ M; 10-min application) had no significant effect on the current amplitude (Fig. 2, B and C).

PKC Is Involved in PMA-Induced Inactivation of $\rho 1$ and $\alpha\beta\gamma$ Receptors. To verify the role of PKC in the PMA-induced inactivation of GABA receptors, the degree of inactivation was determined in the presence of the PKC inhibitor calphostin C (1 μ M), injected into the oocyte 1 h before PMA (100 nM) treatment (Fig. 3, A and B). Calphostin C, on its own, did not significantly modify the amplitude of the GABA-activated current (0.96 ± 0.06 ; $n = 5$; data not shown), although it decreased the degree of PMA-induced inactivation of $\rho 1$ and $\alpha\beta\gamma$ receptors (Fig. 3, B and C, respectively). It is possible that calphostin C slowed the rate of the PMA effect. Nevertheless, these data confirm that PKC is involved in the time-dependent inactivation.

PMA-Induced Inactivation Was Not Caused by Direct Phosphorylation of $\rho 1$ Receptor. We next set out to determine whether the PMA-induced inactivation was the result of a direct phosphorylation of the GABA receptor. The

$\rho 1$ subunit has three consensus PKC sites in the IC loop between the third and fourth transmembrane domains (S410, S419, and S426) (Cutting et al., 1991) and it has been demonstrated that mutation of these three sites does not prevent the PMA-induced inactivation (Kusama et al., 1998). However, it is not known which, if any of these three sites can be phosphorylated. In addition, other nonstandard PKC consensus sites may exist (Kennelly and Krebs, 1991). To identify potential PKC sites, we carried out an in vitro phosphorylation assay with a GST $\rho 1$ IC loop fusion protein. A summary autoradiogram is presented in Fig. 4A. Mutation of the serines at positions 419 and 426 to alanines eliminated a majority of the phosphorylation (compare lanes 1 and 2). Most of the phosphorylation occurred at residue 419 (data not shown). Mutation of the serine doublet at positions 422 and 423 to alanines eliminated the remainder of the phosphorylation (lane 3). At present, we do not know which of these two serines (422 or 423) is the actual site of phosphorylation (perhaps it is both), but nevertheless, mutation of these four serines produced an IC loop that was not phosphorylated in vitro.

We next tested if these four mutations altered the PMA-induced inactivation of the $\rho 1$ receptor. PMA (100 nM; 10 min) induced inactivation of the GABA-activated current of the $\rho 1$ mutant (0.29 ± 0.05 ; $n = 5$) after 50 min of recording following PMA treatment (Fig. 4B) similar to that observed for wild-type $\rho 1$ receptors (0.27 ± 0.11 ; $n = 3$).

For these studies, we have assumed the four transmembrane domain model of the GABA receptor (Schofield et al., 1987) and that only domains accessible from inside the cell

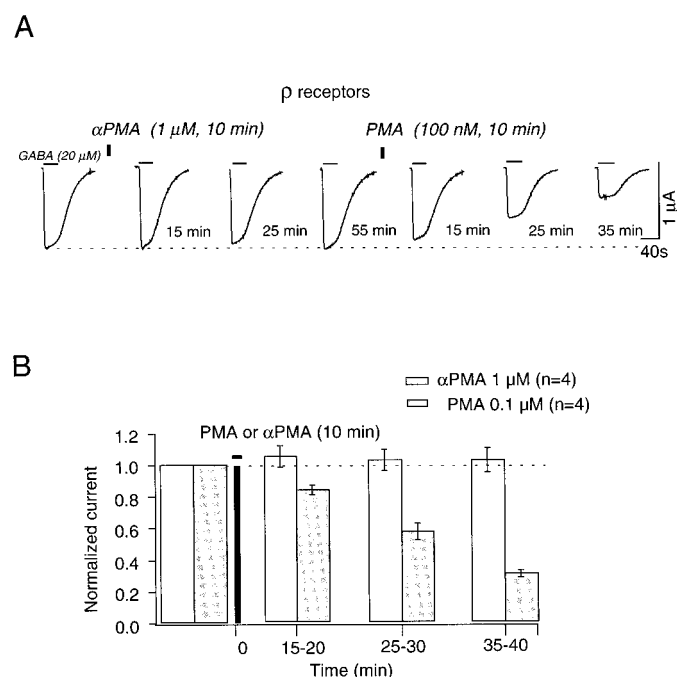


Fig. 1. PMA-induced inactivation of recombinant $\rho 1$ receptors. A, PMA (100 nM; 10 min) decreased the GABA-evoked current to 0.36 of its original value after 35 min of recording. The inactive analog of PMA, α PMA (1 μ M; 10 min), was ineffective during 55 min of recording. B, average of the normalized GABA-activated current amplitude after α PMA (\square) and PMA (\blacksquare) treatment during prolonged recording. Note the amplitude of the GABA-activated current after α PMA treatment did not significantly change (1.03 ± 0.06 ; $n = 4$, $P < .05$). After PMA treatment, however, the amplitude declined to 0.32 ± 0.02 ($n = 4$) of the initial value.

have the potential to be phosphorylated by intracellular kinases. Besides the M3-M4 linker, the M1-M2 linker is assumed to face the intracellular environment. In this 11-residue domain, there is one serine (S281) residue with nearby basic amino acids (R303 and R304). Although this is not a PKC consensus sequence, we mutated this serine to alanine and examined the PMA-induced inactivation. PMA decreased the maximum current amplitude of the S281A $\rho 1$ mutant to a similar fraction (0.13 ± 0.1 ; $n = 3$) as that of the wild-type $\rho 1$ receptor (0.10 ± 0.1 ; $n = 3$). These results, along with the data in Fig. 4, suggest the PMA-induced inactivation was not the result of direct phosphorylation of the $\rho 1$ receptor.

PMA Does Not Alter Sensitivity for GABA. One possible explanation for the PMA-induced inactivation could be a change in the sensitivity for GABA. To investigate this possibility, we recorded the GABA sensitivity of $\rho 1$ and $\alpha\beta\gamma$ receptors before and after inactivation (Fig. 5). In control conditions, $\rho 1$ and $\alpha\beta\gamma$ receptors have a GABA EC_{50} of 51 ± 5 and $0.87 \pm 0.05 \mu M$, respectively (Fig. 5A). Based on these mean EC_{50} values, we selected two GABA concentrations (dashed lines in Fig. 5A) and compared the ratio of the amplitudes at these concentrations before and after inactivation. The ratio between the current amplitude activated at 1 and 5 μM GABA for $\rho 1$ receptors was similar before and after PMA treatment (Fig. 5B and Fig. 5C, left), although the

current amplitude decreased to 0.23 ± 0.06 ($n = 5$) of its pretreatment value (Fig. 5C, right). The normalized traces in Fig. 5B are the scaled and superimposed responses to 1 μM GABA before and after PMA treatment. Note the responses are identical, indicating that there was no change in the activation and deactivation kinetics. The ratio between current amplitude activated at 20 and 50 μM GABA for $\alpha\beta\gamma$ receptors was similar before and after PMA treatment (Fig. 5D, left), although the current amplitude decreased to 0.04 ± 0.02 ($n = 4$) of its pretreatment value (Fig. 5D, right). Thus, PMA does not alter the GABA sensitivity of $\rho 1$ and $\alpha\beta\gamma$ receptors.

Internalization Is a Possible Mechanism of PMA-Induced Inactivation of $\rho 1$ and $\alpha\beta\gamma$ Receptors. A second possible mechanism of the PMA-induced inactivation of the GABA-activated current is removal of GABA receptors from the membrane surface. To estimate changes in the number of receptors on the membrane surface, we examined [3H]GABA binding to surface $\rho 1$ receptors and fluorescence of GFP-

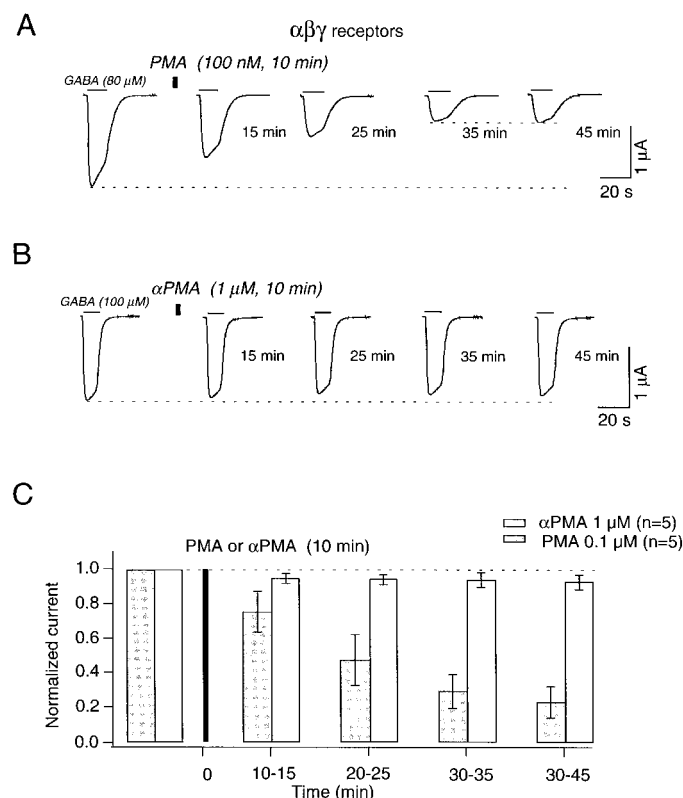


Fig. 2. PMA-induced inactivation of recombinant $\alpha\beta\gamma$ receptors. A, PMA (100 nM; 10 min) decreased the GABA-evoked current to 0.1 of the original value during 45 min of recording. B, inactive analog of PMA, α PMA (1 μM ; 10 min), was ineffective during 45 min of recording. C, average of the normalized GABA-activated current amplitude after PMA and α PMA treatment. Note the amplitude of the GABA-activated current declined to 0.22 ± 0.06 ($n = 5$) of the initial value during 45 min of recording after PMA treatment. With α PMA treatment, the amplitude was 0.95 ± 0.03 ($n = 5$) after 45 min of recording.

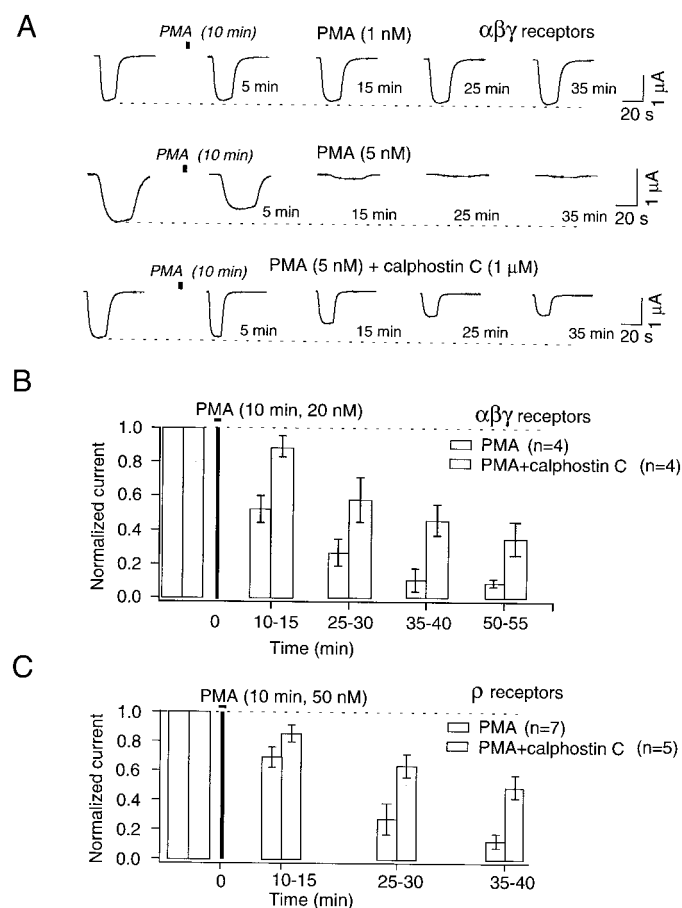


Fig. 3. PKC is involved in the modulation of GABA receptors. A, current traces evoked by GABA (20 μM) from $\alpha\beta\gamma$ receptors after 1 nM (top) and 5 nM (middle) PMA treatment. The lower traces are in the presence of 5 nM PMA and 1 μM calphostin C. B, plot of the amplitude of the GABA-activated current from $\alpha\beta\gamma$ -GABA receptors as a function of time in the presence of 20 nM PMA either with (■) or without (□) 1 μM calphostin C. After 55 min of recording, the normalized amplitudes were 0.12 ± 0.05 ($n = 4$) and 0.37 ± 0.11 ($n = 4$) in the absence and presence of calphostin C, respectively. C, plot of the amplitude of the GABA-activated current from $\rho 1$ GABA receptors as a function of time in the presence of 50 nM PMA either with (■) or without (□) 1 μM calphostin C. After 40 min of recording, the normalized amplitudes were 0.10 ± 0.05 ($n = 7$) and 0.44 ± 0.10 ($n = 5$) in the absence and presence of calphostin C, respectively.

tagged $\alpha\beta\gamma$ -GABA_A receptors during PMA-induced inactivation.

Measurement of the surface binding of [³H]GABA and functional inactivation in individual (the same) oocytes expressing $\rho 1$ receptors demonstrated that PMA decreased the current and binding with a similar time course and magnitude. Normalized to the initial value, the current amplitude and [³H]GABA binding were 0.18 ± 0.03 ($n = 5$) and 0.16 ± 0.03 ($n = 5$) after 30 min from the end of PMA treatment, respectively (Fig. 6). The current and surface binding decayed along a similar time course, with time constants of 71 and 50 min, respectively (single exponential component fitted to the mean of five oocytes). Previous studies have demon-

strated that the degree of surface binding in oocytes expressing $\rho 1$ receptors not treated with PMA was stable over extended time periods (Chang and Weiss, 1999). The concomitant reduction in surface binding and current amplitude suggests an internalization of $\rho 1$ receptors as a potential mechanism of PMA-induced inactivation.

To visualize changes in surface GABA_A receptors, GFP was attached to the C terminus of the β subunit. Figure 7A shows examples of GABA-activated currents formed by $\alpha\beta$ -GFP γ receptors. Except for a reduced expression level, the properties of the $\alpha\beta$ -GFP γ receptors were indistinguishable from the wild-type $\alpha\beta\gamma$ receptors. The top micrograph in Fig. 7B shows a patch of fluorescence on the surface of an oocyte expressing $\alpha\beta$ -GFP γ receptors. After PMA treatment (100

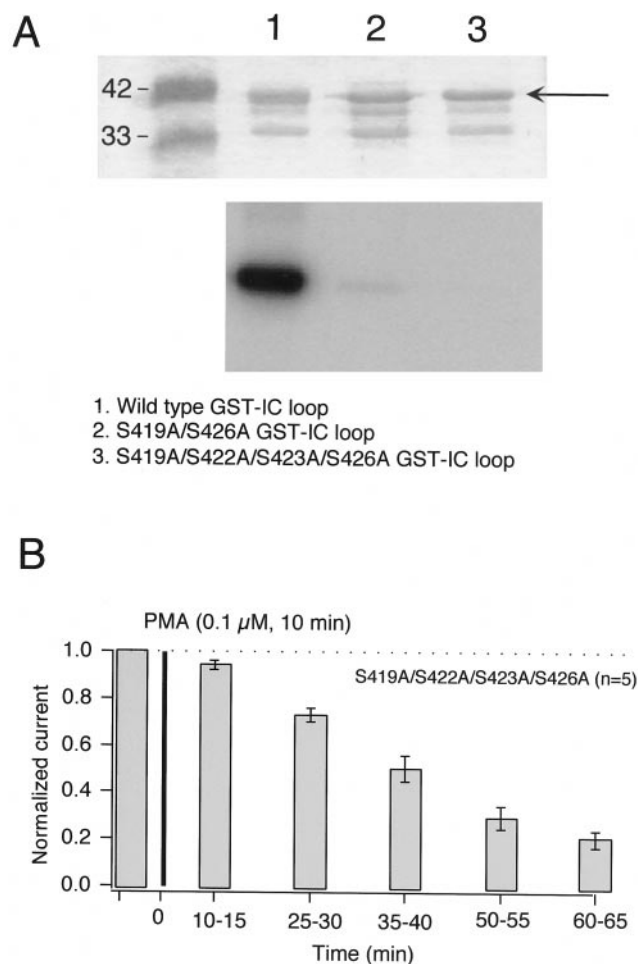


Fig. 4. Mutation of the PKC phosphorylation sites on the $\rho 1$ receptor did not prevent the inactivation of I_{GABA} . **A**, lower gel is an autoradiogram from an in vitro phosphorylation assay of the GST- $\rho 1$ IC loop fusion protein. Note that the S419A and S426A mutations eliminated a majority of the phosphorylation, whereas the S422SA and S423A mutations eliminated the remainder of the phosphorylation. The gel on top is the corresponding Coomassie stain of the autoradiogram, indicating similar amounts of protein were loaded in each lane. Alignment of the autoradiogram with the stained gel, as well as a comparison of the predicted and observed molecular weight of the GST-IC loop fusion protein, indicated that the upper band (indicated by the arrow) corresponded to the phosphorylated band in the autoradiogram. Additional nonphosphorylated bands could be identified as smaller fragments of the fusion protein without the PKC phosphorylation sites. **B**, average of the normalized GABA-activated current amplitude from S419A/S422A/S423A/S426A receptors after PMA (100 nM; 10 min) treatment. Note the decline in the current amplitude was similar to that of the wild-type $\rho 1$ receptor, indicating that these mutations did not alter the PMA-induced inactivation of I_{GABA} .

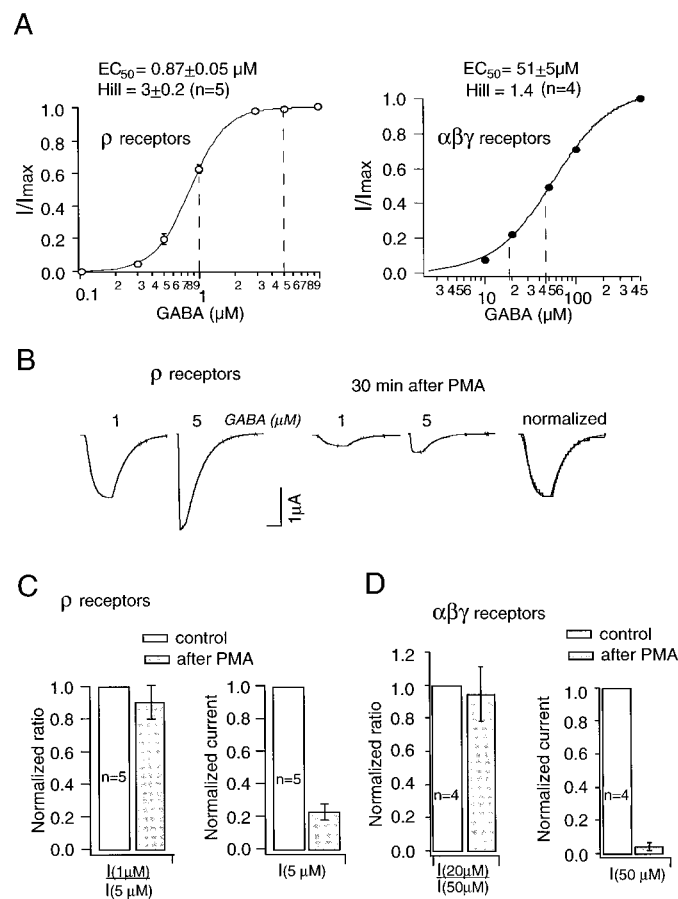


Fig. 5. PMA did not alter agonist sensitivity. **A**, dose-response relationship for $\rho 1$ (left) and $\alpha\beta\gamma$ (right) receptors. The continuous line is the best fit of the Hill equation (eq. 1) to the data. The vertical dashed lines are the responses at 1 and 5 μM GABA for $\rho 1$ and 20 and 50 μM GABA for $\alpha\beta\gamma$ used to assess changes in sensitivity. **B**, current traces from $\alpha\beta\gamma$ receptors at 1 and 5 μM GABA before (left) and after (middle) PMA exposure (100 nM; 10 min). On the right are the 1 μM GABA traces before and after PMA treatment superimposed. **C**, graph on the left plots the ratio of the 1 and 5 μM response before and after PMA treatment; the right graph plots the normalized amplitude of the current in response to 5 μM GABA before and after PMA treatment. Note the ratio did not significantly change after PMA-induced inactivation with a normalized value of 0.9 ± 0.11 ($n = 5$) after PMA exposure. The current amplitude, however, decreased to 0.23 ± 0.06 ($n = 5$) of the initial value. **D**, graph on the left plots the ratio of the 20 and 50 μM response before and after PMA treatment. The right graph plots the normalized amplitude of the current in response to 50 μM GABA before and after PMA treatment. Note the ratio did not significantly change after PMA-induced inactivation with a normalized value of 0.93 ± 0.15 ($n = 4$) after PMA exposure. The current amplitude, however, decreased to 0.04 ± 0.02 ($n = 4$) of its initial value.

nM; 10 min) the intensity of the fluorescence continuously decreased and disappeared from the surface. Similar results were observed in five oocytes. The data in Figs. 6 and 7 suggest that PMA induced the internalization of $\rho 1$ and $\alpha\beta\gamma$ receptors expressed in *Xenopus* oocytes.

Specificity of PMA-Induced Internalization of GABA Receptors. To examine the specificity of PMA-induced internalization, we coexpressed nACh receptors (α_7 or $\alpha_4\beta_2$) along with $\rho 1$ receptors. Carbachol (1 mM) or GABA (20 μ M) were applied at 15-min intervals to oocytes expressing both nACh and GABA receptors. PMA (100 nM; 10-min treat-

ment) produced inactivation of $\rho 1$ receptors (Fig. 8A) but did not significantly change the current amplitude from α_7 or $\alpha_4\beta_2$ receptors (Fig. 8, B and C). After 30 min of recording, the amplitude normalized to the initial value was 0.36 ± 0.05 ($n = 4$) for $\rho 1$ receptors, 1.24 ± 0.18 ($n = 4$) for α_7 receptors, and 1.01 ± 0.1 ($n = 5$) for $\alpha_4\beta_2$ receptors, respectively (Fig. 8D). Thus, PMA specifically internalizes GABA receptors but does not modulate the amplitude of neuronal nACh receptors. These data suggest that the internalization of GABA receptors was not simply due to a PKC-dependent nonspecific plasma membrane retrieval.

Coexpression or Coinjection of Large IC Loop. The IC loop of $\rho 1$ and $\gamma 2$ subunits has been shown to interact with the cytoskeleton (Hanley et al., 1999; Wang et al., 1999) and this interaction may be important in the PKC-dependent receptor internalization. To test this possibility, we coexpressed $\rho 1$ receptors with the M3-M4 IC loop in *Xenopus* oocytes. If the IC loop of the $\rho 1$ receptor interacts with a component of the cytoskeleton and this interaction was necessary for the PMA-induced inactivation, then overexpression of the IC loop should compete with the PMA-induced inactivation. Figure 9A shows a Western blot from oocytes confirming the expression of the IC loop in the cytosol. With PMA treatment (0.1 μ M; 10 min), the amplitude of the GABA-activated current normalized to the initial value was 0.22 ± 0.03 ($n = 5$) and 0.24 ± 0.08 ($n = 4$) after 50 min of

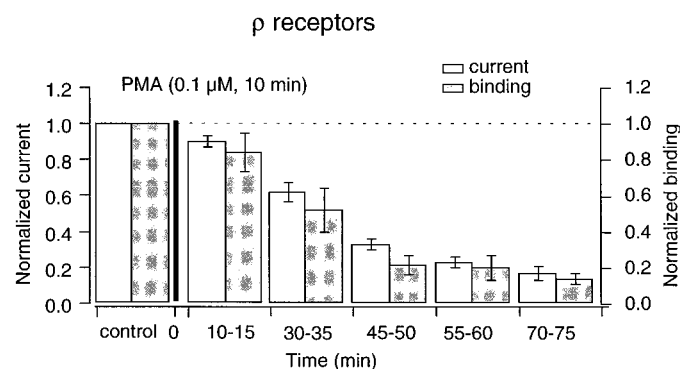


Fig. 6. PMA reduced surface binding of [3 H]GABA to $\rho 1$ receptors. Oocytes were exposed to PMA (100 nM; 10 min) and the current amplitude and surface binding of [3 H]GABA (Chang and Weiss, 1999) were examined concomitantly at 10-min intervals. The average of the normalized amplitude (□) and [3 H]GABA binding (■) are plotted. Note the similar time course of the decline.

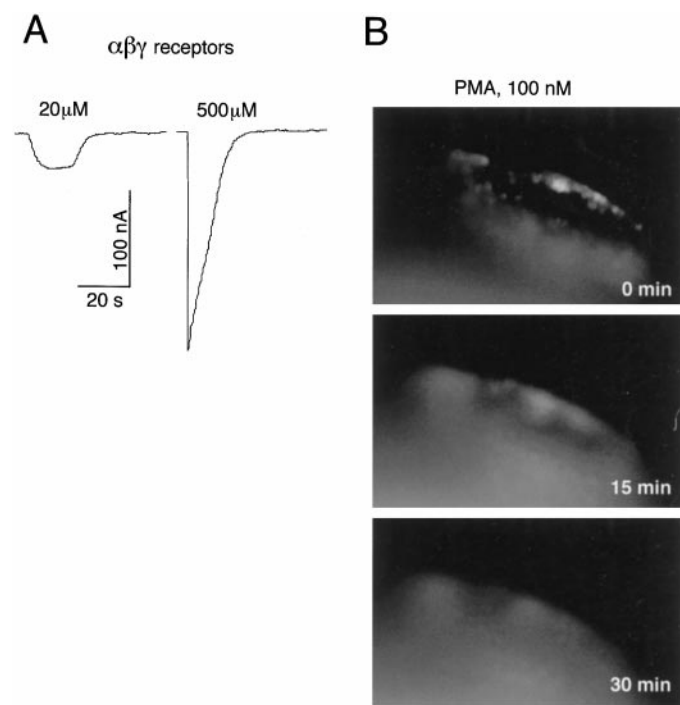


Fig. 7. PMA-induced internalization of $\alpha\beta$ -GFP- γ -receptors. A, GFP was fused to the C terminus of the β subunit and the α , β -GFP, and γ subunits were coexpressed in *Xenopus* oocytes. Representative currents in response to 20 and 500 μ M GABA from the $\alpha\beta$ -GFP- γ construct. Although the expression level was reduced somewhat in comparison to wild-type $\alpha\beta\gamma$ receptors, the activation features of the GFP-tagged receptors were similar to that of the wild type. B, surface fluorescence from an oocyte expressing $\alpha\beta$ GFP- γ receptors. Note the continuous decline of fluorescence after PMA treatment.

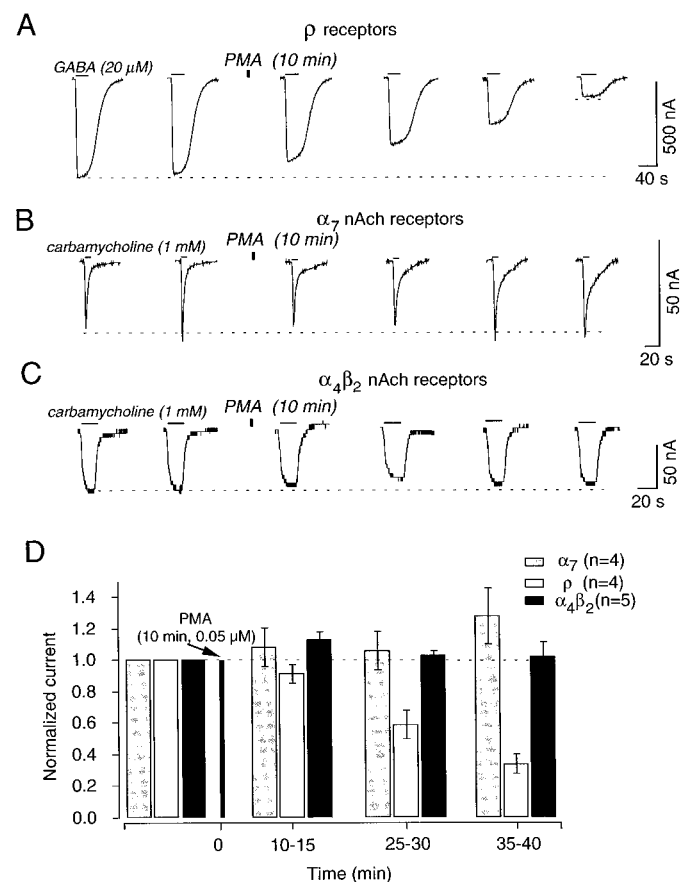


Fig. 8. PMA induced inactivation of $\rho 1$ receptors but did not decrease the current amplitude of neuronal nACh receptors. A–C, traces are currents activated by 20 μ M GABA for $\rho 1$ receptors and 1 mM carbachol for α_7 and $\alpha_4\beta_2$ nACh receptors, respectively. D, normalized averages of the current amplitudes from $\rho 1$, α_7 , and $\alpha_4\beta_2$ receptors after PMA treatment (50 nM; 10 min).

recording with and without the coexpression of the IC loop, respectively (Fig. 9B, right). Similar results were obtained with direct injection of recombinant IC loop protein. These data suggest that the PMA-induced inactivation of $\rho 1$ receptors may not require a specific interaction with the M3-M4 IC loop. We cannot rule out the possibility, however, that for reasons of aberrant IC loop conformation or improper subcellular location, the IC loop protein was unable to compete with the intact IC loop.

Receptors Could Return to Cell Surface after PMA-Dependent Internalization. We next examined if the receptors that were internalized by PKC activation could return to the cell surface. To answer this question, we assessed the recovery of functional GABA-activated currents after PMA-induced internalization. The amplitude of the GABA-evoked current from $\rho 1$ receptors normalized to the initial value recovered from 0.08 ± 0.03 to 0.8 ± 0.1 ($n = 9$) after 24 h (PMA = 100 nM; 10 min) (Fig. 10, A and B). The GABA-activated current did not significantly change during 24 h in oocytes without PMA treatment [amplitude normalized to the initial value was 0.98 ± 0.1 ($n = 5$); Fig. 10B, right]. Twenty-four hours after the PMA-induced internalization, the GABA-activated current from $\alpha\beta\gamma$ receptors recovered from 0.06 ± 0.05 ($n = 4$) to 0.60 ± 0.12 ($n = 4$) of the initial value. These data suggest that receptors internalized by PMA activation can exist as a pool that can be returned to the cell surface.

Further evidence that it was the internalized receptors that returned to the cell surface is provided by the data in Fig. 11. The 6HIS receptor includes 10 glycines followed by six histidines at the C terminus of the wild-type $\rho 1$ receptor. This subunit has a GABA sensitivity identical with that of the wild type (data not shown), but displays a higher Zn^{2+} sensitivity, probably due to an additional Zn^{2+} binding site(s) formed by the histidine residues (Fig. 11A). At $2.5 \mu M$ Zn^{2+} , the fractional block of the 6HIS receptor was 0.25 ± 0.02 ($n = 4$) (Fig. 11B, left traces; Fig. 11C, open column) compared with the wild-type fractional block of 0.04 ± 0.02 ($n = 4$) (Fig. 11C, striped column). After exposure of the 6HIS receptor to

the exoprotease carboxypeptidase A, the maximum amplitude of the current was unchanged but the Zn^{2+} sensitivity reverted to that of the wild-type receptor, 0.04 ± 0.002 ($n = 4$; Fig. 11B, middle traces; Fig. 11C, stippled column), indicating histidines were cleaved from the C terminus. After carboxypeptidase exposure, the oocytes were exposed to 100 nM PMA and the maximum current amplitude decreased to 0.22 ± 0.04 ($n = 4$) of the prePMA value (Fig. 11D, stippled column). Twenty-four hours later, the current amplitude recovered to 0.70 ± 0.1 ($n = 4$) of the original value (Fig. 11D, filled column). If it is new receptors that have come to the cell surface (either newly synthesized or maintained in an intracellular compartment), they should be intact 6HIS receptors and therefore display the higher Zn^{2+} sensitivity. In contrast, if it is the internalized receptors that returned to the cell surface, the Zn^{2+} sensitivity should be similar to that of the wild-type receptor. Figure 11B (right traces) and Fig. 11C (filled column) demonstrate that the Zn^{2+} sensitivity was similar to that of the wild type. Assuming carboxypeptidase A does not cross the cell membrane, the most straightforward interpretation of these results is that receptors that were previously on the cell surface and cleaved by extracellular carboxypeptidase A were reinserted into the plasma membrane.

Discussion

Previous investigations on the PKC-dependent regulation of GABA_A receptors have been controversial because both an inhibition (Sigel and Baur, 1988; Kellenberger et al., 1992; Chapell et al., 1998) and potentiation (Lin et al., 1996) have been observed. For GABA_C receptors, a negative modulation by PKC has been reported for both native GABA_C (Feigenspan and Bormann, 1994) and recombinant $\rho 1$ (Kusama et al., 1998; Filippova et al., 1999) receptors.

Our results document a role for PKC in the modulation of recombinant GABA receptors expressed in *Xenopus* oocytes. PKC induces receptor internalization as revealed by a decrease in surface binding of [³H]GABA, and the disappearance of GFP-tagged GABA receptors from the cell surface. This confirms previous findings that activation of PKC can reduce the amplitude of GABA-activated currents in oocytes (Sigel and Baur, 1988) and is in agreement with the conclusion that receptor internalization is the mechanism of this inactivation for GABA_A receptors expressed in oocytes (Chapell et al., 1998) and $\rho 1$ receptors expressed in HEK293 cells (Filippova et al., 1999). That a similar effect of PKC activation was observed for native GABA_C receptors (Feigenspan and Bormann, 1994) as well as recombinant $\rho 1$ receptors transiently expressed in HEK293 cells (Filippova et al., 1999) indicates that this effect is not unique to the oocyte expression system.

Internalization of membrane receptors by PKC activation is not without precedent because PMA has been shown to internalize the transferrin receptor, epidermal growth factor receptor, sodium, K-ATPase, a retinal taurine transporter, and the Na⁺/glucose transporter (for review, see Backer and King, 1991). In many cases, and similar to our findings, this internalization does not depend on receptor phosphorylation (Backer and King, 1991). Internalization of receptors in *Xenopus* oocytes is not, however, a nonspecific phenomenon because PKC-internalized GABA receptors but not nACh

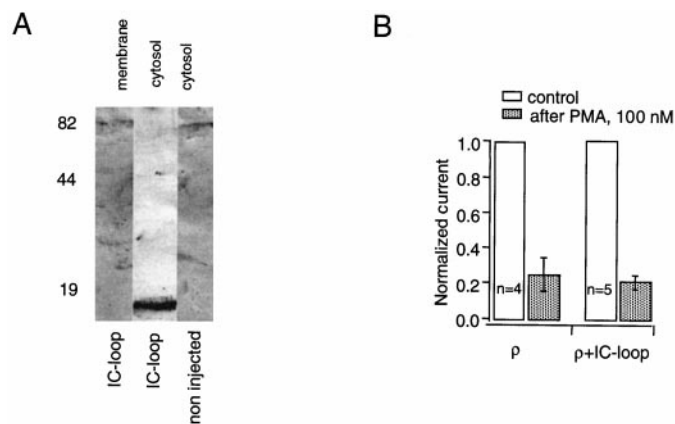


Fig. 9. Coexpression of the IC loop with $\rho 1$ receptors did not prevent the PMA-mediated inactivation. **A**, Western blot from oocytes expressing the $\rho 1$ IC loop. An ~ 15 -kDa band was detected in the cytosol that was not present in the membrane fraction or the cytosolic fraction of nonRNA-injected oocytes. **B**, current amplitude before and after PMA treatment in oocytes expressing the $\rho 1$ receptor and oocytes expressing the $\rho 1$ receptor along with the IC loop. The degree of PMA-induced inactivation was similar, indicating that the IC loop did not compete with the receptor internalization.

receptors expressed in the same oocyte. Although this phenomenon may reflect a specificity for interaction with the internalization machinery or with PKC (receptor for activated C kinase proteins; for review, see Mochly-Rosen, 1995), we cannot rule out the possibility that the nACh and GABA receptors are clustered in different regions of the oocyte and that endocytosis is region- (e.g., pole-) specific. Although we have not mapped the location of the GABA and nACh receptors, the $\alpha\beta$ -GFP γ receptors are clearly clustered on the cell surface (Fig. 7).

The details of PKC-dependent modulation of GABA_C receptors are still unclear. Assuming the residues identified in the *in vitro* phosphorylation studies are the same as in the full-length $\rho 1$ receptor, direct phosphorylation of the $\rho 1$ receptor itself was not required for the PKC-dependent internalization. Our present working hypothesis is that other protein(s) along the pathway for internalization require phosphorylation by PKC. Phorbol esters have been shown to alter the organization of microtubules, microfilaments, and other components of the cytoskeletal network (Phaire-Washington et al., 1980; Backer and King, 1991). Presumably, an interaction between GABA receptors and the cytoskeleton could be crucial for receptor shuttling to and from the cell surface. Recently, two proteins have been identified, mitogen-activated protein-1B and GABARAP, that are candidates for linking GABA_C and GABA_A receptors to the cytoskeleton, respectively (Hanley et al., 1999; Wang et al., 1999). The cytoskeleton has already been implicated in membrane receptor modulation (Connolly, 1984; Rosenmund and Westbrook, 1993), including recombinant $\rho 1$ GABA_C receptors expressed in HEK293 cells (Filippova et al., 1999).

Concerning the particular domains of the GABA receptor that could interact with the machinery responsible for inter-

nalization and retrieval, the obvious candidate was the putative large IC loop between M3 and M4. This region has already been implicated in the interaction with mitogen-activated protein-1B (Hanley et al., 1999). Coexpression or coinjection of the $\rho 1$ IC loop did not interfere with the PMA-induced internalization although several technical problems could account for these findings. First, the amount of IC loop could have been insufficient to block the requisite protein-protein interactions. Second, the coexpressed IC loop may exist in a different subcellular compartment than the $\rho 1$ receptors (e.g., cytosol versus membrane). Third, the conformation of the IC loop protein could be different from that of the IC loop in the $\rho 1$ receptor. Alternatively, other putative intracellular regions such as the loop between the first and second membrane-spanning domains could serve as a tether for internalization. And last, the present picture of the membrane topology of the GABA receptor subunit is only a model based on a hydrophobicity analysis. It is possible that other regions of the subunit are available for intracellular protein-protein interactions.

The reported half-life of receptors on the membrane surface of native neurons varies from 12 to 30 h at different stages of development (Steinbach, 1981; Killisch et al., 1991; Mammen et al., 1997). During this time, receptors can change their surface distribution (Mammen et al., 1997; O'Brien et al., 1997). In addition to the surface receptors, a second intracellular pool exists (Mammen et al., 1997). Whether receptors can be dynamically shuttled between the membrane surface and an intracellular pool during their lifetime is still controversial. Concerning GABA receptors, activation of tyrosine kinases has been shown to recruit GABA_A receptors to the postsynaptic region (Wan et al.,

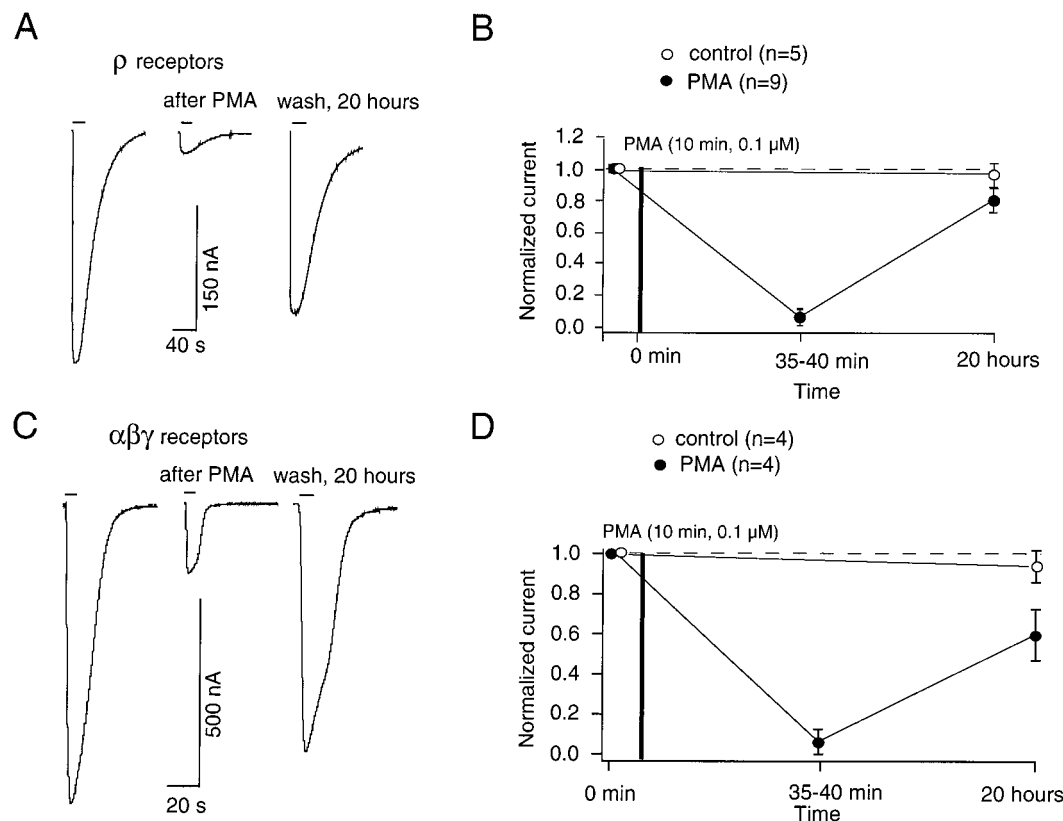


Fig. 10. Recovery from PMA-induced internalization of GABA receptors. A, GABA-activated current from $\rho 1$ receptors before and after PMA treatment (100 nM; 10 min). Note that the current almost completely recovered within 20 h. B, plot of the normalized current amplitude before and after PMA treatment. \circ represent the GABA-activated current amplitude in a group of oocytes that were not exposed to PMA. Note that in the absence of PMA, the current amplitude remained stable over this time course. C, GABA-activated current from $\alpha\beta\gamma$ receptors before and after PMA treatment (100 nM; 10 min). As for the $\rho 1$ receptors, the current almost completely recovered within 20 h. D, plot of the normalized current amplitude before and after PMA treatment in $\alpha\beta\gamma$ receptors. \circ represent the GABA-activated current amplitude in a group of oocytes that were not exposed to PMA.

1997). In this study, we demonstrated a PKC-dependent internalization of GABA receptors from the cell surface and, as evidenced by functional recovery, these internalized receptors can be reinserted in the plasma membrane. A similar recovery of internalized receptors has been observed for $\rho 1$ receptors expressed in HEK293 cells (Filippova et al., 1999). Thus, by controlling surface expression, PKC may play a key role in regulating GABA-mediated inhibition in the CNS.

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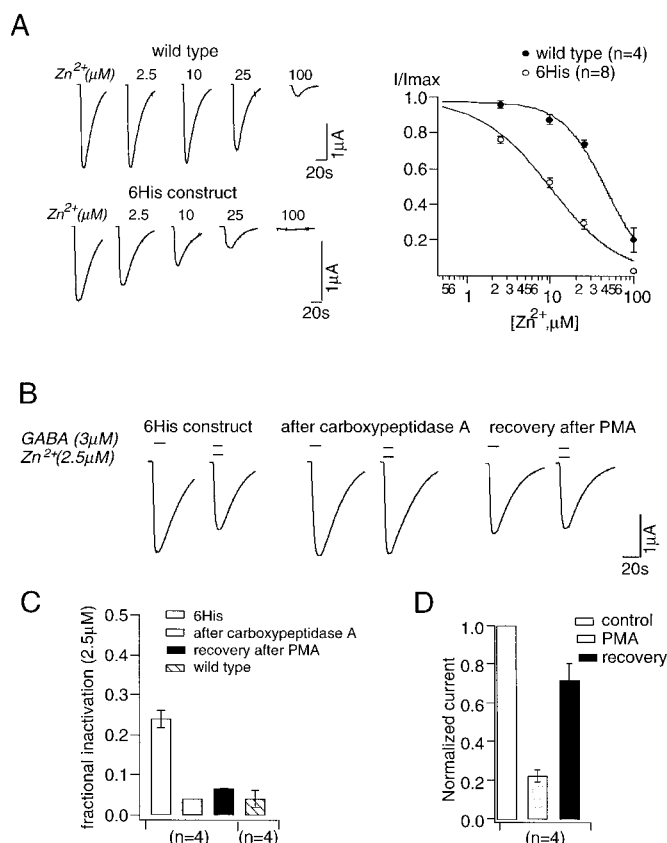


Fig. 11. Internalized receptors can return to the cell surface. **A**, traces show the block of a 3 μM GABA-activated current by different concentrations of Zn^{2+} for the wild-type (top) and 6HIS (bottom) constructs. The graph to the right plots the fractional block as a function of the Zn^{2+} concentration. The continuous lines are fits of eq. 2 to these data, which yielded IC_{50} values/Hill coefficients of $44 \pm 3/1.8 \pm 0.2$ ($n = 4$) and $10.2 \pm 0.1/1.0 \pm 0.05$ ($n = 8$) for the wild-type and 6HIS receptors, respectively. **B**, block of the 6HIS receptor by 2.5 μM GABA before (left) and after (middle) a 120-min incubation with the exoprotease carboxypeptidase A that cleaved the histidines and reverted the Zn^{2+} sensitivity to that of the wild-type receptor. The rightmost traces show the Zn^{2+} sensitivity after the same oocyte was exposed to 100 nM PMA causing internalization and was then allowed to recover for 24 h. Note that the Zn^{2+} sensitivity was similar to that of the wild type (cleaved 6HIS), indicating the internalized receptors were returned to the cell surface. **C**, averages from four oocytes showing the fractional activation of a 3 μM GABA-activated current by 2.5 μM Zn^{2+} before exposure to carboxypeptidase A (\square), after exposure (\blacksquare), and after exposure to 100 nM PMA with a subsequent 24-h recovery period (\blacksquare). Percentage of inactivation by Zn^{2+} is shown for the wild-type $\rho 1$ receptor for comparison (\square). **D**, plot of the fraction of current remaining after 100 nM PMA (\square) and after the 24-h recovery period (\blacksquare). The averages are for the same data set as in Fig. 11C.

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Send reprint requests to: David S. Weiss, Ph.D., Department of Neurobiology, University of Alabama School of Medicine, 1719 Sixth Ave. South CIRC 410, Birmingham, AL 35294. E-mail: dweiss@nrc.uab.edu
