The inhibition of γ-aminobutyric acid (GABA)-gated chloride currents by the protein kinase C (PKC) activator 4β-phorbol 12-myristate 13-acetate (PMA) was investigated using recombinant human GABA\(_{A}\) receptors expressed in Xenopus oocytes. PMA (5 nM) reduced the GABA response in oocytes expressing the \(\alpha_1\beta_2\gamma_2\) receptor construct, as measured by the two-electrode voltage-clamp method. GABA responses declined to approximately 25% of their pretreatment value within 45 min. GABA responses in oocytes expressing a receptor construct from which the known PKC phosphorylation sites were absent, \(\alpha_1\beta_2(S410A)\), were comparably inhibited. Phorbol 12-myristonystrate (PMM; 5 nM), which does not activate PKC, did not alter the GABA response in either construct, while the PKC inhibitor calphostin C (0.5 \(\mu\)M) prevented the PMA effect. To further investigate PMA inhibition of the GABA response, a GABA\(_{A}\) receptor \(\alpha_1\) subunit/green fluorescent protein (GFP) chimera (\(\alpha_1\text{GFP}\)) was used to visualize GABA\(_{A}\) receptor distribution. Similar to the wild type constructs, PMA robustly decreased GABA responses in oocytes expressing \(\alpha_1\text{GFP}\beta_2\gamma_2\) and \(\alpha_1\text{GFP}\beta_2(S410A)\) receptor constructs. Following PMA treatment, GFP fluorescence in the oocyte plasma membrane was decreased to approximately 45% of the pretreatment values indicating GABA\(_{A}\) receptor internalization. This effect of PMA was prevented by calphostin C and was not produced by PMM. Experiments with bd24, a monoclonal antibody which recognizes an extracellular epitope of the \(\alpha_1\) subunit, were used to demonstrate that PMA, but not PMM, decreases \(\alpha_1\) subunit immunoreactivity in the plasma membrane of intact oocytes expressing the \(\alpha_1\beta_2\gamma_2\) construct, thus confirming the results obtained with the chimeric receptor. It is concluded that, in Xenopus oocytes, PMA induces an internalization of the GABA\(_{A}\) receptor through PKC-mediated phosphorylation of an unidentified protein(s) and that this contributes to the decrease in electrophysiological responses to GABA following PKC activation.

\(\gamma\)-Aminobutyric acid (GABA) is the principal inhibitory neurotransmitter in the vertebrate central nervous system. The fast inhibitory actions of GABA are mediated by the GABA\(_{A}\) receptor, a postsynaptic ligand-gated chloride channel. At least 17 receptor subunit subtypes have been identified (\(\alpha_1–4, \beta_1–4, \gamma_1–4, \delta, \pi_1,\pi_2\)), and the receptor is thought to form a heteropentamer (1). The subunit composition of the receptor determines agonist potency (2, 3), desensitization kinetics (3), phosphorylation (4–6), and allosteric properties (2, 7, 8).

Calcium-phospholipid-dependent protein kinase (PKC) phosphorylates purified GABA\(_{A}\) receptors on polypeptides corresponding to \(\beta\) subunits (9), and PKC phosphorylation sites have been identified on a variety of GABA\(_{A}\) subunits including \(\beta_1\) serine 409 (5, 6), \(\gamma_2\) serine 327, and \(\gamma_2\)L serine 343/327 (4, 5). Activation of PKC by phorbol esters such as 4β-phorbol 12-myristate 13-acetate (PMA) inhibits GABA\(_{A}\) receptor function in mouse brain cerebellar microsomes (10), cultured cervical ganglion neurons (6), retinal rod bipolar cells (11), cultured kidney cells expressing recombinant GABA\(_{A}\) receptor subunits (6), and Xenopus oocytes expressing brain mRNA (10, 12, 13) and recombinant GABA\(_{A}\) receptor constructs (6, 10, 14–16). This effect is not induced by structural analogs of PMA that do not activate PKC and is antagonized by specific PKC inhibitors calphostin C (11), tamoxifen (12), and the inhibitory peptide fragment of PKC (10), indicating that activation of PKC inhibits GABA\(_{A}\) receptor function. In contrast, catalytically active PKC increases GABA responses in fibroblasts expressing recombinant GABA\(_{A}\) receptors (17). The basis of these contradictory findings has not been resolved.

The mechanism by which PKC activation inhibits GABA\(_{A}\) receptor function in Xenopus oocytes remains to be fully explained. Phorbol esters decrease GABA\(_{A}\) receptor responses in both A293 cells and Xenopus oocytes expressing rat wild type receptor constructs but not corresponding mutant constructs lacking known PKC phosphorylation sites (6). In contrast, in oocytes expressing mouse receptor constructs, PMA inhibits both wild type receptor constructs and also constructs that contain PKC phosphorylation site mutations (15). These data indicate that, under some experimental conditions, PKC-mediated inhibition of GABA responses may be due to: 1) phosphorylation of the receptor on unidentified sites, 2) phosphorylation of receptor-associated proteins, or 3) alteration of PKC-dependent processes within the oocyte, which may decrease GABA responses.

In the current set of experiments, the effect of PKC activation on the function and plasma membrane distribution of recombinant human GABA\(_{A}\) receptor constructs expressed in Xenopus oocytes was investigated using the two-electrode voltage-clamp technique and confocal microscopy. Our results demonstrate that PMA causes GABA\(_{A}\) receptor internalization by a mechanism that is PKC-dependent but does not involve phosphorylation of the known PKC phosphorylation sites on the
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receptor. Furthermore, receptor internalization occurs concomitantly in the decrease in GABA<sub>A</sub> receptor function observed following PMA treatment.

**EXPERIMENTAL PROCEDURES**

**Materials**—Female Xenopus laevis were purchased from Xenopus 1 (Ann Arbor, MI). Drugs and reagents were obtained from the following sources: GABA and THDOC (3α, 21-dihydroxy-α-pregnan-20-one), Research Biochemicals, Inc. (Natick, MA); propofol, Aldrich; calphostin C, Calbiochem (San Diego, CA); monoclonal anti-α1 subunit antibody (bd24), Boehringer Mannheim; Texas Red-conjugated goat anti-mouse IgG, Molecular Probes (Eugene, OR). The β2 subunit mutant, β2(S410A) was constructed using polymerase chain reaction methodology by National Biosciences (Plymouth, MN) and was confirmed by sequencing. The β2/S65T GFP variant. For this construct, GFP was fused to the C terminus of the α1 subunit GFP chimera was constructed using the F64L S65T GFP variant. For this construct, GFP was fused to the C terminus of the α1 subunit as described elsewhere. 2 GABA<sub>A</sub> receptor subunit cDNAs (in the pCDM8 vector) were obtained originally from Dr. Paul Whiting (Merck Sharp and Dohme Research Laboratories). All other chemicals and drugs were of analytical grade and purchased from Sigma.

PMA, PMM, calphostin C, and THDOC were dissolved in 100% dimethyl sulfoxide (Me<sub>2</sub>SO), stored at −20 °C at stock concentrations, then diluted to working concentrations with ND96. The Me<sub>2</sub>SO concentration in working solutions was 0.1%. Propofol was dissolved in ethanol, then diluted to working concentration with ND96. The final ethanol concentration was 0.05%.

**Expression of Recombinant GABA<sub>A</sub> Receptors**—Oocytes were isolated from mature female X. laevis anesthetized in a cold 0.2% tricaine solution as described previously (16). Following a topical application of lidocaine (4%), a small abdominal incision was made and a portion of the ovary was removed. Individual oocytes were separated by placing small pieces of ovary in a solution of 85 mM NaCl, 2 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.5, containing collagenase type 2A (200 μg/ml), for 3 h at room temperature with gentle agitation. Defolliculated oocytes were placed in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 5 mM HEPES, pH 7.5) and the oocyte nucleus was injected with cDNAs encoding human GABA<sub>A</sub> receptor subunits (30 μl of sterile H<sub>2</sub>O containing 0.5 ng of cDNAs/subunit). Injected oocytes were maintained in room temperature in ND96 supplemented with 2 mM pyruvate, 0.5 mM theophylline, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin.

**Two-electrode Voltage-clamp Experiments**—Two-electrode voltage-clamp experiments were performed as described previously (16). At least 24 h following injection, oocytes were placed in a perfusion chamber (100 μl volume) and perfused with ND96 at a rate of either 5 or 1 ml/min as indicated. Cells were voltage-clamped at −60 mV with a model OC-725B Oocyte Clamp (Warner Instruments Corp., Hamden, CT) using two glass microelectrodes filled with 3 M KCl (resistance 0.5–1.5 megohms). GABA responses were recorded using a chart recorder, and peak current amplitudes were measured.

Oocytes were perfused with GABA (10 μM), THDOC (10 μM), propofol (100 μM), or pentobarbital (300 μM) until the peak current amplitude of the response was obtained. THDOC, propofol, and pentobarbital were perfused at 10-min intervals, while GABA was perfused at either 5- or 10-min intervals. Each experiment was begun after three successive responses to the agonist did not vary by more than 10%. Differences between treatment groups were analyzed by a Student’s t test using InStat 2.0. All results are expressed as mean ± S.E.

PMA or PMM (5 nM in 0.1% Me<sub>2</sub>SO) was perfused for 10 min beginning 5 min after a stable agonist response. The beginning of phorbol ester perfusion is defined as 0 min in all experiments, and agonist application following PMA treatment was begun 5 min after the cessation of the 10-min PMA perfusion. Response of the receptor to agonist was assessed for a period of 1 h following phorbol ester treatment. The perfusion rate was 5 ml/min, except for experiments in which calphostin C was used. Because of the expense of continuous perfusion with calphostin C, the perfusion rate was 1 ml/min for these experiments. The change in perfusion rate did not alter the effect of PMA in the absence of calphostin C. In antagonism experiments, oocytes were perfused with 0.5 μM calphostin C in 0.05% Me<sub>2</sub>SO for 30 min prior to PMA treatment, during which time responses to GABA (10 μM) were assessed at 5-min intervals. GABA solutions for these experiments also contained 0.5 μM calphostin C. After the 30-min perfusion with calphostin C, oocytes were perfused with 5 nM PMA + 0.5 μM calphostin C (final concentration of 0.1% Me<sub>2</sub>SO) for 10 min. Oocytes were then perfused continuously with calphostin C for an additional 35 min, and GABA (10 μM) responses were assessed in the presence of calphostin C (0.5 μM) at 5-min intervals.

**GABA<sub>A</sub> Receptor-GFP Chimera Fluorescence**—The effect of phorbol esters on GFP fluorescence in the oocyte plasma membrane was assessed in oocytes expressing α1GFPβ2L or α1GFPβ2(S410A) receptor constructs using confocal microscopy. For these experiments, a Student’s t test using

**RESULTS**

PMA (5 nM) treatment of oocytes expressing the α1β2γ2L receptor construct decreased the peak amplitude of the GABA (10 μM) response to approximately 25% of the pretreatment control response by 1 h (Fig. 1A). No effect of PMM (5 nM) treatment was observed throughout this time course. The effects of PMA (5 nM), PMM (5 nM), and PMA (5 nM) + the PKC inhibitor calphostin C (0.5 μM) on the GABA response at the 40° change in perfusion rate did not alter the effect of PMA in the absence of calphostin C. In antagonism experiments, oocytes were perfused with 0.5 μM calphostin C in 0.05% Me<sub>2</sub>SO for 30 min prior to PMA treatment, during which time responses to GABA (10 μM) were assessed at 5-min intervals. GABA solutions for these experiments also contained 0.5 μM calphostin C.
ment GABA response. The inactive PMA analog PMM did not affect GABA responses of either receptor construct, with GABA responses of 103 ± 8% and 91.5 ± 2% (mean ± S.E., n = 6 each construct) of the pretreatment GABA response. GABA responses following PMA treatment were significantly different from those in the PMM treatment group (p ≤ 0.0001, unpaired t test).

FIG. 1. PMA inhibition of oocytes expressing α1β2γ2L or α1β2(S410A) receptor constructs. A, Oocytes expressing α1β2γ2L receptor constructs were treated with PMM (5 nM; n = 3) or PMA (5 nM; n = 4) for 10 min. Responses to GABA (10 μM) were measured every 10 min. Responses to GABA are expressed as a percentage of the pretreatment GABA response (mean ± S.E.). PMA produces a large decrease in the GABA response, which is maximal by 45 min. B, Oocytes expressing α1β2γ2L or α1β2(S410A) receptor constructs were treated with PMM (5 nM; n = 6 each construct), PMA (5 nM; n = 3, 6), or PMA (5 nM) + calphostin C (0.5 μM; n = 3 each construct) for 10 min. Oocytes treated with PMA in the presence of calphostin C were pretreated with calphostin C for 30 min prior to PMA treatment, and calphostin C was present thereafter for the duration of the experiment. The GABA response average shown was collected 45 min after the onset of phorbol ester treatment and is expressed as a percentage of pretreatment response (mean ± S.E.). An asterisk (*) indicates a significant difference between PMA treatment and both PMM and PMA + calphostin C treatments in oocytes expressing the same receptor construct (p ≤ 0.0001, unpaired t test).

FIG. 2. PMA inhibition of GABA responses in oocytes expressing α1GFPβ2γ2L or α1GFPβ2(S410A) receptor constructs. Oocytes were treated with PMM (5 nM; n = 6 each construct), PMA (5 nM; n = 9, 6), or PMA (5 nM) + calphostin C (0.5 μM; n = 3, 5) for 10 min. Oocytes treated with PMA in the presence of calphostin C were pretreated with calphostin C for 30 min prior to PMA treatment, and calphostin C was present thereafter for the duration of the experiment. The GABA response average shown was collected 45 min after the onset of phorbol ester treatment and is expressed as a percentage of pretreatment GABA response (mean ± S.E.). An asterisk (*) indicates a significant difference between PMA treatment and both PMM and PMA + calphostin C treatments in oocytes expressing the same receptor construct (p ≤ 0.0005, unpaired t test).
In the presence of calphostin C, PMA treatment of oocytes expressing α1β2γ2L and α1β2(S410A) receptor constructs resulted in GABA responses that were 86 ± 7% and 77 ± 3% (mean ± S.E., n = 3 each construct), respectively, of the pretreatment GABA responses. For each construct, GABA responses following PMA treatment in the presence of calphostin C were significantly different from responses following treatment with PMA alone (p ≤ 0.0001, unpaired t test).

PMA inhibition of GABA responses was also observed in oocytes expressing constructs containing the α1 subunit/GFP chimera (Fig. 2). This chimera has been previously characterized in Xenopus oocytes and shown to function in a manner indistinguishable from wild type receptors with respect to receptor desensitization, agonist potency, allosteric modulation and rectification.2 GABA responses in oocytes expressing α1GFPh2γ2L or α1GFPh2(S410A) receptor constructs were reduced to 17 ± 3% and 9 ± 5% (mean ± S.E., n = 9 and 6, respectively), of pretreatment GABA responses 45 min following PMA treatment. PMM treatment of oocytes expressing these receptor constructs yielded GABA responses at 45 min which were 108 ± 7% (α1GFPh2γ2L) and 88 ± 2% (α1GFPh2(S410A)) (mean ± S.E., n = 6 each construct) of the prePMM GABA responses. For each chimeric construct tested, GABA responses in the presence of PMA were significantly inhibited when compared with GABA responses in the presence of PMM (p ≤ 0.0001, unpaired t test). In the presence of calphostin C, PMA treatment in oocytes expressing either α1GFPh2γ2L or α1GFPh2(S410A) receptor constructs resulted in GABA responses that were 90 ± 7% (n = 3) and 68 ± 10% (n = 5) (mean ± S.E.) of the pretreatment GABA responses. These values were significantly different from the corresponding responses in oocytes treated with PMA in the absence of calphostin C (p ≤ 0.0005, unpaired t test).

The effect of PMA on the surface expression of the GABA A receptor/GFP chimera was investigated using confocal microscopy at a time point corresponding to maximal PMA inhibition of the GABA response (Fig. 3, A and B). The distribution of the α1GFPh2γ2L receptor construct, as indicated by fluorescence at 509 nm, was restricted to the animal pole. No such fluorescence was observed in oocytes expressing wild type receptors (data not shown). Forty-five min after a 10-min treatment with PMA (5 nM), the presence of receptor on the oocyte membrane surface, as indicated by GFP fluorescence, was reduced to 43 ± 7% (mean ± S.E.) of pretreatment levels (paired t test, p ≤ 0.0001, n = 18). GFP fluorescence in PMM-treated oocytes was 107 ± 9% (mean ± S.E.) of the pretreatment levels (n = 12). In the presence of calphostin C, PMA treatment resulted in fluorescence that was 112 ± 13% (mean ± S.E.) of the fluorescence present prior to PMA treatment (n = 6).

Surface expression of the receptor was similarly affected in oocytes expressing the α1GFPh2(S410A) construct (Fig. 4, A and B). PMA decreased GFP fluorescence to 45 ± 8% (mean ± S.E.) of pretreatment levels following PMA treatment (paired t test, p ≤ 0.0001, n = 16) (Fig. 4B). GFP fluorescence in oocytes treated with PMM (5 nM) was 101 ± 16% (mean ± S.E.) of pretreatment fluorescence (n = 12). In oocytes treated with PMA in the presence of calphostin C, GFP fluorescence was 74 ± 10% (mean ± S.E.) of pretreatment levels (n = 6). There was no difference between PMM and PMA + calphostin C treatment groups.

Interestingly, no intracellular GFP fluorescence was observed in the oocytes under any conditions. This may be due to 1) the inability of the laser to penetrate far into a large 1 mm cell, 2) the quenching of the GFP signal by the oocyte yolk/pigment or 3) the fact that the intracellular distribution of the GFP signal may be so diffuse that it is below detection levels.

Although we cannot distinguish between these possibilities, potassium channel/GFP chimeras expressed in oocytes also show a GFP signal restricted to the plasma membrane (22) similar to that observed here. In the case of the potassium channel/GFP chimera, fluorescence microscopy was performed

![Fig. 3. PMA produces GABA A receptor internalization in oocytes expressing the α1GFPh2γ2L receptor construct.](http://www.jbc.org/).
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**DISCUSSION**

In the present study, activation of PKC by PMA decreases GABA responses in oocytes expressing \(\alpha1\beta2\gamma2L\) and \(\alpha1\beta2\gamma2L(S410A)\) receptor constructs, indicating that this effect is not due to phosphorylation of known PKC sites on the receptor.

PMA also decreases GABA responses in oocytes expressing \(\alpha1\text{GFP}\beta2\gamma2L\) and \(\alpha1\text{GFP}\beta2\gamma2L(S410A)\) receptor constructs. The decrease in electrophysiological responses to GABA is accompanied by GABA\textsubscript{\textalpha} receptor internalization as determined using receptor-GFP chimeric constructs and immunofluorescence experiments were performed on oocytes expressing the \(\alpha1\beta2\gamma2L\) receptor construct (Fig. 5). The presence of this construct on the oocyte surface was assessed using a monoclonal antibody to an extracellular epitope of the \(\alpha1\) subunit (19, 20). Indirect anti-\(\alpha1\) subunit antibody immunofluorescence was visualized by confocal microscopy with a Texas Red-conjugated secondary antibody using confocal microscopy. Shown are representative oocytes from PMM (left) and PMA (right) treatment groups. In PMA-treated oocytes \((n = 14)\), immunofluorescence was 45 \(\pm\) 6\% (mean \(\pm\) S.E.) of that observed in PMM-treated oocytes \((n = 13)\). This difference was statistically significant \((p \leq 0.0001,\) unpaired \(t\) test).

**FIG. 5.** PMA decreases GABA\textsubscript{\textalpha} receptor immunofluorescence on the oocyte surface. Oocytes expressing the \(\alpha1\beta2\gamma2L\) receptor construct were treated with PMA (5 nM) or PMM (5 nM). Forty-five minutes later, intact oocytes were incubated with a mouse monoclonal anti-\(\alpha1\) subunit antibody, which recognizes an extracellular epitope on the \(\alpha1\) subunit. GABA\textsubscript{\textalpha} receptors on the oocytes surface were then visualized by indirect immunofluorescence with a Texas Red-conjugated secondary antibody using confocal microscopy. Shown are representative oocytes from PMM (left) and PMA (right) treatment groups. In PMA-treated oocytes \((n = 14)\), immunofluorescence was 45 \(\pm\) 6\% (mean \(\pm\) S.E.) of that observed in PMM-treated oocytes \((n = 13)\). This difference was statistically significant \((p \leq 0.0001,\) unpaired \(t\) test).

To determine if PMA treatment would decrease the surface immunoreactivity of a wild type receptor construct, immunofluorescence experiments were performed on oocytes expressing the \(\alpha1\beta2\gamma2L\) receptor construct (Fig. 5). The presence of this construct on the oocyte surface was assessed using a monoclonal antibody to an extracellular epitope of the \(\alpha1\) subunit (19, 20). Indirect anti-\(\alpha1\) subunit antibody immunofluorescence was visualized by confocal microscopy with a Texas Red-conjugated goat anti-mouse IgG antibody. Texas Red fluorescence in oocytes treated with PMA (5 nM) was 45 \(\pm\) 6\% (mean \(\pm\) S.E.) of that observed in oocytes treated with PMM (5 nM). This difference was statistically significant \((p \leq 0.0001,\) unpaired \(t\) test) and similar in magnitude to the percentage decrease in GABA\textsubscript{\textalpha} receptor observed using the GABA\textsubscript{\textalpha} receptor-GFP chimeric construct.

The GABA\textsubscript{\textalpha} receptor is allosterically modulated by a variety of compounds that have distinct binding sites on the receptor complex. At higher concentrations, many of these compounds can directly gate the chloride channel in the absence of GABA. These compounds include propofol, pentobarbital, and THDOC (23–25). While the mechanisms by which these compounds directly gate the channel are not well defined, evidence exists that they activate the channel through sites distinct from the GABA-binding site (25–27). If PMA induces GABA\textsubscript{\textalpha} receptor internalization, it would predicted that PMA treatment would also decrease the direct channel-gating effects of THDOC, propofol and pentobarbital. Following PMA (5 \(\mu\)M) treatment, the electrophysiological responses of \(\alpha1\beta2\gamma2L\) receptor constructs to these compounds were 5 \(\pm\) 0.4\%, 13 \(\pm\) 6\%, and 9 \(\pm\) 2\% (mean \(\pm\) S.E.) of the prePMA responses, respectively (Fig. 6). These values were significantly different from those observed in oocytes treated with PMM (5 nM) \((p \leq 0.05,\) unpaired \(t\) test, \(n = 3\) each compound).

**FIG. 4.** PMA produces GABA\textsubscript{\textalpha} receptor internalization in oocytes expressing the \(\alpha1\text{GFP}\beta2\gamma2L(S410A)\) construct. A, confocal images of oocytes expressing the \(\alpha1\text{GFP}\beta2\gamma2L(S410A)\) construct taken before (Pre) and after (Post) 45 min of treatment with PMM (5 nM; top), PMA (5 nM; middle), or PMA (5 nM) + calphostin C (0.5 \(\mu\)M; bottom). Oocytes treated with PMA in the presence of calphostin C were pretreated with calphostin C for 30 min prior to PMA treatment, and calphostin C was present thereafter for the duration of the experiment. B, PMA decreases GFP fluorescence on the oocyte surface in the absence \((n = 16)\) but not the presence of calphostin C \((n = 6)\), while PMM has no effect \((n = 12)\). On the ordinate is mean \((\pm\) S.E.) GFP fluorescence following treatment with phorbol esters expressed as the percentage of the pretreatment GFP fluorescence. An asterisk \((*)\) indicates significant difference from pretreatment fluorescence \((p \leq 0.0001,\) paired \(t\) test). Open bar, pre-treatment; hatched bar, post-treatment.

On fixed, sectioned oocytes. Using this protocol, limited laser penetration and the quenching of the GFP signal by the yolk/pigment would have been minimized, thus supporting the possibility that the intracellular GFP signal is too diffuse for detection.
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![Graph showing the effects of THDOC, propofol, and pentobarbital on GABA<sub>A</sub> receptor inhibition by PMA.](image)

**Fig. 6.** PMA inhibits the direct channel-gating of the GABA<sub>A</sub> receptor by THDOC, propofol, and pentobarbital. Responses to THDOC (10 μM), propofol (100 μM), or pentobarbital (300 μM) were assessed in oocytes expressing the α1β2/2L receptor construct. Oocytes were then treated with either PMA (5 nM) or PMM (5 nM) for 10 min. Forty-five min after the onset of phorbol ester treatment, responses to THDOC, propofol, or pentobarbital were again measured. Data are shown on the ordinate as the percentage of the pretreatment response (mean ± S.E.). Electrophysiological responses to all three compounds were inhibited in PMA-treated oocytes compared with responses obtained in PMM-treated oocytes (p ≤ 0.05, unpaired t test, n = 3 for each compound). Open bar, PMA; hatched bar, PMM.

Experiments with wild type receptor constructs. Consistent with receptor internalization, PMA also decreases the direct channel-gating effects of pentobarbital, propofol, and THDOC. A decrease in the presence of surface receptors is consistent with previous findings in *Xenopus* oocytes, showing that PMA decreases the efficacy of GABA (10, 16).

PKC modulation of membrane protein trafficking has been demonstrated previously in *Xenopus* oocytes. Activation of PKC decreases the number of endogenous sodium/potassium ATPase transporters (28) and recombinant rabbit and rat sodium/glucose cotransporters (29) in the plasma membrane. In contrast, PKC activation increases the number of GAT1 transporters in the plasma membrane (30), an effect that is dependent on the presence of a leucine heptad repeat in the GAT1 transporter (31). PKC activation does not affect the trafficking of all heterologously expressed proteins since RCK1 potassium channel activity is decreased by PKC without an alteration in the number of surface channels (32). The above studies suggest that PKC-mediated internalization of GABA<sub>A</sub> receptors in oocytes is not due to a general effect of PKC in stimulating internalization of surface receptors. Further evidence that PKC does not stimulate the internalization of general membrane proteins is suggested by PKC-mediated increases in the activity of NMDA receptors (33–35) and the human sodium/glucose cotransporter (29).

In a previous study, PMA inhibited GABA responses in *Xenopus* oocytes expressing rat GABA<sub>A</sub> receptors. One and a half hours after phorbol ester application, the α1β2γ2S receptor construct was inhibited by 30% while α1β2γ2S(S327A) or α1β2γ2S(S327A) receptor constructs, which possess mutated PKC phosphorylation sites, were inhibited to a significantly lesser extent (15). By 13.5 min, PMA inhibited the α1β2γ2S construct by 60%, an effect that was only slightly reduced in the α1β2γ2S(S327A) receptor construct and not at all in the α1β2γ2S(S327A) receptor construct. These data indicate that PKC-mediated inhibition of the GABA<sub>A</sub> receptor occurs in part at known PKC phosphorylation sites on the receptor but also independently of these sites. In contrast, phorbol esters inhibit GABA responses in *Xenopus* oocytes expressing mouse α1β1, α1β1γ2S, or α1β1γ2L GABA<sub>A</sub> receptor constructs but do not affect α1β1(S409A), α1β1(S409A)γ2S(S327A), or α1β1(S409A)γ2L(S327A, S343A) receptor constructs, indicating that PKC phosphorylation sites on the receptor mediate phorbol ester inhibition of GABA<sub>A</sub> receptors (6). The reason for the discrepancy between studies examining phorbol ester effects on GABA<sub>A</sub> receptors expressed in *Xenopus* oocytes is unclear. Differences in phorbol ester concentrations and treatment length may be important.

Krishek et al. (6) treated *Xenopus* oocytes with PMA (250 nM) for 30–90 min (although effects were apparent within 10 min), Kellenberger et al. (15) treated oocytes with 10 nM PMA for 1 min every 4 min throughout the experiment, and in the present study oocytes were perfused with 5 nM PMA for 10 min. In addition to PMA protocol differences, the various studies expressed subunits from different species with rat (15), mouse (6), and human (present study) subunits used. Furthermore, β1 subunits were used in the Krishek study (6), while β2 subunits were used here and by Kellenberger et al. (15). Our results agree more closely with those of Kellenberger et al. (15), where the same β2 subunit isoform was used and similar PMA concentrations and application times were employed.

None of the previous studies which show that PKC activation inhibits GABA<sub>A</sub> receptors assessed whether GABA<sub>A</sub> receptor internalization may underlie the decrease in GABA response. It is possible that receptor internalization may account in part for the inhibition of GABA responses observed by Kellenberger et al. study (15) since, similar to the present results, PMA decreased GABA responses in oocytes expressing receptors lacking known PKC phosphorylation sites. Receptor internalization may not explain the PMA-induced decrease in receptor responsiveness observed by Krishek et al. (6) since PMA inhibition of GABA responses was not observed in GABA<sub>A</sub> receptors that lack PKC phosphorylation sites and therefore is different from the present results. Thus, it appears that PKC activation modulates GABA<sub>A</sub> receptor function both by a direct effect of phosphorylation on ion channel function and also by controlling the number of GABA<sub>A</sub> receptors in the plasma membrane. Which mechanism(s) is operable in a given experiment may depend on the experimental parameters. The existence of these two distinct mechanisms may contribute to the disparate results in the literature.

Although our results demonstrate that phosphorylation of previously identified PKC phosphorylation sites on the GABA<sub>A</sub> receptor are not involved in receptor internalization, we cannot rule out the possibility that there may be other phosphorylation sites on the receptor that are important. These sites may be phosphorylated either by PKC or secondarily to PKC activation by other kinases. In this regard, the GABA<sub>A</sub> receptor is phosphorylated by tyrosine kinase (36–38), protein kinase A (5), and calcium/calmodulin-dependent kinase II (39–40). Since PMA stimulates internalization of receptor constructs lacking the γ2L subunit, phosphorylation of this subunit can be ruled out as being required for PMA-induced internalization of the receptor. Additionally, we do not rule out the possibility that, in our experiments, the receptors were phosphorylated on the known PKC phosphorylation sites but only conclude that phosphorylation of these sites is not required for receptor internalization.

Although the decrease in GABA<sub>A</sub> receptor responses noted in the present study may be explained, at least in part, by recep-
phosphorylation appears to play a role in GABA<sub>A</sub> receptor trafficking in neurons. It is possible that serine/threonine phosphorylation may also regulate neuronal GABA<sub>A</sub> receptor trafficking in living cells. Finally, the known PKC sites on the receptor. Additionally, our results validate the use of a GABA<sub>A</sub> receptor-GFP chimera for studying GABA<sub>A</sub> receptor trafficking in living cells. Finally, the present findings highlight the importance of considering the role of receptor trafficking in the interpretation of data obtained when studying the effects of phosphorylation on neurotransmitter receptor function.

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REFERENCES

Activation of Protein Kinase C Induces γ-Aminobutyric Acid Type A Receptor Internalization in *Xenopus* Oocytes

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