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Protein Kinase C Modulates the Activity of a Cloned γ-Aminobutyric Acid Transporter Expressed in *Xenopus* Oocytes via Regulated Subcellular Redistribution of the Transporter*

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We report that activators and inhibitors of protein kinase C (PKC) and protein phosphatases regulate the activity of a cloned rat brain γ-aminobutyric acid (GABA) transporter (GAT1) expressed in *Xenopus* oocytes. Four compounds known to activate PKC increased GABA uptake 2–3.5-fold over basal control levels. Inhibition of PKC by bisindolylmaleimide reduced basal GABA uptake 80% and blocked the phorbol 12-myristate 13-acetate (PMA)-induced stimulation of transport. Okadaic acid, a protein phosphatase inhibitor, stimulated transport 2.5-fold; a 4-fold increase in GABA uptake occurred when oocytes were treated with cyclosporin A, a specific inhibitor of protein phosphatase 2B. Modulation resulted in changes to V_{max} but not to K_m and was influenced by the functional expression level of the transporter protein; as expression level increased, the ability to up-regulate transporter activity decreased. Down-regulation of transporter activity was independent of expression level. Modulation did not occur through phosphorylation of the three consensus PKC sites predicted by the primary protein sequence since their removal had no effect on the susceptibility of the transporter to modulation by PMA or bisindolylmaleimide. Subcellular fractionation of oocyte membranes demonstrated that under basal level conditions, the majority of GAT1 was targeted to a cytoplasmic compartment corresponding to the trans-Golgi or low density vesicles. Stimulation of PKC with PMA resulted in a translocation of transporters from this compartment to the plasma membrane. At higher expression levels of GAT1 protein, a larger portion of GAT1 was found on the plasma membrane during basal level conditions and treatment with bisindolylmaleimide resulted in removal of these transporters from the plasma membrane. At expression levels demonstrated to be resistant to modulation by PMA, PMA-treatment still resulted in translocation of transporters from the cytoplasm to the plasma membrane. Thus, the inability of PMA to increase uptake at high expression of the GAT1 protein is due to saturation at a step subsequent to translocation. These findings 1) demonstrate the presence of a novel regulated secretory pathway in oocytes and 2) suggest a modulatory mechanism for neurotransmitter transporters that could have significant effects upon synaptic function.

Transporters are integral membrane proteins that use a chemical or electrochemical gradient to move molecules across membranes. The Na^+-dependent neurotransmitter transporters have been localized to the presynaptic terminals of neurons and surrounding glia, suggesting that one of their functions is to remove neurotransmitter from the synaptic cleft. This action could contribute to the termination of synaptic transmission (Iversen, 1975); however, electrophysiological analysis shows these transporters to have slow kinetic properties that could substantially limit their ability to shape synaptic events (Sarantis *et al.*, 1993; Mager *et al.*, 1993). This implies that, if neurotransmitter transporters are to affect the time course of synaptic transmission, they need to be present at high density at the synapse or increase their turnover rate. Either requirement suggests that modulation of transport could be critical to transporter function since regulatory mechanisms have been shown to affect both of these processes. For example, modulation of activity occurs for the facilitated glucose transporter via insulin-stimulated translocation of the protein from intracellular stores to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). Alternatively, modulation of function occurs for other membrane proteins (*e.g.*, ion channels) via alterations of various unitary properties of the protein (Kaczmarek, 1988; Sardet *et al.*, 1990). Identification of the genes encoding neurotransmitter transporters has shown them to possess putative phosphorylation sites (for review see Amara and Kuhar (1993)) which renders them candidates for modulatory mechanisms that could affect their activity.

There is some evidence for protein kinase-dependent modulation of endogenous neurotransmitter transporters. Using primary cultures from rat brain cortex, Casado *et al.* (1991) reported that phorbol esters produced a 50% increase in glutamate transport in glial cells while having no effect on glutamate transport in neurons. Additionally, Gomeza *et al.* (1991) showed that PKC[1] activation reduced GABA transport in glial cells, with no effect on neuronal GABA uptake. In both of these reports, the failure of phorbol esters to affect GABA and glutamate transport in neurons was not attributed to a lack of PKC or to a non-functional PKC. Rather, the dissimilar effects of phorbol esters on neuronal and glial transport were postu-

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[1] The abbreviations used are: PKC, protein kinase C; GABA, γ-aminobutyric acid; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PMA, phorbol 12-myristate 13-acetate; 4αPDD, 4α-phorbol 12,13-didecanoate; SC-10, *N*-heptyl-5-chloro-1-naphthalenesulfonamide; RER, rough endoplasmic reticulum.

lated to be due to different isotypes of PKC present in the two cell types (with varying sensitivity to phorbol esters), or to a smaller pool of PKC available in these neurons (Mudd and Raizada, 1990). Thus, it remains to be determined whether the lack of protein kinase-dependent modulation is a general feature of neuronal transporters, whether these transporters have the potential to be regulated via second messenger pathways and the mechanisms underlying such modulation.

We have begun to address these questions by examining the ability of a cloned rat brain neuronal GABA transporter (GAT1) to be modulated by protein kinases and phosphatases. The GAT1 clone (Guastella *et al.*, 1990) is representative of many of the recently cloned genes encoding neurotransmitter transporters in that its primary protein sequence shares significant homology with other members of this transporter subfamily and predicts a number of consensus phosphorylation sites (see Amara and Kuhar (1993)). In this report, we show that the activity of the GAT1 clone expressed in *Xenopus* oocytes is regulated by PKC and protein phosphatases and demonstrate that the observed modulation is mediated by a regulated subcellular redistribution of the transporter.

EXPERIMENTAL PROCEDURES

Materials—[^{3}H]GABA was purchased from DuPont NEN. Bisindolylmaleimide, OAG, PMA, 4αPDD, and SC-10 were purchased from Calbiochem (San Diego, CA). Okadaic acid and cyclosporin A were gifts of Dr. Joseph Farley (Indiana University, Bloomington). Horseradish peroxidase-conjugated donkey anti-rabbit Ig was purchased from Amersham Corp. All other reagents were obtained from Sigma. Cyclosporin A, okadaic acid, PMA, and 4αPDD were dissolved at high concentration in 100% ethanol; bisindolylmaleimide, (−)-indolactam V, OAG, and SC-10 were dissolved at high concentration in 100% Me_2SO. All drugs were then serially diluted in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.4) at least 100-fold prior to oocyte injection.

Antisera—The polyclonal rabbit antisera 342J was generated against a peptide corresponding to the carboxyl terminus (amino acids 588–599) of the rat brain GABA transporter GAT1 (Guastella *et al.*, 1990).

Synthesis of mRNA and Mutagenesis of GAT1—The DNA template encoding GAT1, which was used for *in vitro* transcription, was generated by polymerase chain reaction from: 1) linearized GAT1 in pBluescript; 2) a sense primer consisting of the T7 promoter, 6 bases of the alfalfa mosaic virus 5′-untranslated leader sequence (Kain *et al.*, 1991), an optimum translation initiation sequence (Kozak, 1987), and the first 18 nucleotides of the GAT1 coding sequence; and 3) an antisense primer consisting of (5′ to 3′) a T_{20} sequence and the terminal 18 bases of the GAT1 coding sequence. Mutations of the three putatively intracellular PKC phosphorylation sites were performed using the Clontech Transformer Site-Directed Mutagenesis Kit (Palo Alto, CA). Single bases at each of the three sites, Ser^{24}, Thr^{46}, and Ser^{562}, were mutated to create codons encoding Ala. The mutations were verified by sequencing through the appropriate regions. cRNA encoding both the wild-type and mutant transporters was generated using the Ambion Megascript *in vitro* Transcription Kit (Austin, TX).

Xenopus Oocyte Expression and [^{3}H]GABA Uptake Assays—These methods are described in detail elsewhere (Quick and Lester, 1994). Briefly, oocytes were defolliculated and maintained at 18 °C in ND96, supplemented with 2.5 mM sodium pyruvate, 50 μg/ml gentamycin, and 5% horse serum (Quick *et al.*, 1992). Either 1 or 5 ng of GAT1 cRNA was injected into each oocyte and [^{3}H]GABA uptake assays were performed 2–5 days post-injection. Unless otherwise noted, all modulation experiments were performed on GAT1 oocytes expressing basal GABA uptake levels of less than 10 fmol [^{3}H]GABA/oocyte/min. Vehicle solutions and drugs were administered by microinjection in 25-nl volumes. To measure GABA uptake, oocytes were incubated in ND96 containing 260 nM [^{3}H]GABA, after which the oocyte was washed three times in ND96, solubilized in 10% SDS, and quantified by liquid scintillation counting. Unless otherwise noted, values in the text are expressed as the mean ± S.D.

Effect of Microinjection on GABA Uptake in GAT1-expressing Oocytes—To standardize the drug delivery procedure, all compounds were introduced into the oocyte by microinjection. Two control experiments were performed to verify that there were no changes in uptake due to the microinjection technique: 1) 25 nl of ND96 medium were injected to examine the effect of microinjection on GABA uptake, and 2) ethanol and $MeSO_4$ (other vehicles used for drug solubilization) were compared to ND96-injected oocytes. The results of these two experiments showed no effect of ND96, ethanol, or $MeSO_4$ control injections on GABA uptake (data not shown). The approximate final concentrations of ethanol and $MeSO_4$ inside the oocyte, assuming an oocyte volume of 0.5 μl, were 2 and 0.2%, respectively; these concentrations were at least 4-fold higher than the concentrations used for drug solubilization in the experiments described below.

We find that microinjection provides a reliable method for drug administration and provides several advantages over incubation methods. First, injection of the drug into the oocyte eliminates the requirement for diffusion across the oocyte membrane, thus shortening the preincubation period. This allows the focus to be on effects which are primarily post-transcriptional/post-translational. Microinjection into the cytoplasm also reduces the possibility that the compound or solvent is affecting the transporter indirectly by diffusion into the membrane bilayer. Ethanol, $MeSO_4$, and fatty acids can intercalate into the membrane and alter membrane fluidity (Swann, 1984; Klausner *et al.*, 1980) and such action has, in turn, been shown to affect the Cl^- transporter in corneal epithelium (Schaeffer and Zadunaisky, 1979), and the Na^+,K^+-ATPase in bovine brain microsomes (Swann, 1984).

Fractionation of Membranes—Total membrane protein was isolated from uninjected and GAT1-expressing oocytes by homogenization in 0.32 M sucrose in TE buffer (50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 200 μM phenylmethlysulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) with 15 strokes of a tight-fitting pestle in a chilled Dounce homogenizer. The homogenate was centrifuged twice at 1000 × *g* and membranes were pelleted at 40,000 rpm in a Beckman SW 41 rotor for 30 min at 4 °C. The pellets were resuspended in 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, and the protein content was determined by Bio-Rad Protein Assay (Richmond, CA). The fractionation protocol used was a modification from Corey and Stallcup (1992). Oocytes expressing GAT1 were injected with either PMA (final concentration 400 nM) or bisindolylmaleimide (final concentration 100 nM) (150 oocytes were injected within 20 min) and were incubated for an additional 10 min prior to homogenization. Groups of 150 oocytes each were homogenized in 2 ml of 0.32 M sucrose and centrifuged as described above. The final supernatant was immediately layered on a discontinuous sucrose gradient of 2.0 M (2 ml), 1.3 M (3.2 ml), 1.0 M (3.2 ml), and 0.6 M (2.0 ml) sucrose in TE buffer containing 5 mM $MgCl_2$. After centrifugation at 40,000 rpm in a Beckman SW 41 rotor for 4 h at 4 °C, 1-ml fractions were collected from the bottom of the gradient, diluted 4-fold with 0.15 M sucrose in TE buffer, and the microsomal membranes in each fraction were pelleted at 48,000 rpm in a Beckman SW 50.1 rotor for 3 h at 4 °C. The pellets were resuspended in either 80 μl of 50 mM Tris-Cl, pH 7.5, 1 mM EDTA (for membrane marker assays) or 15 μl of sample buffer (Laemmli, 1970) (for gel electrophoresis). All samples were stored at −80 °C.

Marker Enzyme Assays for Rough Endoplasmic Reticulum, Plasma Membrane, and Trans-Golgi—The rough endoplasmic reticulum membrane marker, glucose 6-phosphatase, was assayed according to Corey and Stallcup (1992). Identification of plasma membranes and Golgi membranes were by assays of Na,K-ATPase and galactosyltransferase activity, respectively, and were performed by the methods of Hensley *et al.* (1989).

Electrophoretic and Immunoblotting Analysis—Microsomal membrane fractions stored at −80 °C were thawed at room temperature. Proteins were separated on 10% SDS-polyacrylamide gels (Laemmli, 1970), transferred to nitrocellulose membranes (Schleicher & Scheull) for 4 h at 50 V, and then blocked overnight at 4 °C in PBS containing 0.1% Tween-20, 5% skim milk, and 1% donkey serum. Antibodies were diluted and the membranes were washed in phosphate-buffered saline containing 0.1% Tween-20 and 5% skim milk. The immunoblotting procedure consisted of the following steps at room temperature: incubation for 60 min with the polyclonal antibody against GAT1 (342J) diluted 1:200, three 10-min washes, incubation for 30 min with horseradish peroxidase-conjugated donkey anti-rabbit Ig antibody diluted 1:1000, three 10-min washes, followed by detection using ECL reagents (Amersham Corp.). The antibody recognized a 67-kDa band in oocytes expressing GAT1 that was not present in uninjected oocytes (Fig. 1) and the size is in agreement with the size of the product from an *in vitro* translation of GAT-1 in the absence of microsomal membranes (Guastella *et al.*, 1990). This is consistent with the conclusion that GAT-1 synthesized in oocytes does not contain *N*-linked sugars and was confirmed by lack of digestion of the 67-kDa band by endoglycosidase F/N-glycanase (Boehringer Mannheim) (data not shown). Quantification of band densities was done on the LKB Ultrascan laser densitometer.

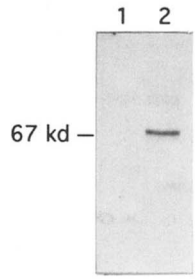

FIG. 1. **Immunoblot analysis of GAT1 expressed in *Xenopus* oocytes.** Membranes (60 μg of protein/lane) prepared from uninjected oocytes (*lane 1*) and oocytes expressing GAT1 (*lane 2*) were subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose filter. The filter was incubated with rabbit antisera raised against synthesized peptide corresponding to the COOH-terminal domain of GAT1 (amino acids 588–599). Secondary antibody conjugated to horseradish peroxidase was incubated with the filters, and the bands were visualized by enhanced chemiluminescence.

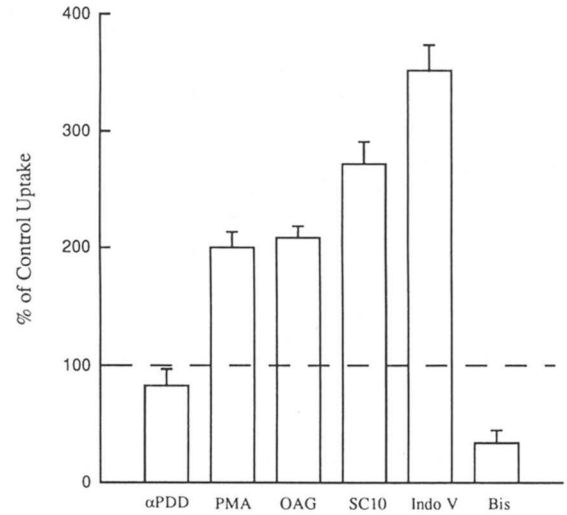

FIG. 2. **Effects of PKC activation and inhibition on [^{3}H]GABA uptake.** Oocytes were injected with 1 ng of GAT-1 cRNA and assayed for [^{3}H]GABA uptake 2–4 days post-injection. All drugs were injected into the oocyte in 25-nl volumes to the following final concentrations: αPDD (0.4 μM), PMA (0.1 μM), OAG (0.5 μM), SC-10 (0.3 μM), indolactam V (2.5 μM), and bisindolylmaleimide (0.1 μM). The assay time was 15 min and was performed 30 min after microinjection of drugs. [^{3}H]GABA uptake data for the drug-injected oocytes are listed as a percentage of the uptake in vehicle-only injected oocytes. Each value represents the mean ± S.D. for four or five oocytes/condition; data shown for individual drugs were taken from a single batch of oocytes tested on the same day.

RESULTS

GABA Uptake in GAT1-expressing Oocytes Is Modulated by Activation and Inhibition of PKC—Four different PKC activators were found to increase GABA transport (Fig. 2). In GAT1-expressing oocytes injected with 0.1 μM (final concentration) of the phorbol ester PMA, GABA transport was increased to 206 ± 14% of basal values. This stimulation of GABA transport 1) did not occur following injection of 0.4 μM of the inactive phorbol ester 4αPDD (83 ± 14% of basal values) and 2) was not due to drug-induced changes in protein synthesis, since co-injection of 60 μg/ml cycloheximide, which resulted in greater than 80% inhibition of protein synthesis as determined by trichloroacetic acid precipitation, did not inhibit the PMA-induced stimulation (data not shown). In addition to PMA, three other activators of PKC were found to increase GABA transport over control values: 0.5 μM OAG (Gilmore and Martin, 1983), 208 ± 10%; 0.3 μM SC-10 (Nishino *et al.*, 1986), 272 ± 19%; and 2.5 μM (−)-indolactam V (Sugimura, 1982), 352 ± 22%. In support of the role of PKC in modulating GABA transport activity, injection of 0.1 μM bisindolylmaleimide, an inhibitor of PKC (Toullec *et al.*, 1991), reduced basal GABA uptake to 30 ± 13% of control values. Additionally, GAT1-expressing oocytes were injected with either 0.1 μM bisindolylmaleimide or 0.4 μM PMA alone, or in combination (Table I). The 2-fold stimulation of GABA transport produced by PMA was completely blocked by co-injection of bisindolylmaleimide.

TABLE I
GABA uptake in GAT1-expressing oocytes

Drug	Concentration	Percent of control uptake
	μM	*mean ± S.D.*
PMA	0.4	212 ± 15
Bisindolylmaleimide	0.1	23 ± 21
PMA + bisindolylmaleimide	0.4 + 0.1	22 ± 10
PMA	0.1	212 ± 10
Cyclosporin A	0.1	226 ± 14
PMA + cyclosporin A	0.1 + 0.1	284 ± 12

Inhibition of Protein Phosphatases Increases GABA Transport—The finding that PKC activators stimulated GABA transport suggested that maintenance of proteins in a phosphorylated state might be responsible for increasing transport. Therefore, we tested whether inhibition of cellular phosphatases would also result in an increase in GABA transport (Fig. 3). Injection of 100 nM okadaic acid (*open circles*) had no effect on uptake in GAT1-expressing oocytes. However, transport was increased to 154 ± 14% and 260 ± 5% of basal values with injection of 1 and 10 μM, respectively. Injection of 25 nM cyclosporin A, an inhibitor of PP2B (calcineurin) (Friedman and Weissman, 1991), increased GABA uptake to 121% (*closed circles*), and maximal 5-fold increases were observed with 400 nM. Furthermore, we injected GAT1-expressing oocytes with either cyclosporin A or PMA, or both. Transport was increased approximately 2-fold when each drug at 100 nM was injected alone; combined injection stimulated transport almost 3-fold over control values suggesting the effects are partially additive (Table I).

Modulatory Effects of PMA and Bisindolylmaleimide Are Concentration- and Time-dependent—PMA had no effect on GABA transport at 10 nM while 50 nM increased transport to approximately 130% of control values (Fig. 4, *open circles*). Maximal increases were achieved with injections of 100 nM. Injection of 1 nM bisindolylmaleimide decreased GABA uptake approximately 20%, and maximal inhibition of 75% occurred with 50 nM (Fig. 4, *closed circles*).

The time course of GABA uptake stimulation by PMA (400 nM), okadaic acid (10 μM), or cyclosporin A (1.0 μM), and inhibition by bisindolylmaleimide (100 nM), is shown in Fig. 5. Okadaic acid (*open squares*) and cyclosporin A (*closed squares*) showed maximal stimulation of transport (approximately 280 and 200%, respectively) 30 min after injection, and this increase remained stable for the duration of the 50-min assay. PMA (*circles*) produced peak stimulation (approximately 225% of control) after 40 min, followed by a slight decline thereafter. Bisindolylmaleimide (*triangles*) inhibited uptake by 40% within 15 min, and maximal inhibition of 80% was observed by 35 min.

Kinetic Analyses of GABA Transport during Modulation—The mechanism underlying the observed modulation was examined by kinetic analyses of GABA transport in the presence or absence of bisindolylmaleimide or PMA. Analysis of an Eadie-Hofstee plot for ND96-injected GAT1-expressing oocytes determined a K_m of 4.1 μM and a V_{max} of 58.4 fmol/min (Fig. 6*A*). Treatment of a parallel group of oocytes with bisindolylmaleimide had little effect on K_m (5.2 μM) but decreased V_{max} by 56% (25.6 fmol/min). In a separate experiment examining the effects

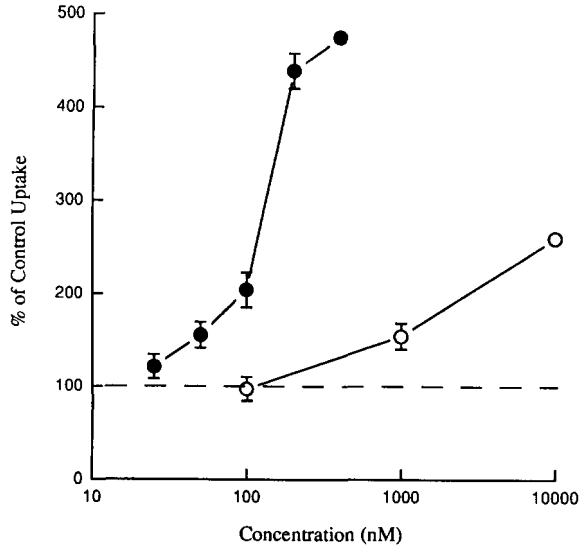

FIG. 3. **Effects of okadaic acid and cyclosporin A on [^{3}H]GABA uptake.** Oocytes were injected with 1 ng of GAT-1 cRNA and assayed for [^{3}H]GABA uptake 2–3 days post-injection. Okadaic acid (*open circles*) or cyclosporin A (*closed circles*) was injected into the oocyte in 25-nl volumes to final concentrations as shown. [^{3}H]GABA uptake was assayed 30 min after microinjection. [^{3}H]GABA uptake data for the drug-injected oocytes are listed as a percentage of the uptake in vehicle-only injected oocytes. Each value represents the mean ± S.D. for four or five oocytes/condition. The data are representative of three experiments.

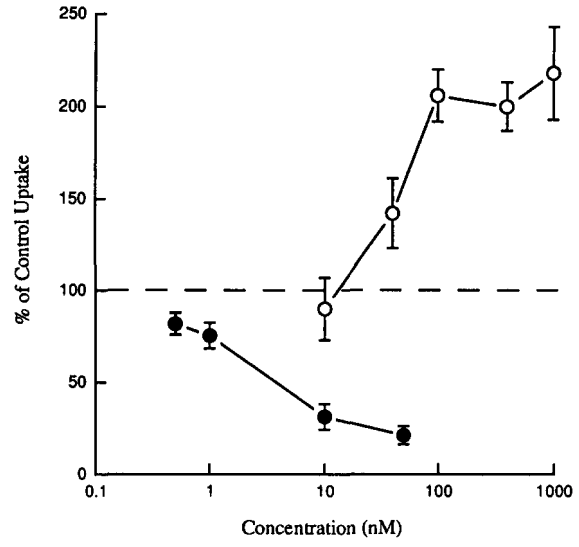

FIG. 4. **Concentration curves for PMA and bisindolylmaleimide.** Oocytes were injected with 1 ng of GAT-1 cRNA and assayed for [^{3}H]GABA uptake 2–3 days post-injection. PMA (*open circles*) or bisindolylmaleimide (*closed circles*) was injected into the oocyte in 25-nl volumes to final concentrations as shown. [^{3}H]GABA uptake was assayed 30 min after microinjection. [^{3}H]GABA uptake data for the drug-injected oocytes are listed as a percentage of the uptake in vehicle-only injected oocytes. Each value represents the mean ± S.D. for four or five oocytes/condition. The data are representative of three experiments.

of PMA, the K_m and V_{max} values determined for control oocytes were 5.8 μM and 1.1 fmol/min, respectively (Fig. 6*B*). Treatment of oocytes with PMA had no effect on K_m (5.3 μM) but increased V_{max} by almost 200% (2.1 fmol/min). These results indicate that the PMA- and bisindolylmaleimide-induced effects on GABA transporter activity are primarily due to changes in the maximum velocity of transport, with only minimal changes to the apparent ligand affinity.

Effect of GAT1 Expression Level on Modulation of [^{3}H]GABA Uptake—Throughout the concentration and time course experiments, it was noted that the expression level of the transporter protein had a significant effect on the modulation of GABA uptake. We examined this effect more closely through *post hoc* analyses of our PMA and bisindolylmaleimide data as a function of various GAT1 expression levels. The results of these analyses are shown in Fig. 7. Since there is currently no suitable ligand for measuring B_{max}, the expression level of the transporter was quantified by measuring GABA uptake (femtomoles of [^{3}H]GABA/oocyte/min) in vehicle-injected (control) oocytes expressing GAT1. Injections of 400 nM PMA (*filled circles*) produced a 1.5–3.2-fold increase over control transport at expression levels of 5 fmol of [^{3}H]GABA/oocyte/min or less. This up-regulation of activity decreased as expression level increased. At expression levels greater than approximately 13 fmol of [^{3}H]GABA/oocyte/min, PMA had little effect on transporter activity compared to control oocytes. Across this same range of expression, there was no effect of expression level on transporter activity inhibition by bisindolylmaleimide (*open circles*).

This effect was more readily observed within the same batch of oocytes in which varying expression levels were achieved by performing uptake assays at different times after GAT1 injection. The effect of PMA (Fig. 7, *connected circles*) was highest at an expression level of approximately 2 fmol/oocyte/min (220%) and decreased (to 150%) as expression increased to 4 fmol of [^{3}H]GABA/oocyte/min. At an expression level of 14 fmol of [^{3}H]GABA/oocyte/min, PMA increased transporter activity by only 20% and the effect was no longer observed when expression reached 16 fmol/oocyte/min. The concentration of [^{3}H]GABA was in at least 10-fold excess; therefore, the absence of stimulation of transporter activity at high GAT1 expression levels was not due to a limiting amount of substrate.

Effect of Removal of Putative PKC Sites on Modulation of GAT1 by PMA and Bisindolylmaleimide—To determine if modulation was occurring via direct phosphorylation of the putative PKC sites on GAT1, we generated a mutant trans-

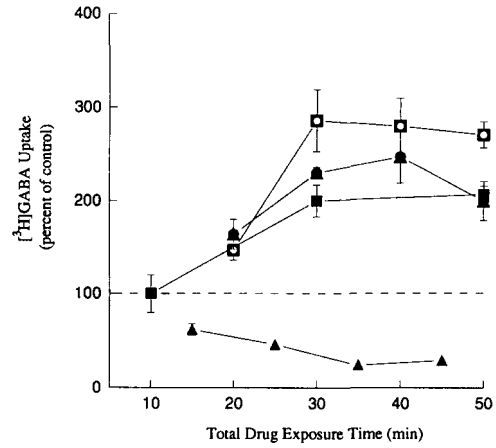

FIG. 5. **The time course of increases and decreases in [^{3}H]GABA uptake subsequent to drug application in GAT-1 injected oocytes.** Oocytes were injected with 1 ng GAT-1 cRNA and assayed for [^{3}H]GABA uptake 2–3 days post-injection. All drugs were injected into the oocyte in 25-nl volumes to the following final concentrations: 0.1 μM bisindolylmaleimide (*triangles*), 1 μM cyclosporin A (*squares*), 0.4 μM PMA (*circles*), and 10 μM okadaic acid (*open squares*). The total exposure time (*abscissa*) refers to the time following drug injection, including the assay time. Assay times ranged from 5–30 min. [^{3}H]GABA uptake data for the drug-injected oocytes are plotted on the *ordinate* as a percentage of the uptake in vehicle-only injected oocytes. Each data point represents the mean ± S.D. for four or five oocytes/condition; data shown for individual curves were taken from within the same batch of oocytes tested on the same day. The S.D. for the vehicle-injected control oocytes ranged from ±9 to ±19%.

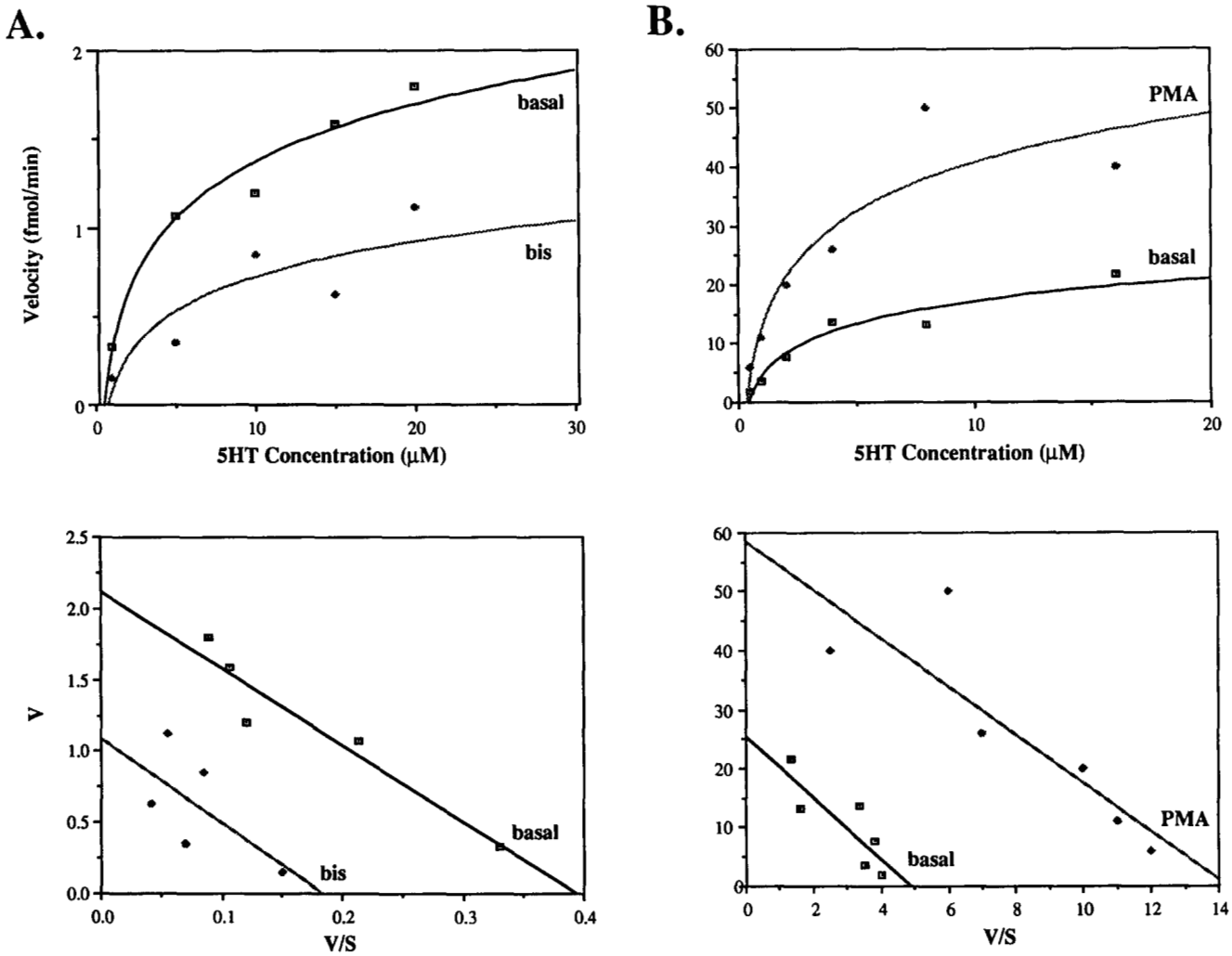

FIG. 6. **Saturation curves and Eadie-Hofstee transformations for control, bisindolylmaleimide-, and PMA-treated oocytes.** [^{3}H]GABA accumulation was measured for GABA concentrations from 0.5 μM to 20 μM. Oocytes were either untreated (*basal*), injected with 100 nM bisindolylmaleimide (*A*) or injected with 400 nM PMA (*B*) 20 min prior to the 15 min assay. Eadie-Hofstee plots were obtained with the data sets from the saturation curves. *V*, GABA uptake (femtomoles/oocyte/min); and *S*, free substrate concentration. The average standard error of the mean for both experiments was 14.6%. Experiments shown are representative of four separate experiments.

porter in which all three of the intracellular consensus PKC sites were eliminated. Removal of the consensus PKC sites did not affect the modulation of the transporter by either PMA or bisindolylmaleimide compared to the wild-type GABA transporter (Fig. 8). Similar results were obtained from three experiments across three different batches of oocytes. In each experiment, the expression level of the wild-type and mutant proteins were approximately equal (data not shown). It is possible that modulation could result from direct phosphorylation of non-consensus PKC sites; however, we chose to investigate other potential mechanisms for modulation.

Effects of PMA and Bisindolylmaleimide on Oocyte Membrane Potential—The transporter encoded by GAT1 is electrogenic (Kavanaugh *et al.*, 1992; Mager *et al.*, 1993) and thus the driving force for GABA transport is dependent upon the resting membrane potential of the oocyte. Therefore, one hypothesis is that the observed modulation is due to drug-induced changes in membrane potential. To test this, we used a standard two-electrode voltage-clamp configuration (Quick and Lester, 1994) and made measurements of oocyte membrane potentials 5 min prior to microinjection and then continuously 5–40 min post-injection. The oocytes were injected with ND96, ethanol, $MeSO_4$, PMA, or bisindolylmaleimide (three to five oocytes/condition; three-oocyte batches). Pre-injection membrane potentials of all oocytes measured were between −35 and −50 mV. In 70% of the oocytes tested, a slight change from the base line resting potential (<8 mV) to less negative potentials was observed, but only within the first 10 min post-injection. These changes appeared to be related to the microinjection procedure and were not specific to any of the injected modulatory drugs. Therefore, PMA and bisindolylmaleimide are not modulating GABA transport by exerting an effect on membrane potential.

Effects of PMA and Bisindolylmaleimide on GAT1 Subcellular Localization—The PKC-mediated changes in V_{max}, the dependence of modulation on expression level, and the inability to eliminate the observed modulation by removal of the consensus PKC sites suggested to us that the effect could be due to movement of transporters to and from the plasma membrane. This type of modulation has been well characterized for the facilitated glucose transporters: insulin-activated glucose uptake into fat cells is due to translocation of transporters from intracellular vesicles to the plasma membrane (Cushman and Wardzala, 1980; Suzuki and Kono, 1980). To determine if GAT1 is modulated by a similar mechanism it was necessary to identify the intracellular compartment(s) in which GAT1 resides. This was done by subcellular fractionation of the oocyte to separate the membranes of the rough endoplasmic reticulum (RER), plasma membrane, and trans-Golgi. Membrane prepa-

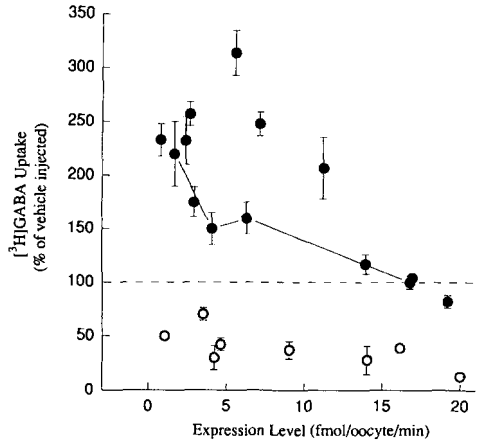

FIG. 7. **Effect of GAT-1 expression level on modulation of [^{3}H]GABA uptake by PMA and bisindolylmaleimide in oocytes.** Oocytes were injected with 1 or 5 ng of GAT-1 cRNA and assayed for [^{3}H]GABA uptake 2–5 days post-injection. The assay time was 15 min. Prior to assay, the oocytes were injected with 25 nl of a vehicle solution, or a solution containing PMA (0.4 μM, 15–25 min before assay) or bisindolylmaleimide (100 nM, 0–10 min before assay). The expression level was quantified as the amount of [^{3}H]GABA uptake in the vehicle-injected oocytes and is plotted on the *abscissa*. The percent change in uptake compared to the vehicle-injected oocytes is plotted on the *ordinate* for PMA-injected (*closed symbols*) and bisindolylmaleimide-injected (*open symbols*) oocytes. Each data point represents the mean ± S.E. for three to five oocytes. Data points connected by the solid line represent an experiment performed on the same batch of injected oocytes; different expression levels for this batch were achieved by performing the assay at varying times after cRNA injection. The standard error of the means for the vehicle-injected oocytes varied from ±2 to ±24% (mean = ±10.7%).

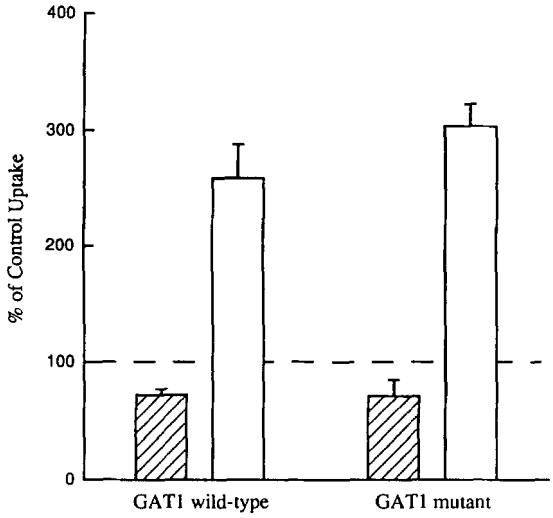

FIG. 8. **Effect of removal of PKC sites on modulation of GAT1 activity by PMA and bisindolylmaleimide.** Single bases at each of the three PKC consensus sites, Ser^{24}, Thr^{46}, and Ser^{562}, were mutated to create codons encoding Ala. The mutations were verified by sequencing through these regions. cRNA encoding both the wild-type and mutant transporters was generated and 1 ng was injected into oocytes which were assayed 2–3 days post-injection. Either PMA or bisindolylmaleimide was microinjected to a final concentration of 400 and 100 nM, respectively. The oocytes were assayed for [^{3}H]GABA uptake 15 min after drug injection; the assay time was 30 min. [^{3}H]GABA uptake data for the drug-injected oocytes are plotted on the *ordinate* as a percentage of the uptake in vehicle-only injected oocytes. Each data point represents the mean ± S.D. for four or five oocytes/condition; data shown for individual curves were taken from within the same batch of oocytes tested on the same day.

rations from uninjected oocytes that were untreated (basal level), PMA-injected, or bisindolylmaleimide-injected were fractionated on a discontinuous sucrose gradient. Fractions collected from the gradient were assayed for specific enzymes and a typical gradient profile is presented in Fig. 9. As expected, each marker enzyme was enriched at the appropriate gradient interface: glucose 6-phosphatase (identifying RER membranes) at the lowest gradient interface corresponding to *fractions 2* and *3*, Na^+/K^+ ATPase (plasma membrane) at the middle interface or in *fraction 5*, and galactosyltransferase (trans-Golgi) at the upper interface (*fractions 9* and *10*). Distribution of the marker enzymes, and thus the corresponding membranes, were unaffected by PMA and bisindolylmaleimide treatment (Fig. 9).

To determine if PMA was inducing a translocation of cytoplasmic transporters to the plasma membrane, we performed subcellular fractionation of basal and PMA-treated oocytes and a representative experiment is shown in Fig. 10. In the basal untreated state all of the detectable GAT1 protein was found in a subcellular compartment corresponding to the trans-Golgi network (Fig. 10*A*, *fractions 8–10*). This indicates that the transporter is primarily targeted to a cytoplasmic compartment for storage rather than expressed constitutively on the plasma membrane. After PMA treatment, the population of transporters previously found in the Golgi fractions was present on the plasma membrane (Fig. 10*B*, *fraction 5*), leaving no detectable transporter in the Golgi fractions (Fig. 10*B*, *fractions 9* and *10*). A band of approximately 80 kDa is also observed in several of the fractions and was determined to be nonspecific due to its presence in uninjected oocytes (data not shown). [^{3}H]GABA uptake assays using oocytes taken prior to homogenization confirmed that PMA treatment increased transport activity as expected and indicated the presence of functional transporters on the plasma membrane below the level of detection by immunoblot analyses.

We next investigated whether the reduction in uptake activity seen with bisindolylmaleimide was due to removal of transporters from the cell surface. Oocytes were either untreated or microinjected with bisindolylmaleimide (100 nM final concentration) 30 min prior to homogenization. Since the effect of bisindolylmaleimide on transport activity was found to be independent of expression level (see Fig. 7 above), we expressed GAT1 at higher levels (confirmed by uptake assays performed in parallel) to ensure detectability of the transporter on the plasma membrane in the basal state. The amount of transporter in each fraction was quantified by densitometry and calculated as a percentage of total transporter in all of the fractions. Localization of transporter during the basal state (Fig. 11*A*) demonstrated approximately 16% of total transporters located in *fraction 5*, corresponding to plasma membrane; the remaining 84% were found in *fractions 9* and *10*, corresponding to Golgi-associated membranes. We think it likely that the increased detectability of transporter associated with the plasma membrane fractions was not only due to an increase in overall transporter synthesis at the higher expression level (as determined by prior uptake analyses) but also due to an "overflow" of transporter from a regulated pathway to a constitutive pathway, as discussed in further detail below. In oocytes that had been treated with bisindolylmaleimide (Fig. 11*B*), there were no detectable transporters in *fraction 5*, indicating that the remaining transporters associated with the plasma membrane were below detectability by immunoblot.

The data from the uptake experiments demonstrated that high expression levels of GAT1 protein eliminates the ability of PMA to modulate transport activity. The data from the subcellular fractionation experiments demonstrated that PMA treatment results in translocation of transporters from the cytoplasm to the plasma membrane. These observations raise the question of whether the inability of PMA to modulate transport activity at higher expression levels is related to the translocation of transporters or another mechanism. To address this

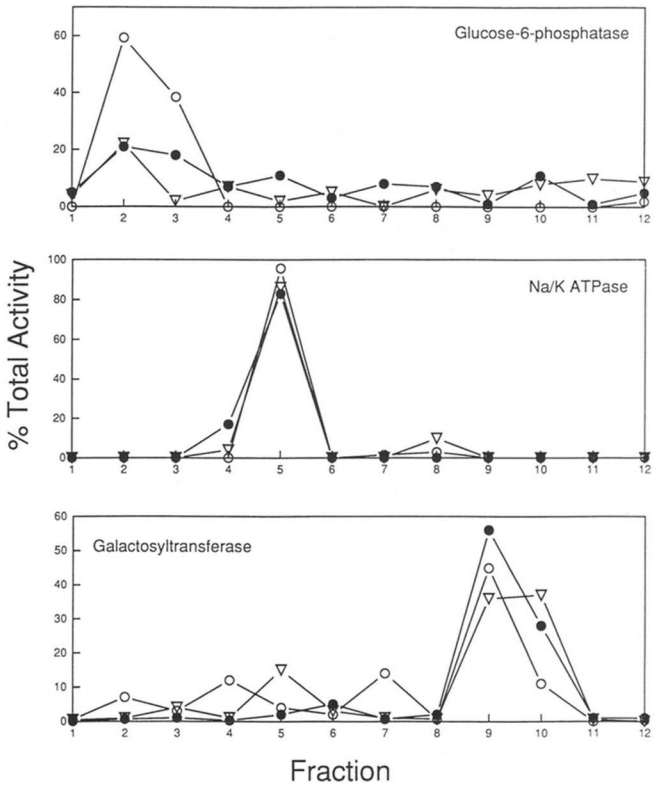

FIG. 9. **Sucrose gradient profiles for marker enzymes identifying RER, plasma membrane, and trans-Golgi.** Membranes were prepared from uninjected oocytes that were untreated (basal level), PMA-injected (0.4 μM), and bisindolylmaleimide-injected (0.1 μM), and analyzed by equilibrium sedimentation on discontinuous sucrose gradients, as described under "Experimental Procedures." Marker enzymes were assayed as described, and the enzyme activity in each fraction was plotted as a percentage of the total enzyme activity found in all fractions. This is a representative example from four separate experiments.

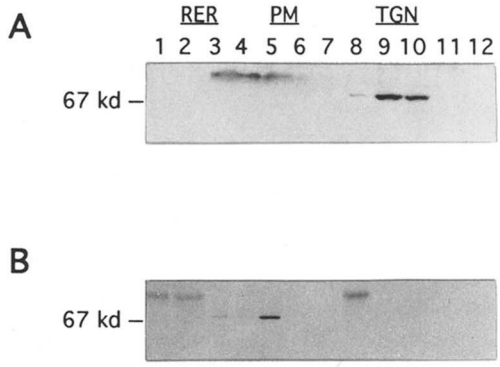

FIG. 10. **The effect of PMA on the subcellular distribution of the GAT1 protein.** Membranes were prepared from GAT1-expressing oocytes that were either untreated (basal level) (*A*) or PMA-injected (0.4 μM) (*B*). They were analyzed by equilibrium sedimentation on discontinuous sucrose gradients as described under "Experimental Procedures." The membrane pellets were resuspended and subjected to SDS-polyacrylamide electrophoresis, transferred to nitrocellulose, and GAT1 protein was visualized by immunoblotting. This is a representative example from four separate experiments. TGN, trans-Golgi network.

question, we performed subcellular localization analyses of the GAT1 protein in oocytes expressing high levels of transporter during the basal state and also after PMA treatment (Fig. 12*A*). By uptake assay, we determined that the expression level was high enough that transporter activity was not altered by PMA treatment (data not shown). During the basal state, 45% of total transporters were found associated with the plasma membranes (*fraction 5*) and 55% were located in fraction 9, corresponding to low density vesicles. After treatment with PMA, 88% of the transporters were found associated with the plasma membranes (*fraction 6*) and 12% of the transporters remained associated with the Golgi fractions (Fig. 12*B*).

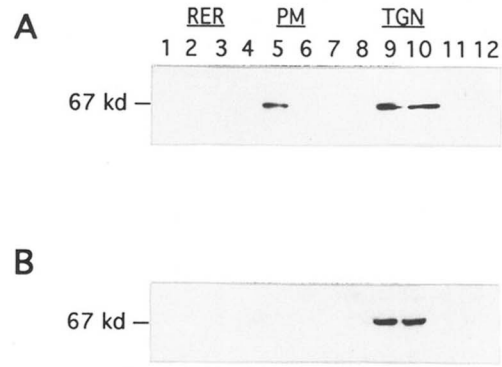

FIG. 11. **The effect of bisindolylmaleimide on the subcellular distribution of GAT1.** Membranes were prepared from GAT1-expressing oocytes that were either untreated (basal level) (*A*) or bisindolylmaleimide-injected (0.1 μM) (*B*). They were analyzed by equilibrium sedimentation on discontinuous sucrose gradients as described under "Experimental Procedures." The membrane pellets were resuspended and subjected to SDS-polyacrylamide electrophoresis, transferred to nitrocellulose, and GAT1 protein was visualized by immunoblotting. This is a representative example from four separate experiments. TGN, trans-Golgi network.

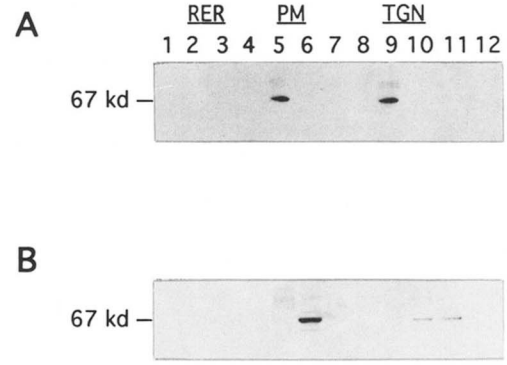

FIG. 12. **The effect of high expression levels of GAT1 protein on PMA-induced translocation of the transporter.** Membranes were prepared from oocytes expressing uptake levels of 34 fmol/min/oocyte that were untreated (basal level) (*A*) or PMA-injected (*B*). They were analyzed by equilibrium sedimentation on discontinuous sucrose gradients, as described under "Experimental Procedures." The membrane pellets were resuspended, subjected to SDS-polyacrylamide electrophoresis, and transferred to nitrocellulose, and GAT1 protein was visualized by immunoblotting. TGN, trans-Golgi network.

DISCUSSION

It is thought that one of the major functions of neurotransmitter transporters is the termination of synaptic transmission by uptake of transmitter from the synaptic cleft into surrounding neurons and glia. Therefore, the stimulation or inhibition of transport via second-messenger pathways could play a significant role in synaptic function. Molecular cloning and characterization of the neurotransmitter transporters reveal putative consensus phosphorylation sites (Amara and Kuhar, 1993), lending support to the possibility of transporter modulation. In primary astrocyte cultures, phorbol esters affect glutamate and GABA uptake differentially, increasing glutamate transport while decreasing GABA transport. Modulation of a cloned neurotransmitter transporter expressed heterologously would provide an opportunity to further these observations and examine potential mechanisms underlying these effects. Through the use of direct cytoplasmic microinjection of second-messenger

activators and inhibitors we have demonstrated the modulation of a GABA transporter expressed in *Xenopus* oocytes and describe the mechanism underlying this modulation.

Activation of PKC by both phorbol esters (PMA and OAG) and other agents (SC-10 and (−)-indolactam V) increased GABA transport. PMA-induced stimulation could be blocked by bisindolylmaleimide, suggesting that PKC was responsible for the increase in transport. The increase in transport elicited by PMA was not due to an increase in transport protein synthesis since the effect was observable in the presence of cycloheximide. In addition, the increase in transport was seen after acute treatment with PMA, lending support to a post-translational effect on the transporter. The phosphatase inhibitors okadaic acid and cyclosporin A also increased GABA transport, implying that the maintenance of proteins in a phosphorylated state is involved in modulation. However, removal of the consensus PKC sites found in the primary amino acid sequence of GAT1 had no effect on the regulation of transporter activity, suggesting that phosphorylation could be occurring at non-consensus sites on the transporter or the PKC effect on transporter activity was via an indirect mechanism.

An indirect modulatory mechanism is observed for the facilitated glucose transporters in that insulin rapidly activates glucose transport in fat cells, primarily by translocating the GLUT1 and GLUT4 isoforms from subcellular compartments to the plasma membrane (Wardzala *et al.*, 1978; Blok *et al.*, 1988; Calderhead *et al.*, 1990). Therefore, we chose to test the hypothesis that PKC was modulating uptake activity by redistribution of the GABA transporter from a cytoplasmic location to the plasma membrane. Subcellular localization of the transporter in the basal state and after PMA treatment demonstrated that modulation was consistent with changes in membrane trafficking. In the basal state, the majority of transporters were found in a compartment corresponding to the trans-Golgi, or low density vesicles, with little or no detectable transporter on the plasma membrane. After PMA treatment, transporters were no longer found in the trans-Golgi compartment but only on the plasma membrane, indicating that PKC activation resulted in transporter translocation. Other data we obtained are consistent with this finding. 1) The time course we observed for the PMA-induced increase in transporter activity is comparable to the time course for the translocation kinetics of the facilitated glucose transporter (Yang *et al.*, 1992), and 2) the kinetic data demonstrate that modulation affected primarily V_{max} rather than K_m, suggesting changes in the number of transporters on the cell surface resulting from increased exocytosis. It is also possible that the observed changes in V_{max} are due to changes in transporter turnover rate rather than changes in the number of surface transporters; it is difficult to quantify the actual number of surface carriers because there is currently no suitable ligand for determining a true B_{max}. In addition, the modulation may not be completely due to translocation of transporters but may also involve conversion of transporter between active and inactive states. This possibility is supported by the evidence indicating that at high expression levels of the transporter, PMA still stimulates the translocation of transporter from the cytoplasm to the plasma membrane yet there is no measurable increase in transport activity.

Inhibition of PKC with bisindolylmaleimide resulted in a decrease in transporter activity during the basal state and blocked transporter stimulation by PMA. This latter effect is most probably due to the inhibition of the PKC-induced translocation of transporters to the plasma membrane. However, the reduction of transporter activity from basal levels requires additional interpretation. The decrease in V_{max} after bisindolylmaleimide treatment is consistent with removal of transporter from the cell surface and this is confirmed by the subcellular localization of the transporter during the basal state and after PKC inhibition. This suggests a low level of transporter that is continuously on or recycled to the plasma membrane and that this is mediated by basal PKC activity. Further activation of PKC may alter this relationship, resulting in an increase in transporters moving to the plasma membrane and/or remaining on the cell surface. Another possibility is that down-regulation of transporter activity by bisindolylmaleimide is due to a selective degradation of surface transporters. However, we think this hypothesis is unlikely since there is still a reservoir of cytoplasmic transporters that can be recruited to the plasma membrane by the same mechanism that places transporters on the cell surface during the basal state. Therefore, inhibition of PKC is most likely affecting a translocation process rather than a degradative one.

Two basic types of exocytotic pathways exists in eukaryotic cells: 1) a constitutive pathway in which macromolecules are transported to the cell surface as soon as they are synthesized and 2) a regulated pathway in which macromolecules are targeted for storage in cytoplasmic vesicles until a triggering event stimulates exocytosis. We found that the GABA transporter expressed in *Xenopus* oocytes is targeted to a regulated pathway for storage until PKC activation triggers translocation of transporters to the plasma membrane. As transporter protein levels increased so did the amount of transporter found on the plasma membrane during the basal state. This finding is consistent with evidence that overexpression of heterologously expressed proteins results in normally regulated proteins being expressed constitutively, as if "overflowing" to the alternate pathway.[2]

An important question resulting from our study is how GAT1 is targeted to a cytoplasmic storage location under basal conditions. Of the facilitated glucose transporters endogenously expressed in adipocytes and muscle cells, GLUT1 is expressed constitutively on the plasma membrane while GLUT4 is targeted to cytoplasmic vesicles (Harrison *et al.*, 1990). This observation extends to expression of these two isoforms in mammalian cell lines (Shibasaki *et al.*, 1992), thus allowing Asano *et al.* (1992) to construct chimeras between the two isoforms in order to identify potential targeting signals. Their results suggest that two domains govern targeting of GLUT4 to cytoplasmic vesicles, of which one domain encompasses a leucine heptad repeat. A leucine zipper motif is also found in GAT1 (Guastella *et al.*, 1990) and appears to be conserved among other members of the neurotransmitter transporter family (Amara and Kuhar, 1993); however, no function has been attributed to this region.

GAT1 expression level influenced the ability of PMA to stimulate transport activity; however, overexpression of transporter protein does not appear to saturate the "machinery" necessary for translocation. Rather it affects a step subsequent to translocation, possibly vesicle fusion with the plasma membrane. It is unclear why this effect occurs since the transporter can be expressed at even higher levels than at that which we found no increase in activity upon PMA treatment. This is an important question that remains to be resolved and which will potentially be elucidated when the steps involved in the observed modulation are described in further detail.

The steps involved in the movement of secretory vesicles has been well studied (for review see Kelly (1993)), and the entire neurotransmitter secretory mechanism has been reconstituted in several systems, including *Xenopus* oocytes (Alder and Poo, 1993). For example, Ca^{2+}-dependent secretion of glutamate has been demonstrated in oocytes injected with total rat brain mRNA (Alder *et al.*, 1992). Furthermore, Scheuner *et al.* (1992) demonstrated that the constituents of chromaffin secretory

[2] S. Pfeffer, personal communication.

vesicle membranes are sufficient to reconstitute exocytosis when injected into *Xenopus* oocytes and propose that the interchangeability of mammalian and amphibian secretory pathway components suggests substantial biochemical conservation of the regulated exocytotic pathway. Our data reveal a novel regulated secretory pathway present in *Xenopus* oocytes that has the ability to recognize a rat neuronal GABA transporter. Therefore, the *Xenopus* oocyte is not only a convenient system for examining the potential for proteins to be modulated, it may provide a useful model for studying the modulatory mechanisms present in other cell systems.

The finding that a neurotransmitter transporter is modulated by a mechanism similar to the modulation of a facilitated glucose transporter is significant since there is no primary amino acid identity between members of the two transporter families. This would suggest a common "signal" in the secondary structures of these transporters which may confer this particular mode of modulation. It is also significant that modulation by membrane trafficking first identified for the facilitated glucose transporter expressed endogenously in adipocytes and muscle cells is also found to modulate a GABA transporter expressed exogenously in *Xenopus* oocytes. It remains to be determined whether modulation by this mechanism is found for endogenously expressed neurotransmitter transporters; however, the presence of a sophisticated exocytotic pathway has been well established in neurons (for review see Kelly (1993)). This particular mode of modulation would have important implications for the proposed functions of neurotransmitter transporters. For example, GABA uptake inhibitors increase and prolong $GABA_B$ transmission (Solis and Nicoll, 1992); therefore, increasing the number of GABA transporters near $GABA_B$ receptors would further shape the time course of these synaptic events. Our data provide evidence that the density of GABA transporters at the plasma membrane has the potential to be regulated by a mechanism involving a second messenger cascade.

We demonstrate PKC-regulated modulation of a cloned neuronal GABA transporter expressed in *Xenopus* oocytes and that modulation is mediated primarily through subcellular redistribution of transporters to and from cytoplasmic storage compartments and the plasma membrane. Thus, expression in *Xenopus* oocytes and microinjection of modulatory compounds will provide a convenient system in which to study the regulation of additional neurotransmitter transporters as well as elucidate the mechanisms involved in protein targeting and regulated exocytosis.

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