

# Regulation of the Neuronal Glutamate Transporter Excitatory Amino Acid Carrier-1 (EAAC1) by Different Protein Kinase C Subtypes

MARCO I. GONZÁLEZ, MARCELO G. KAZANIETZ, and MICHAEL B. ROBINSON

*Departments of Pediatrics and Pharmacology, Children's Hospital of Philadelphia (M.I.G., M.B.R.), and Department of Pharmacology, Center for Experimental Therapeutics (M.G.K.), University of Pennsylvania, Philadelphia, Pennsylvania*

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## ABSTRACT

In previous studies, we have shown that activation of protein kinase C (PKC) rapidly (within minutes) increases the activity and cell surface expression of the glutamate transporter EAAC1 in two systems that endogenously express this transporter (C6 glioma cells and cocultures of neurons and astrocytes). However, the magnitude of the increase in activity is greater than the increase in cell surface expression. In addition, certain compounds completely block the increase in cell surface expression but only partially attenuate the increase in activity. We hypothesized that PKC increases EAAC1 activity by increasing cell surface expression and catalytic efficiency and that two different subtypes of PKC mediate these effects. To address these hypotheses, the PKC subtypes expressed by C6 glioma cells were identified. Of the PKC subtypes that are activated by phorbol esters, only PKC $\alpha$ , PKC $\delta$ , and PKC $\epsilon$  were observed. Gö6976, a compound that blocks PKC $\alpha$  at concentrations that

do not inhibit PKC $\delta$  or PKC $\epsilon$ , partially inhibited the increase in uptake but completely abolished the increase in EAAC1 cell surface expression. The 'Gö6976-insensitive' increase in activity was not associated with a change in total transporter expression but was associated with an increase in the  $V_{\max}$ . Na<sup>+</sup>-dependent glycine transport was not increased, providing indirect evidence that the Gö6976-insensitive increase in activity was not caused by a change in the Na<sup>+</sup> electrochemical gradient required for activity. Finally, by down-regulating different subtypes of PKC, we found evidence that PKC $\epsilon$  mediates the increase in EAAC1 activity that is independent of changes in cell surface expression and found further evidence that PKC $\alpha$  mediates the increase in cell surface expression. The potential relationship of the present work with a previously identified role for PKC $\alpha$  in certain forms of synaptic plasticity is discussed.

Sodium-dependent uptake is the major mechanism for the regulation of synaptic glutamate levels. This uptake is mediated by a family of transporters, including GLAST (EAAT1), GLT-1 (EAAT2), EAAC1 (EAAT3), EAAT4, and EAAT5 (Danbolt, 2001). These transporters share a high degree of sequence identity but show differential patterns of expression. GLAST and GLT-1 are primarily expressed by glial cells throughout the cerebellum and forebrain. EAAC1 and EAAT4 are generally considered neuronal transporters. EAAC1 expression is enriched in the pyramidal cells of cortex and hippocampus and EAAT4 expression is restricted to the

Purkinje cells of the cerebellum (for review, see Sims and Robinson, 1999; Danbolt, 2001). Finally, the expression of EAAT5 is enriched in Müller glia in the retina but has also been detected in photoreceptors and bipolar cells (Eliasof et al., 1998; Pow and Barnett, 2000).

Electrophysiological studies suggest several mechanisms by which these transporters may regulate excitatory signaling. At some synapses, the binding of glutamate to transporters may buffer the amount of glutamate available for the activation of postsynaptic receptors (Tong and Jahr, 1994). At other synapses, transporters may directly control the time course for pre- or postsynaptic receptor activation by active uptake (Otis et al., 1997). Finally, transporters may also limit the activation of receptors by controlling the spillover of glutamate to neighboring synapses (Diamond, 2001). Based on these studies, it is not surprising that changes in gluta-

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**ABBREVIATIONS:** PKC, protein kinase C; PI3K, phosphatidylinositol 3-kinase; EAAC1, excitatory amino acid carrier 1; DAG, diacylglycerol; cPKC, classic PKC; nPKC, novel PKC; PMA, phorbol 12-myristate 13-acetate; Gö6976, 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)pyrrolo(3,4-c)-carbazole; PBS, phosphate-buffered saline; PBS Ca/Mg, phosphate-buffered saline containing 0.1 mM calcium and 1.0 mM magnesium; ANOVA, analysis of variance; Glu, glutamate; DMEM, Dulbecco's modified Eagle's medium; Bryo, bryostatin 1; Tmtx, thymeleatoxin; Bis II, bisindolylmaleimide II.

mate transporter activity have been recently implicated in synaptic plasticity, including long-term potentiation and depression (Brasnjó and Otis, 2001; Levenson et al., 2001). These findings suggest a primary role of glutamate transporters in the control of receptor activation and raise the possibility that regulation of the transporters can ultimately regulate synaptic transmission.

Several intracellular signaling pathways can regulate glutamate transporter activity within minutes through mechanisms that are independent of *de novo* transporter synthesis, including PKC, arachidonic acid, phosphatidylinositol 3-kinase (PI3K), and free radicals (for review, see Sims and Robinson, 1999; Danbolt, 2001). Several studies have shown varied effects of PKC activation on GLT-1 and GLAST (for original citations, see Sims and Robinson, 1999; Danbolt, 2001). Activation of PKC rapidly increases EAAC1-mediated glutamate uptake. This increase in activity is associated with increased EAAC1 cell surface expression; however, the increase in uptake is larger than the increase in EAAC1 cell surface expression. Furthermore, inhibition of PI3K eliminates the PKC-dependent increase in cell surface expression but does not completely block the increase in uptake (Davis et al., 1998). These observations suggest that PKC activation increases EAAC1-mediated uptake by increasing the number of transporter molecules expressed at the cell membrane and/or by altering the catalytic efficiency of the transporter.

It is well established that PKC activity is mediated by a family composed of three subgroups. The first subgroup, referred to as classic PKC (cPKC) subtypes includes three members ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) that are activated by diacylglycerol (DAG) and phorbol esters; these cPKCs require  $\text{Ca}^{2+}$  as a cofactor. The second subgroup, referred to as novel PKC (nPKC) subtypes, includes four members ( $\delta$ ,  $\epsilon$ ,  $\theta$ , and  $\eta$ ) that are  $\text{Ca}^{2+}$ -independent but are activated by DAG or phorbol esters. The third group, referred to as atypical PKCs includes two members,  $\zeta$  and  $\lambda$ . These subtypes are insensitive to  $\text{Ca}^{2+}$ , DAG, and phorbol esters (for review, see Kazanietz et al., 2000; Way et al., 2000; Newton, 2001). The biological significance of the heterogeneity of the PKC family has not been fully clarified. Although some isozymes have overlapping expression patterns that may imply redundancy, expression of some PKC subtypes is restricted to specific cells or subcellular organelles, suggesting that each isoform is involved in the regulation of different cell functions.

The aim of the present study was to determine whether specific PKC subtypes are involved in the regulation of EAAC1-mediated uptake and cell surface expression. Initial studies were conducted to identify the particular PKC subtypes expressed in C6 glioma cells, a cell line that endogenously expresses only EAAC1. Using subtype specific pharmacological agents and down-regulation of the different PKC subtypes, we provide evidence to suggest that two different PKC isozymes increase EAAC1-mediated uptake by different mechanisms. One subtype, PKC $\alpha$ , seems to selectively increase transporter cell surface expression; the second subtype, PKC $\epsilon$ , regulates uptake by a trafficking-independent mechanism, perhaps by increasing the intrinsic activity of the transporter.

## Materials and Methods

**Materials.** C6 glioma cells, a cell line that endogenously and exclusively expresses the EAAC1 subtype of transporter and none of the other subtypes, was used in this study (for original references, see Davis et al., 1998). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was from HyClone (Logan, UT). Culture plates were from Corning (Cambridge, MA). Radioisotopes were from PerkinElmer Life Sciences (Boston, MA). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma-Aldrich (St. Louis, MO). Gö6976 was purchased from Calbiochem (La Jolla, CA). Bryostatins 1 and thymeleatoxin were from Biomol (Plymouth Meeting, PA). Antibodies for PKC $\alpha$  and PKC $\beta$  were purchased from Transduction Laboratories (San Diego, CA) and the antibodies for PKC $\gamma$ , PKC $\delta$ , PKC $\epsilon$ , PKC $\theta$ , and PKC $\eta$  were from Santa Cruz Biotechnology (Santa Cruz, CA). Sulfo-*N*-hydroxysulfosuccinimidobiotin and Immunopure Immobilized Monomeric Avidin were purchased from Pierce (Rockford, IL). Dr. Jeffrey D. Rothstein (Johns Hopkins University) generously provided the EAAC1 antibody.

**Neuronal Cultures.** Primary neuron-enriched cultures were prepared from embryonic cortex. In brief, embryos from pregnant Sprague-Dawley rats were removed on embryonic day 18 or 19. After removal of the meninges, cortex was isolated and incubated in trypsin for 20 min. After trituration in a pasteur pipette, 12 ml of cell suspension (400,000 cells/ml) was plated into a 10 cm dish that had been precoated with poly-L-lysine. Cells were maintained in Neurobasal medium supplemented with 2% B27. Cultures were used after 9–10 days *in vitro*.

**Measurement of  $\text{Na}^+$ -Dependent Transport Activity.** The measurement of glutamate or glycine uptake was conducted in triplicate at 37°C in a water bath, as described previously (Davis et al., 1998). Briefly, cell monolayers were rinsed twice with 1 ml of warm solution of sodium- or choline-containing buffer, before incubation with the radioisotopes for 5 min (0.5  $\mu\text{M}$  L-[ $^3\text{H}$ ]glutamate or 10  $\mu\text{M}$  [ $^3\text{H}$ ]glycine). For the saturation analyses, glutamate concentrations ranged from 1  $\mu\text{M}$  to 100  $\mu\text{M}$ . Uptake was stopped using three washes with ice-cold choline-containing buffer and cells were solubilized in 1 ml of 0.1N sodium hydroxide. An aliquot of cell lysate (500  $\mu\text{l}$ ) was transferred to 5 ml of Cytoscent (ICN; Aurora, OH) and analyzed for radioactivity using a Beckman scintillation counter.  $\text{Na}^+$ -dependent uptake was defined as the difference in radioactivity accumulated in  $\text{Na}^+$ -containing buffer and in choline-containing buffer.

**Biotinylation.** EAAC1 cell surface expression was measured as described previously (Davis et al., 1998). C6 glioma cell monolayers (10-cm dishes) were rinsed twice with PBS containing 0.1 mM calcium and 1.0 mM magnesium (PBS Ca/Mg). Cells were then incubated with 2 ml of biotin solution (1 mg/ml sulfo-*N*-hydroxysulfosuccinimidobiotin in PBS Ca/Mg) for 20 min at 4°C with gentle shaking. The biotin solution was removed and the plates were washed twice with PBS Ca/Mg plus 100 mM glycine. The plates were then incubated in PBS Ca/Mg/glycine for 20 min at 4°C to quench the unreacted biotin. Cells were lysed with 1 ml of radioimmunoprecipitation assay buffer containing protease inhibitors. Lysates were cleared of nuclei and debris by centrifugation at 12,400 rpm for 20 min. Aliquots of the lysate were mixed with an equal volume of Laemmli buffer (62.5 Tris-HCl, pH 6.8, 2% SDS, and 5% 2-mercaptoethanol) and analyzed as the 'lysate fraction'. Another aliquot of lysate was incubated over night at 4°C with avidin conjugated beads. This mixture was centrifuged for 15 min at 12,500 rpm; an aliquot of the supernatant was saved, mixed with the same volume of Laemmli buffer, and used as 'intracellular fraction'. The pellet containing the biotinylated/cell surface proteins was washed four times with radioimmunoprecipitation assay buffer plus protease inhibitors. Finally, the pellet was incubated with Laemmli buffer for 30 min to elute biotinylated proteins. After centrifugation at 12,400 rpm for 15 min, the supernatant was saved as the biotinylated/cell surface fraction.

All three fractions were stored at  $-20^{\circ}\text{C}$  until they were analyzed by Western blot.

**Western Blot.** For the identification of the different PKC subtypes expressed in C6 cells, 5  $\mu\text{g}$  of a synaptosomal preparation from rat cerebellum or cortex or 20  $\mu\text{g}$  of C6 cells total lysate were used. For the biotinylation experiments, 25  $\mu\text{g}$  of total lysate protein and equivalent volumes of nonbiotinylated (intracellular) and biotinylated (cell surface) fractions were loaded on an 8% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes and the membranes were blocked with 5% nonfat milk and 0.1% Tween 20 in Tris buffer (Davis et al., 1998). The blots were probed with specific antibodies for each PKC subtype, EAAC1, or actin and were visualized with chemiluminescence. Immunoreactivity was quantified using NIH Image (<http://rsb.info.nih.gov/ni-image/>) after the scanning of the films. As observed previously (Haugeto et al., 1996), EAAC1 immunoreactivity migrated as monomers and multimers; both signals were quantitated and analyzed for this study. Data are presented as the sum of immunoreactivity in monomers and multimers. The quantities of monomer were also summarized across experiments and the changes were essentially identical to those reported as total immunoreactivity. Under control conditions and across all the experiments, the percentage of biotinylated EAAC1 immunoreactivity was  $34.5 \pm 2.2$  and the percentage of actin biotinylated was  $13.2 \pm 1.9$  (mean  $\pm$  S.E.M.,  $n = 17$ ).

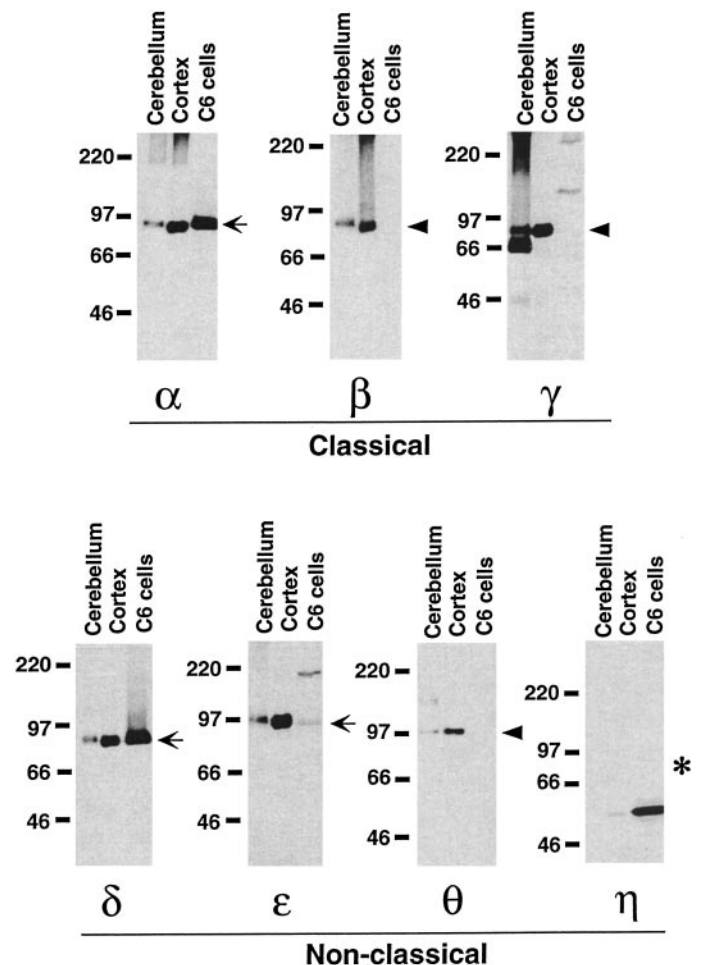
## Results

**Identification of the PKC Subtypes Expressed in C6 Cells.** Based on our earlier studies, we hypothesized that the phorbol ester-induced increase in EAAC1-mediated uptake is induced by different subtypes of PKC and that PKC activation can increase EAAC1 activity by trafficking-dependent and -independent mechanisms (see *Introduction*). As a first step, we used subtype-specific antibodies to identify the PMA-sensitive PKC subtypes expressed by C6 glioma cells. The expression of the atypical PKC members was not examined because these subtypes are not activated by PMA. For these analyses, cerebellar and cortical synaptosomal homogenates were used as positive controls. Immunoreactive bands were observed for one of the cPKCs, PKC $\alpha$ , and for two of the nPKCs, PKC $\delta$  and PKC $\epsilon$ . (Fig. 1). The molecular masses of these immunoreactive bands were approximately 80 to 90 kDa and identical to those observed in brain homogenates and in accordance with the expected size for the PKC subtypes (Kazanietz et al., 1993). PKC $\beta$ , PKC $\gamma$ , and PKC $\theta$  were detected in the homogenates used as positive controls but not in total lysates of C6 cells. As would be predicted from previous studies, PKC $\eta$  was not expressed in C6 glioma cells, but a 'cross-reacting' band of approximately 50 kDa was observed (Chen and Wu, 1995; Moreton et al., 1995). These data suggest that the C6 glioma cells used in the present study express only PKC $\alpha$ , PKC $\delta$ , and PKC $\epsilon$ .

**Effects of Gö6976, a cPKC Inhibitor, on PMA-Induced Increases in EAAC1-Mediated Uptake.** Gö6976 is a compound that inhibits cPKC subtypes at lower concentrations than those required to inhibit the nPKC subtypes and has been used to discriminate the effects of cPKC from those of nPKCs (for review, see Way et al., 2000). Based on this, we used Gö6976 to determine whether activation of PKC $\alpha$  may be involved in the regulation of EAAC1-mediated uptake. As was observed previously, a 15-min treatment with PMA increased uptake activity to approximately 230% of that observed in vehicle-treated cells (Davis et al., 1998). At concentrations between 1 nM and 10  $\mu\text{M}$ , Gö6976 inhibited the

PMA-induced increase in activity in a concentration-dependent fashion (Fig. 2A). These data were best fit to a 'Gö6976-sensitive' component ( $\text{IC}_{50}$  value = 45 nM) and a component that was essentially 'Gö6976-insensitive' (not inhibited at 10  $\mu\text{M}$ ). This  $\text{IC}_{50}$  value is in the range of that previously reported for the inhibition of the cPKCs (Way et al., 2000). The maximal inhibition of the PMA-induced increase was to 50% of the increase induced by PMA. By itself, Gö6976 had no effect on transport activity, suggesting that Gö6976 does not have a direct effect on transporter function.

To determine how Gö6976 may influence the PMA-induced increase in uptake, we examined the effects of this compound on the kinetic parameters of the glutamate transport (Fig. 2B). As seen previously, PMA increased the  $V_{\text{max}}$  of glutamate uptake by 2.4-fold (Davis et al., 1998). Gö6976, by itself, did not change the  $V_{\text{max}}$  of the transporter, but Gö6976 reduced the increase in  $V_{\text{max}}$  induced by PMA to 1.7-fold compared with control. In all the treatments, no changes in the  $K_m$  values were observed (see legend to Fig. 2). Together,



**Fig. 1.** Immunoblot analysis of the PKC subtypes expressed in C6 cells. Proteins extracted from crude synaptosomal preparations of cerebellar or cortical tissue or total lysate from C6 cells were probed with isoform-specific antibodies against the different PKC subtypes. PKC $\alpha$ , PKC $\delta$ , and PKC $\epsilon$  were detected in C6 cell lysates (arrowhead). PKC $\beta$ , PKC $\gamma$ , and PKC $\theta$  (arrowhead) were detected in cerebellar and cortical tissues but not in C6 cells. PKC $\eta$  was not detected in cerebellum, cortex, or C6 cells (asterisk). Although PKC $\eta$  was not observed under these conditions, a faint band of the appropriate molecular mass ( $\sim 90$  kDa) was observed in cortical tissue when 30  $\mu\text{g}$  of protein was analyzed (data not shown). These immunoblots are representative of at least two independent experiments.

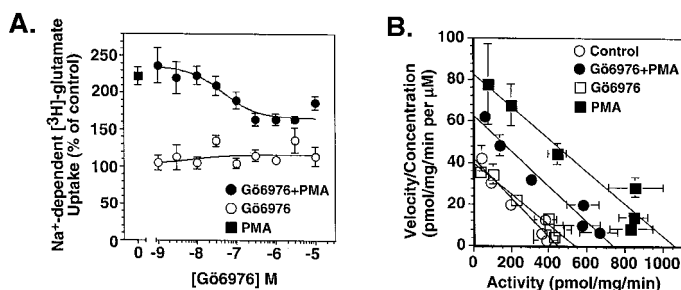


these results suggest that there are G6976-sensitive and -insensitive components of the PMA-induced increase in transport activity and that both are related to increases in the maximal transport capacity.

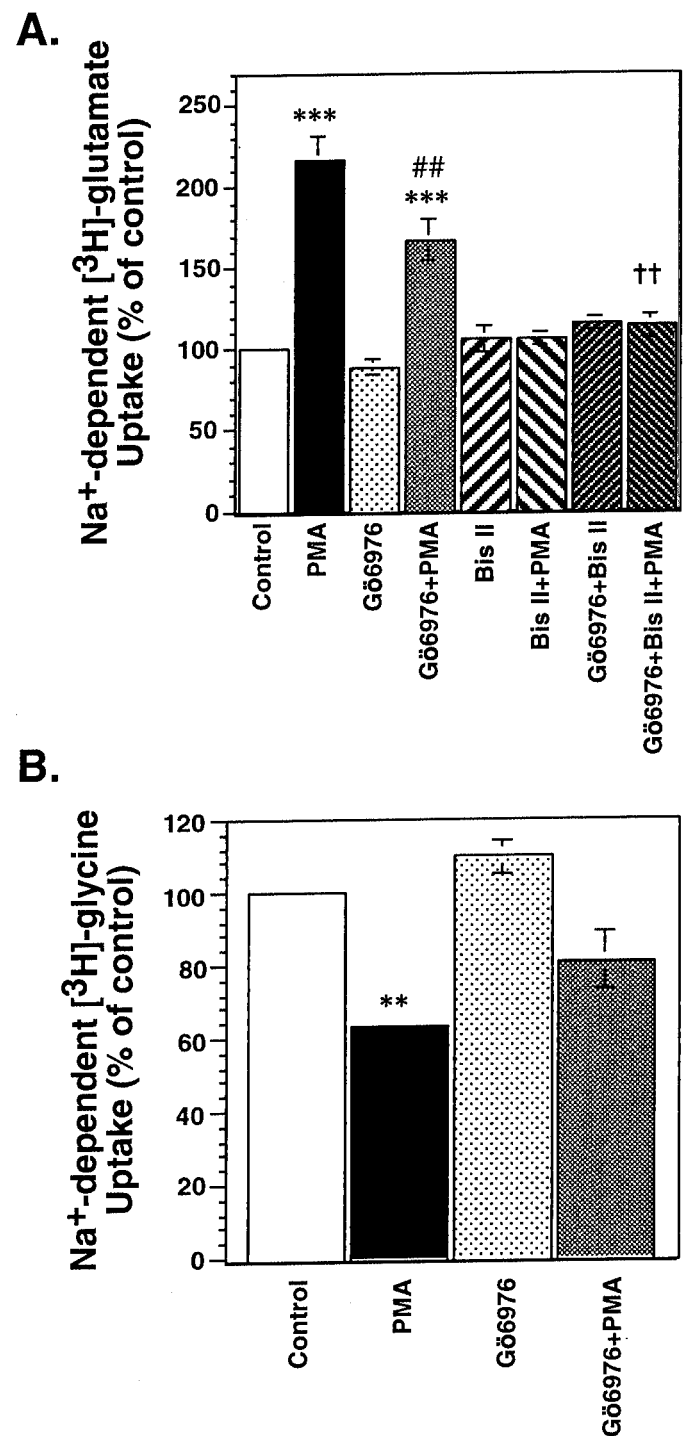
Because PMA is known to interact with non-PKC targets (Kazanietz et al., 2000), the effects of a general inhibitor of PKC, bisindolylmaleimide II (Bis II) were examined. Coapplication of Bis II and G6976 completely blocked the G6976-insensitive increase in activity induced by PMA, suggesting that this G6976-insensitive component is PKC dependent (Fig. 3A). It is also theoretically possible that PMA could increase transport activity by altering the  $\text{Na}^+$  electrochemical gradient required for transporter function. To address this possibility, the effects of PMA on  $\text{Na}^+$ -dependent glycine uptake were examined. As shown previously (see Davis et al., 1998 for original reference), a 15-min incubation with PMA significantly reduced the  $\text{Na}^+$ -dependent glycine transport (Fig. 3B). By itself, G6976 had no effect on glycine transport, and G6976 partially blocked the decrease in uptake induced by PMA. These data provide indirect evidence that the G6976-insensitive increase in  $\text{Na}^+$ -dependent Glu transport can not be attributed to a generalized change in the  $\text{Na}^+$  electrochemical gradient required for transporter function. They also suggest that the G6976-insensitive increase in uptake is mediated by a PKC, presumably one of the other subtypes expressed in C6 glioma cells, either PKC $\delta$  or PKC $\epsilon$ .

**Effects of G6976 on the PMA-Induced Change in EAAC1 Cell Surface Expression.** In previous studies, we have shown that activation of PKC with PMA increases EAAC1 cell surface expression (Davis et al., 1998). To determine whether cPKCs or nPKCs are involved in this redistribution, the effects of G6976 on the PMA-induced increase in EAAC1 cell surface expression were examined. The amount of EAAC1 present at the cell surface was assessed using a membrane-impermeant biotinylation reagent combined with batch extraction of biotinylated proteins and Western blot-

ting (Fig. 4). A 15-min preincubation with PMA increased the amount of biotinylated/cell surface EAAC1 to ~150% of control and had no effect on the total amount of EAAC1 immu-



**Fig. 2.** Effects of G6976 on the PMA-induced increase in  $\text{Na}^+$ -dependent Glu transport activity. **A**, C6 cells were preincubated with the indicated concentrations of G6976 for 10 min, followed by addition of PMA (100 nM) for 15 min.  $\text{Na}^+$ -dependent glutamate uptake was measured. The data are mean  $\pm$  S.E.M. of at least four independent experiments and are expressed as a percentage of vehicle-treated cells. Data for the inhibition of the PMA-induced increase in transport activity were best fit to sensitive and insensitive components. The  $\text{IC}_{50}$  value was 45 nM and the maximal level of inhibition was to 165% of control. **B**, C6 glioma cells were treated with combinations of treatments as described above (vehicle, 100 nM PMA, 10  $\mu\text{M}$  G6976) and the concentration-dependence of Glu transport activity was measured. Data are mean  $\pm$  S.E.M. of four independent experiments and were fit by linear regression analysis. The  $V_{\text{max}}$  values were: control,  $440 \pm 60$ ; PMA,  $1040 \pm 110$ ; G6976 with PMA,  $750 \pm 130$ ; and G6976,  $550 \pm 80$ . Control versus PMA,  $p < 0.001$ ; control versus G6976 with PMA and PMA versus G6976 with PMA,  $p < 0.01$  compared by ANOVA. The  $K_m$  values were control,  $10.4 \pm 0.3$ ; PMA,  $13.7 \pm 2.1$ ; G6976 with PMA,  $12.3 \pm 0.9$ ; and G6976,  $14.2 \pm 2.0$ ; with no significant differences compared with control.

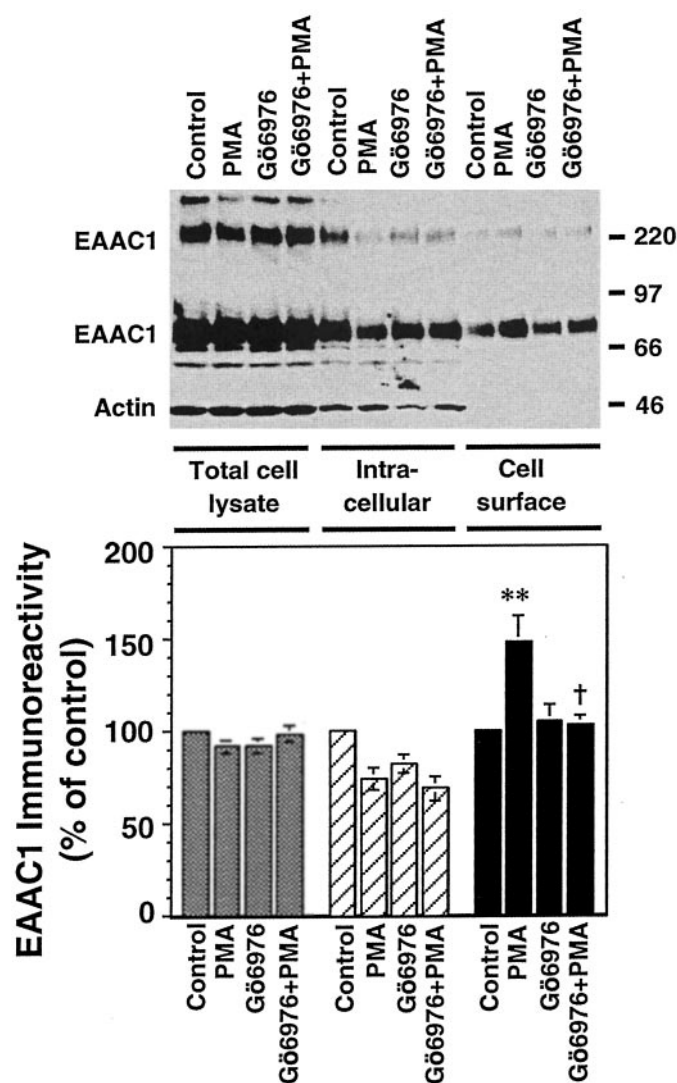


**Fig. 3.** Effect of PKC inhibitors on  $\text{Na}^+$ -dependent glutamate and glycine uptake. **A**, cells were treated with G6976 (10  $\mu\text{M}$ ) and/or Bis II (10  $\mu\text{M}$ ) for 10 min before the addition of 100 nM PMA for 15 min, followed by measurement of  $\text{Na}^+$ -dependent glutamate (0.5  $\mu\text{M}$ ) uptake. Data are mean  $\pm$  S.E.M. of at least three independent experiments. \*\*\*,  $p < 0.01$ , compared with control; ##,  $p < 0.01$ , compared with PMA treated cells; ††,  $p < 0.01$  compared with G6976 with PMA; all comparisons were made by ANOVA. **B**, effects of PMA and G6976 on  $\text{Na}^+$ -dependent glycine (10  $\mu\text{M}$ ) transport. Data are mean  $\pm$  S.E.M. of three independent experiments. \*\*,  $p < 0.01$ , control versus PMA, compared by ANOVA.

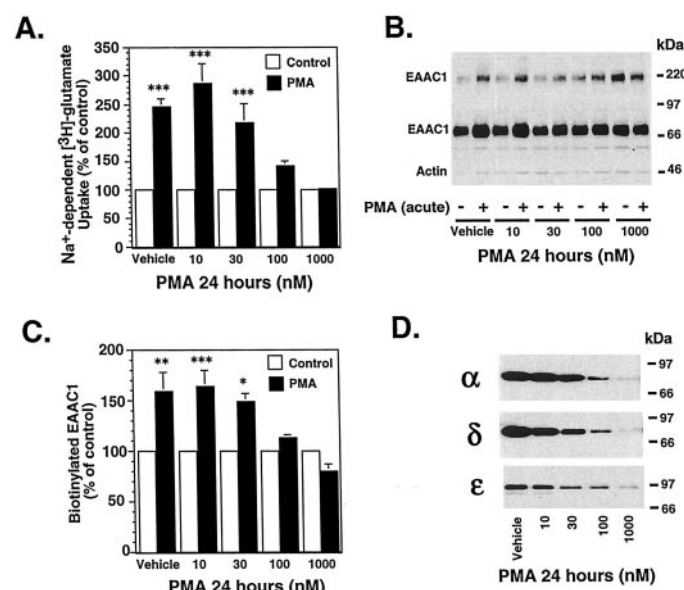
noreactivity (total cell lysate). At a concentration that maximally reduces but does not completely block the PMA-induced increase in transport activity, Gö6976 (10  $\mu$ M) completely blocked the PMA-induced increase in EAAC1 cell surface expression. In these studies, the amount of biotinylated and nonbiotinylated actin was also examined as a control for the different treatments and the biotinylation procedure and none of the treatments increased the amount of biotinylated actin (data not shown). Together, these data suggested that the Gö6976-sensitive increase in Glu transport activity is associated with an increase in the cell surface expression of EAAC1. Because PKC $\alpha$  is the only PKC subtype expressed in C6 glioma cells that is inhibited by low

concentrations of Gö6976, these data also suggest that PKC $\alpha$  increases EAAC1-mediated activity by increasing EAAC1 cell surface expression.

**Effects of Chronic Phorbol Ester Incubation on PMA-Induced Regulation of EAAC1.** It was suggested previously that PMA preferentially down-regulates PKC $\delta$  in C6 glioma cells, but has minimal effects on PKC $\alpha$  (Chen and Wu, 1995). Therefore, to investigate the possible involvement of PKC $\delta$  in the regulation of EAAC1, the effects of prolonged incubation with increasing concentrations of PMA on the PKC-dependent increase in transport activity, EAAC1 cell surface expression, and PKC subtype expression was examined (Fig. 5). C6 glioma cells were treated with several concentrations of PMA (10, 30, 100, and 1000 nM) for 24 h. These long-term incubations with PMA decreased the effects (activity and cell surface expression) of short-term (15 min) PMA



**Fig. 4.** Effects of Gö6976 on the PMA-induced increase in EAAC1 cell surface expression. C6 cells were preincubated with 10  $\mu$ M Gö6976 for 10 min followed by the addition of PMA (100 nM) for 15 min. Cell surface expression was examined using a membrane impermeant biotinylation reagent. The top shows a representative Western blot from these analyses. The bottom contains the summary of four independent experiments (mean  $\pm$  S.E.M.). Data are presented as the sum of the immunoreactivity detected in both monomers and multimers. Cell surface expression in PMA-treated cells was significantly different compared with control (\*\*,  $p < 0.001$ , compared by ANOVA). In cells treated with Gö6976 and PMA, EAAC1 cell surface expression was significantly different from PMA-treated cells (†,  $p < 0.05$ , compared by ANOVA). The levels of monomer were also summarized separately with similar results.



**Fig. 5.** Effect of long-term treatment with PMA on the short-term effects of PMA and on expression of PKC subtypes. A, C6 glioma cells were treated with increasing concentrations of PMA (10–1000 nM) for 24 h. After a 2-h rinse in DMEM, cells were treated with fresh PMA (100 nM) for 15 min (acute). The solid bars represent the activity measured in cells treated in the short term with PMA (100 nM) after the long-term treatment. These data were normalized to the activity measured in cells that underwent short-term incubation with vehicle after an identical long-term treatment. Data are the mean  $\pm$  S.E.M. of four independent observations. \*\*\*,  $p < 0.001$ , cells undergoing short-term treatment with PMA were compared with its respective control by ANOVA. The long-term treatment with PMA had no significant effect on activity, except at the highest concentration (10 nM PMA,  $99 \pm 11$ ; 30 nM PMA,  $84 \pm 7$ ; 100 nM PMA,  $123 \pm 10$ ; and 1000 nM PMA,  $149 \pm 7$ ,  $p < 0.05$ , data expressed as a percentage of that observed in cells treated long-term with vehicle). B, representative Western blot of the effects of long-term incubation with PMA on short-term regulation of biotinylated/cell surface EAAC1 immunoreactivity. C, quantitation of the effects of long-term incubation with PMA on EAAC1 cell surface expression. These data are presented as the percentage of the cell surface expression measured in cells that underwent short-term incubation with vehicle after an identical long-term treatment. Data are the mean  $\pm$  S.E.M. of four independent observations. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ , comparing cells undergoing short-term treatment with PMA with its respective control. The long-term treatment with PMA had no significant effect on cell surface expression, except at the highest concentration (10 nM PMA,  $100 \pm 4$ ; 30 nM PMA,  $91 \pm 9$ ; 100 nM PMA,  $123 \pm 6$ ; 1000 nM PMA,  $158 \pm 5$ ;  $p < 0.01$ , data expressed as a percentage of that observed in cells chronically treated with vehicle). D, effects of long-term PMA incubation on the expression of the PKC subtypes. A representative Western blot of four independent experiments is shown.

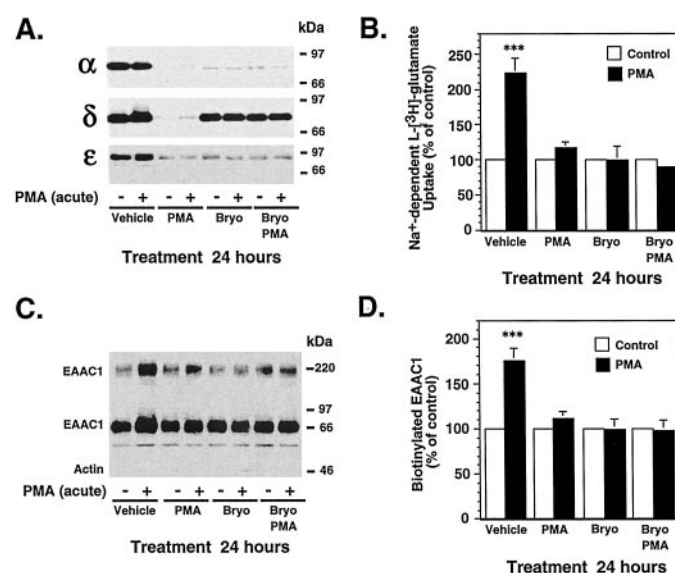


treatment in a dose-dependent manner (Fig. 5). When lower concentrations of PMA were used to down-regulate PKC, there was a parallel down-regulation of all three PKC subtypes. Chronic incubation with 1000 nM PMA almost completely abolished (10–20% of control) expression of all the PKC subtypes, and fresh addition of PMA did not produce any increase in uptake activity or cell surface expression. These long-term incubations with PMA had no significant effect on Glu uptake, and had no significant effect on cell surface expression at concentrations up to 100 nM (see legend to Fig. 5). However, long-term incubation with 1000 nM PMA significantly increased Glu uptake and EAAC1 cell surface expression without changing the total amount of EAAC1 immunoreactivity ( $114 \pm 6\%$  of vehicle-treated cells,  $n = 4$ ). Therefore, we were not able to selectively down-regulate individual PKC subtypes as referenced by Chen and Wu (1995). Although long-term down-regulation of PKCs with phorbol esters is sometimes used to provide evidence that a phenomenon is PKC dependent, we acknowledge that the long-term exposure to phorbol esters could have indirect effects.

**Effects of Chronic Treatment with Bryostatin 1 on PMA-Induced Regulation of EAAC1.** Bryostatin 1 (Bryo) is a nonphorbol ester activator of PKC, but this compound only induces a subset of the effects induced by phorbol esters. In addition, Bryo also blocks some of the effects induced by phorbol esters (Szallasi et al., 1994). It has been previously shown that Bryo induces down-regulation of PKC $\alpha$  and PKC $\epsilon$ , but protects PKC $\delta$  from phorbol ester-induced down-regulation (Lorenzo et al., 1997; Lu et al., 1997). In fact, in rat fibroblasts protection of PKC $\delta$  from down-regulation with Bryo blocks the tumor-promoting effects of PMA, suggesting that after protection with Bryo PKC $\delta$  is still active (Lu et al., 1997). To deplete C6 cells of PKC $\alpha$  and PKC $\epsilon$ , and to isolate the effects of PKC $\delta$ , C6 cells were chronically treated with Bryo (100 nM) or with Bryo plus PMA (1000 nM) for 24 h. After this long-term incubation, cells were rinsed in DMEM for 2 h. After long-term treatment with Bryo, both PKC $\alpha$  and PKC $\epsilon$  were down-regulated while PKC $\delta$  was essentially preserved (Fig. 6A). It is theoretically possible that long-term treatment with Bryo could induce expression of the other PKC subtypes. To rule out this possibility, the expression of the other PMA-sensitive PKC subtypes ( $\beta$ ,  $\gamma$ ,  $\theta$ , and  $\eta$ ) was examined. Long-term treatment with Bryo or Bryo with PMA did not induce the expression of any of these subtypes (data not shown,  $n = 2$ ), suggesting that the only phorbol ester-sensitive PKC subtype is PKC $\delta$ . Under these conditions, a fresh application of PMA had no effect on Na<sup>+</sup>-dependent Glu transport activity or on EAAC1 cell surface expression, suggesting that PKC $\delta$  is not involved in the regulation of EAAC1 (Fig. 6B–D). Combined with our demonstration that only three PKC subtypes are expressed in C6 glioma cells, this would suggest that PKC $\alpha$  and PKC $\epsilon$  are candidates for the regulation of EAAC1 cell surface expression and activity. Because our data with G66976 suggested that PKC $\alpha$  is involved in the regulation of EAAC1 cell surface expression, PKC $\epsilon$  is the only other subtype that could explain the PMA-dependent increase in the intrinsic activity of the transporter (the G66976-insensitive component).

**Effects of Chronic Treatment with Thymelatoxin on PMA-Induced Regulation of EAAC1.** Thymelatoxin (Tmtx) has a lower affinity for nPKC subtypes than for the

cPKCs; therefore, it has been used to discriminate between the differential roles of specific PKC isozymes (Kazanietz et al., 1993). In HT-29 cells, Tmtx activates and down-regulates PKC $\alpha$  but does not activate or down-regulate PKC $\epsilon$  (Llosas et al., 1996). To investigate whether Tmtx produces similar effects in C6 glioma cells, cell monolayers were chronically treated with Tmtx (100 and 1000 nM) and the expression of the different PKC subtypes was analyzed by Western blot. As shown in Fig. 7A, treatment with 100 nM Tmtx for 24 h caused a dramatic down-regulation of PKC $\alpha$  and PKC $\delta$ , with a minimal reduction in the levels of PKC $\epsilon$ . We also probed these samples for expression of the other phorbol ester-activated PKC subtypes ( $\beta$ ,  $\gamma$ ,  $\theta$ , and  $\eta$ ) to rule out the possibility that these treatments induced expression of other PKC subtypes. Long-term treatment with Tmtx did not induce the expression of any other phorbol ester-sensitive PKC subtypes (data not shown,  $n = 2$ ), suggesting that the predominant

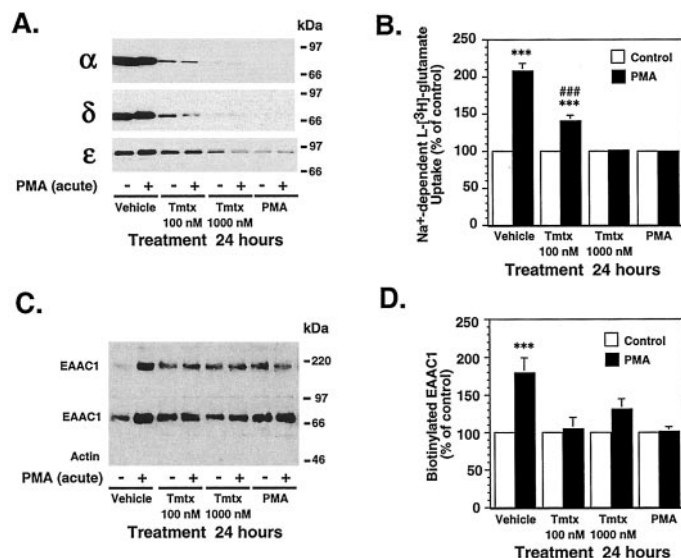


**Fig. 6.** Effects of PKC $\delta$  protection on the increase in EAAC1 activity or cell surface expression induced by PMA. A, PKC $\alpha$  and PKC $\epsilon$  were down-regulated by a 24-h treatment with Bryo (100 nM) or Bryo (100 nM) plus PMA (1000 nM). After a 2-h rinse in DMEM, cells underwent short-term treatment with fresh PMA (100 nM) for 15 min. To confirm the presence of PKC $\delta$ , total cell lysates of C6 glioma cells were separated on an 8% polyacrylamide gel and the polyvinylidene difluoride membranes were probed with isoform-specific antibodies against PKC $\alpha$ , PKC $\delta$ , and PKC $\epsilon$ . B, after the down-regulation of PKC $\alpha$  and PKC $\epsilon$ , cells underwent short-term treatment with vehicle or 100 nM PMA for 15 min followed by the measurement of Na<sup>+</sup>-dependent glutamate uptake activity. The solid bars represent the activity measured in cells undergoing short-term treatment with PMA (100 nM) after the long-term treatment. These data were normalized to the activity measured in cells that underwent short-term incubation with vehicle after an identical long-term treatment. None of these long-term treatments had a significant effect on transport activity (PMA,  $114 \pm 14$ ; Bryo,  $85 \pm 17$ ; Bryo with PMA,  $97 \pm 20$ ; data expressed as a percentage of that observed in cells chronically treated with vehicle). C, after PKC $\alpha$  and PKC $\epsilon$  down-regulation, the effects of PKC activation on EAAC1 cell surface expression were assessed using an impermeant biotin reagent. A representative blot of the biotinylated fraction is shown. D, the quantitation of four independent biotinylation experiments is presented. Data (solid bars) are presented as the percentage of cell surface expression measured in cells chronically incubated with the same treatment and incubated short-term with vehicle. \*\*\*,  $p < 0.001$ , comparing cells undergoing short-term treatment with PMA with its respective control. None of these long-term treatments had a significant effect on cell surface expression (PMA,  $118 \pm 16$ ; Bryo,  $85 \pm 18$ ; Bryo with PMA,  $84 \pm 25$ ; data expressed as a percentage of that observed in cells chronically treated with vehicle). The levels of monomer were also summarized separately with similar results.

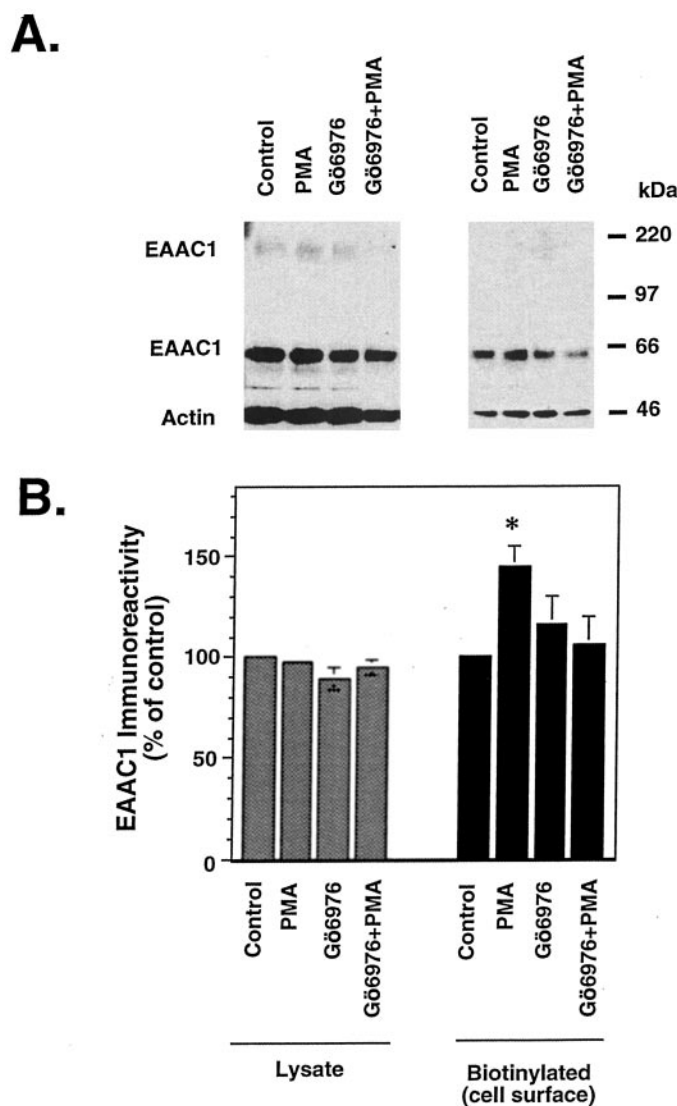
PMA-activated PKC subtype was PKC $\epsilon$ . Under these conditions, a fresh application of PMA caused a significant increase in Glu transport activity, but had no effect on EAAC1 cell surface expression (Fig. 7, B-D). In contrast, when cells were chronically treated with 1000 nM Tmtx, all the PKC subtypes were down-regulated essentially to the same extent as was observed with PMA (Fig. 7A), and a fresh application of PMA did not induce changes in EAAC1 activity or cell surface expression. These results suggest that PKC $\epsilon$  induces an increase in EAAC1 activity by a trafficking-independent

mechanism. This PKC $\epsilon$ -dependent increase in activity may be correlated with the Gö6976-insensitive increase in transport activity, because both are independent of EAAC1 redistribution, suggesting that these phenomena share a common mechanism that requires PKC $\epsilon$  activation.

**Effects of Gö6976 Application on the Regulation of EAAC1 Cell Surface Expression in Cortical Neurons.** To determine whether a classic PKC also regulates EAAC1 cell surface expression in a cellular milieu that is more similar to the intact brain, cortical neurons were incubated with PMA and PMA plus Gö6976. As shown in Fig. 8, PMA treatment increased EAAC1 cell surface expression by ~45%.



**Fig. 7.** Effect of PKC $\alpha$  and PKC $\delta$  down-regulation on the PMA-induced increase of EAAC1-mediated uptake and cell surface expression. A, C6 cells were chronically treated with thymeleatoxin (Tmtx) (100 nM or 1000 nM) or PMA (1000 nM) for 24 h. After a 2-h rinse in DMEM, cells underwent short-term treatment with fresh PMA (100 nM) for 15 min. To assess the effects of the different long-term treatments on the expression of PKC subtypes, total C6 glioma cell lysates were probed with isoform-specific antibodies against PKC $\alpha$ , PKC $\delta$  and PKC $\epsilon$ . Chronic treatment with 100 nM Tmtx dramatically reduced the expression of PKC $\alpha$  and PKC $\delta$ ; but the immunoreactivity detected for PKC $\epsilon$  was similar to the levels observed in vehicle treated cells. B, after PKC $\alpha$  and PKC $\delta$  down-regulation, Na<sup>+</sup>-dependent glutamate uptake activity was measured. The solid bars represent the activity measured in cells undergoing short-term treatment with PMA (100 nM) after the long-term incubation. These data were normalized to the activity measured in cells that underwent short-term incubation with vehicle after an identical long-term treatment. Data are the mean  $\pm$  S.E.M. of four independent experiments. PMA significantly increased activity in cells chronically treated with vehicle or 100 nM Tmtx (\*\*\*,  $p < 0.001$ , by ANOVA). Compared with cells chronically treated with vehicle, long-term treatment with 100 nM Tmtx significantly reduced the acute effect of PMA (###,  $p < 0.001$ , by ANOVA). Chronic treatment with 100 nM Tmtx had no significant effect on transport activity, but 1000 nM PMA or 1000 nM Tmtx increased transport activity (100 nM Tmtx,  $120 \pm 7$ ; 1000 nM Tmtx,  $145 \pm 14$ ,  $p < 0.001$ ; PMA,  $143 \pm 6$ ,  $p < 0.001$ ; data expressed as a percentage of that observed in cells chronically treated with vehicle). C, in cells depleted of PKC $\alpha$  and PKC $\delta$ , the changes on cell surface expression induced by fresh application of PMA (100 nM) were studied using a biotinylation assay. A representative Western blot of the biotinylated fraction is shown. D, the data represent the quantitation of four independent biotinylation experiments. Data (solid bars) are presented as the percentage of cell surface expression measured in cells chronically treated with the same treatment and incubated short-term with vehicle. Comparing cells undergoing short-term treatment with PMA with its respective control, none of these values is significantly different ( $p > 0.05$ , compared by ANOVA). None of the long-term treatments significantly changed EAAC1 cell surface expression (100 nM Tmtx,  $123 \pm 20$ ; 1000 nM Tmtx,  $118 \pm 20$ ; 1000 nM PMA,  $117 \pm 18$ ; data are expressed as a percentage of cell surface expression observed in vehicle-treated cells). The levels of monomer were also summarized separately with similar results.



**Fig. 8.** Effects of PMA and Gö6976 on EAAC1 cell surface expression in cortical neurons. Neuron-enriched cultures were preincubated with 10  $\mu$ M Gö6976 for 10 min followed by the addition of PMA (100 nM) for 30 min. After treatment, EAAC1 cell surface expression was examined using a membrane impermeant biotinylation reagent. A, a representative Western blot from these analyses. B, the summary of four independent experiments (mean  $\pm$  S.E.M.). Data are presented as the sum of the immunoreactivity detected as both monomers and multimers. Cell surface expression in PMA-treated cells was significantly different compared with control (\*,  $p < 0.05$ , compared by Bonferroni comparison test). In cells treated with Gö6976 and PMA, EAAC1 cell surface expression was not significantly different from control cells ( $p > 0.05$ , compared by Bonferroni comparison test).



When PMA was added after preincubation with Gö6976, no increase in EAAC1 cell surface expression was detected. This suggests that the regulation of EAAC1 by a Gö6976-sensitive PKC is not restricted to C6 glioma cells.

## Discussion

Activation of PKC by PMA produces an increase in EAAC1-mediated uptake that is associated with an increase in EAAC1 cell surface expression (Davis et al., 1998). Because PMA activates cPKC and nPKC subtypes, it is not clear whether a particular PKC subtype regulates EAAC1-mediated uptake and cell surface expression or if these effects are mediated by different PKC subtypes. To address this possibility, the expression of PMA-sensitive PKC subtypes was analyzed in C6 glioma cells and the only subtypes expressed were PKC $\alpha$ , PKC $\delta$ , and PKC $\epsilon$ . Gö6976, a compound that selectively blocks cPKCs at nanomolar concentrations, partially inhibited the PMA-induced increase in activity, but completely abolished the increase in EAAC1 cell surface expression, suggesting that these effects are mediated by PKC $\alpha$ , because it is the only cPKC expressed by C6 cells. Na<sup>+</sup>-dependent glycine transport activity was not increased under these conditions, suggesting that the Gö6976-insensitive increase in activity cannot be attributed to a change in the Na<sup>+</sup> electrochemical gradient. When PKC $\delta$  was protected from down-regulation, short-term PKC activation had no effect on EAAC1 activity or cell surface expression; however, when PKC $\epsilon$  was preserved, short-term PKC activation increased EAAC1-mediated activity but had no effect on cell surface expression. These results suggest that PKC $\alpha$  is selectively involved in the regulation of EAAC1 cell surface expression and that PKC $\epsilon$  regulates EAAC1 activity by a trafficking-independent mechanism.

The expression patterns of PKC subtypes have been previously examined in C6 glioma cells, but the results vary between studies (Chen and Wu, 1995; Moreton et al., 1995; Brodie et al., 1998). Despite the discrepancies, PKC $\alpha$ , PKC $\delta$ , and PKC $\epsilon$  were observed in all these prior studies, and this pattern of expression is in agreement with the subtypes detected in the current study. The differences in the expression pattern may be attributed to differences in the source of C6 glioma cells, culture conditions, or other variables. For example, confluence/cell cycle and differentiation are both thought to alter the patterns of PKC expression in C6 cells (Moreton et al., 1995; Brodie et al., 1998).

Recent studies suggest that the cell surface expression and the intrinsic activity of some transporters can be regulated independently by different mechanisms, suggesting that this dual mode of regulation may emerge as a general phenomenon. In adipocytes, the insulin-induced increase in glucose uptake is associated with increased cell surface expression of the GLUT4 subtype of glucose transporter, but the number of transporters delivered to the cell membrane may not be sufficient to account for the increase in glucose uptake (Zierler, 1998). It has also been shown that GLUT4 translocation occurs much faster than the increase in transporter activity, suggesting that nonfunctional transporters may be redistributed to the plasma membrane before activation (Somwar et al., 2001). To explain these observations, it has been proposed that insulin stimulates two independent signaling pathways. The first involves the activation of PI3K and reg-

ulates GLUT4 translocation. The second requires the activation of p38 mitogen-activated protein kinase and leads to stimulation of the GLUT4 molecules located at the cell membrane (Sweeney et al., 1999; Somwar et al., 2001). Similarly, an insulin-dependent increase in norepinephrine transport has been observed in SK-N-SH cells, this increase in activity is independent of transporter redistribution but also requires the activation of p38 mitogen-activated protein kinase (Apparsundaram et al., 2001). In a final example, the intrinsic activity and the cell surface expression of the GAT1 subtype of GABA transporter can also be regulated by PKC. It seems that the change in intrinsic activity requires a PKC-regulated interaction between GAT1 and syntaxin1A, and this interaction alters the rate of GABA flux through the transporter (Deken et al., 2000; Horton and Quick, 2001). These data suggest that transporter activity and cell surface expression may be independently regulated; however, to our knowledge, there are no earlier examples of two different PKC subtypes specifically regulating the cell surface expression and catalytic efficiency of a transporter.

PKC regulates the activity and cell surface expression of several plasma membrane proteins, including neurotransmitter transporters (serotonin, dopamine, norepinephrine, and GABA) and G-protein coupled receptors; however, there are few examples in which specific PKC subtypes have been associated with a particular type of regulation. For example, in cultured fibroblasts, the regulation of Na<sup>+</sup>-dependent L-aspartate uptake parallels the activation and translocation of PKC $\epsilon$ , suggesting a specific role of PKC $\epsilon$  in the regulation of transport activity in these cells (Franchi-Gazzola et al., 1994). However, it is not known which Glu transporters are regulated under these conditions (Cooper et al., 1998). In NIH 3T3 cells, the Pit-2 subtype of phosphate transporter is specifically regulated by PKC $\epsilon$ , but it is not clear whether this effect is related to redistribution of the transporter or a change in intrinsic activity (Jobbagy et al., 1999). The regulation of the Na<sup>+</sup>/H<sup>+</sup> antiporter is produced by the activation of PKC $\alpha$  and PKC $\epsilon$ , but the mechanisms of these effects have not been identified (Karim et al., 1995).

In the present study, we found that pharmacological blockade or down-regulation of PKC $\alpha$  blocked the PMA-induced increase in EAAC1 cell surface expression, providing evidence that PKC $\alpha$  specifically regulates trafficking of EAAC1. Although it is not clear whether PKC $\alpha$  activates translocation of EAAC1 from a subcellular compartment to the plasma membrane or inhibits constitutive endocytosis, there is some evidence that PKC $\alpha$  may be a common mediator of the regulated trafficking of proteins. In HepG2 cells, PKC $\alpha$  is localized to the Golgi/trans-Golgi network and activation of PKC increases vesicle budding at the endoplasmic reticulum/Golgi/trans-Golgi network and promotes the release of proteoglycans (Buccione et al., 1996; Westermann et al., 1996). In MCF-7 cells, it seems that PKC $\alpha$  activation results in formation of  $\beta$ 1 integrin-PKC $\alpha$  complexes and translocation of these complexes to the plasma membrane (Ng et al., 1999). In vivo and in vitro, there is a large nonplasma membrane pool of EAAC1 (Davis et al., 1998; He et al., 2000). In vitro, under baseline conditions, EAAC1 is concentrated in vesicular structures that are often perinuclear, but after PKC activation, EAAC1 forms discrete clusters on the cell surface (Davis et al., 1998). These observations suggest that it is possible that PKC $\alpha$  increases the rate of translocation of



EAAC1 from a subcellular compartment to the plasma membrane.

Previously we suggested that PKC might regulate the activity of EAAC1 by a mechanism independent of changes in cell surface expression (Davis et al., 1998). In the present report, we provide further evidence that PKC regulates EAAC1 catalytic efficiency and identify PKC $\epsilon$  as a likely mediator of this regulation. Although it is possible that the trafficking-independent increase in transport activity is a nonspecific effect of the conditions employed, there are two observations that reduce this likelihood. First, the Gö6976-insensitive increase in activity is blocked by a general PKC inhibitor, providing evidence that this effect is PKC-dependent. Second, the Gö6976-insensitive increase is not accompanied by an increase in Na<sup>+</sup>-dependent glycine transport, reducing the likelihood that this effect is related to a generalized change in the Na<sup>+</sup> gradients required for transporter function. Because EAAC1 contains PKC phosphorylation sites, it is possible that PKC $\epsilon$  directly phosphorylates EAAC1. Consistent with this idea, PKC activation also increases the activity of GLT-1, another glutamate transporter (Casado et al., 1993). This effect depends on the phosphorylation of a PKC consensus site (Ser113) that is conserved in EAAC1. It is also possible that PKC $\epsilon$  indirectly promotes a change in catalytic efficiency by inducing the interaction of an accessory protein with EAAC1, but this possibility has not been explored. In fact, PKC activation regulates the GAT1 subtype of GABA transporter by increasing an interaction between GAT1 and syntaxin 1A (see Deken et al., 2000 for original citation). GTRAP3-18, a recently identified protein that interacts with EAAC1, alters the  $K_m$  value for the transport but not the  $V_{max}$  (Lin et al., 2001); therefore, it seems unlikely that this particular interaction explains the effects mediated by PKC $\epsilon$ .

Based on recent physiological studies (see *Introduction*), defining the mechanisms that regulate Glu transporters may have important implications for understanding the mechanisms that underlie changes in synaptic plasticity. EAAC1 protein is targeted to both perisynaptic and synaptic regions that are also enriched with the GluR2 subunit of the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (He et al., 2000). It has been suggested that long-term depression is dependent upon a reduction in the number of postsynaptic glutamatergic receptors, mainly the  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid subtype. Recent studies suggest that PKC $\alpha$  may be involved in this regulation, by a mechanism that requires both GluR2 phosphorylation and a physical interaction between PKC $\alpha$  and GluR2 (Chung et al., 2000; Perez et al., 2001). Although clearly speculation at this time, it would be interesting if PKC $\alpha$  activation were to result in the simultaneous regulation of EAAC1 and GluR2, because these two molecules might have a complementary effect on excitatory signals. In preliminary studies, we have observed that PKC activation induces an association between PKC $\alpha$  and EAAC1 (M. I. González and M. B. Robinson, unpublished observations), suggesting that similar mechanisms may regulate receptors and transporters. Although one might predict that increases in EAAC1 cell surface expression might contribute to long-term depression, recent studies have found evidence that long-term potentiation and contextual fear conditioning are associated with increased EAAC1 cell surface expression (Levenson et al.,

2001). At present, it is unclear whether changes in EAAC1 are required for either long term potentiation or long term depression, but it is assumed that defining the mechanisms that regulate EAAC1 will provide additional tools to address this issue.

In summary, we have found that although C6 glioma cells express several PKC subtypes, only PKC $\alpha$  is required for the PKC-dependent increase in EAAC1 cell surface expression. Furthermore, we found evidence that PKC $\epsilon$  increases EAAC1-mediated uptake by a trafficking-independent mechanism that is consistent with an increase in the catalytic efficiency of EAAC1. These results suggest that EAAC1 cell surface expression and intrinsic activity can be regulated independently by different PKC subtypes and suggests the existence of a novel mechanism for the regulation of glutamate transport that may contribute to the modulation of synaptic efficacy.

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**Address correspondence to:** Dr. Michael B. Robinson, 502N Abramson Pediatric Research Building, 3615 Civic Center Boulevard, Philadelphia, PA 19104-4318. E-mail: robinson@pharm.med.upenn.edu