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# Protein Kinase C Isoform δ Is Involved in the Stimulation of the $Na^+$-$H^+$ Exchanger in $C_6$ Glioma Cells

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**SUMMARY**

Protein kinase C (PKC), the major receptor for tumor-promoting phorbol esters, is a family consisting of at least 12 distinct isoforms. In $C_6$ glioma cells, short term (10 min) treatment with 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) results in a dose-dependent translocation of PKCα, PKCδ, and PKCθ. Long term (24 hr) treatment with appropriate doses of TPA results in the complete down-regulation of PKCδ but not of PKCα and PKCθ. This property was used to determine which isoform might be involved in the activation of the glial cell $Na^+$-$H^+$ exchanger. It was found that (a) TPA has dose-dependent effects on PKCα, PKCδ, and PKCθ translocation and on $Na^+$-dependent, EIPA-sensitive $Na^+$-$H^+$ exchanger stimulation; (b) the antiporter is blocked both by staurosporine and in cells in which PKCα, PKCδ, and PKCθ have been completely down-regulated; (c) the inactive form (α-TPA) of TPA, which does not induce translocation of the three isoforms (α, δ, and θ) has no effect on the antiporter; (d) Western blot analysis demonstrated dose-dependent TPA (10, 30, 100, or 1000 nM)-induced translocation of PKCα, PKCδ, and PKCθ. TPA (10, 30, 100, and 1000 nM)-induced $Na^+$-$H^+$ exchanger activation was also found to be dose dependent. There was no difference in TPA-induced $Na^+$-$H^+$ exchanger activation between 30 and 100 nM; it correlated with the extent of TPA-induced PKCδ translocation over the same concentration range, suggesting that isoform responsible for the exchanger activation is PKCδ; (e) When 1 μM TPA is added after prior treatment of the cells with 10 nM TPA, an additive effect on $Na^+$-$H^+$ exchanger is seen that is not observed when the initial stimulus is 30 nM TPA, as would be expected if the PKCδ isoform were responsible for exchanger activation; (f) Finally, when PKCδ, but not PKCα and PKCθ, is completely down-regulated by 24-hr pretreatment with 10, 30, or 100 nM TPA, the $Na^+$-$H^+$ exchanger can no longer be stimulated by 1 μM TPA; when 1 nM TPA pretreatment is used, no down-regulation occurs and the exchanger still responds to 1 μM TPA. We have shown that the $Na^+$-$H^+$ exchanger in $C_6$ glioma cells can be stimulated by TPA-induced PKC activation and, for the first time, that PKCδ is involved in the activation of this antiporter. Our results also suggest that different members of the PKC family within a single cell elicit specific physiological responses.

The $Na^+$-$H^+$ exchanger is an electroneutral membrane glycoprotein that exchanges external $Na^+$ ions for internal $H^+$ ions when the $pH_i$ falls (1). It is a "housekeeping" $pH_i$ regulator and is the most widely studied antiporter as it is ubiquituously expressed; it is rapidly activated in response to various extracellular signals, leading to sustained cytoplasmic alkalinization. These signals can be grouped into three categories: mitogenic signals, including phorbol esters, growth factors (2–4) and cytokines (5); hormones or peptides, which activate second messenger systems (6–9); mechanical signals, including osmotic shock (10, 11) and shear stress (12). Recent molecular cloning studies have demonstrated the existence of at least four isoforms of the $Na^+$-$H^+$ exchanger (NHE-1 through NHE-4). The first complete amino acid sequence deduced was that of NHE-1, the human growth factor-activatable $Na^+$-$H^+$ exchanger, or housekeeping $pH_i$ regulator (1). When compared with the NHE-1 isoform, the NHE-2 isoform has a 10–50-fold lower affinity for EIPA, whereas the NHE-3 isoform is much more resistant than NHE-1 to amiloride or EIPA (13). Unlike other $Na^+$-$H^+$ exchanger isoforms, NHE-4 transfected into mutant fibroblasts demonstrated amiloride sensitivity (14). $Na^+$-$H^+$ exchanger has been shown to be stimulated by PKC activation in many cells or tissues, such as cardiac myocytes and Purkinje fibers (8, 15–17), leukocytes (18), fibroblasts (19), and epidermal cells (20). During PKC-induced $Na^+$-$H^+$ exchanger stimulation, it is suggested that the exchanger is phosphorylated at, or near, the cytoplasmic modifier site on the regulatory domain of the antiporter (1, 21–23), thereby leading to an alkaline shift of the resting $pH_i$.

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**ABBREVIATIONS:** PKC, protein kinase C; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EIPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; TTBS, Tris-buffered saline/Tween 20; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; $pH_i$, intracellular pH.

The PKC family, which consists of phospholipid-dependent serine/threonine kinases, is believed to play a major role in cellular functions. Molecular cloning has shown that it consists of at least 12 isoforms with different tissue expressions (24–26). All isoforms have closely related structures but differ in their individual properties, and they are divided into three groups: group A, consisting of four conventional PKCs (cPKC$\alpha$, cPKC$\beta_I$, cPKC$\beta_{II}$, and cPKC$\gamma$) and requiring calcium and phospholipid for enzymatic activity; group B, consisting of four new PKCs (nPKC$\delta$, nPKC$\epsilon$, nPKC$\eta$(L), and nPKC$\theta$), which are calcium independent but phospholipid dependent; and group C, consisting of four atypical PKCs (aPKC$\zeta$, aPKC$\lambda$, aPKC$\iota$, and aPKC$\mu$). Groups A and B, but not group C, can be activated by diacylglycerol or phorbol esters (e.g., TPA) (24). Activation of PKC is believed to involve its translocation from the cytosol to the membrane by endogenous diacylglycerol. This membrane association/activation event can be mimicked by TPA, which elicits irreversible insertion of PKCs into the lipid bilayer, causing cumulative, long term stimulation of the enzyme (27), eventually terminated by its proteolytic degradation (down-regulation) (28).

Although cells generally contain more than one PKC isoform, little is known about their distinct roles (24). In our previous study using Western blot analysis and antibodies specific for each of the isoforms PKC$\alpha$, PKC$\beta$, PKC$\gamma$, PKC$\delta$, PKC$\epsilon$, and PKC$\zeta$, we found the isoforms PKC$\alpha$, PKC$\delta$, and PKC$\zeta$ to be abundant in $C_6$ glioma cells, whereas PKC$\beta$ and PKC$\gamma$ are not expressed and PKC$\epsilon$ is expressed at a very low level (29, 30). In the present study, we showed that PKC$\theta$, originally found to be abundantly expressed in skeletal muscle (31), is also expressed in $C_6$ glioma cells. Using different doses of TPA, we also found conditions under which differential translocation and down-regulation of PKC$\alpha$, PKC$\delta$ (32), and PKC$\theta$ can be induced in these cells and chose the conditions under which PKC$\delta$ is completely down-regulated while the levels of PKC$\alpha$ and PKC$\theta$ are not affected. TPA is, therefore, a useful tool for distinguishing among the roles of these different PKC isoforms in situations such as activation of the $Na^+$-$H^+$ exchanger in glial cells. A somewhat similar method was used by Puceat *et al.* (33) to determine the functional roles of PKC isoforms in rat cardiac myocytes.

## Experimental Procedures

**Chemicals and solutions.** Unless indicated otherwise, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies against PKC$\alpha$-specific and PKC$\delta$-specific peptide sequences, DMEM, fetal calf serum, penicillin, and streptomycin were purchased from GIBCO-RBL (Gaithersburg, MD). Affinity-purified rabbit polyclonal antibodies against PKC$\eta$-specific and $\theta$-specific peptide sequences were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-labeled donkey anti-rabbit second antibody and the ECL detecting reagent were purchased from Amersham International (Arlington Heights, IL). TPA and $\alpha$-TPA were purchased from L.C. Services Corp (Woburn, MA) and dissolved in 0.1% DMSO. Reagents for SDS-PAGE were obtained from Bio-Rad (Hercules, CA), $^{125}$I-protein A from DuPont-NEN (Boston, MA), and EIPA from Research Biochemicals International (Natick, MA). All of the pH-related experiments were performed in HEPES-buffered solution containing (in mM): NaCl 118, KCl 4.7, $KH_2PO_4$ 1.2, $MgCl_2$ 1.2, $CaCl_2$ 2.0, glucose 10, and HEPES 20, pH adjusted to 7.4 at 37° with NaOH.

**Cell culture and cell treatment.** $C_6$ glioma cells from the American Type Culture Collection (Rockville, MD) were grown in DMEM supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 $\mu$g/ml streptomycin in an atmosphere of 5% $CO_2$/95% humidified air at 37° on 24-mm-diameter coverslips in 35-mm dishes for experiments involving $pH_i$ measurement and directly in 145-mm Petri dishes for PKC isoform assay.

**Preparation of cell extracts and immunoblot analysis.** For PKC isoform assay, confluent cells were treated with different doses of TPA or DMSO (control) in growth medium for 10 min or 24 hr before harvesting; the cells were then rapidly washed with ice-cold phosphate-buffered saline, scraped off the plates, and collected by centrifugation for 10 min at 1000 × *g*.

Cell extracts were prepared and immunoblot analyses were performed as described previously (29). In brief, cytosolic extracts and membrane fractions (100 $\mu$g of protein) were denatured by heating in Laemmli stop solution (SDS plus mercaptoethanol) and subjected to SDS-PAGE on a 10% running gel. The proteins were then transferred to a nitrocellulose membrane, which was incubated successively at room temperature with 1% bovine serum albumin in TTBS (50 mM Tris·HCl, 0.15 M NaCl, 0.05% Tween-20, titrated to pH 7.5) for 1 hr, with rabbit anti-PKC$\alpha$ and anti-PKC$\delta$ antibodies diluted 1:250 in TTBS containing 1% bovine serum albumin for 3 hr, and with [$^{125}$I]protein A (0.4 $\mu$g, 4–6 $\mu$Ci/20 ml) in TTBS for 1 hr. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands were visualized using a Phosphor Imager-Image Quant apparatus (Molecular Dynamics, Sunnyvale, CA). For immunoblotting experiments on PKC$\eta$ and $\theta$, ECL detection was performed as previously described (34); the membrane was incubated successively at room temperature with 0.1% dry milk in TTBS for 1 hr, with rabbit anti-PKC$\eta$ or PKC$\theta$ antibodies for 1 hr, and with horseradish peroxidase-labeled antibody for 1 hr. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands detected by ECL reagents were developed using Hyperfilm-ECL (Amersham International).

**Measurement of $pH_i$.** Measurement of $pH_i$ has been described in detail previously (35). In brief, glioma cells, grown on a coverslip, were loaded with 5 $\mu$M 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein for 20–30 min at room temperature in HEPES-buffered solution. The cells were then washed with the same solution and excited alternately by 490- and 440-nm wavelength light using a filter wheel (Cairn Research, Kent, England) rotating at 32 Hz. The overall sampling rate was 0.5 Hz. The emission ratio 490/440 from the intracellular 2′,7′-bis(carboxyethyl)-5,6-carboxyfluorescein was calculated and converted to a linear pH scale using *in situ* calibration

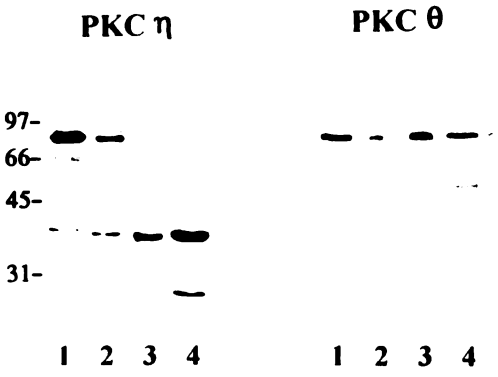



**Fig. 1.** Expression of PKC$\theta$ in $C_6$ glioma cells. PKC$\eta$ and $\theta$ in whole-cell lysates of rat brain (*lane 1*), mouse heart (*lane 2*), mouse skeletal muscle (*lane 3*), and $C_6$ glioma cells (*lane 4*) were detected by protein immunoblotting. Whole-cell lysates of various tissues and $C_6$ glioma cells were prepared, and 100-$\mu$g samples of protein were separated by 10% SDS-PAGE, transferred to nitrocellulose paper, and immunodetected with PKC$\eta$- and PKC$\theta$-specific antibodies (1:50) as described in Experimental Procedures.

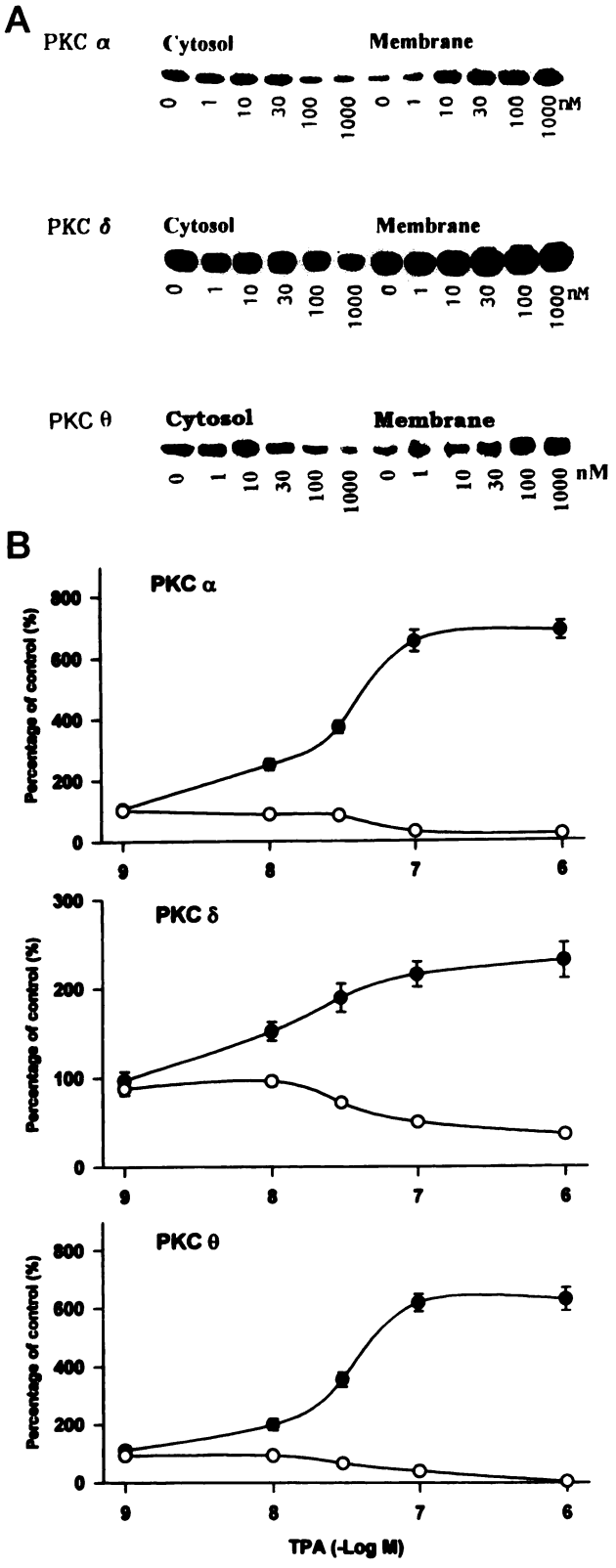



**Fig. 2.** A, TPA dose-dependent translocation of PKC$\alpha$, PKC$\delta$, and PKC$\theta$ in $C_6$ glioma cells. Data from Western blots are shown. Cells were treated with 0.1% DMSO or various doses (1, 10, 30, 100, or 1000 nM) of TPA for 10 min and then fractionated into cytosolic and membrane fractions as described in Experimental Procedures. Proteins (100 $\mu$g) were separated by 10% SDS-PAGE, transferred to nitrocellulose paper, and immunodetected with PKC$\alpha$-, PKC$\delta$-, and PKC$\theta$-specific antibodies. B, Quantitative data of TPA dose-dependent translocation of PKC$\alpha$, PKC$\delta$, and PKC$\theta$ in $C_6$ glioma cells. Western blots were analyzed quantitatively by Phosphor Imager-Image Quant. Quantitative data are presented as mean ± standard error for at least four experiments. Cytosolic (○—○) and membrane (●—●) fractions were fractionated as described in Experimental Procedures.

data obtained at the end of the experiment by the nigericin technique, as described elsewhere (36). A calibration curve similar to that used in our previous work (35) was established by measuring the fluorescence ratio values between $pH_i$ 4.5 and 9.5. Between $pH_i$ 6.0 and 8.0, the response is linear and fits the equation: $pH_i = pK + \log [(R_{max} - R)/(R - R_{min})] + \log (F_{440\ min}/F_{440\ max})$, where $R$ is the ratio of 530-nm fluorescence at 490-nm excitation divided by 530-nm fluorescence at 440-nm excitation. $R_{max}$ and $R_{min}$ are the maximum and minimum ratio values from the data curve and p$K$ is the dissociation constant for the dye, taken as 55 nM (pH 7.26).

**Statistical Analysis.** All data are expressed as the mean ± standard error.

## Results

In our previous study, we tested $C_6$ glioma cells for the presence of PKC isoforms $\alpha$, $\beta$, $\gamma$, $\delta$, $\epsilon$, and $\zeta$ and found the isoforms $\alpha$, $\delta$, and $\zeta$ to be abundant (29, 30). We have since tested for the presence of two additional new type isoforms, PKC$\eta$ and PKC$\theta$, and have therefore tested all known conventional and new isoforms. We did not test for atypical PKC isoforms in the present study because it is known that these isoforms, as well as isoform $\zeta$, are not activated by TPA (24, 29).

**Expression of PKC$\theta$ in $C_6$ glioma cells.** PKC$\eta$ and PKC$\theta$ are known to be abundantly expressed in heart and skeletal muscle, respectively (31, 37). We therefore used these two tissues from the mouse as positive controls in our comparative study. We found PKC$\eta$ to be abundantly expressed in rat brain and mouse heart but not in mouse skeletal muscle or $C_6$ glioma cells (Fig. 1, PKC$\eta$, *lanes 1–4*). The 36–40-kDa bands (Fig. 1, *left*) represent an unknown protein that is apparently cross-reactive with PKC$\eta$ antibody. PKC$\theta$ was found to be expressed in rat brain, mouse skeletal muscle, and $C_6$ glioma cells (Fig. 1, PKC$\theta$, *lanes 1, 3, and 4*) but only weakly in mouse heart (Fig. 1, *right lane 2*). $C_6$ glioma cells, therefore, also express the new type PKC isoform $\theta$ but not the $\eta$ isoform.

**Dose-dependent TPA-induced translocation of PKC$\alpha$, PKC$\delta$, and PKC$\theta$.** After a 10-min treatment of cells with different doses of TPA (1, 10, 30, 100, and 1000 nM), the translocation of all three isoforms, PKC$\alpha$, PKC$\delta$, and PKC$\theta$ (Fig. 2), was found to be dose dependent over the range of 10–1000 nM. TPA 1 nM had no effect, whereas at 1000 nM, the response plateaued at a value slightly higher than that at 100 nM TPA (Fig. 2B). The extent of translocation induced by 10, 30, and 100 nM of TPA, calculated as a multiple of the resting membrane level, was 2.5 ± 0.2, 3.7 ± 0.2, and 6.5 ± 0.4 times control for PKC$\alpha$; 1.5 ± 0.1, 1.9 ± 0.2, and 2.2 ± 0.1 times control for PKC$\delta$, as reported previously (32); and 2.0 ± 0.2, 3.6 ± 0.3, and 6.2 ± 0.3 times control for PKC$\theta$. Thus, after a 10-min treatment with 30 or 100 nM TPA, the translocation of PKC$\alpha$ and PKC$\theta$ was increased to a greater extent (3.7 increasing to 6.5 times control; 3.6 increasing to 6.2 times control, respectively) than that of PKC$\delta$ (1.9 increasing to 2.2 times control) (Fig. 2B).

**Differential TPA-induced down-regulation of PKC$\alpha$, PKC$\delta$, and PKC$\theta$.** We studied the translocation and down-regulation of PKC$\alpha$, PKC$\delta$, and PKC$\theta$ after a 10-min or 24-hr

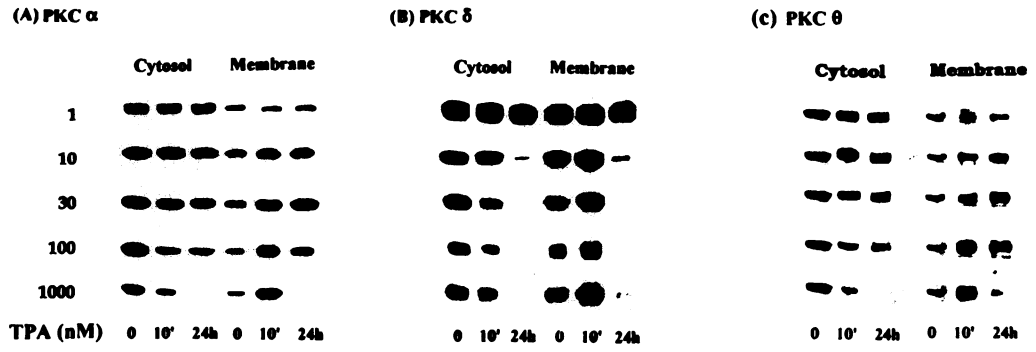



**Fig. 3.** Protein immunoblots showing translocation and down-regulation of PKC$\alpha$ (A), PKC$\delta$ (B), and PKC$\theta$ (C). $C_6$ glioma cells were treated with 1, 10, 30, 100, or 1000 nM TPA (10 min or 24 hr) or 0.1% DMSO (24 hr) and separated into cytosolic and membrane fractions that were then subjected to SDS-PAGE. Results are representative of more than four experiments.

treatment with 1, 10, 30, 100, and 1000 nM TPA. The results for the 10-min treatment (Fig. 3) were identical to those presented in Fig. 2. After a 24-hr treatment with 1 nM TPA, no effect on any of the three isoforms was seen, whereas at 1000 nM TPA, all three isoforms showed complete disappearance; however, at TPA concentrations of 10, 30, or 100 nM, a differential effect was seen, with PKC$\delta$ being almost completely down-regulated and PKC$\alpha$ and PKC$\theta$ being unaffected (Fig. 3). The differential down-regulation of PKC$\alpha$ and PKC$\delta$ and the quantification data have been demonstrated previously (32), and this finding was used in the subsequent studies to identify the isoform that is involved in $Na^+$-$H^+$ exchanger stimulation.

$\alpha$-TPA, the inactive form of the phorbol ester, was used to confirm the TPA specificity of PKC$\alpha$, PKC$\delta$, and PKC$\theta$ activation. When cells were treated for 10 min or 24 hr with 1 $\mu$M $\alpha$-TPA, neither translocation nor down-regulation of the three PKC isoforms occurred (data not shown).

**PKC activation stimulates the $Na^+$-$H^+$ exchanger.** In HEPES-buffered solution, the mean resting $pH_i$ of $C_6$ glioma cell monolayers was 7.20 ± 0.1 (44 preparations). Fig. 4A shows that after a 2–4-min latency period, the addition of 1 $\mu$M TPA irreversibly alkalinized the $pH_i$ by 0.15 ± 0.01 pH unit (20 preparations; Table 1). $\alpha$-TPA alone had no effect on the resting $pH_i$ (Fig. 4B, *first arrow*) and did not affect the subsequent alkalinization induced by 1 $\mu$M TPA (Fig. 4B, *second arrow*, five preparations). DMSO 0.1% (the solvent for TPA and $\alpha$-TPA) had no effect on the $pH_i$ (three preparations, data not shown). To test whether the the alkalinizing effect of TPA on the resting $pH_i$ was due to activation of the amiloride-sensitive $Na^+$-$H^+$ exchanger, the effect of amiloride (1.0 mM, three preparations) was tested (Fig. 4C). After the addition of amiloride, the resting $pH_i$ slowly acidified (Fig. 4C, *first*

TABLE 1

**Dose dependent $pH_i$ increase induced by short term (10 min) application of TPA**

| | TPA (nM) | | | |
|---|---|---|---|---|
| | 1000[a] | 100[a] | 30 | 10 |
| Increase in $pH_i$ (unit) | 0.15 ± 0.01 | 0.15 ± 0.02 | 0.13 ± 0.01 | 0.073 ± 0.003 |
| *n* | 20 | 5 | 6 | 14 |

[a] Significant difference ($p < 0.05$, Student's *t* test) compared with the 10 nM response.

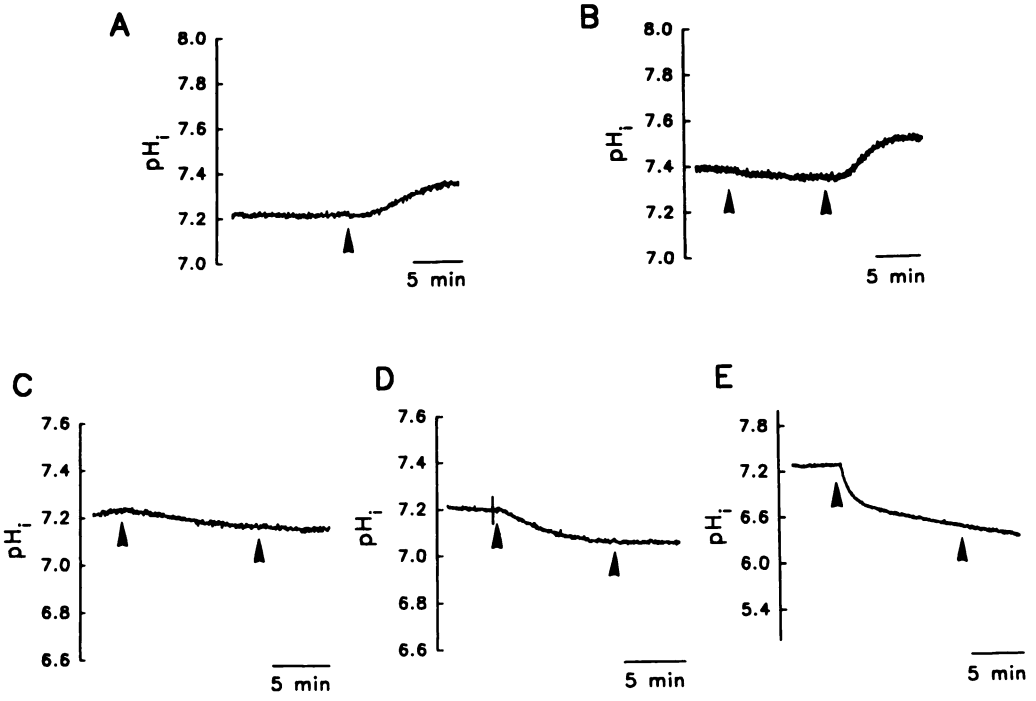



**Fig. 4.** PKC activation stimulates the Na-H exchanger in $C_6$ glioma cells. *Arrow*, time of addition. A. *Arrow*, 1 $\mu$M TPA. B. *First arrow*, 1 $\mu$M $\alpha$-TPA; *second arrow*, 1 $\mu$M TPA. C. *First arrow*, 1 mM amiloride; *second arrow*, 1 $\mu$M TPA. D. *First arrow*, 10 $\mu$M EIPA; *second arrow*, 1 $\mu$M TPA. E. *First arrow*, $Na^+$-free, NMDG substituted for external $Na^+$ ions; *second arrow*, 1 $\mu$M TPA.

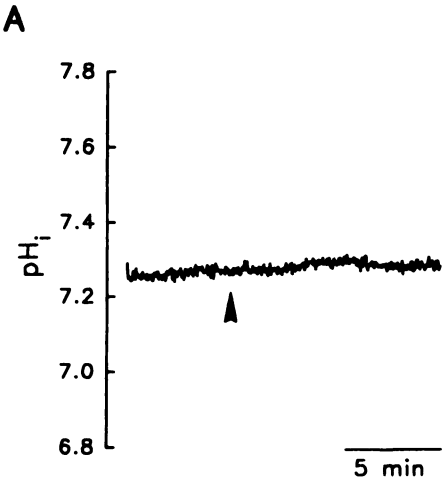
*arrow*), possibly because of accumulation of metabolic acid due to $Na^+$-$H^+$ exchanger inhibition. Once $pH_i$ started to stabilize, the addition of 1 μM TPA no longer had an effect on $pH_i$ (Fig. 4C, *second arrow*). Furthermore, EIPA (10 μM), a more potent and selective inhibitor of the antiporter than amiloride (38, 39), also caused acidification of the resting $pH_i$ (Fig. 4D, *first arrow*) and blocked the TPA effect on the $Na^+$-$H^+$ exchanger (Fig. 4D, *second arrow*). In a third approach involving the blockade of the $Na^+$-$H^+$ exchanger, external sodium ions were completely replaced by N-methyl-D-glucamine (i.e., $Na^+$-free conditions, Fig. 4E, four preparations); under these conditions, the reverse mode of exchange of external $H^+$ ions for internal $Na^+$ ions was immediately activated, resulting in an initial rapid acidification (Fig. 4E, *first arrow*) and a subsequent slower acidification phase due to the accumulation of internal metabolite through antiporter blockade; again, TPA (1 μM) had no effect on $pH_i$ (Fig. 4E, *second arrow*). TPA-induced alkalinization in $C_6$ glioma cells can therefore be attributed to activation of the $Na^+$-$H^+$ exchanger as the alkaline shift of the resting $pH_i$ is $Na^+$ dependent and EIPA sensitive.

As shown in Figs. 2 and 3, TPA is clearly a potent PKC activator in $C_6$ glioma cells. Fig. 5A shows that its effect is directly due to activation of PKC and not to a nonspecific effect of TPA as it is completely inhibited by a potent PKC inhibitor, staurosporine (10 nM, four preparations). To rule out the possibility that staurosporine might have a nonspecific inhibitory effect on the antiporter (Fig. 5A), the cells were pretreated with 10 nM (four preparations) or 100 nM (four preparations) staurosporine for 10 min before the addition of 10 μM EIPA (Fig. 5B). Under these conditions, the $Na^+$-$H^+$ exchanger was still active after staurosporine treatment as the blocking effect of EIPA on the antiporter was identical to that seen in Fig. 4D.

We then tested the effect of 1 μM TPA on cells in which all three isoforms (PKCα, PKCδ, and PKCθ) had been completely down-regulated. Cells were treated for 24 hr with either 1 μM TPA or 1 μM α-TPA. As shown in Fig. 6A (eight preparations), the addition of 1 μM TPA to down-regulated cells (pretreated with 1 μM TPA) did not cause an alkaline shift, whereas in non-down-regulated cells (pretreated with 1 μM α-TPA), the $Na^+$-$H^+$ exchanger was still TPA sensitive (Fig. 6B, four preparations). These results suggest that at least one of the three isoforms, PKCα, PKCδ, or PKCθ, is involved in exchanger activation. In the presence of EIPA, the resting $pH_i$ of down-regulated cells slowly acidified due to inhibition of the $Na^+$-$H^+$ exchanger (Fig. 6C; *first arrow*); when 1 μM TPA was added (Fig. 6C, *second arrow*, four preparations), there was no effect on $pH_i$ (compare with Fig. 6A). These results show that down-regulation of PKCα, PKCδ, or PKCθ has no inhibitory effect on the antiporter itself as the EIPA-sensitive component was still present in the down-regulated cells.

**Dose-dependent TPA-stimulation of the $Na^+$-$H^+$ exchanger.** As with TPA-induced PKCα, PKCδ, or PKCθ translocation (Fig. 2), TPA-induced stimulation of $Na^+$-$H^+$ exchange was also dose-dependent. Table 1 summarizes the effect of TPA on the resting $pH_i$; a statistically significant difference is seen between the results for 10 and 100 nM TPA, which correlates with the significant increase in PKCα, PKCδ, and PKCθ translocation seen between these two doses (Fig. 2B: 1.5 ± 0.1 versus 6.5 ± 0.4 times control for PKCα; 1.5 ± 0.1 versus 2.2 ± 0.1 times control for PKCδ; 2.0 ± 0.2 versus 6.2 ± 0.3 times control for PKCθ). However, the results using 30 and 100 nM TPA were not significantly different in terms of either the TPA-induced $pH_i$ increase (Table 1: 0.13 ± 0.01 versus 0.15 ± 0.02 pH units) or PKCδ translocation (Fig. 2B: 1.9 ± 0.2 versus 2.2 ± 0.1 times control), whereas the difference in translocation of PKCα and θ is marked (Fig. 2B: 3.7 ± 0.2 versus 6.5 ± 0.4 times control for PKCα; 3.6 ± 0.3 versus 6.2 ± 0.3 times control for PKCθ), indicating that it is probable that PKCδ is involved in the stimulation of the $Na^+$-$H^+$ exchanger.

To further investigate any possible differential role of PKCα, PKCθ, or PKCδ in activation of the $Na^+$-$H^+$ exchanger, the following experiments were performed. After a 10-min pretreatment of the cells with 10 nM TPA, the addition of 1 μM TPA (Fig. 7A, 14 preparations) resulted in a further alkaline shift; this effect was not seen after pretreatment with either 30 or 100 nM TPA (Fig. 7, B and C). Because the difference in translocation seen between 30 and 100 nM TPA is much less marked for PKCδ than for PKCα and PKCθ (Fig. 2B), this also suggests that it is PKCδ, and not PKCα or PKCθ, that might stimulate the antiporter.



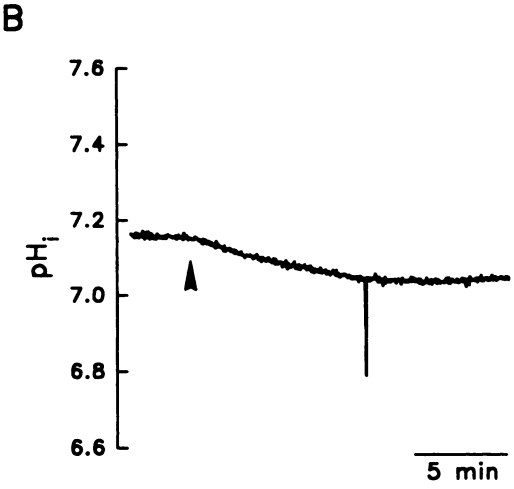




**Fig. 5.** Staurosporine inhibits the effect of TPA on the $Na^+$-$H^+$ exchanger. The cells were pretreated with 10 nM staurosporine for 15 min before (A) 1 μM TPA and (B) 10 μM was added (*arrows*).

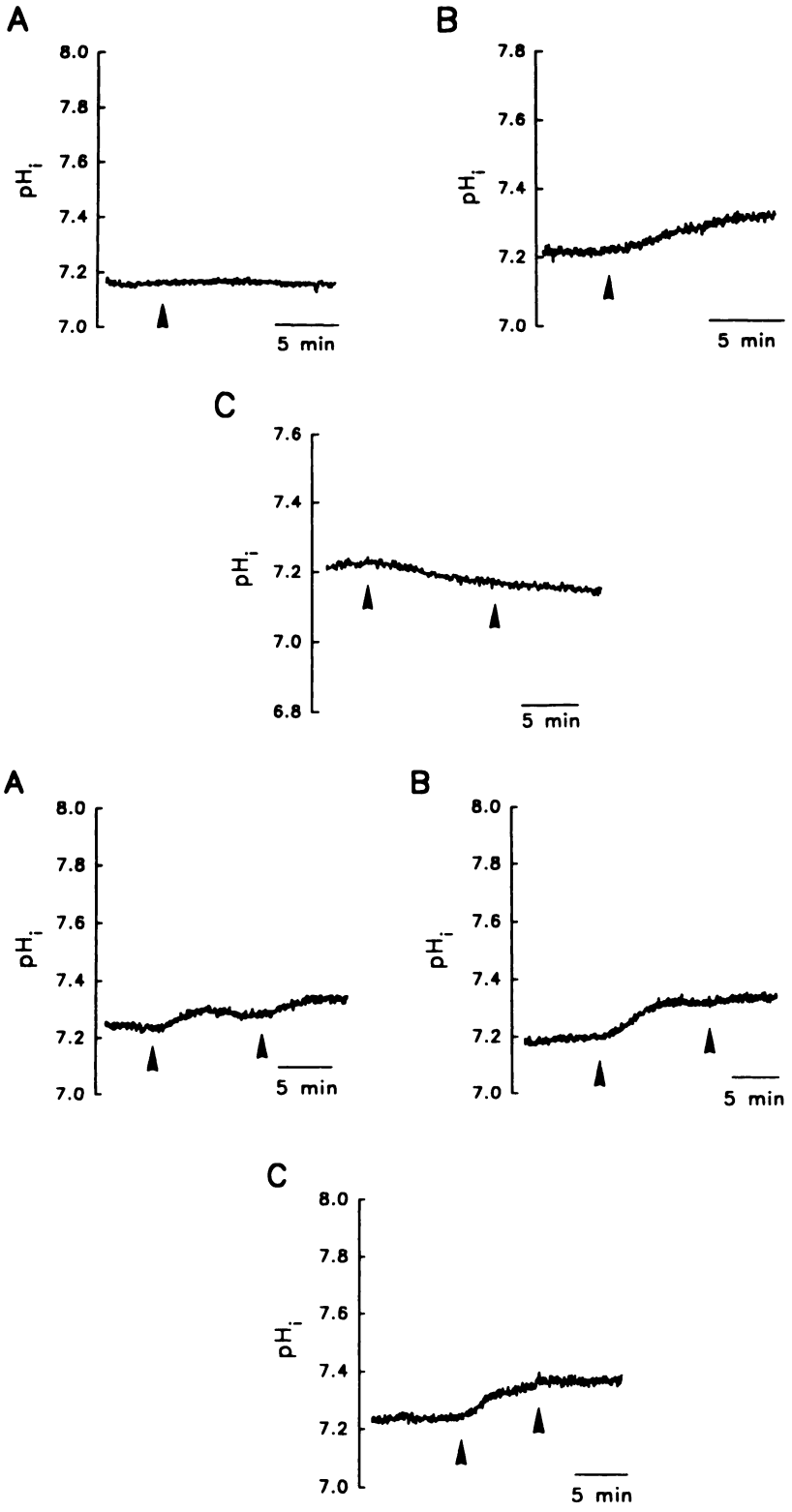



**Fig. 6.** Down-regulation of PKC blocks the effect of TPA on $pH_i$. The addition of 1 $\mu$M TPA to cells pretreated for 24 hr with (A) 1 $\mu$M TPA, (B) 1 $\mu$M $\alpha$-TPA or (C) 10 $\mu$M EIPA. C. *First arrow*, EIPA; *second arrow*, 1 $\mu$M TPA.




**Fig. 7.** The additive effect of TPA on internal pH. TPA (1 $\mu$M) (*second arrow*) was added to cells pretreated with (A) 10 nM TPA, (B) 30 nM TPA, or (C) 100 nM TPA (*first arrow*).

**TPA activation of the $Na^+$-$H^+$ exchanger is abolished after differential down-regulation of PKC$\delta$.** To further confirm that PKC$\delta$ is involved in antiporter activation, the cells were treated with 10, 30, or 100 nM TPA for 24 hr, conditions under which PKC$\delta$ is completely down-regulated but PKC$\alpha$ and PKC$\theta$ levels were unaffected (see Fig. 3). Under these conditions, the antiporter could no longer be stimulated by 1 $\mu$M TPA (Fig. 8, A through C), indicating that PKC$\alpha$ and PKC$\theta$ are not involved in the activation of $Na^+$-$H^+$ exchanger. However, if 1 nM TPA was used for the 24-hr pretreatment (no PKC translocation or down-regulation), the antiporter was still responsive to 1 $\mu$M TPA (0.15 $\pm$ 0.01 pH units, six preparations; Fig. 8D). This is a strong evidence that PKC$\delta$

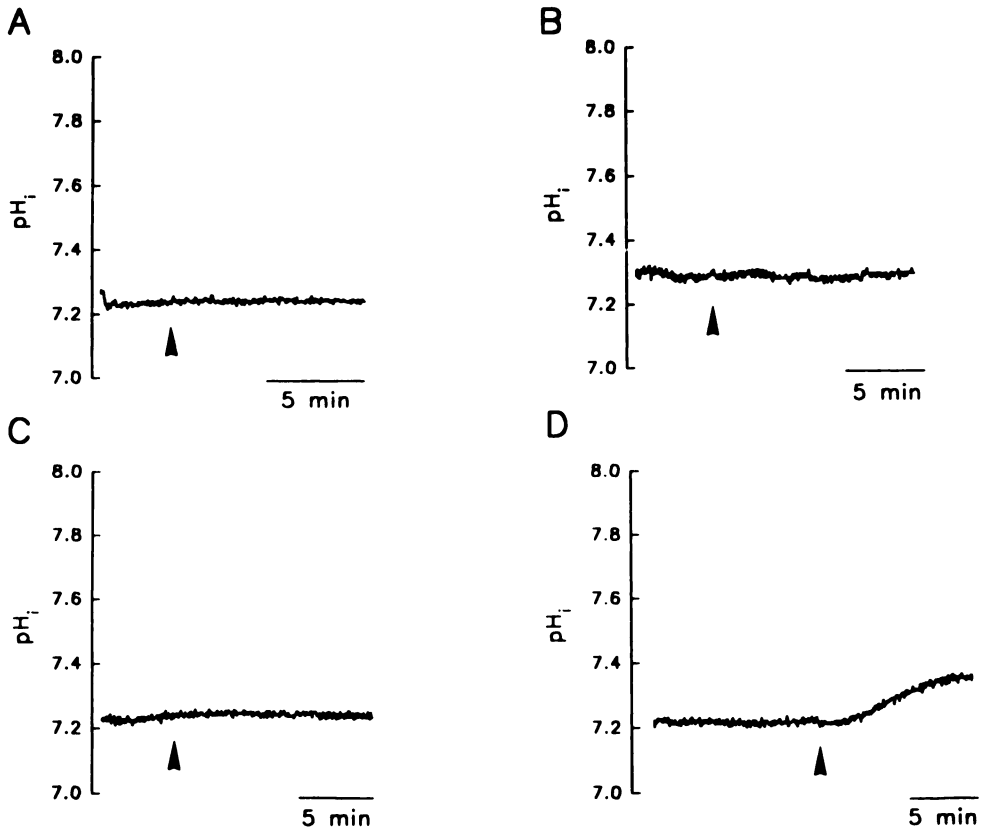



**Fig. 8.** Down-regulation of PKCδ abolishes TPA-induced cell alkalinization. Cells were pretreated for 24 hr with (A) 10 nM TPA, (B) 30 nM TPA, (C) 100 nM TPA, or (D) 1 nM TPA before the addition of 1 μM TPA (*arrow*).

alone, and not PKCα or PKCθ, is sufficient to stimulate the antiporter in $C_6$ glioma cells.

## Discussion

The $Na^+$-$H^+$ exchanger has been shown to play a pivotal role in regulating $pH_i$ during cellular acidification. Through regulation of $pH_i$, the exchanger plays an essential role in cell growth, mitogenesis, receptor-mediated signal transduction, secretion, volume regulation, and fertilization (14, 40, 41). In glial cells, we have found that the antiporter is very sensitive to both amiloride and EIPA and that, as shown in other cells (22, 42), it can be activated by phorbol esters (see below). We therefore suggest that $C_6$ glioma cells also have the housekeeping or ubiquitous isoform NHE-1.

We recently demonstrated that $C_6$ glioma cells express large amounts of PKCα, PKCδ, and PKCζ and trace amounts of PKCε and that TPA can induce translocation and down-regulation of the conventional and new, but not atypical, isoforms in this cell type (29). Although the known number of isoforms is increasing, the most recently cloned isoforms belong to the atypical group (λ, ι, and μ), as does PKCζ, the other abundant isoform in $C_6$ glioma cells. These atypical isoforms are not activated by TPA (43, 44) and can therefore be ruled out in the present study. The expression of two additional new types of isoforms, PKCη and θ, was examined in the present study; this means that we have tested for all known conventional and new isoforms. PKCθ was found to be expressed in $C_6$ glioma cells, whereas PKCη was not found (Fig. 1). Furthermore, the short term and long term effects of TPA on PKCθ and PKCα were found to be similar (Figs. 2 and 3). Therefore, with the use of different doses of TPA, differential translocation and down-regulation of PKCα, PKCθ, and PKCδ can be induced, making it possible to choose conditions under which PKCδ is completely down-regulated without any effect on PKCα and PKCθ. We have therefore demonstrated that it is the PKC isoform δ, and not the isoforms α and θ, which participates in stimulation of the $Na^+$-$H^+$ exchanger in $C_6$ glioma cells.

In the present study, three lines of evidence support the idea that it is PKC activation and not other nonspecific effects of TPA that stimulates the $Na^+$-$H^+$ exchanger in $C_6$ glioma cells. First, in these cells, TPA has a dose-dependent effect on both the translocation of PKCα, PKCθ, and PKCδ and the $Na^+$-dependent, EIPA-sensitive stimulation of the $Na^+$-$H^+$ exchanger, suggesting that PKCα, PKCθ, and/or PKCδ might be responsible for antiporter stimulation. Second, TPA activation of the antiporter is blocked in cells treated with staurosporine, a known PKC inhibitor, or in cells in which PKCα, PKCθ, and PKCδ have all been completely down-regulated by long term application of 1 μM TPA. Third, α-TPA, the inactive form of TPA, which does not induce PKCα, PKCθ, or PKCδ translocation or down-regulation, has no effect on the resting $pH_i$.

Further lines of evidence suggest that it is the isoform PKCδ that might mediate $Na^+$-$H^+$ exchanger activation in $C_6$ glioma cells. First, a statistically significant difference on the $pH_i$ is seen between the results for 10 and 100 nM TPA but not for 30 and 100 nM, which correlate well with the extent of TPA-induced PKCδ translocation seen over these two sets of concentration range, respectively (Table 1 and Fig. 2B). However, beyond a certain degree of activation of any PKC isoforms, no further effect on the antiport may be observed, and the present correlation may be coincidental; therefore, the following evidence is provided. Second, addi-

tive effects on $Na^+$-$H^+$ exchange are seen when 1 $\mu$M TPA is added after prior short term treatment of the cells with 10 nM but not with 30 nM TPA (Fig. 7, A and B), which correlates well with the observation that although PKC$\alpha$, PKC$\delta$, and PKC$\theta$ show significant increases in translocation between 10 and 100 nM TPA, the increase over the range of 30–100 nM TPA is much less pronounced for PKC$\delta$ than for PKC$\alpha$ and PKC$\theta$ (Fig. 2B). Third and most important, under conditions (24-hr pretreatment with 10, 30, and 100 nM TPA) in which PKC$\delta$, but not PKC$\alpha$ and PKC$\theta$, is completely down-regulated, the $Na^+$-$H^+$ exchanger is no longer susceptible to activation by 1 $\mu$M TPA (Fig. 8, A through C). Fourth, under conditions (24-hr pretreatment with 1 nM TPA) in which PKC$\alpha$, PKC$\theta$, and PKC$\delta$ are not down-regulated, 1 $\mu$M TPA can still activate the antiporter (Fig. 8D). We therefore conclude that PKC$\delta$ alone, and not PKC$\alpha$ or PKC$\theta$, is sufficient to activate the antiporter in $C_6$ glioma cells.

The tissue-specific expression of the 12 different PKC isoforms has recently been shown to relate to specialized cell functions. PKC$\alpha$ is involved in up-regulation of phospholipase D, gene expression, and cellular differentiation in many cells (45–46). Both PKC$\alpha$ and PKC$\epsilon$ are probably involved in neuritogenesis in SH-SY5Y neuroblastoma cells (47). In the NIH 3T3 fibroblast, overexpression of PKC$\epsilon$ may cause tumorigenesis (48). PKC$\alpha$ and PKC$\beta_1$ modulate phospholipase C and calcium channel activity in mouse pancreatic $\beta$ cells (49), and PKC$\beta_{II}$ is involved in the proliferation of erythroleukemia cells (50). Most of the above studies involved the overexpression of the specific isoform in cells that either do not normally express it or express it to only a small degree. In contrast, we have shown that $C_6$ glioma cells express considerable amounts of PKC$\alpha$, PKC$\delta$ (29), and PKC$\theta$ (present study) and that the levels of PKC$\alpha$, PKC$\theta$, and PKC$\delta$ show differential TPA susceptibility. The $C_6$ glioma cell is therefore a good model for studying the specific biological function of these PKC isoforms in a situation in which they are endogenous and abundant. Recently, Puceat *et al.* (33) (also using selective TPA-induced down-regulation of PKC$\alpha$, PKC$\delta$, and PKC$\epsilon$) found that PKC$\epsilon$ is responsible for phosphorylation of the MARCKS protein and that PKC$\alpha$ is responsible for expression of c-*fos* in neonatal rat cardiomyocytes.

A specific functional role for PKC$\delta$ has been demonstrated in Chinese hamster ovary, NIH 3T3, and myeloid progenitor line 32D cells, all of which overexpress this isoform (46, 48, 51). The overexpression and subsequent stimulation of PKC$\delta$ lead to cell cycle arrest in Chinese hamster ovary cells (51) and to complete growth inhibition in NIH 3T3 cells (48). The untransfected mouse myeloid progenitor cell line does not detectably respond to TPA treatment; however, transfectants overexpressing PKC$\delta$ acquire the ability to differentiate into mature macrophages after TPA stimulation (46). These three studies involved long term (>6 hr) TPA treatment of the PKC$\delta$-overexpressing cells, conditions under which PKC$\delta$ is down-regulated in overexpressing NIH 3T3 cells (48). Furthermore, it has been shown that tyrosine phosphorylation of PKC$\delta$ occurs after a 10-min TPA treatment and may positively influence the function of PKC$\delta$ in regulating the differentiation of myeloid progenitor transfectants (52). PKC$\delta$ has been found to be abundant in $C_6$ glioma cells (29; present study), oligodendrocytes (53), and primary cultures of rat astrocytes (54), and it is possible that this isoform plays an important role in regulating cellular functions in glial cells. The present study shows that PKC$\delta$ mediates $Na^+$-$H^+$ exchange in $C_6$ glioma cells and suggests that this isoform is probably responsible for the phosphorylation state of the house-keeping $Na^+$-$H^+$ exchanger.

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