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Role of Protein Kinase C Subtypes α and δ in the Regulation of Bradykinin-Stimulated Phosphoinositide Breakdown in Astrocytes

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SUMMARY

Cultured astrocytes express bradykinin (BK) receptors coupled to phospholipase C (PLC)-mediated phosphoinositide (PI) hydrolysis. Short term (10- or 90-min) treatment of cells with 1 μM 12-*O*-tetradecanoylphorbol-13-acetate (TPA) decreased BK-induced PI breakdown, but this inhibitory action was lost after 3-hr TPA treatment. Extended (6- or 24-hr) pretreatment resulted in marked potentiation of the BK response. Western blot analysis using protein kinase C (PKC) isozyme-specific antibodies indicated that astrocytes express PKC-α, PKC-δ, and PKC-ζ. With TPA treatment of the cells for various times (10 min, 90 min, 3 hr, 6 hr, or 24 hr), translocation of PKC-α and PKC-δ from the cytosol to the membrane was seen after 10- or 90-min treatment and restoration to basal levels in the membrane fraction was seen after 3-hr treatment. However, partial or complete down-regulation of PKC-α and PKC-δ was seen after 6- or 24-hr treatment, respectively. No translocation or down-regulation of PKC-ζ was seen after either short term or long term TPA treatment. The inactive phorbol ester α-TPA had no effect on BK-induced PI hydrolysis or on the translocation or down-regulation of PKC-α and PKC-δ. These results suggest that, in unstimulated astrocytes, both PKC-α and PKC-δ, but not PKC-ζ, may exert tonic inhibition of BK-mediated PI turnover. After 10- or 90-min TPA treatment, AlF_4^-- but not Ca^{2+} ionophore-induced PI hydrolysis was inhibited, whereas [^{3}H]BK binding was unaffected, indicating that the site of action of PKC-α and PKC-δ in the BK receptor/G protein/PLC pathway is after the receptor and before PLC, i.e., the G protein. After down-regulation of PKC-α and -δ, increases in both AlF_4^--induced inositol phosphate formation and [^{3}H]BK binding contributed to marked potentiation of BK-induced PI responses. Scatchard plot analysis showed an increase in both the maximal number of binding sites and the binding affinity. Both the up-regulation of [^{3}H]BK binding and the subsequent BK-induced PI turnover were blocked by 0.5 μM cycloheximide, a protein synthesis inhibitor. The increase in AlF_4^--induced PI hydrolysis after 24-hr TPA treatment was also inhibited by cycloheximide, indicating that new synthesis of BK receptors and G proteins was required after down-regulation of PKC-α and PKC-δ. The present study is the first to show that PKC-α and PKC-δ, but not PKC-ζ, are involved in the regulation of receptor-mediated PI turnover in astrocytes.

It has been proposed that the PKC family of phospholipid-dependent serine/threonine kinases plays a major role in cellular functions. Molecular cloning analysis has shown that this is a family of at least 12 isozymes, with different patterns of tissue expression (1–3). The isoforms have closely related structures but differ in their individual properties, and they are divided into conventional (PKC-α, -β_I, -β_{II}, and -γ), new (PKC-δ, -ε, -η, and -θ), and atypical (PKC-ζ, -λ, -ι, and -μ) isoforms. Activation of PKC is thought to involve its translocation (stimulated by endogenous diacylglycerol) from the cytosol to the membrane, where it becomes tightly associated. This membrane association/activation event can be mimicked by the phorbol ester TPA, which acts by binding to the diacylglycerol binding sites of PKC (4). Due to the potency and stability of TPA, it is able to irreversibly insert PKC into the lipid bilayer, thereby causing cumulative and long term stimulation of the enzyme (5). This activation is eventually terminated by the subsequent proteolytic degradation (down-regulation) of PKC (6). Although cells generally contain more than one PKC isozyme, little is known about their individual roles. The differential localization and activation properties of the PKC subtypes have prompted studies aimed at determining the roles played by individual PKC

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ABBREVIATIONS: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PI, phosphoinositide; PLC, phospholipase C; BK, bradykinin; PTX, pertussis toxin; GFAP, glial fibrillary acidic protein; PSS, physiological salt solution; IP, inositol phosphate(s); FCS, fetal calf serum; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; PBS, phosphate-buffered saline; TTBS, Tris-buffered saline/Tween-20; DMSO, dimethylsulfoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; α-TPA, 4α-12-*O*-tetradecanoylphorbol-13-acetate.

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isozymes in a number of cellular functions, including cell differentiation and proliferation (7, 8), transmitter release and exocytosis (9, 10), regulation of phospholipase D, PLC, and phospholipase A_2 activity (11–14), melanogenesis (15, 16), and mitogenesis (17).

Brain astrocytes are known to possess many types of receptors for classical neurotransmitters and neuropeptides (18, 19). PLC-mediated PI hydrolysis in these cells is stimulated by classical neurotransmitters (18) and neuropeptides (20, 21), including BK, endothelin-1, angiotensin, and substance P. A common observation in receptor-mediated PI turnover is that PKC activation by phorbol esters can induce a substantial decrease in agonist responsiveness (22, 23). Cultured astrocytes have been reported to express BK receptors coupled to PLC via PTX-insensitive G proteins and, in these cells, PKC has been shown to play a prominent role in the regulation of BK-evoked PI responses (24). In the present study, we have examined which isoform is involved in this regulation. Cells were treated with TPA for various times (10 min, 90 min, 3 hr, 6 hr, and 24 hr), BK-induced PI hydrolysis was then measured, and the amounts of different PKC isoforms were determined by Western blotting using PKC isoform-specific antibodies. The effects of TPA on AlF_4^-- and Ca^{2+} ionophore (A23187 and ionomycin)-induced PI hydrolysis and on [^{3}H]BK binding were also studied, to investigate the possible site of action of PKC isozymes in the BK receptor/G protein/PLC pathway.

Experimental Procedures

Materials. Rabbit polyclonal antibodies raised against peptide sequences unique to PKC-α, -β, -γ, -δ, -ε, or -ζ, basal modified Eagle's medium, FCS, glutamine, and gentamicin were purchased from Gibco-BRL (Gaithersburg, MD). TPA and α-TPA were from LC Services Corp. (Woburn, MA). BK, A23187, ionomycin, poly-L-lysine, and phenylmethylsulfonyl fluoride were from Sigma Chemical Co. (St. Louis, MO). Reagents for SDS-PAGE were from Bio-Rad. *myo*-[^{3}H]Inositol (23.5 Ci/mmol), [^{3}H]BK (82.3 Ci/mmol), and ^{125}I-Protein A were from DuPont-New England Nuclear. Horseradish peroxidase-labeled donkey anti-rabbit second antibody and the ECL detecting reagent were purchased from Amersham International.

Primary cultures of astrocytes. Glial cell cultures were prepared from the cerebellum of 8-day-old Wistar rats as described by Gallo *et al.* (25). Briefly, cerebella were dissected and dissociated by mechanical chopping and trypsinization, to obtain a cell suspension. Cells were plated at a density of 3×10^6 cells/poly-L-lysine-precoated, 35-mm dish for the PI hydrolysis assay and at a density of 5×10^7 cells/145-mm dish for the PKC isoform assay. Cultures were maintained in basal modified Eagle's medium supplemented with 10% FCS, 2 mM glutamine, and 50 μg/ml gentamicin, which was changed twice each week. The cells were grown in an atomosphere of 5% CO_2/95% humidified air at 37° and were used after 10–12 days in culture, at which time they consisted of confluent glial cells, which stained positively for GFAP.

Immunohistochemistry. Cells were cultured on 15-mm glass coverslips in 35-mm dishes. Ten days later, astrocyte cultures were stained for immunofluorescence, as described by Morrison and De Vellis (26). Briefly, astrocytes were washed with PBS and fixed at room temperature for 15 min in 2% (w/v) paraformaldehyde, followed by acetone for 5 min. The cells were incubated for 30 min with mouse anti-GFAP antibody (Boehringer Mannheim), washed extensively, and then stained for 30 min with anti-mouse immunoglobulin-fluorescein. After additional washes, the coverslips were rinsed in distilled water and mounted on glass slides using glycerol/PBS (1:9, v/v); the cells were then examined using a Nikon fluorescence microscope equipped with fluorescein filters, with mercury vapor epi-illumination. In negative controls, the primary antibody was omitted and no background staining was seen.

Measurement of PI hydrolysis. PI hydrolysis was assessed by measuring the accumulation of [^{3}H]IP in cells labeled by a 24-hr incubation in growth medium containing *myo*-[^{3}H]inositol (2.5 μCi/ml). Cells were then washed with PSS (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose, 20 mM HEPES, pH 7.4) containing 10 mM LiCl and were incubated at 37° for 20 min. After this preincubation, BK (1 μM), AlF_4^- (10 μM) (10 mM $AlCl_3$ and 10 μM NaF), A23187 (10 μM), or ionomycin (1 μM) was added and incubation was performed for another 30 min. The reaction was terminated by aspiration of the reaction solution and addition of ice-cold methanol, and the accumulated [^{3}H]IP was measured by using an AG-1X8 (formate form, 100–200 mesh; Bio-Rad) column and was eluted with 0.2 N ammonium formate/0.1 N formic acid, as originally described by Berridge *et al.* (27). Each experiment was performed in triplicate.

In all experiments (other than the 10-min incubation with TPA or α-TPA) in which cells were treated with TPA, α-TPA (1 μM), or cycloheximide (0.5 μM), these reagents were added to the growth medium for the appropriate time during the *myo*-[^{3}H]inositol labeling step. Control cells were preincubated in parallel with 0.1% DMSO (solvent for TPA and α-TPA) for 24 hr. They were then washed three times with 1 ml of PSS and stimulated with BK, AlF_4^-, A23187, or ionomycin in the presence of LiCl. In experiments involving 10-min pretreatment with TPA or α-TPA, the reagents were added in PSS 10 min before stimulation with BK, AlF_4^-, A23187, or ionomycin. The incorporation of *myo*-[^{3}H]inositol was not affected by 24-hr TPA treatment but was inhibited 32% by 24-hr cycloheximide treatment.

Preparation of cell extracts and immunoblot analysis of PKC isozymes. Cells were treated with TPA in the growth medium for periods of 10 min, 1.5 hr, 3 hr, 6 hr, or 24 hr or with α-TPA for 10 min or 24 hr before harvesting. DMSO (0.1%) was added to control cells for 24 hr. The cells were then rapidly washed with ice-cold PBS, scraped, and collected by centrifugation at 1000 × *g* for 10 min.

The preparation of cell extracts and immunoblot analyses were performed as described previously (28). Briefly, the collected cells were lysed in ice-cold homogenization buffer containing 20 mM Tris·HCl, pH 7.4, 1 mM dithiothreitol, 5 mM EGTA, 2 mM EDTA, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin. The homogenates were centrifuged at 45,000 × *g* for 1 hr at 4° to yield the supernatants (cytosolic extracts) and pellets (membrane fractions). Samples from these two fractions (100 μg of protein) were denatured and subjected to SDS-PAGE using a 10% running gel. Proteins were transferred to nitrocellulose paper, and the membrane was incubated successively with 1% BSA in TTBS (50 mM Tris·HCl, pH 7.5, containing 0.15 M NaCl and 0.05% Tween-20) at room temperature for 1 hr, with rabbit antibodies specific for PKC-α, PKC-β, PKC-γ, PKC-δ, PKC-ε, or PKC-ζ (diluted 1/250 in TTBS containing 1% BSA) for 3 hr, and with ^{125}I-Protein A (0.4 μg, 4–6 μCi/20 ml) for 1 hr. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands were visualized and quantitated by using a PhosphorImager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA). In some experiments, ECL detection was performed as described previously (29); the membrane was incubated successively at room temperature with 0.1% dry milk in TTBS for 1 hr, with rabbit antibodies specific for PKC isoforms for 2 hr, and with horseradish peroxidase-labeled anti-rabbit antibody for 1 hr. After each incubation, the membrane was washed extensively with TTBS. The immunoreactive bands detected with ECL reagents were developed with Hyperfilm-ECL (Amersham International) and quantitated by using a computing densitometer with ImageQuant software (Molecular Dynamics).

[^{3}H]BK binding assay. To measure total binding of [^{3}H]BK to intact cells, monolayer cultures grown on 24-well plates were washed with PSS and then incubated at 4° for 4 hr with 0.1–10 nM [^{3}H]BK,

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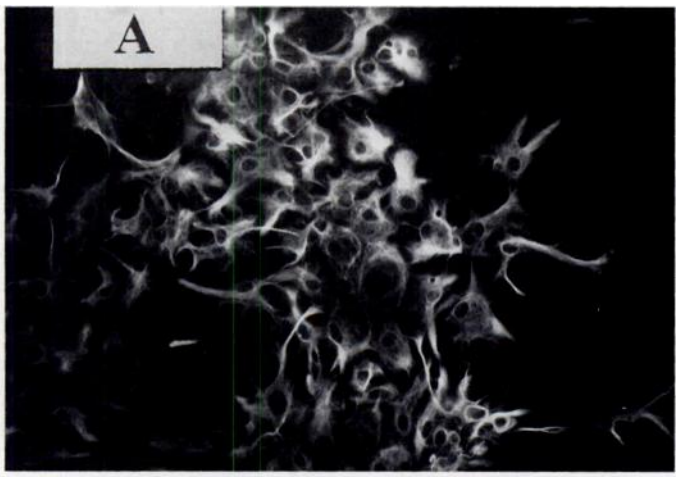
in 0.2 ml of binding buffer containing 17 mM NaCl, 5.4 mM KCl, 0.44 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.21 mM $MgCl_2$, 0.34 mM Na_2HPO_4, 110 mM *N*-methylglucamine, 0.1 mM bacitracin, 0.1% BSA, and 20 mM HEPES, pH 7.4. Nonspecific binding was determined in the presence of 10 μM BK and subtracted from total binding to give specific binding (approximately 90% of total binding). In experiments involving 1.5-, 3-, 6-, or 24-hr TPA treatment or 24-hr cycloheximide treatment, the reagents were added to the growth medium for the appropriate time before the binding assay was performed. The binding reaction was terminated by aspiration of the binding buffer, followed by three rapid washes with ice-cold PSS. The cells were then solubilized with 0.5 ml of 0.2 N NaOH, scraped, and transferred to vials for counting. Radioligand binding data were analyzed with the LIGAND program.

Results

Characterization of astrocytes by immunohistochemistry. The cells were identified as astrocytes by labeling with anti-GFAP antibodies (Fig. 1). Most had a flat polygonal morphology (Fig. 1B) and were GFAP positive (Fig. 1A).

Effects of TPA on the BK-induced PI response. As shown in Fig. 2, 1 μM BK alone elicited an approximately 10-fold increase in IP accumulation. The time course of the effect of 1 μM TPA on the BK-induced PI response was bidirectional. Short term (10- or 90-min) pretreatment with TPA resulted in inhibition of 30 ± 4% (10 experiments) or 28 ± 5% (four experiments), respectively. After 3-hr pretreatment the inhibitory effect of TPA was lost (123 ± 13%, seven experiments), whereas after longer TPA preincubation periods (6 and 24 hr) marked potentiation was seen (240 ± 45%, seven experiments, and 363 ± 47%, 11 experiments, respectively). TPA had no effect on the basal level of IP accumulation at any time of treatment (data not shown). To establish how quickly the marked potentiation of the BK-induced PI response by 24-hr TPA treatment occurred, detailed time courses of BK-induced [^{3}H]IP accumulation under both normal and TPA-treated (24 hr) conditions were determined (Fig. 3). This increase in [^{3}H]IP formation was related to BK incubation time. It occurred with as little as 30 sec of BK incubation (24% increase) and continued to increase as the incubation time was extended (Fig. 3).



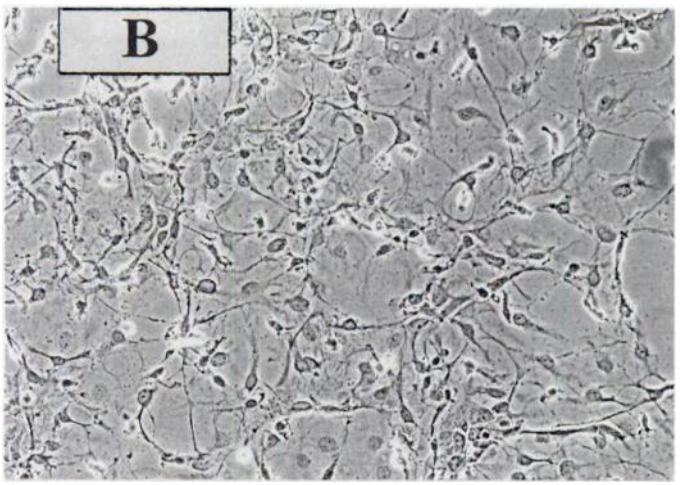


Fig. 1. GFAP staining of 10-day astrocyte cultures. The same field from a representative culture was visualized by indirect immunofluorescence for GFAP (A) and by phase-contrast microscopy (B) (magnification, 200×).

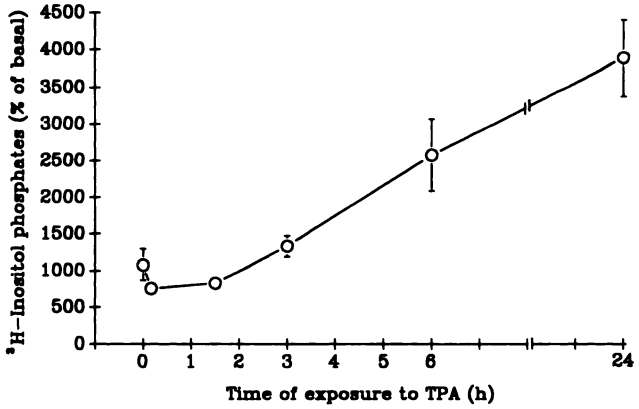


Fig. 2. Time-dependent effects of TPA on BK-induced [^{3}H]IP formation in astrocytes. Cells were pretreated with 1 μM TPA for the indicated periods of time before challenge with 1 μM BK. The data presented are the mean ± standard error of a minimum of four independent experiments performed in triplicate. Basal [^{3}H]IP accumulation was 800 ± 40 dpm/dish.

Expression of PKC isozymes in astrocytes after short term and long term treatment with TPA. To determine which PKC isoform is involved in the regulation of the BK-induced PI response, the expression of PKC isoforms in astrocytes was characterized by Western blot analysis. Using isoform-specific antibodies, PKC-α, PKC-δ, and PKC-ζ were found to be expressed in astrocytes (Fig. 4, *right lanes*). PKC-α, PKC-β, PKC-γ, PKC-δ, PKC-ε, and PKC-ζ were detected in cell lysates of 8-day-old rat cerebella (Fig. 4, *left lanes*). The effect of TPA on the levels of PKC-α, PKC-δ, and PKC-ζ in astrocytes was evaluated. A 10- or 90-min exposure of cells to 1 μM TPA induced marked translocation of PKC-α and PKC-δ from the cytosol to the particulate fraction (Fig. 5). However, after longer periods of TPA treatment a progressive decline in the total amount of PKC-α and PKC-δ immunoreactivity in the membrane and cytosol was seen. The membrane levels of both PKC-α and PKC-δ were identical to those in controls after 3-hr treatment and were down-regulated to values below controls after 6-hr treatment. Almost complete down-regulation of both isozymes was found after 24-hr treatment with TPA (Fig. 5). In contrast, the expression of PKC-ζ in both cytosol and membranes was not altered by TPA treatment. To confirm the specific action of TPA on

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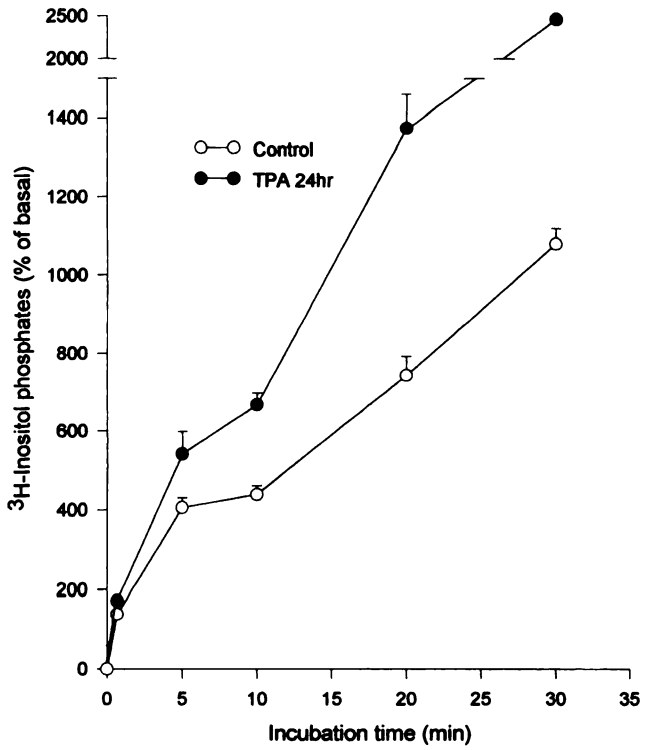

Fig. 3. Time course of BK-induced [^{3}H]IP accumulation under normal and TPA-treated (24 hr) conditions. Cells were pretreated with 0.1% DMSO (○) or 1 μM TPA (●) for 24 hr, washed with PSS containing 10 mM LiCl, preincubated at 37° for 20 min, and then incubated for the indicated periods of time in the presence of 1 μM BK. Data are presented as the mean ± standard error from a typical experiment performed in triplicate.

PKC, the inactive phorbol ester α-TPA was used. When cells were treated with 1 μM α-TPA for 10 min or 24 hr, neither translocation nor down-regulation of PKC-α or PKC-δ was seen (data not shown).

Correlation between the effects of TPA on the BK-induced PI response and on the activation of PKC-α and -δ. The correlation between the BK-induced PI response and the translocation and down-regulation of PKC isozymes α and δ after different periods of TPA treatment is shown in Fig. 6. The extent of TPA-induced translocation of PKC-α and PKC-δ to the membrane was quantitated with ImageQuant software. Ten minutes of TPA treatment resulted in 200% and 290% translocation of PKC-α and PKC-δ, respectively, whereas the BK-induced PI response was inhibited by 30%. After 90 min of treatment, 217% and 172% translocation of PKC-α and PKC-δ, respectively, was seen and the BK-induced PI response was inhibited by 28%. After 3 hr of TPA treatment, both PKC-α and PKC-δ were restored to control values (104% and 123 ± 8%, respectively, 16 experiments) and the inhibitory effect of TPA on the BK-induced PI response was lost (123 ± 13%, seven experiments). After 6 hr of TPA treatment, PKC-α and PKC-δ were down-regulated to 32% and 55% of control levels, respectively, whereas the BK-induced PI response was potentiated to 240%. After 24 hr of TPA treatment, both PKC-α and PKC-δ were almost completely down-regulated (5.6% and 8.2%, respectively) and the potentiation of BK-induced PI response was increased to 363%.

Correlation between the effects of TPA on the AlF_4^--induced PI response and on the activation of PKC-α and -δ. To determine the site of action of PKC-α and PKC-δ in the pathway of BK-induced PI hydrolysis, AlF_4^- was used to directly stimulate G proteins to generate IP (30). AlF_4^- (10 μM) alone elicited an approximately 4-fold increase in IP formation. After pretreatment of the cells with 1 μM TPA for different periods of time, bidirectional formation of IP induced by AlF_4^- was also seen (data not shown). The correlation between the AlF_4^--induced PI turnover and the translocation and down-regulation of PKC-α and PKC-δ after TPA treatment is shown in Fig. 7. Under conditions where PKC-α and -δ were translocated (10- or 90-min TPA treatment), AlF_4^--induced PI hydrolysis was inhibited by 37% or 35%, respectively. After these two isoforms were restored to their control values (3-hr TPA treatment) the inhibitory effect on the AlF_4^--induced PI response was lost (83.3 ± 10.0%, five experiments), whereas when they were down-regulated below control values (6- or 24-hr TPA treatment) the AlF_4^--induced PI response was potentiated to 118% or 172%, respectively (Fig. 7). Neither 10-min or 24-hr treatment with TPA had any effect on [^{3}H]IP formation induced by A23187 or ionomycin, which stimulate PLC independently from activation of G proteins (data not shown).

Effect of TPA on [^{3}H]BK binding in astrocytes. The results described above indicated that the G protein, and not PLC, was possibly the site of action of PKC-α and -δ in the BK-induced PI hydrolysis pathway. However, in this pathway the BK receptor was also a possible target site, and [^{3}H]BK binding was therefore examined. Binding studies, using 0.1–10 nM [^{3}H]BK, showed saturable binding, reaching a B_{max} of 147 fmol/mg of protein with a K_d of 114 pM (Fig. 8). The correlation between TPA-induced translocation and down-regulation of PKC-α and PKC-δ and the effect of TPA on [^{3}H]BK binding is shown in Fig. 9. When PKC-α and PKC-δ were translocated or restored to control levels, [^{3}H]BK binding was not affected. However, marked increases in

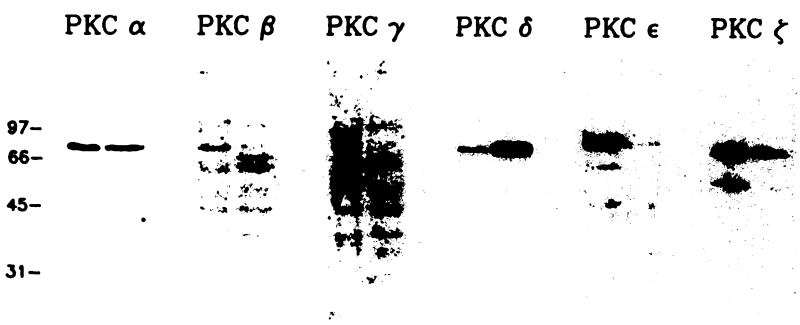

Fig. 4. Expression of six PKC isoforms in whole-cell lysates of 8-day-old rat cerebellum (*left lanes*) and astrocytes (*right lanes*), as determined by protein immunoblotting. Whole-cell lysates were prepared and samples of 100 μg of protein were separated by 10% SDS-PAGE, transferred to nitrocellulose paper, and immunodetected with PKC-specific antibodies (1/250 dilution), as described in Experimental Procedures.

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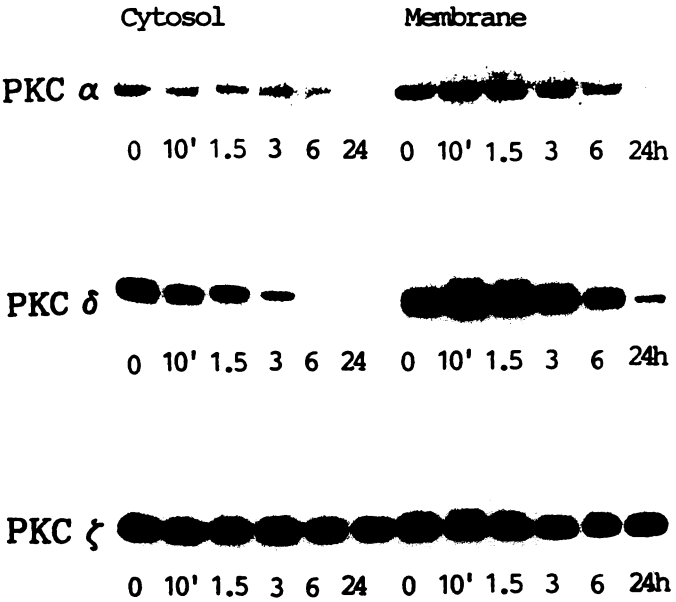

Fig. 5. Translocation and down-regulation of PKC isoforms in astrocytes in response to TPA. Cells were treated with 0.1% DMSO or 1 μM TPA for different times (10 min, 1.5 hr, 3 hr, 6 hr, or 24 hr), and then cytosolic and membrane fractions were prepared. The proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose paper, and immunodetected with PKC-α-, -δ-, or -ζ-specific antibodies, as described in Experimental Procedures. The immunoreactive bands were visualized by using a PhosphorImager (PKC-α) or ECL detection (PKC-δ and PKC-ζ).

[^{3}H]BK binding (186 ± 8%, four experiments, and 246 ± 22%, five experiments) were seen when both isoforms were down-regulated by pretreatment with TPA for 6 or 24 hr, respectively. Scatchard plot analysis of the increase in [^{3}H]BK binding after 24-hr TPA treatment showed altered B_{max} and K_d values, with the maximal number of binding sites being increased from 153 ± 9 to 432 ± 10 fmol/mg of protein (three experiments) and the K_d being decreased from 121 ± 7 to 73 ± 18 pM (three experiments).

The increase in B_{max} after 24-hr TPA treatment suggests that new receptor protein might be synthesized. To test this, cycloheximide, a protein synthesis inhibitor, was used. Twenty-four-hour treatment with 0.5 μM cycloheximide had no effect on the basal level of [^{3}H]IP formation but inhibited both BK- and AlF_4^--induced [^{3}H]IP formation and [^{3}H]BK binding, indicating that new BK receptors or G proteins were normally synthesized during this period (Fig. 10). After 24-hr TPA treatment of the cells, the increases in BK- and AlF_4^--induced [^{3}H]IP formation and [^{3}H]BK binding were still inhibited by cycloheximide, as shown in Fig. 10, indicating that synthesis of BK receptors and G proteins occurred under these conditions.

Discussion

Cultured astrocytes are reported to express BK_2 receptors coupled to PLC via PTX-insensitive G proteins (24, 31). Short term (10- or 90-min) TPA treatment of the cells, which induced translocation of PKC-α and PKC-δ, attenuated BK-induced IP formation. When the translocated PKC-α and -δ were restored to basal levels (3-hr treatment with TPA), the inhibitory action was lost. Extended (6- or 24-hr) pretreatment, which induced down-regulation of these two isoforms, resulted in marked potentiation of the BK response. Neither translocation nor down-regulation of PKC-ζ was observed after either short term or long term TPA treatment, and α-TPA had no effect on either the BK-induced PI hydrolysis or the translocation or down-regulation of PKC-α and PKC-δ. Thus, in astrocytes, PKC-α and PKC-δ, but not PKC-ζ, might exert tonic control over BK-mediated PI turnover in unstimulated cells. AlF_4^--induced PI hydrolysis was similarly affected by TPA. However, A23187- or ionomycin-induced IP formation was not affected. Furthermore, because AlF_4^--induced PI hydrolysis, but not [^{3}H]BK binding, was inhibited when PKC-α and -δ were translocated (10- or 90-min TPA treatment), the site of action of these two isoforms in the BK receptor/G protein/PLC pathway must be after the receptor and before PLC, i.e., the G protein.

Activation of PKC by phorbol esters causes a substantial decrease in PI turnover mediated by agonists (23), e.g., by endothelin, angiotensin, and BK in neuroblastoma cells and by muscarinic agonists in astrocytes (22, 32). We show here a similar effect on BK-induced PI turnover in astrocytes. To determine which PKC isoform was involved, Western blot analysis was performed using PKC isoform-specific antibodies. The PKC-α, -δ, and -ζ isoforms were found to be expressed in rat astrocytes; similar results have been seen in C_6

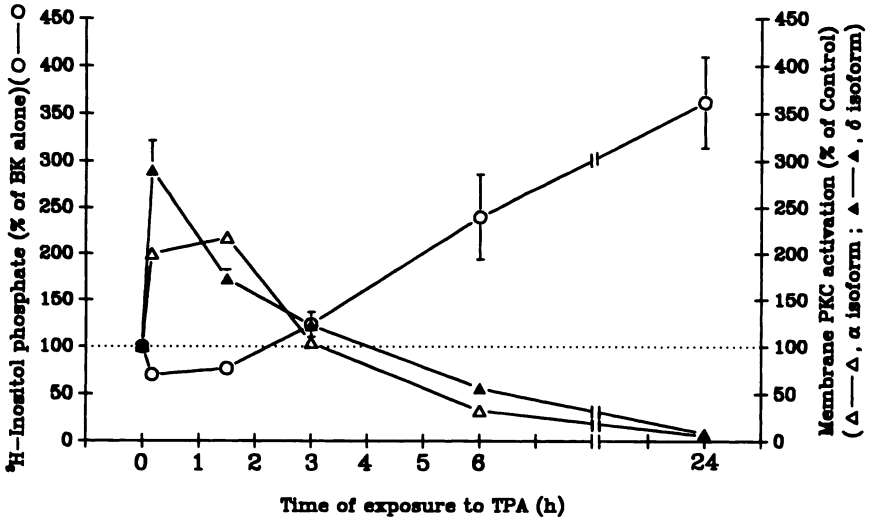

Fig. 6. Correlation between the effects of TPA on BK-induced [^{3}H]IP formation and on membrane PKC-α and -δ expression in astrocytes. For BK-induced [^{3}H]IP formation, cells were pretreated with 1 μM TPA for the indicated periods of time and then challenged with 1 μM BK. For translocation and down-regulation of PKC-α and -δ, cells were treated with 1 μM TPA for the indicated periods of time and then fractionated into cytosolic and membrane fractions as described in the legend to Fig. 5. The extent of translocation and down-regulation of these two isoforms in the membrane was quantitated using a PhosphorImager with ImageQuant software or a densitometer with ImageQuant software. Data are presented as the mean ± standard error of a minimum of four independent experiments.

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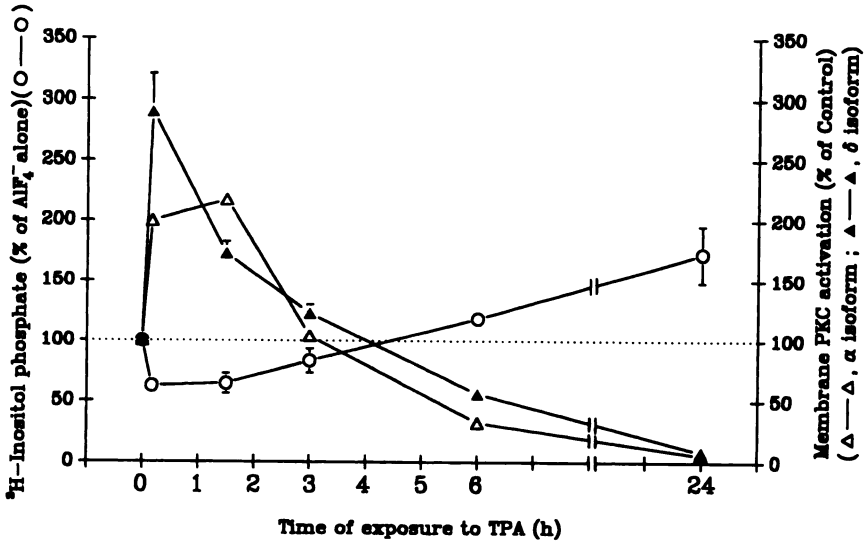

Fig. 7. Correlation between the effects of TPA on AlF_4^--induced [^{3}H]IP formation and on membrane PKC-α and -δ expression in astrocytes. The methods used were the same as in Fig. 6.

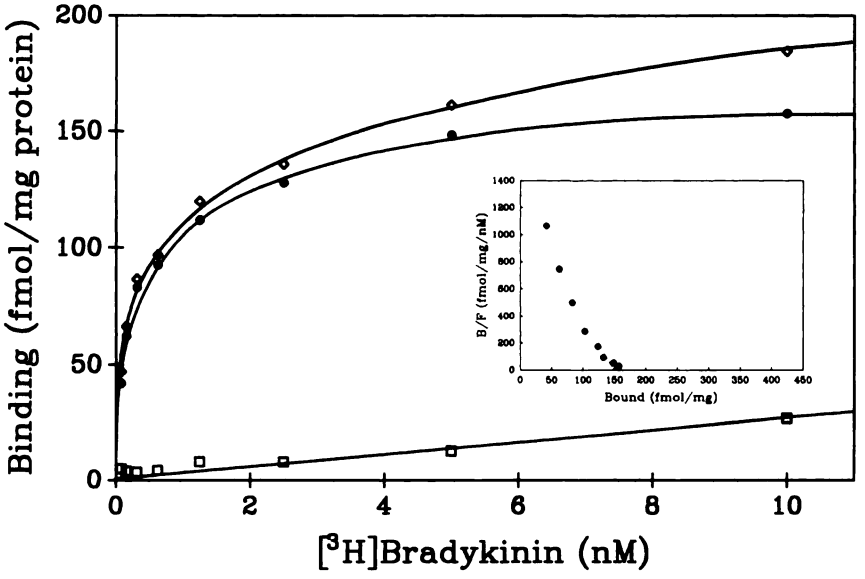

Fig. 8. Binding of [^{3}H]BK to intact astrocytes. Total binding (◇) was determined in binding buffer containing 0.1–10 nM [^{3}H]BK. Nonspecific binding (□) was determined in binding buffer containing 10 μM BK plus 0.1–10 nM [^{3}H]BK. The difference between the two values gives the specific binding (●). *Inset*, Scatchard analysis of specific [^{3}H]BK binding to BK receptors in astrocytes. The values presented are the mean of triplicate determinations from a typical experiment.

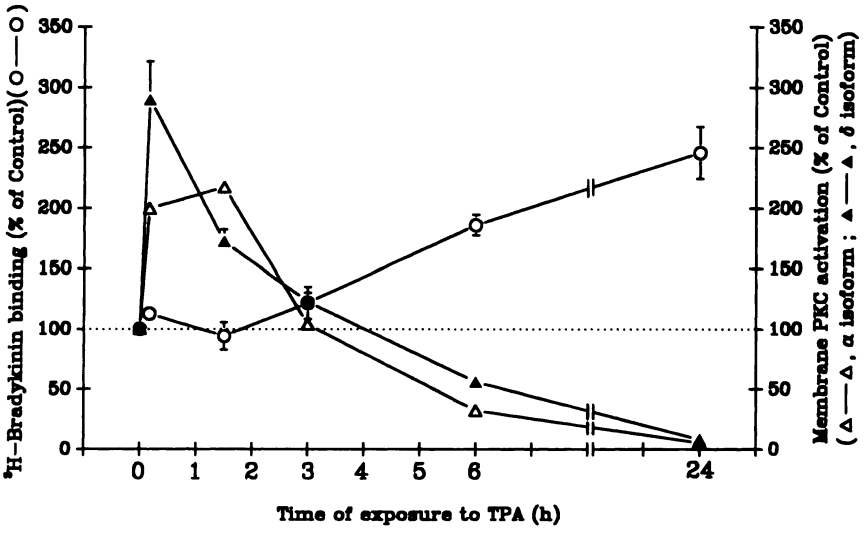

Fig. 9. Correlation between the effects of TPA on [^{3}H]BK binding and on membrane PKC-α and -δ expression in astrocytes. For [^{3}H]BK binding, cells were pretreated with 1 μM TPA for the indicated periods of time and then [^{3}H]BK binding was performed in binding buffer containing 5 nM [^{3}H]BK, in the presence or absence of 10 μM BK. Specific [^{3}H]BK binding was obtained from the difference between total and nonspecific binding. The methods for translocation and down-regulation of PKC-α and PKC-δ were as described for Fig. 6. Data are presented as the mean ± standard error of a minimum of four independent experiments.

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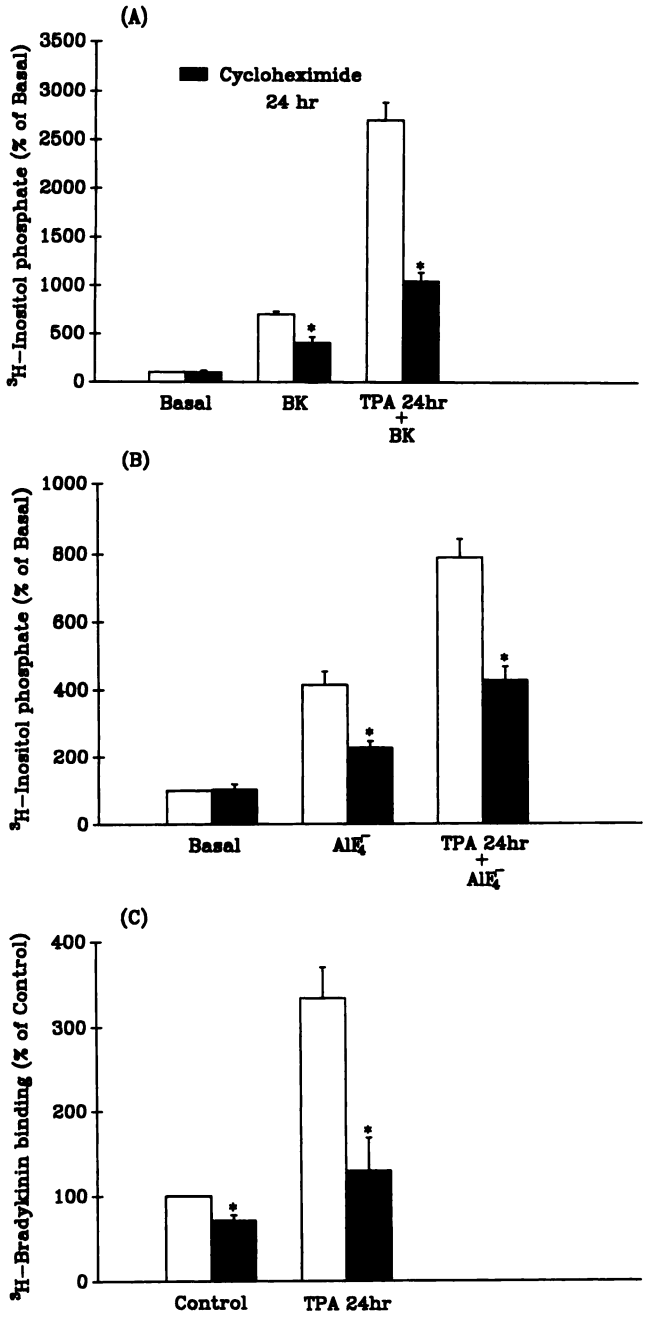

Fig. 10. Effects of cycloheximide on BK-induced (A) and AlF_4^--induced (B) [^{3}H]IP formation and on [^{3}H]BK binding (C) after 24-hr TPA treatment of cells. Cells were pretreated for 24 hr with 0.5 μM cycloheximide, 1 μM TPA, or cycloheximide plus TPA. BK or AlF_4^- was added to induce [^{3}H]IP formation, or [^{3}H]BK binding was determined using 5 nM [^{3}H]BK in the presence or absence of 10 μM BK. Data are the mean ± standard error of three independent experiments. *, $p < 0.05$, compared with the response in the absence of cycloheximide.

glioma cells (28) and oligodendrites (33). PKC-ε was expressed at high levels in the cerebellum of 8-day-old rats. However, in astrocyte cultures it was absent and an increase in PKC-δ was seen (Fig. 4). Therefore, glial cells, in contrast to neuronal cells, express little or no PKC-ε (34). To determine the possible site of action of TPA in the BK-induced PI hydrolysis pathway, AlF_4^-, which directly activates G_q protein to induce PI hydrolysis (35, 36), was used. When PKC-α and -δ were translocated (10- and 90-min TPA treatment), AlF_4^--induced PI hydrolysis was inhibited; this inhibition was lost when these two isoforms returned to basal levels (3-hr TPA treatment). However, [^{3}H]BK binding and A23187- or ionomycin-induced PI hydrolysis were not affected by TPA, indicating that the site of action of PKC-α and -δ in this PI pathway is after the receptor and is the G protein and not PLC. There are three classes of PI-specific PLC isozymes (β, γ, and δ), which are activated by different mechanisms upon receptor stimulation (37, 38). Agonists that act through G_q protein-coupled PI-specific PLC to mediate PI hydrolysis are known to activate the β isoforms, and their action is PTX insensitive (38, 39). BK receptors are thought to be coupled to PLC-β_1 via interaction with the G_q family of G proteins (40). Because the catalytic activities of PLC-β_1, PLC-β_2, and PLC-β_3 were dependent on Ca^{2+} (41–43), we measured Ca^{2+} ionophore-stimulated IP production as an index of PLC activity distal to regulation by BK receptors and G proteins. In the astrocytes used here, the inhibition by TPA of BK- or AlF_4^--stimulated, but not A23187- or ionomycin-stimulated, IP formation suggests that the main inhibitory effect of PKC-α and PKC-δ is on the G protein or the coupling of the G protein to PLC. Because elevated calcium concentrations stimulate PLC activity directly (44, 45), the lack of inhibition of Ca^{2+} ionophore-induced PLC activity by TPA indicates that regulation by PKC occurs upstream of the PLC enzyme. Similar conclusions were reached by Pachter *et al.* (14) using PKC overexpression in fibroblasts. It has been shown that phorbol ester treatment of several cell types stimulates phosphorylation of PLC-β but not PLC-γ or PLC-δ (46). PLC-β is the only PLC isoform shown to be regulated by a heterotrimeric G protein (38, 39). The observation that *in vitro* phosphorylation of PLC-β by PKC has no effect on PLC-β activity led Ryu *et al.* (46) to propose that this phosphorylation may prevent the activation of PLC-β by G protein. It is probable that, in astrocytes, activation of PKC-α and PKC-δ by TPA directly inhibits G proteins or phosphorylates PLC-β, causing interference with the coupling between PLC-β and G_q protein and subsequent inhibition of PI hydrolysis.

A marked potentiation of the BK-induced PI response was seen after 6-hr TPA pretreatment of the cells, conditions under which PKC-α and -δ are partially down-regulated. Moreover, when these two isoforms were completely down-regulated (24-hr TPA treatment), the potentiation was even more marked. A similar potentiation of the AlF_4^--induced PI response was also seen under these conditions, whereas A23187- or ionomycin-induced IP formation was unaffected. We therefore suggest that, in rat astrocytes, both PKC-α and PKC-δ might exert tonic inhibition of the BK receptor-coupled G protein. When these two isoforms are down-regulated, the removal of this tonic inhibition leads to potentiation of BK- and AlF_4^--induced PI hydrolysis. An increase in [^{3}H]BK binding was also seen. These two factors might explain the relatively greater potentiation of the BK-induced response, compared with the AlF_4^--induced response, after treatment with TPA for 6 or 24 hr. Scatchard plot analysis revealed that both the maximal number of binding sites and the binding affinity of the BK receptors were increased. This increase in [^{3}H]BK binding was inhibited by cycloheximide, indicating that newly synthesized BK receptor protein was involved. Phorbol esters have been reported to desensitize κ-opioid, α_2-adrenergic, muscarinic, β-adrenergic, and atrial natri-

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uretic peptide receptors (47–49). TPA also causes internalization of M_3 muscarinic receptors in the colon (50). However, these phenomena did not occur in astrocytes, inasmuch as [^{3}H]BK binding was unaffected after short term (10- or 90-min) TPA treatment. The increase in [^{3}H]BK binding after down-regulation of PKC-α and -δ is therefore probably due to the increased expression of receptor protein. Moreover, increased expression of G protein might also occur, because cycloheximide also inhibits the increase in the AlF_4^--induced PI response after 24-hr TPA treatment. The precise mechanism for the increased expression of BK receptor and G protein is unknown. Yong (51) and Sawada *et al.* (52) have reported that PKC plays an important role in the regulation of proliferation of astrocytes, and treatment with 100 nM phorbol-12,13-dibutyrate for 4 days or with TPA for 5 days results in increased [^{3}H]thymidine uptake (49, 50). In our experiments, [^{3}H]thymidine uptake was also slightly increased after 24-hr treatment with 1 μM TPA in 10% FCS-containing medium but not in serum-free medium (data not shown); this contributes slightly to the increased DNA synthesis and might increase the expression of BK receptor and G protein under our experimental conditions where 10% FCS-containing medium was used. Increased BK-induced PI hydrolysis after long term treatment with TPA has also been seen in tracheal smooth muscle cells (53).

In summary, in rat astrocytes, PKC-α and PKC-δ might exert tonic inhibition of the BK receptor-coupled G protein in the BK-induced PI hydrolysis pathway. When these two isoforms were translocated (10- or 90-min TPA treatment) the BK-induced PI response was inhibited, whereas when they were down-regulated (6- or 24-hr TPA treatment), marked potentiation of the BK response was seen. The increased expression of both BK receptors and G proteins also contributed to this marked potentiation. A proposed model to explain the possible site of action of PKC-α and -δ in the BK receptor/G_q protein/PLC-β pathway is shown in Fig. 11. This is the first study to determine which PKC isoform is involved in the regulation of receptors coupled to PLC via G protein-mediated PI turnover in astrocytes. Additional experiments, using antisense oligonucleotides, will determine whether it is PKC-α and/or PKC-δ that is involved in the regulation of this signal transduction pathway.

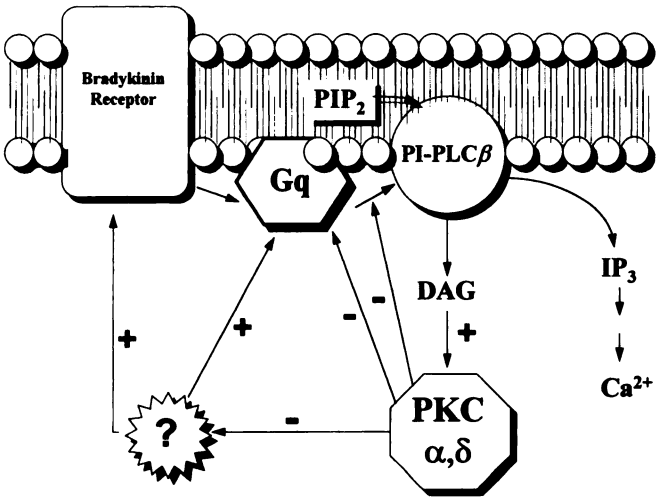

Fig. 11. Schematic representation of PKC-α and -δ in the regulation of BK receptor-mediated PI hydrolysis in rat astrocytes. Activation of the BK receptor results in the stimulation of PLC-β via coupling to a PTX-insensitive G_q protein. PKC-α and -δ exert tonic inhibition of the G_q protein or of the coupling of G_q to PLC-β. An unknown factor, which increases the expression of the BK receptor and G_q protein, is inhibited by activation of PKC-α and -δ. Down-regulation of these two isoforms leads to the relief of tonic inhibition of the coupling of G_q to PLC-β and increased expression of the BK receptor and G_q protein, resulting in marked potentiation of the BK response. *PIP_2*, phosphatidylinositol bisphosphate; *DAG*, diacylglycerol; *IP_3*, inositol trisphosphate.

Acknowledgments

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