Inhibition of Gap Junction Activity through the Release of the C1B Domain of Protein Kinase Cγ (PKCγ) from 14-3-3

IDENTIFICATION OF PKCγ-BINDING SITES*

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We have shown previously that insulin-like growth factor-I or lens epithelium-derived growth factor increases the translocation of protein kinase Cγ (PKCγ) to the membrane and the phosphorylation of Cx43 by PKCγ and causes a subsequent decrease of gap junction activity (Nguyen, T. A., Boyle, D. L., Wagner, L. M., Shinohara, T., and Takemoto, D. J. (2003) Exp. Eye Res. 76, 565–572; Lin, D., Boyle, D. L., and Takemoto, D. J. (2003) Investig. Ophthalmol. Vis. Sci. 44, 1160–1168). Gap junction activity in lens epithelial cells is regulated by PKCγ-mediated phosphorylation of Cx43. PKCγ activity is stimulated by growth factor-regulated increases in the synthesis of diacylglycerol but is inhibited by cytosolic docking proteins such as 14-3-3. Here we have identified two sites on the PKCγ-C1B domain that are responsible for its interaction with 14-3-3. Two sites, C1B1 (residues 101–112) and C1B5 (residues 141–151), are located within the C1 domain of PKCγ. C1B1 and/or C1B5 synthetic peptides can directly compete for the binding of 14-3-3, resulting in the release of endogenous cellular PKCγ from 14-3-3, in vivo or in vitro, in activation of PKCγ enzyme activity, phosphorylation of PKCγ, and in the subsequent translocation of PKCγ to the membrane, and in inhibition of gap junction activity. Gap junction activity was decreased by at least 5-fold in cells treated with C1B1 or C1B5 peptides when compared with a control. 100 μM of C1B1 or C1B5 peptides also caused a 10- or 4-fold decrease of Cx43 plaque formation compared with control cells. The uptake of these synthetic peptides into cells was verified by using high pressure liquid chromatography and matrix-assisted laser desorption ionization time-of-flight-mass spectrometry. We have demonstrated that the activity and localization of PKCγ are regulated by its binding to 14-3-3 at the C1B domain of PKCγ. Synthetic peptides corresponding to these regions of PKCγ successfully competed for the binding of 14-3-3 to endogenous PKCγ, resulting in inhibition of gap junction activity. This demonstrates that synthetic peptides can be used to exogenously regulate gap junctions.

14-3-3 proteins comprise a group of acidic proteins of about 29–33 kDa, originally identified as brain-specific (3). 14-3-3 proteins continue to generate interest because of their roles in signal transduction pathways that control cell cycle checkpoints, MAP kinase activation, and apoptosis (4–6). The 14-3-3 protein family is highly conserved and is found in all eukaryotic cells. At least 100 proteins have been found to interact with 14-3-3 isoforms, including various protein kinases (PKCs* (7)), Raf family members (8), receptor proteins (glucocorticoid receptor (9) and insulin-like growth factor I receptor (10)), scaffolding molecules (IRS-1 (11)), and proteins involved in control of apoptosis (BAD (12)). In mammalian cells, seven subtypes of 14-3-3 (β, ε, γ, η, σ, τ, and θ) have been identified (13, 14). 14-3-3 proteins are most abundant in the cytoplasm, and all actions of 14-3-3 proteins involve binding to target proteins.

Binding of 14-3-3 to its partners depends on the phosphorylation of specific Ser or Thr residues in the recognition domains. By using peptides derived from Raf-1, Muslin et al. (15) identified the optimal motif for association of target proteins with 14-3-3 proteins as RXSxP, where P represents phosphorylated Ser and X represents any amino acid. Yaffe et al. (16) and Rittinger et al. (17) reported that there are two preferred 14-3-3-binding motifs, RXSxP and RXXXP. Most of the 14-3-3 partners identified to date contain one of these motifs. PKCγ has two potential 14-3-3-binding motifs within the C1B domain.

PKC plays an integral part in the cell signaling machinery. PKC comprises a family of serine/threonine kinases, which contain at least 11 isoforms and can be found in most cell types (18). PKCs phosphate growth factor receptors, ion channels, structural proteins, and gap junction connexin proteins (19). Classical PKCs (including α, γ, β1, and β2) translocate to membranes after both diacylglycerol (DAG) and calcium binding to C1 and C2 domains, respectively (18).

For activation to occur, the PKCs are often covalently modified by phosphorylation on serine and threonine residues. This is thought to induce a conformational change in the PKC protein, resulting in an enhanced interaction with membranes (21). Several signaling proteins use two membrane-targeting modules to reversibly regulate their activation state and cellular distribution (21, 22). Each module of the classical PKC binds membranes with low affinity, with tight membrane binding achieved when both domains are membrane-bound. When the affinity of one module for membranes is dependent on stimulus-dependent changes in membrane composition (generation of lipid secondary messengers) or protein structure (phosphorylation), the interaction with the membrane can be revers-

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ibly regulated (21). PKC has served as a model for the reversible regulation of membrane location by the concerted action of two membrane-targeting modules. PKCγ has two C1 domain repeats, C1A and C1B. However, unlike the classical PKCα isoform, both the C1A and C1B domain have high affinity for DAG, even at low Ca²⁺ levels (23). This enzyme can be activated by very low DAG levels (2). In addition, the C1 domain of PKCγ has been hypothesized to act as an oxidative stress switch through modifications of the Cys residues without a DAG or Ca²⁺ signal (48). We have recently identified that PKCγ from whole lens is activated by exogenous hydrogen peroxide (20). This activation could be regulated by the interaction of the C1 domain of PKCγ with membrane docking proteins such as 14-3-3.

The C1 domain of PKCγ is a Cys-rich region from 36 to 151 of PKCγ residues. It is present as a tandem repeat, designated C1A and C1B (24). The NMR structure of the C1B domain of PKCγ reveals a globular domain with two pulled β-sheets forming the ligand binding pocket (25). Two Zn²⁺ atoms are coordinated by His and Cys residues at opposite ends of the primary sequence, helping to stabilize the domain. Several reports (26, 27) suggest that docking of PKC to 14-3-3 involves the C1B domain of PKCγ.

There are two possible 14-3-3 consensus binding regions in the C1B of PKCγ for 14-3-3 binding, residues 101–112 and 141–151. In order to identify which 14-3-3 isoform interacts with PKCγ, we first examined the interaction between PKCγ and 14-3-3 isoforms, ε, γ, and ζ. PKCγ is usually cytoplasmic and inactive and translocates to membranes upon binding of lipids and Ca²⁺ (28, 29). But as reported previously (2, 23), PKCγ translocates with only a DAG signal. PKCs can bind to a scaffold protein, such as 14-3-3, which is thought to bind in the cytoplasm and can either activate or inhibit an individual PKC protein, such as 14-3-3, which is thought to bind in the cytoplasm and can either activate or inhibit an individual PKC protein (26).

Previously in our laboratory, we have shown that IGF-I or LEDGF decreases gap junction activity through the activation of PKCγ, causing a PKC-mediated phosphorylation ofconnexion 43 (Cx43), and then a decrease in gap junction activity (1, 2). PKC-mediated phosphorylation of Cx43 and subsequent decrease of gap junction activity could be regulated through the release of PKCγ from 14-3-3, followed by the activation and translocation of PKC to the membrane, and then by subsequent PKC-catalyzed phosphorylation of Cx43. In this study, we have investigated the interaction of PKCγ with 14-3-3ε and have identified the residues on PKCγ which interact with 14-3-3ε. We report the competition of endogenous PKCγ binding to 14-3-3ε in live cells or in vitro by C1 domain peptides. This resulted in the endogenous PKCγ translocation to the plasma membrane, in the phosphorylation Cx43, and in subsequent decreases in gap junction activity and gap junction plaque formation in vitro.

EXPERIMENTAL PROCEDURES

Reagents—N/N1003A rabbit lens epithelial cells were obtained from Dr. John Reddan (Rochester, MI). Monoclonal antibodies against PKCγ were purchased from BD Biosciences. Polyclonal antibodies against C1B of PKCγ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against IGF-I and protease inhibitor mixture were purchased from Sigma. Lucifer yellow and rhodamine dextran were purchased from Molecular Probes (Eugene, OR).

Cells and Cell Cultures—N/N1003A rabbit lens epithelial cells were cultured in 75-cm² flasks (Midwest Scientific) in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 50 μg/ml gentamicin. The cells were grown at 37 °C in an atmosphere of 90% air and 10% CO₂ and used for experiments when they reached 90% confluency.

Endogenous DAG Assay—Cells were cultured in 75-cm² flasks and dosed at 10 ng/ml LEDGF for various times. Sample preparation and radiometric assays were performed according to the manufacturer's instructions for the sn-1,2-diacylglycerol assay reagents system (Amer sham Biosciences). Total lipids were extracted with chloroform/methanol and used as the substrates for DAG kinase. Phosphatidic acid (PA), which was labeled by 32P, was produced by the reaction. The product mixtures were separated by thin layer chromatography, and zones corresponding to 32P-PA were visualized by autoradiography of the dried plates overnight. The spots containing 32P-PA were quantitated by scintillation counting. Endogenous DAG levels were calculated from DAG standard curves.

PKC Enzyme Activity—Activation of the PKCγ isoform was determined by measurement of enzyme activity. Cells were grown to confluent LEDGF for 10, 20, or 30 min. For peptide studies, cells were grown to 100 μM peptide present at a concentration of 100 μM 14-3-3. LEDGF was determined by use of a PepTag assay kit. Equal protein from whole cell extracts was immunoprecipitated with PKCγ antisera at 4 °C for 4 h as described previously (2). Immunoprecipitated PKCγ-agarose bead complexes were incubated with the PKC reaction mixture according to the manufacturer's instructions for the PepTag assay kit. Reactions were stopped, and fluorescent PepTag peptides (PKC reaction products) were resolved by agarose gel electrophoresis and visualized under UV light. The phosphorylated peptide bands were excised and measured as described at 570 nm (2).

Synthetic Peptides—Two possible sites of the C1 domain of PKCγ, residues 101–112 and 141–151, were synthesized by Sigma Genosys. Synthetic peptide C1B1 sequence is RCVRSVPSLCG, and nontargeting peptide is SFGKCHLYPKV.

NOTES

Measurement of Synthetic Peptides Using HPLC and Mass Spectrometry—7.2 × 10⁶ cells were cultured in 75-cm² flasks and treated with 100 μM of C1B5 (equivalent to 11.76 μg per flask) for 2 h. 5 μl of 250 μl of whole cell extract of treated cells was dissolved in 295 μl of 0.1% (v/v) trifluoroacetic acid in water and then used for HPLC purification and quantification, using a 1.6 mm × 25 cm C₁₈ reverse phase column (Vydac, Torrance, CA) with a 5–60% (v/v) linear gradient of acetonitrile in 0.1% (v/v) trifluoroacetic acid, over a period of 1 min. Identification was confirmed by MALDI-TOF mass spectrometry. The amount of uptake of peptides by the cells was determined from the standard curve of C1B5 peptide alone, using the identical HPLC gradient.

Western Blot Analyses—Cell lysates were prepared by lysing cells in ice-cold lysis buffer consisting of 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% (v/v) Triton X-100, 25 μg/ml apronin, and 25 μg/ml leupeptin. 20 μg of total protein were boiled and resolved on 12% SDS-PAGE minigels under reducing conditions. Proteins were electroblotted onto nitrocellulose membranes. The membranes were blocked with 5% (w/v) nonfat dried milk and probed with diluted antibodies overnight as indicated. Bands were revealed by chemiluminescence reaction (ECL, Pierce). Blots were routinely stripped in a denaturing buffer (0.5 M Tris-HCl (pH 8.6), 10% SDS, 1% β-mercaptoethanol) and reprobed with antibodies for loading control. In this and subsequent experiments Western blots are quantified by image analyses using Un-Scan-it gel software (Orem, UT).

Co-immunoprecipitation—Cells were cultured to 90% confluency in 75-cm² tissue culture flasks and then treated with 10 ng/ml LEDGF for 30 min (1). The cells were harvested with 1 ml of ice-cold cell lysis buffer, containing 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 0.5% (v/v) Triton X-100, 25 μg/ml apronin, and 25 μg/ml leupeptin. The cells were homogenized and centrifuged at 13,000 rpm (20,000 × g) for 30 min at 4 °C. 500 μg of total soluble proteins (cell lysate) from treated or control cell cultures were used for co-immunoprecipitation. The precipitates were washed three times with phosphate-buffered saline.
Interaction of C1B Domain of PKCγ with 14-3-3ε

PKCγ was transcribed/in vitro and purified using anti-GST-agarose beads (Santa Cruz Biotechnology). In vitro transcribed/translated PKCγ was made by using TNT-coupled reticulocyte systems (Promega). GST-14-3-3ε-agarose beads were washed and incubated with 5 μl of in vitro transcribed/translated PKCγ for 1 h. Various concentrations of C1B5 were added to the complex for an additional 1 h. The complex was then washed and separated on 12% (w/v) PAGE as described above.

Translocation—Cells were treated with 100 μM of peptides for 2 h prior to addition of LEDGF. As a negative control, cells were treated with a nonspecific peptide at the same dose and time. Cells were harvested in 50 mM Tris (pH 7.5) and 20 mM MgCl₂ (2). The cell lysates were centrifuged for 1 h at 35,000 rpm (100,000 × g) at 4 °C. The sample was separated into supernatant (cytosolic) and pellet (membrane) fractions and analyzed by Western blot as described previously above. Immunoprecipitated proteins were transferred to nitrocellulose membranes and probed with anti-PKCγ antibodies (1:1000) or other antisera as indicated.

Gap Junction Activity—Scrape loading/dye transfer assay (SL/DT) is a common technique to measure gap junction activity. The SL/DT assay provides a rapid and simple measurement of the gap junctional communication between cultured cells in a monolayer, and it is based on introduction of tracer dye in the plasma membrane and transferring cell viability or colony-forming ability. Lucifer Yellow, which is an intensely fluorescent 4-aminophthalimide, is used as the tracer dye in the SL/DT assay. It does not diffuse through intact plasma membranes, and its low molecular weight (M, 457.2) permits its transmission from one cell to another presumably through patent gap junctions. Rhodamine dextran is a high molecular weight polymer (M, 10,000), and it cannot diffuse through intact plasma membranes nor cross gap junctions; therefore, it is used as a control to differentiate dye transfer from diffusion because of cell damage (30). Cells were then fixed with 2.5% (w/v) paraformaldehyde and the number of cells transferring dye were counted using a standard fluorescent or laser-scanning confocal microscope.

Cells were grown to 90% confluency on glass coverslips and washed three times with PBS. Then 2.5 ml of 1% (w/v) lucifer yellow and 0.75% (w/v) rhodamine dextran control for dye transfer in PBS were added at the center of the coverslip. Using a razor blade, two cuts crossing each other were made on the coverslip, passing through the dye in the center of the coverslip. These cells were incubated with the dye for 1 min, and then the cells were washed three times with PBS. This was followed by incubating the cells in tissue culture medium for 10 min at room temperature, allowing dye to transfer from cell to cell. The cells were then washed with PBS and fixed with 2.5% (w/v) paraformaldehyde in PBS for 30 min at room temperature. Dye transfer was evaluated by examining the cells under a fluorescent microscope. For quantitation, the extent of dye transfer was estimated by counting the number of fluorescent-labeled cells, lucifer yellow and rhodamine dextran, in the microscopic field under >20 magnification in the center intersection of the cut (31). Each treatment was repeated three times, and each slide was counted three times. Results are expressed as the average number of cells that allows dye transfer from cell to cell in 10 min.

Gap Junction Plaques—Another method of measuring gap junction activity is by measurements of plaques, large cluster of up to 10,000 gap junction hexamer channels (connexons). Plaques form more functional activity is by measurements of plaques, large cluster of up to 10,000 gap junctions; therefore, it is used as a control to differentiate dye transfer from diffusion because of cell damage (30). Cells were then fixed with 2.5% (w/v) paraformaldehyde in PBS. This was followed by incubation of 5 μl of 0.15% (v/v) Triton X-100 for 20 min and blocking of the plasma membrane with 1 ml of 0.15% (v/v) bovine serum albumin in PBS for 2 h at room temperature. Anti-Cx43 monoclonal antibodies were diluted in PBS (0.1% (v/v) bovine serum albumin), and the cells were then fixed for 10 min with 2.5% (w/v) paraformaldehyde in PBS. This was followed by incubation with 1 ml of 0.15% (v/v) Triton X-100 for 20 min and blocking of the non-specific sites with 3% (w/v) bovine serum albumin in PBS for 2 h at room temperature. Anti-Cx43 monoclonal antibodies were diluted in blocking buffer (3% bovine serum albumin) and then added to the fixed cells, which were incubated for 1 h at 4 °C. The working dilution of the secondary antibody, goat anti-rabbit, and anti-Cx43 (both 1:250) was detected using a direct fluor 568 (Molecular Probes) which is goat anti-rabbit and has an excitation/emission wavelength of 578/603 nm, was used with the working concentration of 5 μg/ml in blocking buffer. The cells were mounted on slides with 1% (v/v) glycerol in PBS. Slides were examined using a laser-scanning confocal microscope (Nikon C1), focusing on the top of the plasma membrane. The number of plaques per cell was determined under >100 objective lens.

Statistical Analysis—The level of significance (see * in figure legends) was considered at p < 0.05 using Student’s t test analysis. All data are presented as mean ± S.E. of at least three independent experiments from different batches of cultures.

RESULTS

LEDGF Increases DAG Levels, PKCγ Enzyme Activity, and PKCγ Autophosphorylation—We have shown previously that PKCγ is a major regulator of gap junction assembly/disassembly when both PKCδ and γ are activated in lens epithelial cells (34) and that LEDGF can cause the translocation of PKCγ, not PKCα, from the cytosol to the membrane. Additionally, the activation of PKCγ by LEDGF induced increases in DAG can cause a decrease in gap junction activity through the phosphorylation of Cx43 (1). A schematic diagram of PKCγ activation is shown in Fig. 10.

Cells were treated with 10 ng/ml of LEDGF for 10, 15, or 30 min. DAG level was measured using the sn-1,2-diacylglycerol assay system (Amersham Biosciences). The results show that 10 ng/ml LEDGF for 10 min causes a significant increase in DAG level (Fig. 1A). This suggests that LEDGF can increase DAG levels to ~200 pmol/mg of fresh tissue, a 2-fold increase when compared with basal levels of ~100 pmol/mg.

PKCγ is quite sensitive to small changes in the natural activator, DAG, because of the presence of two tandem zinc-binding motifs in the C1 domain which bind DAG at very low levels (1–6 nm) (33). Because LEDGF can increase DAG levels, does the elevation of DAG in the presence of LEDGF increase PKCγ enzyme activity as well? Cells were treated with 10 ng/ml of LEDGF for 10, 20, or 30 min. The results show a consistent 2-fold increase in PKCγ enzyme activity similar to a 2-fold increase of DAG levels compared with basal levels (Fig. 1B) within a similar time range. We also examined the effects of an increase in concentration of LEDGF. 20 ng/ml LEDGF for 10 min increase PKCγ enzyme activity by 2.75-fold compared with control (Fig. 1C).

PKCγ activation is often accompanied by phosphorylation of the specific PKCγ on serine residues (2). To demonstrate PKCγ, phosphorylation cells were treated with 0, 10, or 20 ng/ml of LEDGF for 10 min. Co-immunoprecipitation was performed. The results show a 1.75- and 2.0-fold increase in PKCγ phosphorylation in the presence of 10 and 20 ng/ml LEDGF compared with control, respectively (Fig. 1D). Thus, production of DAG, activation of PKCγ enzyme activity, and PKCγ phosphorylation occur within a similar time and dose range after LEDGF addition to the cells.

14-3-3ε Protein Co-immunoprecipitates with PKCγ, and This Is Reversed after LEDGF or IGF-1 Treatment—In order to identify which 14-3-3 isoform interacts with PKCγ, co-immunoprecipitation studies were done. Previously we have shown that growth factors such as IGF-I or LEDGF can induce PKCγ translocation to membranes, interactions of the PKCγ with the gap junction protein, connexin 43, and this results in the inhibition of gap junction activity in lens epithelial cells (1, 2). 14-3-3 has been reported to interact with PKCs and can either cause the inhibition or activation of PKC enzyme activity (26). Here we identify the 14-3-3 isoform that interacts with PKCγ in cells. Cells were treated with growth factors, IGF-I or LEDGF, at optimal times of 15 min and concentrations of 10 ng/ml LEDGF or 25 ng/ml IGF-1 (1, 2). PKCγ was immunoprecipitated from cells by using protein A/G PLUS-agarose beads in the presence of anti-PKCγ antibodies (Fig. 2). The nitrocellulose membranes were probed with anti-14-3-3ε, γ, or δ polyclonal antibodies. Western blots from cells, which were treated with 10 ng/ml LEDGF for 15 min or 25 ng/ml IGF-1 for 15 min, showed a decrease in co-immunoprecipitation of PKCγ and 14-3-3ε compared with control without treatment (Fig. 2A).
DAG levels are increased by LEDGF

PKCγ activation with increasing time after LEDGF treatment

PKC activation with increasing LEDGF

Auto-phosphorylation of PKCγ with increasing LEDGF

PKCγ antibodies at 4 °C for 4 h. Immunoprecipitated PKC-agarose bead complexes were incubated with PKC reaction mixtures of PepTag assay kit according to the manufacturer’s instructions. The reactions were stopped, and fluorescent PepTag peptides (PKC reaction products) were resolved by agarose gel electrophoresis and visualized under UV light. Quantitative analysis of phosphorylated peptide bands was measured using spectrophotometry at 570 nm. The graph represents the average percent of control of three separate experiments (± S.D.). Significant differences between treatment and control (asterisk) are indicated by asterisk (p < 0.05). C, cells were treated with 0 (control), 10, or 20 ng/ml LEDGF for 10 min or 10 ng/ml GST for 10 min. The phosphorylated PepTag peptide bands were excised and measured by spectrophotometry at 570 nm. The graph represents the average percent of control of three separate experiments (± S.D.). Significant differences between treatment and GST (control) are indicated by asterisk (p < 0.05). D, cells were treated with 0, 10, or 20 ng/ml LEDGF or 10 ng/ml GST for 10 min. Cells were harvested in lysis buffer and pelleted at 13,000 rpm (20,000 × g) for 10 min as described under “Experimental Procedures.” Western blots were performed. Immunoprecipitation (IP) is with anti-PKCγ, and immunoblotting (IB) is with anti-phosphoserine antibodies (top band). Quantitative analyses from three experiments (± S.D.) are presented, and significant differences between treatment and control are indicated by asterisk in the graph (p < 0.05).
individual peptides. The results demonstrate that 100 μM peptides for 2 h can compete endogenous PKCγ from binding to endogenous 14-3-3ε. 14-3-3ε was also co-immunoprecipitated with Raf-1, and its association was not affected by the presence of C1B1 or C1B5 peptides (Fig. 3C). The same blot was re-probed with anti-PKCγ antibodies, showing that C1B1 or C1B5 peptides could compete with endogenous PKCγ for the binding of 14-3-3ε (Fig. 3C, bottom). These results suggest that PKCγ binds to 14-3-3ε at the C1B1 and/or C1B5 region within the C1B domain of PKCγ.

To confirm that the 2-h incubation time was sufficient for uptake of synthetic peptides into cells, the uptake of C1B5 peptide into cells was quantitated by using HPLC. Peptide identity of the peaks containing peptides was confirmed by
**Fig. 3.** C1B domain peptides of PKCγ cause release of endogenous PKCγ from endogenous 14-3-3ε. A, schematic diagram of PKCγ with two possible binding sites for 14-3-3. B, cells were treated with 100 μM of C1B1, C1B5, or nonspecific peptides for 2 h. Cells were harvested in lysis buffer and pelleted at 13,000 rpm (20,000 × g) for 30 min as described under “Experimental Procedures.” Supernatants were incubated with PKCγ antibodies at 1:1000 overnight at 4 °C. Protein A/G-agarose beads were added as described under “Experimental Procedures.” Western blot was performed, and immunoblots (IB) were probed with anti-14-3-3ε (top band of B) and anti-PKCγ (bottom band of B). Quantitative analyses from three experiments (±S.D.) are presented showing that both C1B1 and C1B5 can decrease co-immunoprecipitation of endogenous PKCγ and 14-3-3. Significant differences between treatment and control are indicated by asterisk (p < 0.05), and significant differences between nonspecific peptide and C1B1 or C1B5 are indicated by two asterisks. IP, immunoprecipitation. C, cells were treated with 100 μM C1B1, C1B5, or nonspecific peptides for 2 h. Cells were harvested in lysis buffer and pelleted at 13,000 rpm (20,000 × g) for 30 min as described under “Experimental Procedures.” Supernatants were incubated with anti-14-3-3ε antibodies at 1:500 overnight at 4 °C. Protein A/G-agarose beads were added as described under “Experimental Procedures.” Western blot was performed and immunoblot with anti-Raf-1 at 1:1000 (top band of A), and the same blot was stripped and reprobed with PKCγ at 1:1000 (bottom band of A). The results show that there is no significant effect on the interaction of Raf-1 and 14-3-3ε; however, after we stripped and re-hit the same nitrocellulose membrane with anti-PKCγ antibodies, the C1B1 or C1B5 peptides specifically compete for endogenous PKCγ (bottom).
MALDI-TOF mass spectrometry. The results show that ~17% of the C1B5 peptide was internalized (results not shown). This suggests that the cellular concentration after treatment for 2 h with 100 μM peptide was ~4.72 nmol per cell.

**C1 Domain Peptides Cause a Translocation of Endogenous PKCγ to Membrane Fractions**—Because the C1B peptides can compete with endogenous PKCγ for 14-3-3-binding sites, we determined the effects on the cellular location of endogenous PKCγ. Endogenous PKCγ once released from 14-3-3 should translocate from the cytosol to the membrane in the presence of C1B1 and/or C1B5. This could result in enhanced interactions with membrane proteins such as Cx43. Peptides were incubated with cells for 2 h at 100 μM prior to separation of these cells into the supernatant and pellet (membrane) fractions. The results show that C1B1 and/or C1B5 peptides can increase PKCγ translocation from the cytosol (supernatant) to membrane fractions (pellet) by 50–76% (Fig. 4). 100 μM of C1B1 and/or C1B5 peptides is sufficient to translocate PKCγ to the membrane. The total amount of 14-3-3 was not affected in whole cell extracts during this time. (Note: 14-3-3 proteins are always cytosolic.) Quantitation of three independent experiments from different batches of cell cultures is graphed as total average pixel intensity in Fig. 4 (bottom).

**In Vitro Competition Assay**—Synthetic peptide C1B5 competes for in vitro binding of 14-3-3ε to PKCγ. GST-14-3-3ε was expressed in E. coli and purified by using anti-GST-agarose beads (Santa Cruz Biotechnology). GST-4-3-3ε was incubated with in vitro transcribed/translated PKCγ (Promega) with 0, 1, 10, or 100 μM of C1B1 peptides for 1 h. Western blot analysis was performed. Blot was probed with anti-PKCγ, and the quantitative analyses from three experiments are presented in the graph. IP, immunoprecipitation; IB, immunoblot.

**FIG. 5. In vitro competition assay; synthetic peptide C1B5 competes for in vitro binding of 14-3-3ε to PKCγ.** GST-14-3-3ε was expressed in E. coli and purified by using anti-GST-agarose beads (Santa Cruz Biotechnology). GST-4-3-3ε was incubated with in vitro transcribed/translated PKCγ (Promega) with 0, 1, 10, or 100 μM of C1B1 peptides for 1 h. Western blot analysis was performed. Blot was probed with anti-PKCγ, and the quantitative analyses from three experiments are presented in the graph. IP, immunoprecipitation; IB, immunoblot.

**FIG. 6. C1B domain peptides activate PKCγ enzyme activity.** Cells were treated with 100 μM C1B1, C1B5, nonspecific, or both C1B1 and C1B5 peptides for 2 h. Whole cell extracts were immunoprecipitated with anti-PKCγ antibodies at 4 °C for 4 h. Immunoprecipitated PKC-agarose bead complexes were incubated with PKC reaction mixtures of PepTag assay kit according to the manufacturer’s instruction. The reactions were stopped, and fluorescent PepTag peptides (PKC reaction products) were resolved by agarose gel electrophoresis and visualized under UV light. Quantitative analysis of phosphorylated peptide bands was measured using spectrophotometry at 570 nm. The presented data are from three experiments (± S.D.). Significant differences between treatment and control are indicated by asterisk (p < 0.05).
trol without addition of C1B5 peptides (Fig. 5).

C1 Domain Peptides Increase PKCγ Enzyme Activity—To examine the effect of C1B1 or C1B5 peptides on PKCγ enzyme activity, we also tested peptide stimulation of PKCγ enzyme activity after treatment of cells with 100 μM of each peptide. Endogenous PKCγ was immunoprecipitated, and its enzyme activity was measured by use of the PKC peptide substrate assay (2). The enzyme activity was normalized by calibration of the relative level of phosphorylated substrates to the relative amount of PKCγ in the immunoprecipitate, as determined by Western blotting, and was expressed as percentage of non-treated specific PKCγ activity (Fig. 6). The results demonstrate that treatment of cells with 100 μM C1B1 or C1B5 peptides significantly increased PKCγ activity in cells. Cells treated with both C1B1 and C1B5 did not show further increased PKCγ activity compared with treatment with C1B1 or C1B5 alone. Thus, either C1B1 or C1B5 peptides can cause an increase in PKCγ enzyme activity and are not additive for enzyme activation (Fig. 6) or for effects on co-immunoprecipitation (Fig. 3) of a peptide substrate. However, the C1B5 peptide does show more effect on PKCγ translocation (Fig. 4), but the values are not statistically significant.

C1 Domain Peptide Treatment Results in Activation of Endogenous PKCγ, and This Results in an Increase in Phosphorylation of Cx43 on Ser-368—PKCγ interacts with Cx43 and subsequently phosphorylates Cx43 on Ser-368 of the C terminus (1, 2, 34). We examined the effects of peptide treatment of cells on the phosphorylation of Cx43 on Ser-368. Cells were treated with 100 μM of C1B1, C1B5, or nonspecific peptides for 2 h. Western blots demonstrated that C1B1 or C1B5 peptide treatment can increase the phosphorylation of Cx43 on Ser-368 (Fig. 7, top band). The average total pixel intensity of three experiments shows that 100 μM C1B1 or C1B5 peptides can increase phosphorylation of Cx43 on Ser-368 by 3- or 2-fold, respectively (Fig. 7, bottom band). However, the nonspecific peptide has no effect on Cx43 phosphorylation. Cx43 levels did not change during this time.

Inhibition of Gap Junction Activity after Treatment with C1 Domain Peptides—Previous publications (1, 2) have shown that phosphorylation of the gap junction protein, Cx43, results in disassembly of gap junction plaques and that the activation of PKCγ by LEDGF or IGF-I decreases the gap junction dye transfer activity through the phosphorylation of Cx43 in cells. Since C1B1 and/or C1B5 peptide treatment resulted in the release of PKCγ from 14-3-3, does the presence of these peptides also cause a decrease in gap junction activity? Regulation of gap junction activity was measured as dye transfer in cells in the presence and/or absence of peptides (Fig. 8). Cells were treated with 100 μM of C1B1, C1B5, or both peptides for 2 h. The results show that C1B1 or C1B5 treatment can decrease gap junction activity in vivo compared with control cells without peptide or with a nonspecific peptide (Fig. 8). Quantitation of six different experiments are graphed as the average number of cells taking up the dye in the microscopic field under ×20 magnification in the center intersection of the cut (Fig. 8, bottom). Rhodamine dextran has been subtracted for these results.

C1 Domain Peptide Treatment Causes Decreases in Gap Junction Plaques—Cells were treated with 100 μM of C1B1,
C1B5, or nonspecific peptides for 2 h. Cells were fixed and mounted as described under "Experimental Procedures." Fig. 9 demonstrates a decrease in the number of gap junction plaques in the presence of 100 μM of C1B1, C1B5, nonspecific, or both C1B1 and C1B5 peptides. A, the images are from fluorescently labeled cells in the microscopic field under 20 × magnification in the intersection of the cut sites. The results show that gap junction activity was decreased in C1B1 and/or C1B5-treated cells. B, the number of dye transfer cells were counted and graphed in six sets of samples with S.D. Significant differences between treatment and control are indicated by asterisk (p < 0.05).

**DISCUSSION**

It is clear that PKC plays a central role in regulating gap junctional communication of cells as shown in Fig. 10. PKC is generally cytoplasmic and inactive until activation occurs through the binding of lipids to the C1 domain and Ca²⁺ to the C2 domains. PKCγ has a unique activation mechanism for a classical PKC. It appears that both the C1A and C1B domains are exposed and that this PKC isoform can be activated without increased Ca²⁺ (23). Growth factors such as LEDGF or IGF-I can cause the translocation of PKCγ in cells (1, 2). LEDGF, a lens and retina growth factor (36), can increase the endogenous DAG level, PKC enzyme activity, and phosphorylation level of PKCγ (Fig. 1). DAG binds specifically to a hydrophobic groove in the tandem C1 domains, C1A and C1B, with equal affinity for PKCγ, resulting in an increased translocation to membranes (23). Immunoprecipitation assays suggest a physical interaction of PKCγ and Cx43 which results in phosphorylation of Cx43 on Ser-368 (37). PKC directly phosphorylates Cx43 on Ser-368 in vivo, which results in a change in single channel behavior that contributes to a decrease in intercellular communication (37). We have shown that treatment of cells with C1 synthetic peptides results in the activation of endogenous PKCγ resulting in phosphorylation of Cx43 on Ser-368 (Fig. 7). This was accompanied by a decrease in gap junction dye transfer and plaques.

We provide additional evidence that PKCγ binds to the 14-3-3ε isoform (Fig. 2) through the C1B domain of PKCγ. Upon activation of PKCγ after LEDGF or IGF-1 exposure, PKCγ is released from 14-3-3ε (Fig. 2B). PKCγ contains two potential binding sites for 14-3-3ε. The synthetic peptides corresponding to these binding sites were synthesized and shown to be the sites of interaction of PKCγ and 14-3-3ε by both in vivo and in vitro incubation (Figs. 3–5). Either C1B1 or C1B5 appears to compete and is not additive, suggesting that competition at either site on PKCγ may be sufficient to remove endogenous PKCγ from the 14-3-3ε. Removal of the PKCγ from the 14-3-3

![Interaction of C1B Domain of PKCγ with 14-3-3ε](image-url)
could result in an increased interaction with membrane proteins through an as yet undefined mechanism. The PKCγ could undergo a conformational change, or potential phosphorylation sites could be exposed after removal from the 14-3-3. There are several potential phosphorylation sites within the PKCγ/14-3-3-binding sites that could be masked when the enzyme is docked to the 14-3-3. Future studies will entail mutations at these regions.

Among the 14-3-3-binding partners, some proteins, like Raf-1, bind to almost all 14-3-3 isoforms (35, 38). We tested for specificity of the peptides with Raf-1-14-3-3 interactions (Fig. 3C). Neither the C1B1 nor C1B5 peptide altered the interaction of Raf-1 and 14-3-3ε, indicating that these peptides were specific for PKCγ/14-3-3ε interactions. We have demonstrated for the first time that C1B1 and/or C1B5 regions of PKCγ are essential for the interaction with 14-3-3ε. This model is similar to that proposed for the regulation of Raf-1 activity (38) in which 14-3-3 binds to inactive Raf-1 at the consensus sequence RSXpSXP to maintain it in an inactive state, and disruption of this sequence binding is sufficient to permit activation of Raf-1 (37). In addition, Wang et al. (39), using random peptide phage display technology to identify and characterize the peptide for 14-3-3 proteins, demonstrated that an 18-mer (R18) peptide inhibited the interaction of 14-3-3 with Raf-1. Both Raf-1 and PKCγ are then covalently modified by phosphorylation, although PKCγ appears to accomplish some of this via autophos-
Interactions of C1B Domain of PKC with 14-3-3ε

In brain tissues PKCγ appears to prevent brain ischemia and is suggested as a target for ischemic preconditioning (40–44). However, the role of PKCγ in ischemic preconditioning was not clearly understood until recently (1, 2), when we demonstrated that PKCγ is involved in gap junction control. Ischemic damage occurs in part through open gap junctions that allow passage of small molecules (about 1 kDa or less) from damaged cells to healthy ones, resulting in cell death of neighboring cells. Thus, control of gap junctions is essential in the prevention of ischemic damage (45–47). Our data using synthetic peptides of the C1 domain of PKCγ suggest that this approach can be used in preventing ischemic damage, because these peptides specifically target PKCγ and inhibit gap junction activity.

The results of this study have important implications both for the understanding of how PKCγ regulates gap junction activity and for designing novel approaches to modulate gap junction control. Future studies will be directed toward identification of amino acids within the C1B1 and/or C1B5 peptides, which play a direct role in the interaction with 14-3-3ε. Site-directed mutagenesis of Ser-107 in the C1B1 peptide and Ser-145 in the C1B5 peptide will be examined for the analysis of 14-3-3ε-binding sites to the endogenous PKCγ and the roles of phosphorylation at these residues. Moreover, we will examine the effect of these mutants on the inhibition of gap junction activity.

Clearly understanding the role of the C1B binding domain is a key to understanding how PKCγ is involved in the control of gap junction activity in vivo.

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Inhibition of Gap Junction Activity through the Release of the C1B Domain of Protein Kinase Cγ (PKCγ) from 14-3-3: IDENTIFICATION OF PKCγ-BINDING SITES

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