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Phospholipase C-δ1 Is Activated by Capacitative Calcium Entry That Follows Phospholipase C-β Activation upon Bradykinin Stimulation*

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To characterize the regulatory mechanism of phospholipase C-δ1 (PLC-δ1) in the bradykinin (BK) receptor-mediated signaling pathway, we used a clone of PC12 cells, which stably overexpress PLC-δ1 (PC12-D1). Stimulation with BK induced a significantly higher Ca^{2+} elevation and inositol 1,4,5-trisphosphate (IP_3) production with a much lower half-maximal effective concentration (EC_{50}) of BK in PC12-D1 cells than in wild type (PC12-W) or vector-transfected (PC12-V) cells. However, BK-induced intracellular Ca^{2+} release and IP_3 generation was similar between PC12-V and PC12-D1 cells in the absence of extracellular Ca^{2+}, suggesting that the availability of extracellular Ca^{2+} is essential to the activation of PLC-δ1. When PC12-D1 cells were treated with agents that induce Ca^{2+} influx, more IP_3 was produced, suggesting that the Ca^{2+} entry induces IP_3 production in PC12-D1 cells. Furthermore, the additional IP_3 production after BK-induced capacitative calcium entry was detected in PC12-D1 cells, suggesting that PLC-δ1 is mainly activated by capacitative calcium entry. When cells were stimulated with BK in the presence of extracellular Ca^{2+}, [^{3}H]norepinephrine secretion was much greater from PC12-D1 cells than from PC12-V cells. Our results suggest that PLC-δ1 is activated by capacitative calcium entry following the activation of PLC-β, additively inducing IP_3 production and Ca^{2+} rise in BK-stimulated PC12 cells.

Phosphoinositide-specific phospholipase C is classified into three major groups (PLC-β, PLC-γ, and PLC-δ)[1] on the basis of molecular mass, deduced amino acid sequence, and immunological cross-reactivity. So far, 10 different mammalian phosphoinositide-specific PLC isozymes (PLC-β1, -β2, -β3, -β4, -γ1, -γ2, -δ1, -δ2, -δ3, and -δ4) have been characterized (1–4). The δ-type isozymes are smaller (M_r 85,000) than the PLC-β and PLC-γ (M_r 140,000–155,000) isoforms. PLC-β has been shown to be regulated by heterotrimeric GTP-binding proteins (G-proteins) (5). The PLC-β family is regulated by α-subunits of a pertussis toxin-insensitive G_q family of G-protein (6–8) and by βγ subunits of G-proteins (9). PLC-γ is thought to be a cytosolic isozyme that contains two Src homology 2 domains and an Src homology 3 domain and is regulated by tyrosine phosphorylation following binding to either growth factor-activated receptor tyrosine kinases such as the platelet-derived growth factor receptor and the epidermal growth factor receptor (10, 11) or by non-receptor-linked tyrosine kinases of an *src* family (12). In comparison with the PLC-β and PLC-γ isozymes, the physiological role and regulation of the PLC-δ family has been poorly understood despite its wide distribution (13).

The three-dimensional structure of a PLC-δ1 molecule lacking the pleckstrin homology domain revealed the catalytic domains (X and Y regions), which are tightly associated with two accessory modules, an EF-hand domain and a C2 domain (14), the latter of which was previously suggested to mediate Ca^{2+}-dependent binding to lipid vesicles (15). Furthermore, structural studies of the multidomain PLC-δ1 protein suggested that the binding sites for Ca^{2+} ions and the head group of phosphatidylinositol 4,5-bisphosphate are located both within and outside the catalytic domain (14, 15). Other studies of PLC-δ1 also revealed that substances such as Ca^{2+} ions and inositol 1,4,5-trisphosphate (IP_3) could play important roles as positive (16) and negative (17) regulators, respectively.

Although all PLC isozymes are activated by Ca^{2+} *in vitro*, PLC-δ isozymes seem more sensitive to Ca^{2+} than the other isozymes. An increase in Ca^{2+} ion concentration within the physiological range (0.1–10 μM) was sufficient to stimulate PLC-δ1 but not PLC-β1 and PLC-γ1 and to hydrolyze cellular inositol lipids present in permeabilized cells (16). An increase in cytosolic Ca^{2+} to a level sufficient to fix the C2 domain of PLC-δ might therefore trigger the enzyme's activation. Thus, it has been suggested that the activation of the PLC-δ isozymes might occur as an event secondary to the receptor-mediated activation of other PLC isozymes or Ca^{2+} channels (18).

Rat pheochromocytoma (PC12) cells are known to express PLC-δ1 (19). However, its biological function in PC12 cells has not yet been established. In order to elucidate the regulatory mechanism of PLC-δ1, we stably overexpressed PLC-δ1 in PC12 cells. Interestingly, we found that stimulation of G-protein-coupled bradykinin receptors significantly potentiated the responses of the PLC-δ1-overexpressing PC12 cells. Our data demonstrate that PLC-δ1 is mainly activated by capacitative calcium entry following PLC-β activation in the BK receptor-mediated signaling pathway.

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[1] The abbreviations used are: PLC, phospholipase C; BK, bradykinin; G-protein, GTP-binding regulatory protein; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; IP_3, inositol 1,4,5-trisphosphate; NE, norepinephrine; p(NH)ppA, adenyl-5′-yl imidodiphosphate; SK&F 96365 or SK&F, 1-{β-[3-(4-methoxyphenyl)propoxy]-4-methoxyphenethyl}-1H-imidazole hydrochloride; PAGE, polyacrylamide gel electrophoresis; LDB, low detergent blotto; BAPTA, 1,2-bis(2-aminophenoxy)ethane- *N*,*N*,*N*′,*N*′-tetraacetic acid.

EXPERIMENTAL PROCEDURES

Materials—Bradykinin (BK), trichloroacetic acid, IP_3, sulfinpyrazone, nifedipine, dithiothreitol, phenylmethylsulfonyl fluoride, leupeptin, and aprotinin were purchased from Sigma. SK&F 96365, phorbol myristate acetate, and HOE140 were obtained from Research Biochemical International (Natick, MA). Thapsigargin was purchased from Alomone Laboratories (Jerusalem, Israel). Fura-2 pentaacetoxymethyl-ester (Fura-2/AM) and BAPTA/acetoxymethyl ester were purchased from Molecular Probes, Inc. (Eugene, OR). Guanine nucleotides and other nucleotides were purchased from Roche Molecular Biochemicals. [^{3}H]Putrescine dihydrochloride (specific activity, 28.8 Ci/mmol), [α-^{32}P]GTP (3000 Ci/mmol), [^{3}H]norepinephrine ([^{3}H]NE; specific activity, 14.68 Ci/mmol), and [^{3}H]IP_3 were purchased from NEN Life Science Products. The Enhanced Chemiluminescence Detection system was obtained from Amersham Pharmacia Biotech. 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,3-dione and ionomycin were purchased from Calbiochem. Geneticin (G418) was obtained from Life Technologies, Inc.

Cell Culture and Transfection of PLC-δ1 cDNA—PC12 cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone, Logan, UT), 5% heat-inactivated horse serum (Hyclone), and 1% antibiotics (Life Technologies, Inc.) in a humidified atmosphere of 5% CO_2, 95% air at 37 °C. The culture medium was changed every 2 days, and the PC12 cells were subcultured weekly. PLC-δ1 cDNA was cloned in a plasmid vector, pIBI20. The PLC-11/pIBI20 plasmid was then digested with *Not*I. The 2.8-kilobase pair insert obtained was subcloned into a mammalian expression vector, pZipNeo, which contains a viral promoter and the neomycin resistance gene. The constructed plasmid DNA (PLC-δ1/pZipNeo) or the vector DNA (pZipNeo) alone was transfected into PC12 cells using an electroporator (Bio-Rad, 960 microfarads/250 V). One day after transfection, the cells were selectively grown in the presence of 400 μg/ml G418 for a week. The G418-resistant clones were screened for the expression of PLC-δ1 protein by Western blotting and probing with a monoclonal anti-PLC-δ1 antibody using the ECL detection system. Positive clones were then maintained in the presence of 100 μg/ml G418.

$[Ca^{2+}]_i$ Measurement—Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was determined using the fluorescent Ca^{2+} indicator Fura-2 as reported previously (20). In brief, PC12 cell suspensions were incubated in serum-free RPMI 1640 medium containing Fura-2/AM (3 μM) and sulfinpyrazone (250 μM) for 40 min at 37 °C) with continuous stirring. The cells were then washed with Locke's solution (154 mM NaCl, 5.6 mM KCl, 5.6 mM glucose, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES buffer adjusted to pH 7.4) containing sulfinpyrazone (250 μM) and left at room temperature until use. Fluorescence ratios were measured by an alternative wavelength time scanning method (dual excitation at 340 and 380 nm; emission at 500 nm). Calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ was performed as described by Grynkiewicz *et al.* (21).

Mn^{2+} Quenching of Fura-2 Fluorescence—PC12 cells that had been loaded with Fura-2/AM as described above were stimulated with bradykinin in the presence of 25 μM Mn^{2+}, and changes in fluorescence were measured at an excitation wavelength of 360 nm, which is an isosbestic wavelength, and at an emission wavelength of 500 nm, as described by Lee *et al.* (22).

Quantification of Inositol 1,4,5-Trisphosphate—IP_3 concentration in the cells was determined by competition assay with [^{3}H]IP_3 as described previously (23). In brief, to determine agonist-evoked IP_3 production, PC12 cells were stimulated with agonists for the indicated periods of time. The reaction was terminated by aspirating the medium off the cells and adding 15% (w/v) ice-cold trichloroacetic acid containing 10 mM EGTA. The cells were left on ice for 30 min to extract the water-soluble inositol phosphates. Trichloroacetic acid was then removed by extraction with diethyl ether. The final preparation was neutralized with 200 mM Tris, and its pH was adjusted to about 7.4. Assay buffer (0.1 M Tris buffer containing 4 mM EDTA and 4 mg/ml bovine serum albumin), [^{3}H]IP_3 (0.1 μCi/ml), and IP_3-binding protein were added to the cell extract. The mixture was incubated for 15 min on ice and then centrifuged at 2000 × *g* for 10 min. Water and scintillation mixture were added to the pellet to measure radioactivity. IP_3 concentration in the sample was determined based on a standard curve and expressed as pmol/μg of protein in the soluble cell extract. The IP_3-binding protein was prepared from bovine adrenal cortex according to the method of Challiss *et al.* (24).

Measurement of [^{3}H]NE Secretion—Catecholamine secretion by PC12 cells was measured in 24-well plates following the method reported by Park *et al.* (25) with some modification. In brief, cells were loaded with [^{3}H]NE (1 μCi/ml; 68 pmol/ml) while incubating in RPMI containing 0.01% ascorbic acid for 1 h at 37 °C in 5% CO_2, 95% air. The cells were washed with Locke's solution twice and incubated in Locke's solution for 15 min to stabilize. Then the cells were incubated in Locke's solution for 10 min during which basal secretion was measured. The cells were subsequently stimulated with the drugs under test for 10 min. After the incubation, the medium was aspirated from each well and transferred to a scintillation vial. Finally, residual catecholamine in the cells was extracted with 10% trichloroacetic acid, and the extract was transferred to a scintillation vial. The radioactivity in each vial was determined in a scintillation counter. The amount of [^{3}H]NE secreted was calculated as percentage of total [^{3}H]NE content. Net secretion was obtained by subtracting basal secretion from the stimulated secretion. In order to study the effect of SK&F 96365 on the BK-induced [^{3}H]NE secretion, the drug was added to both media used to measure basal and stimulated secretion.

Photoaffinity Labeling of G-protein—Photoaffinity labeling of G-protein with [α-^{32}P]GTP was carried out by the method of Linse and Mandelkow (26) with minor modifications (27). Samples were photolabeled with 5–10 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$ in an ice bath under 254-nm UV irradiation for 5–10 min. After the irradiation, the samples were mixed with Laemmli stopping solution (28) and allowed to stand at room temperature for 1 h. The samples were then subjected to SDS-PAGE using 7.5–12% gels. The gels were dried and exposed to Kodak X-OMAT XAR-5 film using DuPont image-intensifying screens.

Transglutaminase Assay—Transglutaminase activity was determined by quantifying the incorporation of [^{3}H]putrescine into casein as described previously (29). This reaction was carried out in 0.1 ml of buffer containing 50 mM Tris-HCl (pH 8.5), 20% (v/v) glycerol, *N*,*N*′-dimethylcasein (1 mg/ml), 250 μM putrescine, 1 μCi of [^{3}H]putrescine, 20 mM dithiothreitol, 2 mM $MgCl_2$, and the enzyme in the indicated amounts. Where indicated, $CaCl_2$ (1 mM) and GTP (5 mM) were added in the reaction mixture. Glycerol was included in the buffer, because its presence has been found to stabilize the transglutaminase activity (29). The presence or absence of glycerol in the assay had no effect on the GTP-induced inhibition of guinea pig liver transglutaminase activity. Reaction mixtures were incubated for 1 h at 37 °C, and the reaction was stopped by the addition of 0.1 ml of 50% trichloroacetic acid. The precipitate was collected on Whatman GF/C filters and washed three times with 10 ml of 5% trichloroacetic acid. Radioactivity was measured in a liquid scintillation counter.

Immunoblotting and Immunoprecipitation—Cells were grown to confluence and lysed in 500 μl of lysis buffer (20 mM HEPES (pH 7.2), 10% glycerol, 1 mM Na_3VO_4, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM dithiothreitol, 1 μg/ml leupeptin, and 1% triton X-100). After sonication, the cell homogenates were centrifuged at 10,000 × *g* for 10 min. Proteins (50 μg) were separated in 7.5–12% (w/v) gels by SDS-PAGE and transferred to Immobilon-P (Millipore Corp., Bedford, MA). The membranes were blocked for 1 h with low detergent blotto (LDB; 80 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, 0.2% (v/v) Nonidet P-40, and 50 mM Tris/HCl (pH 8.0) containing 5% (w/v) nonfat dry milk) at room temperature and then incubated in LDB containing polyclonal antibody against $G_h\alpha$ (1:500 dilution) for 1 h at room temperature. For immunoblots probed with monoclonal antibody against PLC-β1, PLC-γ1, and PLC-δ1, the antibody was diluted 1:2000, and the incubation was overnight. After being washed with LDB, the membranes were incubated with anti-mouse immunoglobulin peroxidase-linked antibody (1:5000 dilution) in high detergent blotto (2% (v/v) Nonidet P-40 in LDB) for 1 h at room temperature. After three washes, the membranes were subjected to the procedures for enhanced chemiluminescence. For immunoprecipitation, cells were lysed in lysis buffer, and each extract (800 μg/1000 μl) was treated with a preformed complex of *Staphylococcus aureus* goat anti-mouse IgG (Pansorbin, Calbiochem). After an overnight incubation at 4 °C, pellets were obtained by centrifugation at 15,000 × *g* for 1 min and washed three times with lysis buffer. The pellets were then processed by PAGE and Immunoblotting and probing with anti-$G_h\alpha$ antibody, exactly as described above.

Protein Determination—The amount of protein was estimated by the method of Bradford (30) using a Bio-Rad protein determination kit and bovine serum albumin as the standard.

Statistical Analysis—Statistical analysis of the data was done using the unpaired Student's *t* test in comparison between two experimental groups. Differences were considered significant when probability (*p*) values were <0.05.

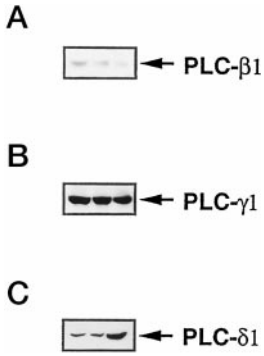

FIG. 1. **Immunoblots with anti-PLC antibodies in PC12 cells.** Wild type PC12 cells (PC12-W, *lane 1*), vector-transfected cells (PC12-V, *lane 2*), and PLC-δ1-overexpressing cells (PC12-D1, *lane 3*) were lysed, and 50 μg of protein was subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal antibodies against PLC-β1 (*A*), PLC-γ1 (*B*), and PLC-δ1 (*C*).

RESULTS

Overexpression of PLC-δ1 in PC12-D1 Cells—PC12 cells were transfected with a construct containing rat brain PLC-δ1 cDNA. Seven clones were obtained. One clone, PLCδ14, exhibiting the highest level of PLC-δ1 as inferred by Western blot analysis was selected and used under the name PC12-D1 throughout the following experiments. A clone of vector-transfected PC12 cells (PC12-V) was used as a control.

Western blot analyses using monoclonal antibodies against mouse PLC-β1, -γ1, and -δ1 revealed a marked overexpression of PLC-δ1 in the PC12-D1 cells (*lane 3* in Fig. 1*C*). Although wild type (PC12-W) and vector-transfected (PC12-V) cells also expressed PLC-δ1, the level of expression was much lower in those cells than in the PC12-D1 cells (*lanes 1* and *2* in Fig. 1*C*). On the other hand, the three kinds of cells all expressed similar amounts of PLC-β1 (Fig. 1*A*) and PLC-γ1 (Fig. 1*B*).

Effect of PLC-δ1 Overexpression on BK-induced $[Ca^{2+}]_i$ Rise—We investigated the effect of PLC-δ1 overexpression on the BK-induced signaling in PC12 cells. BK induced a much greater $[Ca^{2+}]_i$ rise in the PC12-D1 cells than in the PC12-W or PC12-V cells (Fig. 2*A*). The half-maximal effective concentration (EC_{50}) was much lower for the PC12-D1 cells (~10 nM) than the PC12-W or PC12-V cells (both ~100 nM) (Fig. 2*B*). However, the maximal effective concentrations (EC_{100}) were same for the three kinds of cells, namely 5 μM. When the three kinds of PC12 cells were treated with HOE140, an antagonist of B_2 bradykinin receptors, BK-induced $[Ca^{2+}]_i$ rise was completely blocked (data not shown), suggesting that the BK-induced response is entirely dependent on the B_2 receptors.

We also investigated whether BK-induced $[Ca^{2+}]_i$ rise is also potentiated in other PC12 clones that overexpress different levels of PLC-δ1. As shown in Fig. 3, the expression levels of PLC-δ1 in four different PC12 clones (δ5, δ12, δ14, and δ15) differentially affect the BK-induced $[Ca^{2+}]_i$ rise. Two clones, δ5 and δ12, which express intermediate levels of PLC-δ1, exhibited intermediate $[Ca^{2+}]_i$ increases caused by BK. Interestingly, δ14 and δ15 showed similar BK-induced $[Ca^{2+}]_i$ rises, although the expression level of PLC-δ1 in δ14 clone is apparently higher than in δ15 clone. These results suggest that there is some limitation in the activation of PLC-δ1 when the enzyme is expressed over a certain level.

The BK-induced $[Ca^{2+}]_i$ rise in PC12 cells occurs via two routes: Ca^{2+} release from intracellular Ca^{2+} stores and Ca^{2+} influx through Ca^{2+} release-activated calcium channels (31). We tested which route of Ca^{2+} mobilization contributed to the enhanced $[Ca^{2+}]_i$ rise after BK treatment in PC12-D1 cells. As shown in Fig. 4*A*, BK-induced Ca^{2+} release in the absence of extracellular Ca^{2+} was not significantly different in the three

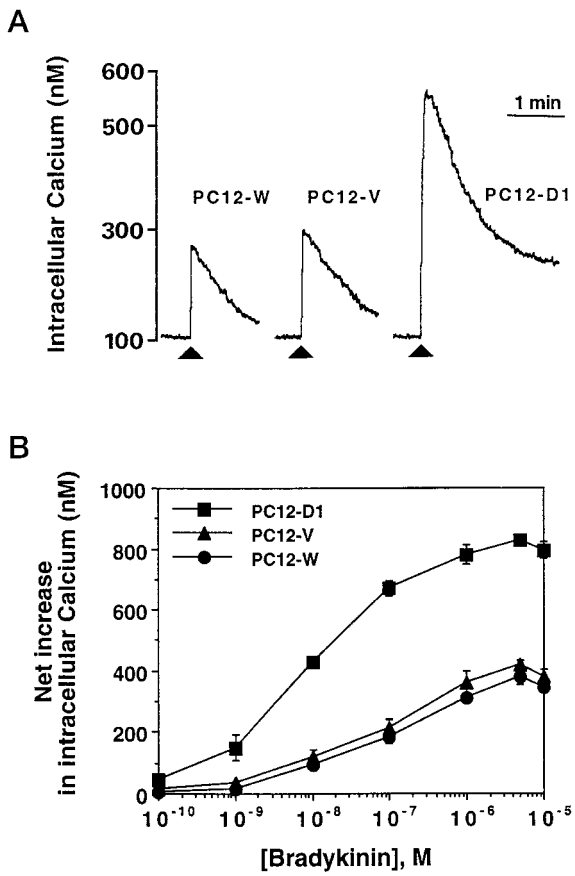

FIG. 2. **BK-induced $[Ca^{2+}]_i$ rise in PC12 cells.** *A*, PC12 cells were stimulated with 10 nM BK (marked by *arrowheads*) in the presence of 2.2 mM extracellular Ca^{2+}. Typical Ca^{2+} transients are presented. *B*, Fura-2-loaded cells were treated with various concentrations of BK in the presence of 2.2 mM extracellular Ca^{2+}, and the peaks in elevated $[Ca^{2+}]_i$ were measured. The experiments were performed five times, and the data are means ± S.E.

kinds of PC12 cells. Both EC_{50} and EC_{100} were similar (Fig. 4*B*). On the other hand, BK-induced Ca^{2+} influx after the addition of extracellular Ca^{2+}, which is thought to occur through Ca^{2+} release-activated Ca^{2+} channels, was greater in the PC12-D1 cells than in the PC12-W or PC12-V cells (Fig. 4*A*). EC_{50} was ~3 and ~30 nM for PC12-D1 and PC12-W or PC12-V cells, respectively (Fig. 4*B*). However, the EC_{100} remained similar (5 μM) among the three kinds of cells. The increased BK-induced Ca^{2+} influx into the PC12-D1 cells was confirmed by Mn^{2+} quenching experiments. Mn^{2+} is a good surrogate for Ca^{2+} ions in these kind of experiments, since it is not pumped out of the cells. Thus, it can be used as a selective tracer for Ca^{2+} influx (22). As shown in Fig. 5, the fluorescence of Fura-2 was gradually quenched by the presence of Mn^{2+}. When PC12 cells were stimulated with BK, fluorescence rapidly decreased, suggesting that BK-induced Mn^{2+} influx had occurred. The fluorescence quenching induced by the BK treatment was greater in the PC12-D1 cells than in PC12-W or PC12-V cells. The results together, therefore, suggest that BK-induced Ca^{2+} influx through Ca^{2+} release-activated Ca^{2+} channels is greatly enhanced in cells overexpressing PLC-δ1.

Effect of PLC-δ1 Overexpression on BK-induced IP_3 Production—Since IP_3 production can be an indicator of PLC activity, BK-induced IP_3 production in PC12-V and PC12-D1 cells was compared. When cells were treated with various concentrations of BK, more IP_3 was formed in the PC12-D1 cells than in the PC12-V cells (Fig. 6*A*). At 5 μM BK concentration, the maximal IP_3 produced occurred 15 s after stimulation, which is in good

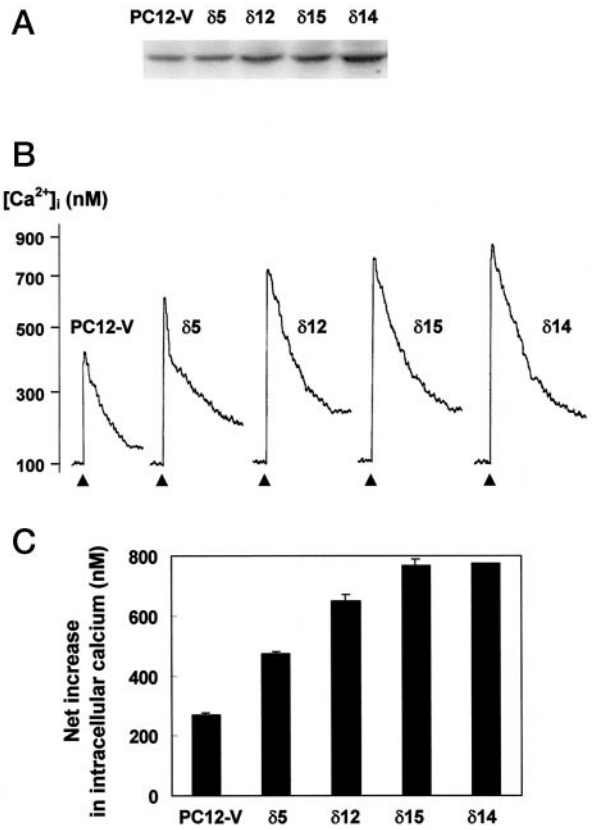

FIG. 3. **Correlation between PLC-δ1 expression and BK-induced $[Ca^{2+}]_i$ rise.** *A*, vector-transfected cells (PC12-V, *lane 1*) and four PLC-δ1-overexpressing cells (δ5, δ12, δ14, and δ15) were lysed, and 50 μg of protein was subjected to SDS-PAGE, transferred to nitrocellulose membrane, and probed with monoclonal antibody against PLC-δ1. *B*, PC12 clones were stimulated with 5 μM BK (marked by *arrowheads*) in the presence of 2.2 mM extracellular Ca^{2+}. Three independent experiments were performed and typical Ca^{2+} transients are presented. *C*, statistical analysis of the $[Ca^{2+}]_i$ rise induced by 5 μM BK. Data are means ± S.E. of triplicate measurements.

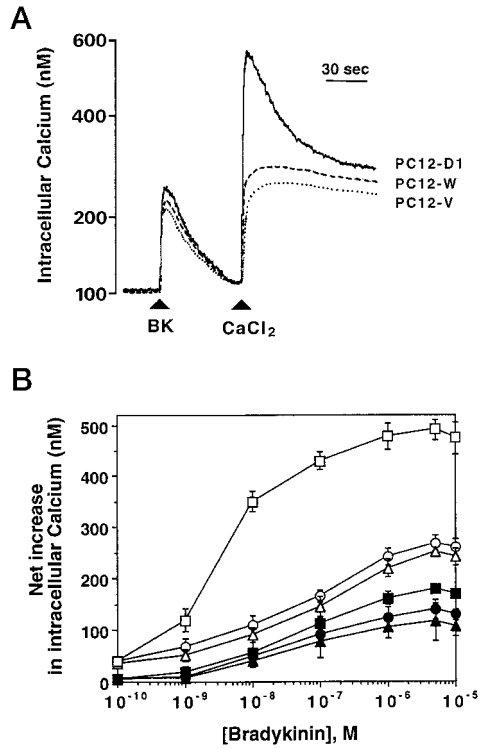

FIG. 4. **BK-induced internal Ca^{2+} release in PC12 cells.** *A*, PC12 cells were treated with 10 nM BK in the absence of extracellular Ca^{2+} 1 min before the addition of 4 mM $CaCl_2$. Typical Ca^{2+} transients in PC12-W (*dashed trace*), PC12-V (*dotted trace*), and PC12-D1 (*continuous trace*) cells are presented. *B*, Fura-2-loaded cells were treated with various concentrations of BK in the absence of extracellular Ca^{2+}, and the peaks in elevated $[Ca^{2+}]_i$ were measured. BK-induced Ca^{2+} release (*closed symbols*) and Ca^{2+} influx (*open symbols*) are shown for PC12-W (*circles*), PC12-V (*triangles*), and PC12-D1 (*squares*) cells. Data are representative of five separate experiments with similar results.

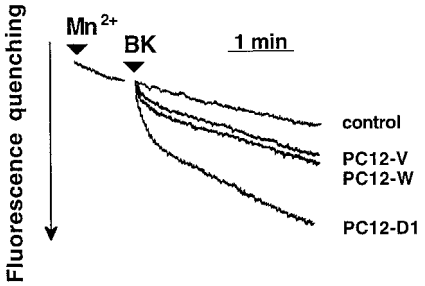

FIG. 5. **Effect of BK on Mn^{2+} quenching.** Fura-2-loaded cells were incubated with 25 μM Mn^{2+} for 3 min prior to the 10 nM BK treatment. The influx of Mn^{2+} was measured in terms of quenching of Fura-2 fluorescence excited at 360 nm and emitted at 500 nm. Traces shown are representative of three separate experiments.

agreement with our previous result (31) (Fig. 6*B*). At this time, the PC12-D1 cells produced ~1.7 times more IP_3 than the PC12-V cells, suggesting that PLC activity is higher in the PC12-D1 cells. Because PC12-D1 cells overexpress PLC-δ1, the difference in the PLC activity between the PC12-V and PC12-D1 cells can be attributed to the activity of overexpressed PLC-δ1. In the most simple scenario, one could assume that the greater IP_3 production in the PC12-D1 cells subsequently induces a greater Ca^{2+} release from the intracellular Ca^{2+} stores. However, the amount of Ca^{2+} release in PC12-V and PC12-D1 cells was similar, which contradicts the assumption of a greater IP_3 production in PC12-D1 cells. A difference in the experimental conditions may provide a clue for the understanding of this discrepancy. Unlike the IP_3 production experiments, which were done in the presence of extracellular Ca^{2+}, BK-induced Ca^{2+} release was determined in the absence of extracellular Ca^{2+}. Therefore, additional IP_3 production by the overexpressed PLC-δ1 in PC12-D1 cells may depend on the availability of extracellular Ca^{2+}. This possibility was tested by measuring IP_3 levels under conditions when extracellular Ca^{2+} was removed and intracellular Ca^{2+} was chelated with BAPTA. In the absence of any Ca^{2+}, the IP_3 production in PC12-V and PC12-D1 cells was similar (Fig. 6, *C* and *D*), suggesting that Ca^{2+} is required for the activation of PLC-δ1.

Enhanced Production of IP_3 by Ca^{2+} Influx—The Ca^{2+} that is necessary for the activation of PLC-δ1 can be supplied by Ca^{2+} release from intracellular Ca^{2+} stores or by Ca^{2+} influx from the extracellular space. When released Ca^{2+} can activate PLC-δ1, then the BK-induced Ca^{2+} release in the PC12-D1 cells should be greater than in PC12-V cells due to the additional IP_3 produced by the overexpressed PLC-δ1. However, released Ca^{2+} can be ruled out as a prominent candidate for PLC-δ1 activator, considering that the BK-induced Ca^{2+} release between the PC12-V and PC12-D1 cells was similar (Fig. 4). Therefore, we tested the possibility that Ca^{2+} could have entered from the extracellular space to activate PLC-δ1. As shown in Fig. 7*A*, Ca^{2+} influx-inducing agents such as high K^+, thapsigargin, and ionomycin activated additional IP_3 production in PC12-D1 cells but not in PC12-V cells. The additional IP_3 production induced by these agents disappeared in the absence of extracellular Ca^{2+} (Fig. 7*B*). The results, therefore, suggest that entry of extracellular Ca^{2+} activates PLC-δ1.

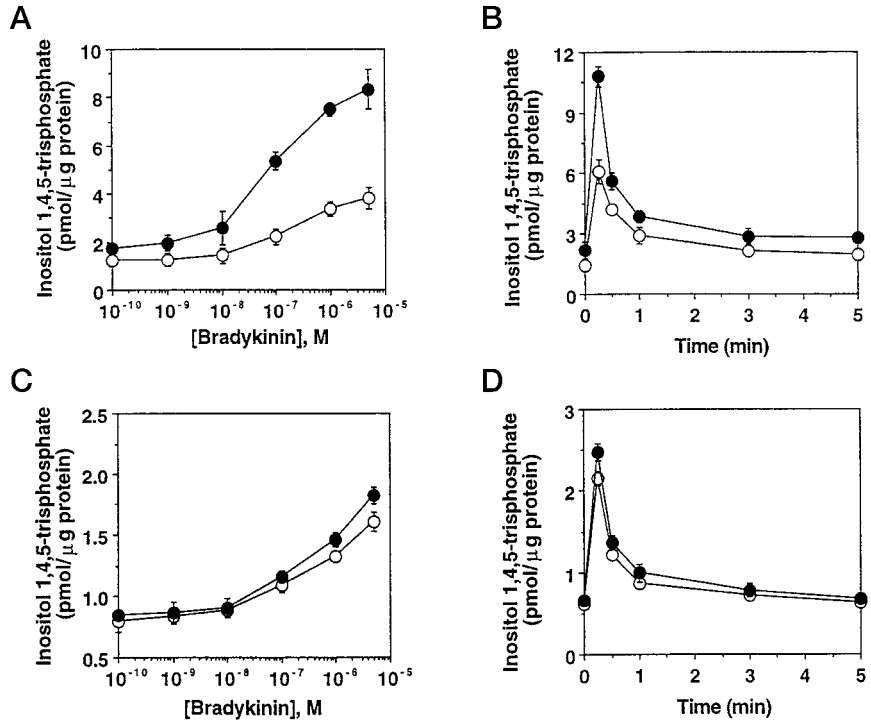

FIG. 6. **BK-induced IP_3 production in PC12 cells.** *A*, PC12-V (*open circles*) and PC12-D1 (*closed circles*) cells were stimulated with the indicated concentrations of BK for 15 s, and the IP_3 produced was measured in the presence of 2.2 mM extracellular Ca^{2+}. *B*, PC12-V (*open circles*) and PC12-D1 (*closed circles*) cells were stimulated with 5 μM BK for the indicated time periods, and the IP_3 produced was measured in the presence of 2.2 mM extracellular Ca^{2+}. *C*, BAPTA-loaded PC12-V (*open circles*) and PC12-D1 (*closed circles*) cells were stimulated with the indicated concentrations of BK for 15 s in the absence of extracellular Ca^{2+}, and the IP_3 produced was measured. *D*, BAPTA-loaded PC12-V (*open circles*) and PC12-D1 (*closed circles*) cells were stimulated with 5 μM BK in the absence of extracellular Ca^{2+} for the indicated time periods, and the IP_3 produced was measured. Three independent experiments were done, and the results were reproducible. Data are means ± S.E.

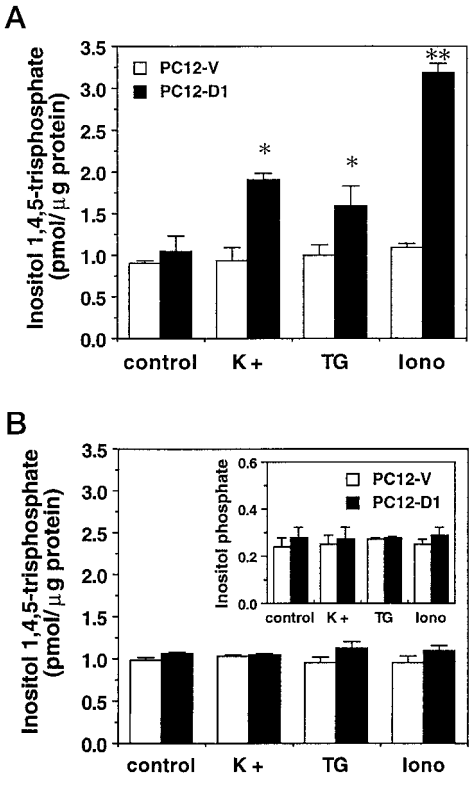

FIG. 7. **IP_3 production stimulated by Ca^{2+} influx-inducing agents.** PC12 cells were stimulated with 70 mM K^+, 300 nM thapsigargin (*TG*), and 500 nM ionomycin (*Iono*) for 15 s, and the IP_3 produced was measured in the presence (*A*) or absence (*B*) of extracellular Ca^{2+}. IP_3 formation in cells loaded with 75 μM BAPTA is shown in the *inset*. *, $p < 0.05$; **, $p < 0.01$, compared with PC12-V cells.

Activation of PLC-δ1 by BK-induced Capacitative Calcium Entry—Since the BK-induced Ca^{2+} influx is generally thought to occur by capacitative calcium entry through Ca^{2+} release-activated Ca^{2+} channels, it is likely that PLC-δ1 activation after BK treatment is mainly due to capacitative calcium entry. To test this hypothesis, the effect of reintroduction of extracellular Ca^{2+} 30 s after stimulation with BK in the absence of extracellular Ca^{2+} on IP_3 production was investigated. In contrast to PC12-V cells (Fig. 8*B*), PC12-D1 cells showed a significant increase in IP_3 after the reintroduction of extracellular Ca^{2+} (Fig. 8*D*). In addition, SK&F 96365, an inhibitor of Ca^{2+} release-activated Ca^{2+} channels, diminished the additional IP_3 production stimulated by the entry of extracellular Ca^{2+} into the PC12-D1 cells (Fig. 8*D*), without affecting the BK-induced IP_3 production into the PC12-V cells (Fig. 8*B*). These results suggest that, when PC12 cells are stimulated with BK, PLC-δ1 is activated by capacitative calcium entry occurring subsequent to IP_3 production and Ca^{2+} release after PLC-β activation. These results also explain greater Ca^{2+} influx induced by BK stimulation in PC12-D1 cells as shown in Fig. 4. Capacitative calcium entry in the PC12-D1 cells triggers serial feedback events such as rapid activation of PLC-δ1, more IP_3 production, further depletion of Ca^{2+} stores, and more capacitative calcium entry.

Effect of PLC-δ1 Overexpression on [^{3}H]NE Secretion—The effect of PLC-δ1 overexpression on catecholamine secretion, in which Ca^{2+} increase plays a key role, was also investigated. Like the Ca^{2+} increase, the BK-induced [^{3}H]NE secretion was much greater in the PC12-D1 cells (Table I). The enhanced secretion was observed in the presence of extracellular Ca^{2+}, but not in the absence of extracellular Ca^{2+}, suggesting that the enhancement of the secretion in PC12-D1 cells is due to the greater influx of extracellular Ca^{2+}. In the presence of SK&F 96365, PC12-V and PC12-D1 cells secreted similar amounts of [^{3}H]NE upon BK stimulation, suggesting that the capacitative calcium entry through Ca^{2+} release-activated Ca^{2+} channels induces PLC-δ1 activation and the subsequent additional increase in $[Ca^{2+}]_i$, leading to the potentiation of [^{3}H]NE secretion.

Lack of Involvement of $G_h\alpha$ in PLC-δ1 Activation—PLC-δ1 has been reported to be linked to $G_h\alpha$ protein in human myometrium (40, 41). In order to elucidate possible involvement of $G_h\alpha$ in the BK receptor-mediated signaling, we investigated whether $G_h\alpha$ is expressed in PC12 cells. For the examination of the nature of the G-proteins involved in the BK receptor-mediated signal transduction, photoaffinity labeling of G-proteins

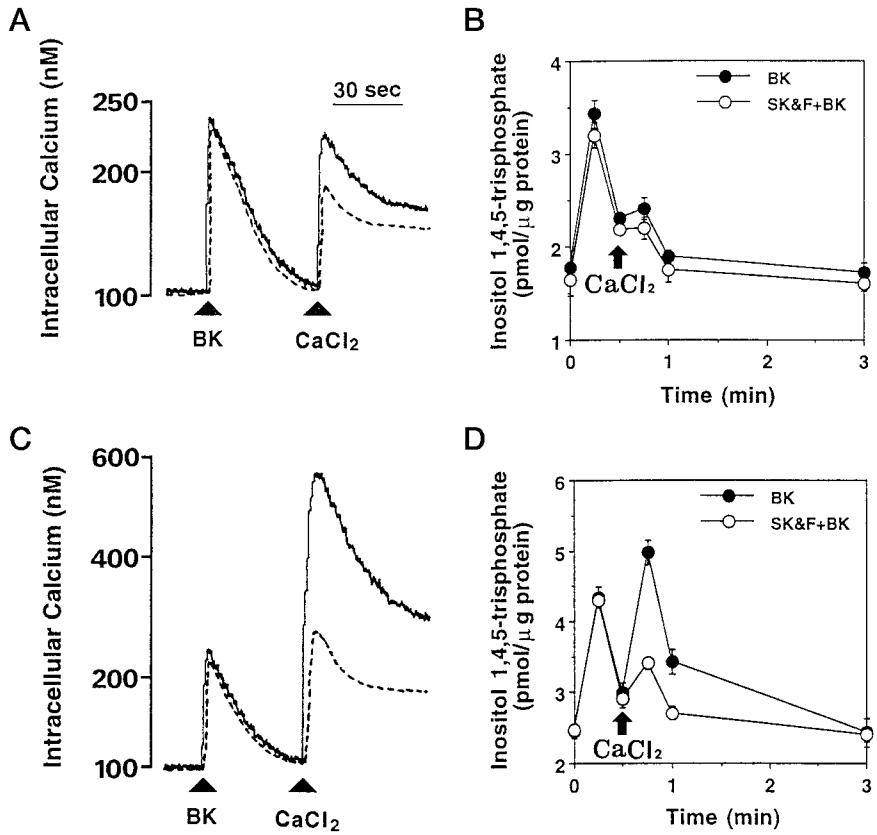

FIG. 8. **Effect of BK-induced capacitative calcium entry on IP_3 production.** *A* and *C*, effects of reintroduced Ca^{2+} and SK&F 96365 on the BK-induced $[Ca^{2+}]_i$ increase. PC12-V (*A*) and PC12-D1 (*C*) cells were stimulated with 5 μM BK in the absence of extracellular Ca^{2+} 1 min before the addition of 4 mM $CaCl_2$. The effect of 10 μM SK&F 96365 on the BK-induced $[Ca^{2+}]_i$ increase (*dashed trace*) is compared with the untreated control (*solid trace*). Typical Ca^{2+} transients are presented. *B* and *D*, effects of reintroduced Ca^{2+} and SK&F 96365 on the BK-induced IP_3 production. PC12-V (*B*) and PC12-D1 (*D*) cells were stimulated with 5 μM BK for 30 s in the absence of extracellular Ca^{2+}, after which 4 mM $CaCl_2$ was added. IP_3 production for the indicated time periods was measured. The effect of 10 μM SK&F 96365 on the BK-induced IP_3 production (*open circles*) was compared with the untreated control (*closed circles*). Data are means ± S.E.

TABLE I
Effect of PLC-δ1 overexpression on BK-evoked [^{3}H]NE secretion

[^{3}H]NE secretion evoked by 5 μM BK in the absence or presence of extracellular Ca^{2+} was measured in PC12-V and PC12-D1 cells as described under "Experimental Procedures" and is expressed as percentage of total radioactivity in the cells. Where indicated, cells were preincubated with 10 μM SK&F 96365 for 10 min and then stimulated with 5 μM BK. Two separate experiments were done, and the results were reproducible. Data are means ± S.E.

Treatment	Net secretion of [^{3}H]NE (percentage of total)			
	Without extracellular Ca^{2+}		With extracellular Ca^{2+}	
	BK	SK&F + BK	BK	SK&F + BK
	%		%	
PC12-V	2.02 ± 0.21	1.98 ± 0.14	6.44 ± 0.65	1.75 ± 0.28
PC12-D1	2.31 ± 0.32	2.07 ± 0.36	13.57 ± 1.10[a]	1.96 ± 0.23

[a] $p < 0.05$, compared with PC12-V cells under the same experimental conditions.

was carried out. As shown in Fig. 9, a labeling of the 74–80-kDa protein, $G_h\alpha$, was not detected, whereas labeling of the 40–50-kDa bands was detected. The labeling of these protein bands was specific for guanine nucleotides, since all of these bands could be blocked by unlabeled GTPγS but not by p(NH)ppA. These results, therefore, suggest that $G_h\alpha$ is not involved in BK receptor signaling. To confirm the above results, we performed a transglutaminase assay, since $G_h\alpha$ has transglutaminase activity in addition to GTPase activity. Transglutaminase activity is known to be increased by Ca^{2+} and blocked by GTPγS alone or by receptor activation in the presence of GTPγS (32). As shown in Table II, the transglutaminase activity of purified $G_h\alpha$ was enhanced by the addition of Ca^{2+}, and the enhanced activity was inhibited by GTP. However, there was no detectable transglutaminase activity even in the presence of 1 mM $CaCl_2$ in the PC12 cells. In addition, immunoblotting analysis also revealed that $G_h\alpha$ is absent from PC12 cells (data not shown). All of these observations strongly suggest that PLC-δ1 is not coupled to $G_h\alpha$ and that Ca^{2+} ion concentration is the main regulator of PLC-δ1 activity in PC12 cells. Therefore, in PC12 cells activation of PLC-δ1 occurs in a second step after the BK receptor-mediated activation of PLC-β isozymes. Furthermore, capacitative calcium entry is important to the activation of PLC-δ1.

DISCUSSION

Our study clearly demonstrates that in PC12 cells PLC-δ1 is activated not by G-protein, $G_h\alpha$, but by Ca^{2+} ions. More importantly, we found that the activation of PLC-δ1 is mainly dependent upon extracellular Ca^{2+} ions that enter by capacitative calcium entry via the BK receptor-mediated PLC-β pathway. PC12 cells contain at least three immunologically

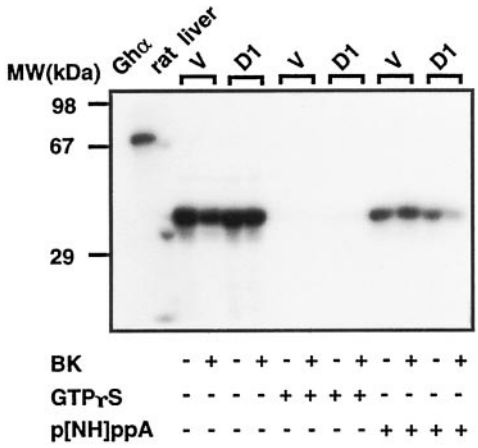

FIG. 9. **BK receptor-stimulated photoaffinity labeling in PC12 cells.** After lysing PC12 cells, the extracts were preincubated with 5 μM BK for 30 min at 4 °C and then further incubated with 5 μCi of [α-^{32}P]GTP, 5 μCi of [α-^{32}P]GTP plus 0.1 mM unlabeled GTPγS, or 5 μCi of [α-^{32}P]GTP plus 0.1 mM p(NH)ppA in the presence of 2 mM $MgCl_2$ and photolabeled with UV light (254 nm) for 5 min. PC12-V (V) and PC12-D1 (D1) cells were lysed, and 50 μg of protein was analyzed by SDS-PAGE (10% gel) and autoradiography, as described under "Experimental Procedures." As a positive control, purified guinea pig $G_h\alpha$ (*lane 1*) and 50 μg of rat liver protein (*lane 2*) were used. The data shown are representative of four independent experiments.

TABLE II
Measurement of transglutaminase activity

PC12-D1 cells were treated with vehicle or 5 μM BK, followed by lysis. $G_h\alpha$ purified from mouse heart membranes was used as the positive control. Basal transglutaminase activity was determined in the absence of GTP and $CaCl_2$. The enzyme activity was evaluated by monitoring the incorporation of [^{3}H]putrescine (0.1 mM) into *N*,*N*-dimethylated casein (1%) in the presence or absence of 1 mM $CaCl_2$ and 5 mM GTP at 20 °C for 30 min. Data are means ± S.E.

Treatment	Transglutaminase activity		
	PC12-D1 (vehicle)	PC12-D1 (BK)	Purified $G_h\alpha$
	cpm		
EGTA	1912 ± 46	1901 ± 277	1009 ± 203
$CaCl_2$	1898 ± 377	1972 ± 198	4374 ± 198
$CaCl_2$ + GTP	ND[a]	2023 ± 28	2700 ± 109

[a] Not determined.

distinct PLC isozymes, PLC-β, PLC-γ, and PLC-δ (33). It has been considered that the BK receptor might be coupled to PLC-β1 through a family of G-proteins, G_q (34). In general, BK can stimulate phosphoinositide hydrolysis in a variety of cell types. However, BK did not lead to production of inositol phosphate in Chinese hamster ovary cells transfected with PLC-δ1 cDNA. This may be due to the absence of PLC-β1 expression in the host Chinese hamster ovary cells (35). Our results clearly show that the BK-induced IP_3 production and $[Ca^{2+}]_i$ increase was markedly enhanced in the PLC-δ1-overexpressing PC12-D1 cells as compared with the vector-transfected PC12-V cells. In contrast to previous studies in which permeabilized cells were mainly used to prove that Ca^{2+} can play the role of PLC-δ1 activator (16, 35), our investigations were performed under physiological conditions without permeabilization. It has been suggested that agonist-induced hydrolysis of phosphoinositides is relatively insensitive to the removal of extracellular Ca^{2+} and that the artificial elevation of Ca^{2+} does not promote phosphoinositide hydrolysis (36). Banno *et al.* (37) suggested that in MC3T3-E1 cells, which contain much higher amounts of PLC-β1 and PLC-γ1 but less PLC-δ1, BK-stimulated IP_3 generation was neither affected by the chelation of extracellular Ca^{2+} with EGTA nor by intracellular Ca^{2+} elevation by ionomycin. This is also the case for our wild type PC12 cells. However, in our PC12-D1 cells, cytosolic $[Ca^{2+}]_i$ rise and IP_3 generation were diminished in the absence of extracellular Ca^{2+}. Therefore, we suggest that extracellular Ca^{2+} is necessary to the activation of PLC-δ1. In permeabilized PLC-δ1-overexpressing Chinese hamster ovary cells, the $[Ca^{2+}]_i$ level up to 1 μM was sufficient to cause significant IP_3 production, whereas no significant IP_3 production was observed at the same Ca^{2+} concentration in vector-transfected cells. These results suggest a preferential association of Ca^{2+} with PLC-δ1 when compared with PLC-β *in vivo* (35). It has been proposed that the initial transient cytosolic $[Ca^{2+}]_i$ induced by IP_3 resulting from receptor/G-protein-mediated PLC activation may in turn contribute to the prolonged activation of PLC in a positive feedback system (38). Our results strongly support this hypothetical model. The BK receptor-mediated signaling in PC12-D1 cells indicates that the activation of PLC-β isozymes leads to a subsequent activation of PLC-δ1. This explains why PLC activity was not affected in PC12-V cells but significantly reduced in PC12-D1 cells in the absence of extracellular Ca^{2+}. Previous studies of PLC-δ1 have suggested that the presence of Ca^{2+} ions is sufficient to activate the enzyme. Changes in Ca^{2+} ion concentration within the physiological range (100 nM to 10 μM) selectively stimulated the activity of PLC-δ1 in permeabilized PC12 cells, and the activity of this enzyme was further enhanced in the presence of phosphatidylinositol transfer protein, which could function in supplying and favorably presenting the substrate directly to the enzymes that hydrolyze or modify PIP_2 (16).

PLC-δ1 was also reported to directly associate with its receptor through a novel type of G-protein, G_h (39). Among the known PLC isozymes, PLC-β1 and PLC-γ1 were not stimulated by activated G_h in a reconstituted system, but a 69-kDa PLC, a proteolytic fragment of PLC-δ1, was found coupled to G_h proteins (32, 39). When an agonist binds to its receptor, PLC-δ1 is directly activated by GTP-bound $G_h\alpha$. The α-subunit of this heterodimeric G-protein is characterized by its transglutaminase activity in addition to its GTP binding function. The regulation of PLC-δ1 by $G_h\alpha$ seems to be different from the regulation of PLC-β isozymes by the subunits of heterotrimeric G-proteins when analyzed in a similar system *in vitro* (39). α_{1B}-Adrenergic receptors activate a 69-kDa PLC by coupling to $G_h\alpha$ (32). Likewise, PLC-δ1 is an effector of oxytocin receptor-mediated signaling via $G_h\alpha$ in human myometrium (40, 41). In these cases, each receptor can independently activate PLC via either G_q or G_h, just as the thrombin receptor simultaneously and directly couples to G_{i2} and $G_{q/11}$ (42). Thus, the same receptor can use multiple G-proteins and effectors to transmit a signal (43, 44). To test for a possible coupling of G_q and G_h with the BK receptors, we investigated whether $G_h\alpha$ is expressed in PC12 cells, but we found $G_h\alpha$ was not detectable.

Our present study clearly indicates that Ca^{2+} ions are the main regulators of PLC-δ1 and PLC-δ1 is secondarily activated by the entry of extracellular Ca^{2+}, in particular by capacitative calcium entry as a downstream effect of PLC-β activation during BK receptor-mediated signaling. This regulation of PLC-δ1 has an important physiological meaning as presenting a positive feedback mechanism in that the signaling mediated by PLC-β-linked receptors can be potentiated and prolonged. This fact explains why the Ca^{2+} entry was much higher in the PC12-D1 cells than in the PC12-W or PC12-V cells when extracellular Ca^{2+} was reintroduced after stimulation with BK in the absence of extracellular Ca^{2+}. Since there are many possible ways in which various PLC isozymes can be activated, this kind of investigation will help to elucidate the role and regulation of PLC-δ1, which still remain an open question in receptor-mediated signaling.

It is interesting that wild type PC12 cells hardly exhibit the Ca^{2+} entry-mediated activation of PLC-δ1, although they express a significant level of PLC-δ1. Comparative analysis of the correlation between the level of PLC-δ1 expression and the magnitude of BK-induced $[Ca^{2+}]_i$ increase in the various PC12 clones suggested that PLC-δ1 can be significantly activated by cytosolic calcium ion when the expression level of PLC-δ1 is higher than that of wild type PC12 cells. In addition, similar potentiation of BK-induced $[Ca^{2+}]_i$ rise was detected in δ15 and δ14 clones, although the expression level of PLC-δ1 was different. The results show a saturating effect in the elevation of cytosolic calcium when the enzyme is expressed higher than a certain level. However, the possibility cannot be ruled out that the initial amount of $[Ca^{2+}]_i$ elevation caused by BK-induced PLC-β activation is a limiting factor. In physiological environments, if there is any tissue in which PLC-δ1 is expressed, PLC-δ1 may play an important role in calcium signaling. Therefore, it will be interesting to investigate the expression level of PLC-δ1 and Ca^{2+} entry-mediated potentiation of phosphoinositide hydrolysis in various tissues and cells.

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Phospholipase C-δ1 Is Activated by Capacitative Calcium Entry That Follows Phospholipase C- β Activation upon Bradykinin Stimulation

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