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\(_{i}\) family of G-protein (6–8) and by β\(_{γ}\) subunits of G-proteins (9). PLC-γ is thought to be a cytosolic isoform 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 other isozymes. An increase in Ca\(^{2+}\) concentration within the physiological range (0.1–10 \(\mu\)M) 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 β-PLC 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 imidodiphosphatase; SK&F 96365 or SR&F 1, [β(34-methoxyphenylpropoxy)-4-methoxyphenethyl]-1H-imidazole hydrochloride; PAGE, polyacrylamide gel electrophoresis; LDB, low detergent blotto; BAPTA, 1,2-bis(aminophenoxy)ethane-N\(_{2}\)N\(_{2}\)N\(_{2}\)N\(_{2}\)tetraacetic acid.
Activation of PLC-δ1 by Capacitative Calcium Entry

EXPERIMENTAL PROCEDURES

Materials—Bradykinin (BK), trichloroacetic acid, IP₃, sulfipyrazone, nifedipine, diethiothreitol, phenylmethanesulfonyl fluoride, leupeptin, and aprotonin 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 pentaacetylxymethyl 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. [¹³⁵]I]Putrescine dihydrochloride (specific activity, 28.8 Ci/mmol), [α-²⁵³]P]GTP (3000 Ci/mmol), [¹²⁵]I]norepinephrine ([¹²⁵]I]NE, specific activity, 14.68 Ci/mmol), and [¹²⁵]I]IP₃ were purchased from NEN Life Science Products. The Enhanced Chemiluminescence Detection system was obtained from Amersham Pharmacia Biotech. 1-6-(17β,3α)-methoxyestradiol-1,3,5(10)-trien-17-ylaminohexyl-1H-pyrrrole-2,3-dione and iomyoncin 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-grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum. The cells were left on ice for 30 min to extract the water-soluble inositol phosphates. Trichloroacetic acid was then removed by aspiration of the medium off the PC12 cells were stimulated with agonists for the indicated periods of time. The reaction was terminated by aspirating the medium off the PC12 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 [¹²⁵]I]NE secreted was calculated as percentage of total [¹²⁵]I]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 [¹²⁵]I]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-²⁵³]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-²⁵³]P]GTP in the presence of 2 mM MgCl₂ 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 centrifuged at 15,000 g for 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 [¹³⁵]I]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'-dimethylcarbamyl (1 mg/ml), 250 μM putrescine, 1 μM of [¹³⁵]I]putrescine, 20 mM diethiothreitol, 2 mM MgCl₂, and the enzyme in the indicated amount. After incubation, the reaction was terminated by adding a monoclonal anti-PLC-δ1 antibody followed by anti-mouse immunoglobulin peroxidase-linked antibody. The membranes were blocked for 1 h with low detergent blotto (LDB; 50 mM NaCl, 2 mM CaCl₂, 0.02% Na₂CO₃, 0.2% (v/v) Nonidet P-40, and 50 mM Tris/HCl (pH 8.0)) containing 5% (v/v) nonfat dry milk) at room temperature and then incubated in LDB containing polyclonal antibody against Gh, 1:50 dilution) for 1 h at room temperature. The antibody was diluted 1:2000, and the incubation was performed 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; 50 mM NaCl, 2 mM CaCl₂, 0.02% Na₂CO₃, 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 Gα₂ (1500 dilution) for 1 h at room temperature. For immunoblots probed with monoclonal antibody against PLC-δ1, 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 a preformed complex of Staphylococcus aureus goat anti-mouse IgG (Pansorbin, Calbiochem). After an overnight incubation at 4 °C, the pellets were then incubated with the indicated Gα₂ 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.
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. 1C). Although wild type (PC12-W) and vector-transfected (PC12-V) cells also expressed PLC-δ1, the level of expression was much lower in those than in the PC12-D1 cells (lanes 1 and 2 in Fig. 1C). On the other hand, the three kinds of cells all expressed similar amounts of PLC-β1 (Fig. 1A) and PLC-γ1 (Fig. 1B).

Effect of PLC-δ1 Overexpression on BK-induced [Ca²⁺]i Rise—We investigated the effect of PLC-δ1 overexpression on the BK-induced signaling in PC12 cells. BK induced a much greater [Ca²⁺]i rise in the PC12-D1 cells than in the PC12-W or PC12-V cells (Fig. 2A). The half-maximal effective concentration (EC₅₀) was much lower for the PC12-D1 cells (~10 nM) than the PC12-W or PC12-V cells (both ~100 nM) (Fig. 2B). However, the maximal effective concentrations (EC₁₀₀) 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 B₂ bradykinin receptors, BK-induced [Ca²⁺]i rise was completely blocked (data not shown), suggesting that the BK-induced response is entirely dependent on the B₂ receptors.

We also investigated whether BK-induced [Ca²⁺]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 [Ca²⁺]i rise. Two clones, δ5 and δ12, which express intermediate levels of PLC-δ1, exhibited intermediate [Ca²⁺]i increases caused by BK. Interestingly, δ14 and δ15 showed similar BK-induced [Ca²⁺]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 PLCδ1 when the enzyme is expressed over a certain level.

The BK-induced [Ca²⁺]i rise in PC12 cells occurs via two routes: Ca²⁺ release from intracellular Ca²⁺ stores and Ca²⁺ influx through Ca²⁺ release-activated calcium channels (31). We tested which route of Ca²⁺ mobilization contributed to the enhanced [Ca²⁺]i rise after BK treatment in PC12-D1 cells. As shown in Fig. 4A, BK-induced Ca²⁺ release in the absence of extracellular Ca²⁺ was not significantly different in the three kinds of PC12 cells. Both EC₅₀ and EC₁₀₀ were similar (Fig. 4B). On the other hand, BK-induced Ca²⁺ influx after the addition of extracellular Ca²⁺, which is thought to occur through Ca²⁺ release-activated Ca²⁺ channels, was greater in the PC12-D1 cells than in the PC12-W or PC12-V cells (Fig. 4A). EC₅₀ was ~3 and ~30 nM for PC12-D1 and PC12-W or PC12-V cells, respectively (Fig. 4B). However, the EC₁₀₀ remained similar (5 μM) among the three kinds of cells. The increased BK-induced Ca²⁺ influx into the PC12-D1 cells was confirmed by Mn²⁺ quenching experiments. Mn²⁺ is a good surrogate for Ca²⁺ ions in these kinds of experiments, since it is not pumped out of the cells. Thus, it can be used as a selective tracer for Ca²⁺ influx (22). As shown in Fig. 5, the fluorescence of Fura-2 was gradually quenched by the presence of Mn²⁺. When PC12 cells were stimulated with BK, fluorescence rapidly decreased, suggesting that BK-induced Mn²⁺ 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 Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ channels is greatly enhanced in cells overexpressing PLC-δ1.

Effect of PLC-δ1 Overexpression on BK-induced IP₃ Production—Since IP₃ production can be an indicator of PLC activity, we investigated whether BK-induced IP₃ production was greater in the PC12-D1 cells than in the PC12-W or PC12-V cells. As shown in Fig. 6A, [IP₃]i was not significantly different in the three kinds of PC12 cells. As inferred by Western blot analyses using monoclonal antibodies against PLC-δ1 (Fig. 1A) and PLC-δ1 (Fig. 1B), the BK-induced release of Ca²⁺ from intracellular Ca²⁺ stores and Ca²⁺ influx through Ca²⁺ release-activated calcium channels (31). We tested which route of Ca²⁺ mobilization contributed to the enhanced [Ca²⁺]i rise after BK treatment in PC12-D1 cells. As shown in Fig. 4A, BK-induced Ca²⁺ release in the absence of extracellular Ca²⁺ was not significantly different in the three kinds of PC12 cells. Both EC₅₀ and EC₁₀₀ were similar (Fig. 4B). On the other hand, BK-induced Ca²⁺ influx after the addition of extracellular Ca²⁺, which is thought to occur through Ca²⁺ release-activated Ca²⁺ channels, was greater in the PC12-D1 cells than in the PC12-W or PC12-V cells (Fig. 4A). EC₅₀ was ~3 and ~30 nM for PC12-D1 and PC12-W or PC12-V cells, respectively (Fig. 4B). However, the EC₁₀₀ remained similar (5 μM) among the three kinds of cells. The increased BK-induced Ca²⁺ influx into the PC12-D1 cells was confirmed by Mn²⁺ quenching experiments. Mn²⁺ is a good surrogate for Ca²⁺ ions in these kinds of experiments, since it is not pumped out of the cells. Thus, it can be used as a selective tracer for Ca²⁺ influx (22). As shown in Fig. 5, the fluorescence of Fura-2 was gradually quenched by the presence of Mn²⁺. When PC12 cells were stimulated with BK, fluorescence rapidly decreased, suggesting that BK-induced Mn²⁺ 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 Ca²⁺ influx through Ca²⁺ release-activated Ca²⁺ channels is greatly enhanced in cells overexpressing PLC-δ1.
agreement with our previous result (31) (Fig. 6B). At this time, the PC12-D1 cells produced ~1.7 times more IP₃ 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 IP₃ production in the PC12-D1 cells subsequently induces a greater Ca²⁺ release from the intracellular Ca²⁺ stores. However, the amount of Ca²⁺ release in PC12-V and PC12-D1 cells was similar, which contradicts the assumption of a greater IP₃ production in PC12-D1 cells. A difference in the experimental conditions may provide a clue for the understanding of this discrepancy. Unlike the IP₃ production experiments, which were done in the presence of extracellular Ca²⁺, BK-induced Ca²⁺ release was determined in the absence of extracellular Ca²⁺. Therefore, additional IP₃ production by the overexpressed PLC-δ1 in PC12-D1 cells may depend on the availability of extracellular Ca²⁺. This possibility was tested by measuring IP₃ levels under conditions when extracellular Ca²⁺ was removed and intracellular Ca²⁺ was chelated with BAPTA. In the absence of any Ca²⁺, the IP₃ production in PC12-V and PC12-D1 cells was similar (Fig. 6C and D), suggesting that Ca²⁺ is required for the activation of PLC-δ1.

Enhanced Production of IP₃ by Ca²⁺ Influx—The Ca²⁺ that is necessary for the activation of PLC-δ1 is supplied by Ca²⁺ release from intracellular Ca²⁺ stores or by Ca²⁺ influx from the extracellular space. When released Ca²⁺ can activate PLC-δ1, then the BK-induced Ca²⁺ release in the PC12-D1 cells should be greater than in PC12-V cells due to the additional IP₃ produced by the overexpressed PLC-δ1. However, released Ca²⁺ can be ruled out as a prominent candidate for PLC-δ1 activator, considering that the BK-induced Ca²⁺ release between the PC12-V and PC12-D1 cells was similar (Fig. 4). Therefore, we tested the possibility that Ca²⁺ could have entered from the extracellular space to activate PLC-δ1. As shown in Fig. 7A, Ca²⁺ influx-inducing agents such as high K⁺, thapsigargin, and ionomycin activated additional IP₃ production in PC12-D1 cells but not in PC12-V cells. The additional IP₃ production induced by these agents disappeared in the absence of extracellular Ca²⁺ (Fig. 7B). The results, therefore, suggest that entry of extracellular Ca²⁺ activates PLC-δ1.

Fig. 3. Correlation between PLC-δ1 expression and BK-induced [Ca²⁺], rise. A, vector-transfected cells (PC12-V, lane 1) and four PLC-δ1-overexpressing cells (65, 812, 814, and 815) 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 Ca²⁺. Three independent experiments were performed and typical Ca²⁺ transients are presented. C, statistical analysis of the [Ca²⁺] rise induced by 5 μM BK. Data are means ± S.E. of triplicate measurements.

Fig. 4. BK-induced internal Ca²⁺ release in PC12 cells. A, PC12 cells were treated with 10 nM BK in the absence of extracellular Ca²⁺ 1 min before the addition of 4 mM CaCl₂. Typical Ca²⁺ 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 Ca²⁺, and the peaks in elevated [Ca²⁺], were measured. BK-induced Ca²⁺ release (closed symbols) and Ca²⁺ 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 Mn²⁺ quenching. Fura-2-loaded cells were incubated with 25 μM Mn²⁺ for 3 min prior to the 10 nM BK treatment. The influx of Mn²⁺ 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.
were stimulated with 50 mM KCl for 15 s, and the IP3 produced was measured in the presence of 2.2 mM extracellular Ca2+. B, PC12-V (open circles) and PC12-D1 (closed circles) cells were stimulated with 5 μM BK for the indicated time periods, and the IP3 produced was measured in the presence of 2.2 mM extracellular Ca2+. 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 Ca2+, and the IP3 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 Ca2+ for the indicated time periods, and the IP3 produced was measured. Three independent experiments were done, and the results were reproducible. Data are means ± S.E.

**Fig. 6. BK-induced IP3 production in PC12 cells.**

A. PC12-D1 (closed circles) cells were stimulated with 50 mM KCl for 15 s, and the IP3 produced was measured in the presence of 2.2 mM extracellular Ca2+. B. PC12-V (open circles) and PC12-D1 (closed circles) cells were stimulated with 5 μM BK for the indicated time periods, and the IP3 produced was measured in the presence of 2.2 mM extracellular Ca2+.

**Effect of PLC-β1 Overexpression on [3H]NE Secretion—**The effect of PLC-β1 overexpression on catecholamine secretion, in which Ca2+ increase plays a key role, was also investigated. Like the Ca2+ increase, the BK-induced [3H]NE secretion was much greater in the PC12-D1 cells (Table I). The enhanced secretion was observed in the presence of extracellular Ca2+, but not in the absence of extracellular Ca2+, suggesting that the enhancement of the secretion in PC12-D1 cells is due to the greater influx of extracellular Ca2+. In the presence of SK&F 96365, PC12-V and PC12-D1 cells secreted similar amounts of [3H]NE upon BK stimulation, suggesting that the capacitative calcium entry through Ca2+ release-activated Ca2+ channels induces PLC-β1 activation and the subsequent additional increase in [Ca2+]i, leading to the potentiation of [3H]NE secretion.

**Lack of Involvement of Gα in PLC-β1 Activation—**PLC-β1 has been reported to be linked to Gα protein in human myometrium (40, 41). In order to elucidate possible involvement of Gα in the BK receptor-mediated signaling, we investigated whether Gα 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...
was carried out. As shown in Fig. 9, a labeling of the 74–80-kDa protein, Gαₐ, 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 Gαₐ is not involved in BK receptor signaling. To confirm the above results, we performed a transglutaminase assay, since Ghₐ has transglutaminase activity in addition to GTPase activity. Transglutaminase activity is known to be increased by Ca²⁺ 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 Gαₐ was enhanced by the addition of Ca²⁺, and the enhanced activity was inhibited by GTP. However, there was no detectable transglutaminase activity even in the presence of 1 mM CaCl₂ in the PC12 cells. In addition, immunoblotting analysis also revealed that Gαₐ is absent from PC12 cells (data not shown). All of these observations strongly suggest that PLC-δ is not coupled to Gαₐ and that Ca²⁺ 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, Gαₐ, but by Ca²⁺ ions. More importantly, we found that the activation of PLC-δ₁ is mainly dependent upon extracellular Ca²⁺ ions that enter by capacitative calcium entry via the BK receptor-mediated PLC-β pathway. PC12 cells contain at least three immunologically
distinct PLC isoforms, 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, Gβγ (34). In general, BK
stimulates 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-81
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 IP3 production and [Ca2+]i increase
was markedly enhanced in the PLC-81-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 Ca2+ can play the role
of PLC-81 activator (16, 35), our investigations were performed
under physiological conditions without permeabilization. It has
been suggested that agonist-induced hydrolysis of phosphoinosities
is relatively insensitive to the removal of extracellular
Ca2+, and that the artificial elevation of Ca2+ 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-stimu-
ulated IP3 generation was neither affected by the chelation
of extracellular Ca2+ with EGTA nor by intracellular Ca2+
elevation by ionomycin. This is also the case for our wild type PC12
cells. However, in our PC12-D1 cells, cytosolic [Ca2+]i rise and
IP3 generation were diminished in the absence of extracellular
Ca2+. Therefore, we suggest that extracellular Ca2+ is
necessary to the activation of PLC-81. In permeabilized PLC-81-
overexpressing Chinese hamster ovary cells, the [Ca2+]i level
up to 1 μM was sufficient to cause significant IP3 production,
whereas no significant IP3 production was observed at the
same Ca2+ concentration in vector-transfected cells. These
results suggest a preferential association of Ca2+ with PLC-81
when compared with PLC-β in vivo (35). It has been proposed
that the initial transient cytosolic [Ca2+]i, induced by IP3
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 isoforms
leads to a subsequent activation of PLC-81. This explains why
PLC activity was not affected in PC12-V cells but significantly
reduced in PC12-D1 cells in the absence of extracellular Ca2+.
Previous studies of PLC-81 have suggested that the presence of
Ca2+ ions is sufficient to activate the enzyme. Changes in Ca2+
ion concentration within the physiological range (100 nM to 10
μM) selectively stimulated the activity of PLC-81 in permeabi-
lized 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 present-
ing the substrate directly to the enzymes that hydrolyze or
modify PIP2 (16).

PLC-81 was also reported to directly associate with its recep-
tor through a novel type of G-protein, Gα (39). Among the
known PLC isoforms, PLC-β1 and PLC-γ1 were not stimulated
by activated Gα in a reconstituted system, but a 69-kDa PLC,
a proteolytic fragment of PLC-81, was found coupled to Gα
proteins (32, 39). When an agonist binds to its receptor, PLC-81 is
directly activated by GTP-bound Gα. The α-subunit of this
diheteromeric G-protein is characterized by its transglutami-
nase activity in addition to its GTP binding function. The
regulation of PLC-81 by Gα seems to be different from the
regulation of PLC-β isoforms by the subunits of heterotrimeric
G-proteins when analyzed in a similar system in vitro (39).
α1B-Adrenergic receptors activate a 69-kDa PLC by coupling
to Gα (32). Likewise, PLC-81 is an effector of oxytocin recep-
tor-mediated signaling via Gα in human myometrium (40, 41).
In these cases, each receptor can independently activate PLC via
either Gα or Gβγ just as the thrombin receptor simultaneously
and directly couples to Gi2 and Gi11 (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 Gα and Gi11
with the BK receptors, we investigated whether Gα was
expressed in PC12 cells, but we found Gα was not detectable.
Our present study clearly indicates that Ca2+ ions are the
main regulators of PLC-81 and PLC-81 is secondarily activated
by the entry of extracellular Ca2+, in particular by capacitative
calcium entry as a downstream effect of PLC-β activation
during BK receptor-mediated signaling. This regulation of PLC-81
has an important physiological meaning as presenting a posi-
tive feedback mechanism in that the signaling mediated by
PLC-β-linked receptors can be potentiated and prolonged. This
fact explains why the Ca2+ entry was much higher in the
PC12-D1 cells than in the PC12-W or PC12-V cells when extracellular Ca2+ was reintroduced after stimulation with BK in
the absence of extracellular Ca2+. Since there are many possi-
ble ways in which various PLC isoforms can be activated, this
kind of investigation will help to elucidate the role and regu-
lation of PLC-81, which still remain an open question in recep-
tor-mediated signaling.

**Table II**

| Treatment          | Transglutaminase activity |
|--------------------|----------------------------|
|                    | PC12-D1 (vehicle) | PC12-D1 (BK) | Purified Gαα |
|                    | cpm              | cpm          | cpm          |
| EGTA               | 1912 ± 46        | 1901 ± 277   | 1009 ± 203   |
| CaCl2 (100 nM)     | 1986 ± 377       | 1972 ± 189   | 4374 ± 189   |
| CaCl2 + GTP        | NDa              | 2023 ± 58    | 2700 ± 109   |

*a* Not determined.

**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-32P]GTP, 5 μCi of [α-32P]GTP plus 0.1 mM unlabeled GTPγS, or 5 μCi of
[α-32P]GTP plus 0.1 mM p[NH]ppA in the presence of 2 mM MgCl2 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 "Exper-
imental Procedures." As a positive control, purified guinea pig Ghα (lane 1)
and 50 μg of rat liver protein (lane 2) were used. The data shown are
representative of four independent experiments.
It is interesting that wild type PC12 cells hardly exhibit the Ca\textsuperscript{2+} entry-mediated activation of PLC-\(\delta\)-1, although they express a significant level of PLC-\(\delta\)-1. Comparative analysis of the correlation between the level of PLC-\(\delta\)-1 expression and the magnitude of BK-induced [Ca\textsuperscript{2+}], increase in the various PC12 clones suggested that PLC-\(\delta\)-1 can be significantly activated by cytosolic calcium ion when the expression level of PLC-\(\delta\)-1 is higher than that of wild type PC12 cells. In addition, similar potentiation of BK-induced [Ca\textsuperscript{2+}], rise was detected in \(\delta\)15 and \(\delta\)14 clones, although the expression level of PLC-\(\delta\)-1 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\textsuperscript{2+}], elevation caused by BK-induced PLC-\(\beta\) activation is a limiting factor. In physiological environments, if there is any tissue in which PLC-\(\delta\)-1 is expressed, PLC-\(\delta\)-1 may play an important role in calcium signaling. Therefore, it will be interesting to investigate the expression level of PLC-\(\delta\)-1 and Ca\textsuperscript{2+} entry-mediated potentiation of phosphoinositide hydrolysis in various tissues and cells.

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