Reconstitution of $\alpha_2D$-Adrenergic Receptor Coupling to Phospholipase D in a PC12 Cell Lysate*

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We have previously shown that $\alpha_2$-adrenergic receptor-mediated coupling to phospholipase D (PLD) in vascular tissues requires a tyrosine kinase activity (Jinsi, A., Paradise, J., and Deth, R. C. (1996) Eur. J. Pharmacol. 302, 183–190). To further clarify this mode of regulation we reconstituted $\alpha_{2D}$-adrenergic receptor-stimulated PLD activity in PC12 cells expressing the cloned receptor. $[^3]H$Myristic acid-labeled cells were lysed by nitrogen cavitation, and aliquots of subnuclear fraction were utilized in the PLD assay. Agonist-stimulated PLD activity was measured in the presence of 0.4% butanol as $[^3]H$phosphatidylbutanol formation. Both GTP and its non-hydrolyzable analog guanosine 5'-O-(thiotriphosphate) stimulated PLD activity in a concentration- and time-dependent manner that required co-activation of protein kinase C by phorbol dibutyrate. Addition of epinephrine produced a 3-fold stimulation of PLD activity in the presence of GTP and GDP. This agonist-stimulated PLD activity was completely blocked by the $\alpha_2$-adrenergic receptor antagonist rauwolscine and by Clostridium botulinum toxin as well as by antibodies directed against either pp60$^{src}$, RhoA, or Ras GTPase-activating protein. These results indicate that coupling of the $\alpha_{2D}$-adrenergic receptor to PLD is complexly regulated by both the tyrosine kinase pp60$^{src}$ and the low molecular weight G protein RhoA.

In recent years, efforts to elucidate the biochemical and molecular mechanisms of phospholipase D (PLD) activation have focused interest on both heterotrimeric and Ras-related low molecular weight G proteins (LMWGs) that have been linked to receptor-mediated signal transduction. Evidence for a functional role of G proteins in PLD activation comes from studies in plasma membranes obtained from mammalian cells (1–4) and in cell-free systems prepared from leukocytes (5) and neutrophils (6). A GTP$^\gamma$S-dependent PLD activity was observed in these preparations only when fractions of both membranes and cytosol were combined. It has been proposed that the cytosol-independent (membrane-dependent) PLD activity represents only a small portion of the total response and that full activation of PLD requires the interplay of cytosolic factors such as PKC, a cytosolic GTP-binding protein, and calcium (7).

A number of studies have provided evidence indicating a role for cytosolic LMWGs such as the Rho and Arf families in regulating membrane-associated PLD activity (8–11). Localization of LMWGs appears to be controlled by their interaction with regulatory proteins such as GTP/GDP dissociation stimulators, GDP dissociation inhibitors, and GTPase-activating proteins (GAPs) (12). Thus reconstitution of receptor-stimulated PLD activity would also be likely to require protein factors from both plasma membrane and cytosolic fractions.

Despite the lack of information about the molecular and cellular distinctions between various isoforms of PLD, the actions of its hydrolytic product (phosphatidic acid) have been documented in a broad spectrum of physiologic events and disease states including metabolic regulation, inflammation, secretion, cellular trafficking, diabetes, mitogenesis, oncogenesis, and senescence (13). The exact mechanism by which such diverse short and long term effects are mediated by the activation of PLD is not yet clearly understood. Recently Hammond et al. (14) identified a highly conserved human PLD gene family whose enzyme product is membrane-associated, stimulated by phosphatidylinositol 4,5-bisphosphate, activated by the monomeric G protein ADP-ribosylation factor-1, and inhibited by oleate.

The $\alpha_{2D}$-adrenergic receptor has been demonstrated to transduce a cellular proliferation response mediated through G proteins (15–17). It has become evident that this mitogenic signal results from activation of the Ras/mitogen-activated protein (MAP) kinase cascade (18, 19), which appears to involve activation by $G_{13}$, subunits (20). The intermediate tyrosine kinase pp60$^{src}$ (a member of the src family of tyrosine kinases) has been proposed to phosphorylate Shc, an adapter protein that associates with Grb2-Sos complexes through the SH2 domain of Grb2, thereby leading to Ras activation (21).

Given these findings we sought to investigate the involvement of Ras-related proteins in the $\alpha_{2D}$-adrenergic receptor cellular signaling process. The $\alpha_{2D}$-adrenergic receptor has been shown to couple to PLD in a tyrosine kinase-dependent manner (22, 23) and is also involved in mediating vasoconstrictor responses that are selectively blocked by tyrosine kinase inhibitors (23, 24). The aim of this study was to directly examine the possible involvement of a LMWG and a tyrosine kinase in regulating $\alpha_2$-adrenergic receptor-mediated PLD activity in PC12/$\alpha_2$ cells by reconstituting agonist-stimulated PLD activity in a crude cell lysate preparation.

EXPERIMENTAL PROCEDURES

Cell Cultures—PC12 cells stably transfected to overexpress the cloned $\alpha_2D$-adrenergic receptor (PC12/$\alpha_2D$ cells, kindly donated by Dr. Stephen Lanier) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum, 5% horse serum, glutamine, and penicillin/streptomycin/Fungizone and were maintained in 5% CO$_2$, 95% O$_2$ at 37 °C.

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1 The abbreviations used are: PLD, phospholipase D; LMWG, low molecular weight G protein; GAP, GTPase-activating protein; MAP, mitogen-activated protein; PKC, protein kinase C; PDBu, phorbol dibutyrate; GTP$^\gamma$S, guanosine 5'-O-(thiotriphosphate); C3 toxin, Clostridium botulinum toxin.
Phospholipase D Assay in Reconstituted PC12/α2D Cell Lysate—PLD activity was measured using a transphosphatidylation reaction as described by Halenda and Rehm (25). PC12/α2D cells were labeled overnight with 2–5 μCi/ml [3H]myristic acid and washed in hypotonic lysis buffer containing 25 mM HEPES (pH 7.4), 1.5 mM MgCl₂, 0.2 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 2 μM pepstatin, and 100 mM phenol dialbutyrate (PDBu). Cells were allowed to swell for 10 min on ice and then were lysed by nitrogen cavitation at 650 p.s.i. for 20 min. The lysate was then centrifuged, and a postnuclear supernatant was obtained. Aliquots of the lysate (approximately 100 μg of total protein/sample) were preincubated for 5 min at 37°C in buffer containing 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol, 25 mM HEPES (pH 7.4), 10 μM CaCl₂, 1 mM ATP, and 1 mM isobutylmethylxanthine. Following this preincubation samples were incubated for the indicated time with the agonist in the presence of antagonist, tyrosine kinase inhibitors, or other additions. To exclude any contribution of residual catecholamines in the lysate, agonist-induced stimulation was compared with a control level that contained 100 mM rauwolscine. All antibodies were incubated with the lysate at a concentration of 1:100 for 1 h at 4°C before carrying out the assay. In some studies antibodies were previously incubated with their control peptides to validate their specificity. In other experiments the lysate was pretreated with 10 μg/ml Clostridium botulinum toxin (C3 toxin) for 1 h at 37°C before carrying out the assay.

**Data Analysis and Analysis of Lipids**—The transphosphatidylation reaction was terminated by adding ice-cold CH₃OH/CHCl₃ (2:1). This mixture was left on ice for 1 h overnight in the freezer. Samples were redissolved in CHCl₃/CH₃OH for spotting on Silica Gel G plates and separated using the following solvent system: benzene/chloroform/pyridine/formic acid (45:38:4:2). Commercial standards were separated in the same lane with the sample or in adjacent lanes and were visualized by iodine vapor staining. Corresponding [3H]phosphatidylbutanol (RF₀<sub>0.45</sub>) bands were scraped off the plate and counted. The pattern of migration was also confirmed by autoradiography in some samples.

Data Analysis—Data are reported as the fraction of [3H]phosphatidylbutanol formed of the total lipid-associated radioactivity. Typical control values of [3H]phosphatidylbutanol formed ranged from 200 to 500 cpm, and total lipid-associated radioactivity was between 75,000 and 100,000 cpm. Data means were analyzed by paired Student’s t tests. A probability of p ≤ 0.05 was selected as the criterion for statistical significance.

Materials—[3H]Myristic acid was purchased from DuPont. Tyrosine kinase inhibitors were kindly provided to us by Dr. Alan Hudson of the Wellcome Foundation Ltd. (Beckenham, Kent, U.K.), and antisera against the α-subunit of G<sub>α</sub>S, G<sub>α</sub>Q, and G<sub>α</sub>R were kindly supplied by Dr. Christopher Lynch. For phospholipid standards, 1-palmitoyl-2-dioleoyl-sn-glycero-3-phosphate and 1,2-dioleoyl-sn-phosphatidylbutanol were purchased from Avanti Polar Lipids (Alabaster, AL). ADP-ribosyltransferase C3 toxin was purchased from Wako Bioproducts (Richmond, VA), anti-RhoA and anti-srb were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti-GAP was from Upstate Biotechnology Inc. (New York, NY). All other chemicals were of the highest reagent grade and were obtained from Sigma.

**RESULTS**

**PKC Dependence of G Protein-stimulated PLD Activity**—G protein-stimulated PLD activity was measured in a PC12/α2D cell lysate using the non-hydrolyzable analog GTPγS. Addition of GTPγS at 100 μM for 20 min produced about a 2-fold increase in [3H]phosphatidylbutanol formation in lysate obtained after pretreatment of cells with PDBu (100 nM) for 20 min (Fig. 1), while control lysate failed to produce a GTPγS-induced stimulation of PLD activity. These results parallel the PKC activation requirement for agonist-stimulated PLD activity that we have observed in intact PC12/α2D cells.2 Additionally, G protein-stimulated PLD activity was measurable only when the incubation buffer contained calcium (1 μM; data not shown).

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2 A. Jinsi-Parimoo and R. C. Deth, submitted for publication.
Fig. 2. A, time-dependent activation of phospholipase D by GTP and GTPγS in PC12/α2D cell lysate. Cells labeled overnight with [3H]myristic acid were used to prepare a cell lysate as described under “Experimental Procedures” after pretreatment with 100 nM PDBu for 20 min. Phospholipase D activity was measured in cell lysate aliquots after incubating with 0.4% butanol, GTP, or GTPγS or epinephrine combined with GTP for the indicated time. Results are expressed as the percentage increase above a control group (363 ± 84 cpm) with no additions at each time point. Each point shown is the mean ± S.E. of at least four determinations. *, denotes significant difference from control group at \( p \leq 0.05 \); #, denotes significant difference from GTP-only group at \( p \leq 0.05 \). B, concentration-dependent activation of phospholipase D by GTP and GTPγS in PC12/α2D cell lysate. Cells labeled overnight with [3H]myristic acid were used to prepare a cell lysate as described under “Experimental Procedures” after pretreatment with 100 nM PDBu for 20 min. Phospholipase D activity was measured in cell lysate aliquots after incubating with 0.4% butanol, GTP, or GTPγS or epinephrine combined with GTP for 20 min. Results are expressed as the percentage increase above a control group (388 ± 167 cpm) with no additions. Each point shown is the mean ± S.E. of at least four determinations. *, denotes significant difference from control group at \( p \leq 0.05 \); #, denotes significant difference from GTP-only group at \( p \leq 0.05 \).

Fig. 3. A, epinephrine-stimulated phospholipase D activity in PC12/α2D cell lysate. Cells labeled overnight with [3H]myristic acid were used to prepare a cell lysate as described under “Experimental Procedures” after pretreatment with 100 nM PDBu for 20 min. Epinephrine (Epi)-stimulated phospholipase D activity was measured in cell lysate aliquots after incubating with 0.4% butanol and epinephrine in buffer containing GTP (10 nM) and GDP (1 μM) for 10 min. Results are expressed as a percentage of a control group (654 ± 6 cpm) with no additions. Each point shown is the mean ± S.E. of at least four determinations. *, denotes significant difference from control group at \( p \leq 0.05 \). B, antagonism of epinephrine-stimulated phospholipase D activity by rauwolscine in PC12/α2D cell lysate. Cells labeled overnight with [3H]myristic acid were used to prepare a cell lysate as described under “Experimental Procedures” after pretreatment with 100 nM PDBu for 20 min. Epinephrine-stimulated phospholipase D activity was measured in cell lysate aliquots after incubating with 0.4% butanol and epinephrine in buffer containing GTP (10 nM) and GDP (1 μM) for 10 min or with epinephrine (1 μM). When present, rauwolscine (1 μM) treatment was carried out 30 min before agonist stimulation. Results are expressed as a percentage of the control group (363 ± 84 cpm) with no additions. Each value shown is the mean ± S.E. of at least four determinations. *, denotes significant difference from control group at \( p \leq 0.05 \); #, denotes significant difference from GTP-only group at \( p \leq 0.05 \).

through stimulation of \( \alpha_2 \)-adrenergic receptors.

G Proteins Involved in Epinephrine-stimulated PLD Activity—To investigate the identity of heterotrimERIC G proteins involved in \( \alpha_2 \)-adrenergic receptor-stimulated PLD activity, the cell lysate was pretreated with antisera raised against the COOH-terminal region of G protein subtypes. As shown in Fig. 4, incubation with control serum or anti-G(αi3) did not significantly reduce the epinephrine-stimulated PLD activity in a PC12/α2D cell lysate; however, both anti-G(αi1) and anti-G(αs) reduced PLD activity measured in the presence of epinephrine to a level not different from GTP/GDP alone. Thus \( \alpha_2 \)-receptors may activate PLD via coupling to either G(αi1) and/or G(αs). Alternatively, the coupling to PLD may be indirectly dependent
on these G proteins.

Involvement of Low Molecular Weight G Proteins and Src in Epinephrine-stimulated PLD Activity—In recent years substantial evidence has accumulated to suggest the involvement of LMWGs in regulating PLD activity, which may underlie the requirement for cytosolic factors that facilitate G protein-mediated activation of PLD (8–11). To probe for the involvement of a Rho-like low molecular weight G protein in epinephrine-stimulated PLD activity we utilized both C3 toxin and specific polyclonal antibodies raised against RhoA. C3 toxin is an exoenzyme produced by C. botulinum that ADP-ribosylates the Rho family of G proteins at an asparagine residue (Asn-41) located within or close to the putative effector domain of the molecule and blocks its downstream signaling (31). Anti-RhoA was an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide corresponding to the GTP-binding domain (residues 119–132) of RhoA, which is the most abundant form of Rho. Incubation of the cell lysate with C3 toxin (10 μg/ml) not only blocked the GTP/GDP-stimulated activation of PLD but reduced activity to significantly below the untreated control level (Fig. 5A). Epinephrine had no effect after C3 toxin pretreatment. Similarly, anti-RhoA pretreatment completely eliminated both the GTP/GDP- and epinephrine-induced increase. Pretreatment of lysate with anti-RhoA that had been preabsorbed with its control peptide had no significant effect on PLD activity. This provides evidence for an important role of a Rho-like LMWG protein, possibly of the RhoA subtype, in regulating both basal and receptor-activated PLD activity.

Polyclonal antibodies against Src and Ras GAP were also used to identify their possible involvement in regulation of PLD. Anti-Src is an affinity-purified rabbit antibody raised against a peptide corresponding to residues 3–18 within the amino-terminal region of pp60src. Anti-Ras GAP is a polyclonal antibody raised against a TrpE fusion protein containing amino acid residues 171–448 corresponding to the SH2/SH3 region of p120 Ras GAP. Incubation of the cell lysate with anti-Src or anti-Ras GAP blocked the PLD activity stimulated by GTP/GDP and also completely blocked epinephrine-induced PLD activity (Fig. 5B). Pretreatment with anti-src preabsorbed with its control peptide had no effect on either basal or epinephrine-stimulated PLD activity. These results indicate a critical role for pp60src and Ras GAP or an associated protein in receptor-mediated PLD activation.

DISCUSSION

We have developed a method to reconstitute agonist-stimulated PLD activity in a crude cell lysate preparation obtained from PC12/α2D cells. Conditions required for activating the receptor-mediated PLD pathway included the presence of calcium and ATP as well as pretreatment with a PKC-activating phorbol ester. Each of these requirements is consistent with prior studies of PLD activity in cell homogenates (8–11) as well as our own studies in intact PC12/α2D cells. A requirement of phosphatidylinositol 4,5-bisphosphate has also been reported (14), but we did not supplement endogenous phosphatidylinositol 4,5-bisphosphate in our studies. Thus stimulation of PLD by α2-adrenergic receptors in this broken cell preparation is characterized by many of the same regulatory features found in intact cells but is amenable to study with reagents such as antibodies that otherwise could not be used with intact cells.

Epinephrine stimulation measured either in the presence of GTP or a combination of GTP and GDP produced an increase in PLD activity (Figs. 2 and 3A), and its sensitivity to the antagonist rauwolscine confirmed its α2-adrenergic receptor origin. As reported for Gs-mediated inhibition of adenylyl cyclase in intact PC12/α2D cells (32), PLD stimulation was diminished at higher agonist concentrations (Fig. 3A), raising the possibility that the receptor was increasingly involved with other coupling pathways at higher levels of occupancy or suggesting the development of a negative influence on the efficiency of PLD coupling. Studies of adenylyl cyclase coupling in intact PC12/α2D cells found that epinephrine caused inhibition at levels up to 100 nM but stimulation at higher concentrations (32), reflecting the now well recognized ability of α2-receptors to activate Gi and Gq pathways with differing efficacy (33). Involvement of a Gq protein in α2-receptor-mediated PLD stimulation was also indicated in earlier studies with rat aorta (23) and in intact PC12/α2D cells by the ability of pertussis toxin pretreatment to block agonist stimulation. In the current study, antisera to Gαq/11 blocked the epinephrine stimulation of PLD while antisera to Gαs did not (Fig. 4), suggesting that one or both of the former G proteins is responsible for agonist-induced activation. The reduction observed with anti-Gq antibodies is unexpected, but since α2-receptors can activate Gi in these cells, it is possible that the βγ subunits common to both types of G proteins may contribute to PLD activation.

Both Arf and Rho LMWGs have been shown to independently and synergistically regulate PLD activity in an isomeric-specific manner (9, 11, 34). The loss of epinephrine-mediated PLD stimulation upon pretreatment with either C3 toxin or anti-RhoA antibodies (Fig. 5A) suggests that activation of PLD in the PC12/α2D cell lysate was under the control of one of the Rho family proteins (presumably RhoA). These findings are in
Fig. 5. A. Involvement of low molecular weight G proteins in epinephrine-stimulated phospholipase D activity in PC12/α2D cell lysate. Cells labeled overnight with [3H]myristic acid were used to prepare a cell lysate as described under “Experimental Procedures” after pretreatment with 100 nM PDBu for 20 min. Epinephrine (Epi)-stimulated phospholipase D activity was measured in cell lysate aliquots after incubating with 0.4% butanol ± epinephrine in buffer containing GTP (10 nM) and GDP (1 μM). C3 toxin and anti-RhoA pretreatment was carried out as described under “Experimental Procedures.” Results are expressed as a percentage of untreated control group values. Each point is the mean ± S.E. of at least four determinations. *, denotes significant difference from control group at p ≤ 0.05. B. Involvement of Src and GAP in epinephrine-stimulated phospholipase D activity in PC12/α2D cell lysate. Cells labeled overnight with [3H]myristic acid were used to prepare a cell lysate as described under “Experimental Procedures” after pretreatment with 100 nM PDBu for 20 min. Epinephrine (Epi)-stimulated phospholipase D activity was measured in cell lysate aliquots after incubating with 0.4% butanol ± epinephrine in buffer containing GTP (10 nM) and GDP (1 μM) as indicated. Anti-Src antibodies (± a preabsorbed control peptide) or anti-GAP antibodies were used as described under “Experimental Procedures.” Results are expressed as a percentage of the untreated control group. Each value is the mean ± S.E. of at least four determinations. *, denotes significant difference from control group at p ≤ 0.05.

agreement with similar observations implicating involvement of RhoA in the activation of membrane-associated PLD from several tissues (10, 34, 35). Since C3 toxin, which has a broader specificity than anti-RhoA, significantly reduced basal PLD activity whereas anti-RhoA did not, it is possible that two or more types of Rho proteins may regulate PLD activity in PC12/α2D cells. While a non-RhoA LMWG might regulate basal activity, RhoA is apparently essential for receptor-stimulated PLD activation.

Inhibition of agonist-induced PLD stimulation by anti-Ras GAP antibody treatment (Fig. 5B) provides evidence for the involvement of multiple LMWGs (i.e. Ras and Rho) in this pathway. p120 Ras GAP not only stimulates Ras GTPase activity but contains SH2 and SH3 domains that allow it to complex with other proteins in a signaling cascade, and thus it may function as a Ras-regulated effector. The antibody we used is directed toward the SH2/SH3 binding domain of Ras GAP, which indicates that these domains may be involved in signal transmission from Ras to Rho. For example, it has been reported that Ras GAP forms a complex with a Rho-specific p190 GAP (36), implying that Rho activity may be subject to Ras regulation as has been previously suggested (37, 38).

Stimulation of α2β-adrenergic receptors has been demonstrated to increase the levels of active Ras, leading to increased MAP kinase activity via a pertussis toxin-sensitive mechanism (18). This signaling pathway has now been shown to involve the pleckstrin homology domains of βγ subunits (possibly via their binding to phosphatidylinositol 4,5-bisphosphate), tyrosine phosphorylation of Shc, and translocation of the Grb2-Sos complex to the plasma membrane where Sos promotes guanine nucleotide exchange on Ras (39). By analogy, Rho-dependent activation of PLD by α2D-receptors may be initiated by a βγ subunit mechanism through Ras, which can simultaneously provide a mitogenic stimulus via Raf and the MAP kinase pathway. This implies a bifurcation of the signaling pathway at the level of Ras GAP or beyond, as illustrated in Fig. 6.

Elimination of α2β-adrenergic receptors to phospholipase D. Agonist stimulation of the receptor releases βγ subunits from pertussis toxin-sensitive G proteins, leading to activation of pp60src (Src) by a yet-to-be-defined mechanism. Src activation of Ras involves adaptor proteins and the guanine nucleotide exchange factor Sos as described previously (19, 39). Activity of Rho is proposed to be regulated by Ras via Ras GAP, possibly via p190 Rho GAP (36). Active Rho provides for both increased PLD activity and inhibition of myosin phosphatase (44), which can promote sustained vascular contraction in concert with activated MAP kinase (45). Prevailing PKC activity is critical for the initial coupling of α2D-adrenergic receptors to PLD after which receptor-induced PLD activity can maintain PKC in its active state. The locus of PKC action is undetermined at present.
Reconstitution of \( \alpha_{2D} \)-Adrenergic Receptor

14561

Receptor tyrosine kinase in the signaling pathway. Jiang et al. (40) earlier reported that overexpression of v-Src leads to increased PLD activity, and the GTP dependence of this increase suggested the involvement of a low molecular weight G protein. Subsequently, it was shown that PLD activation by v-Src did indeed involve Ras, although an additional role for Rho was not investigated (41). Tyrosine phosphorylation of Shc is increased in v-Src-transformed cells, and recently it has been shown that pp60 \( ^{src} \) is responsible for the \( \alpha_S \)-adrenergic receptor-mediated increase in Shc phosphorylation (21). It is also of interest that wortmannin, an inhibitor of phosphatidylinositol 3-kinase, blocks \( \alpha_S \)-receptor-induced phosphorylation of Shc at concentrations that also inhibit \( \alpha_S \)-adrenergic receptor-mediated vasoconstriction (42) and receptor-induced PLD activation (43).

RhoA has recently been shown to reduce the activity of myosin phosphatase (44), which would promote contraction of vascular smooth muscle. The effects of \( \alpha_S \)-receptor-dependent PLD activation in blood vessels (e.g. sustained PKC activation) could therefore be complemented by inhibition of myosin dephosphorylation as well as the phosphorylation of caldesmon by MAP kinase (45) to provide the overall level of contraction. Stimulation of this pathway plays a significant role in \( \alpha_S \)-receptor responses such as the contraction of vascular smooth muscle and may contribute to some forms of hypertension.

In summary, we have for the first time demonstrated the coupling of \( \alpha_S \)-adrenergic receptors to PLD activation in a broken cell preparation. PLD coupling is GTP-dependent and requires the participation of RhoA and pp60 \( ^{src} \) as well as the co-activation of PKC. Stimulation of this pathway plays a significant role in \( \alpha_S \)-receptor responses such as the contraction of vascular smooth muscle and may contribute to some forms of hypertension.

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