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Evidence That the G_h Protein Is a Signal Mediator from α_1-Adrenoceptor to a Phospholipase C

II. PURIFICATION AND CHARACTERIZATION OF A G_h-COUPLED 69-kDa PHOSPHOLIPASE C AND RECONSTITUTION OF α_1-ADRENOCEPTOR, G_h FAMILY, AND PHOSPHOLIPASE C*

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Our studies on the α_1-adrenoceptor signaling have demonstrated that the G_h family is a signal mediator. We report here that a 69-kDa phospholipase C (PLC) is the effector in this signal pathway. The enzyme was isolated by dissociating a G_{h7}-PLC complex which was induced in the bovine liver membranes incubating with (−)-epinephrine and GTP. The enzyme displayed a marked preference hydrolysis for phosphatidylinositol 4,5-bisphosphate over other phosphatidylinositides at micromolar calcium. Reconstitution of PLC with the α_1-adrenoceptor and G_h (G_{h7}) into phospholipid vesicles resulted in a lowered Ca^{2+} requirement for the substrate hydrolysis in the presence of guanosine 5′-3-*O*-(thio)triphosphate (GTPγS) when the receptor was activated with the α_1-agonist. The formation of inositol phosphate was hormone concentration dependent and reached maximal within 3 min which was faster than the formation in the presence of the α_1-antagonist. An $G_{h7\alpha}$ antibody co-immunoprecipitated 80–85% of phospholipase C activity in the presence of GTPγS, but not in the presence of GDP or buffer, showing the association of PLC with the α-subunit of G_h family. Thus, our novel approaches to identify the effector involved in the α_1-adrenoceptor signaling, as well as the reconstitution studies, substantially demonstrate that the α_1-adrenoceptor-mediated transmembrane signaling involves the G_h family and a 69-kDa PLC.

The α_1-adrenoceptors are involved in a variety of important physiological processes, including the control of blood pressure, appetite, and mood. The sequential transmembrane signaling involving the α_1-adrenoceptor and phospholipase C (PLC)[1] is mediated through a pertussis toxin-insensitive GTP binding protein(s) (1, 2). Five phospholipase C isozymes, designated as α, β, γ, δ, and ε, have been purified (3–12), and some of them have been cloned (13, 14). Increasing evidence suggests that PLC-β1 is stimulated by pertussis toxin-insensitive G_q and its family (15–17). Recently, all three α_1-receptor subtypes, α_{1a}, α_{1b}, and α_{1c}, were shown to be capable of coupling to Gqα and its family and stimulating inositol phosphate formation via PLC-β1 in co-transfected cells (18). On the other hand, pharmacological and functional studies with a variety of tissues suggested that the α_1-adrenoceptors may involve multiple signal transduction mechanisms and that different α_1-adrenoceptor subtypes may utilize different mechanisms through which they can initiate signals in their target cells (1). Thus, one subtype, the α_{1a}-receptor, which exists in most tissues, primarily increases extracellular Ca^{2+} influx and arachidonic acid release via phospholipase A_2 through a pertussis toxin-sensitive G-protein(s) (19, 20). The other type, the α_{1b}-receptor subtype, which exists predominantly in liver, spleen, and heart, stimulates phosphatidylinositol breakdown via PLC through a toxin-insensitive G-protein(s), resulting in intracellular calcium mobilization (1, 2). Although the functions of the α_{1c}-receptor subtype have not been determined in tissues, transfection of the receptor into COS-7 or Hela cells stimulates PLC better than the α_{1b}-receptor in the same expression system through a pertussis toxin-insensitive G-protein (21). The hydrolysis of phospholipids other than phosphatidylinositol through PLD(s) has been observed in response to the activation of the α_1-receptors in rat hepatocytes and brain (22, 23). All these observations suggest that the α_1-adrenoceptor-mediated signal transduction mechanism is diverse and that the signal pathways are still controversial.

We previously reported a toxin-insensitive 74-kDa GTP-binding protein ($G_{h\alpha}$) from rat liver which coupled to the α_1-adrenoceptor (24, 25) and stimulated a membrane-bound PLC (26). We also isolated a 78-kDa GTP-binding protein ($G_{h7\alpha}$) from bovine heart. Both proteins were associated with a 50-kDa protein (holoproteins were termed as G_h and G_{h7}). Using the α_1-agonist-receptor-$G_h$ternary complex preparations and $G_{h7\alpha}$ antibody, a G_h family, having different molecular masses in different species and coupling to the α_1-adrenoceptor, was characterized (see companion study (41)).

To extend our understanding of the α_1-adrenoceptor-mediated signal pathway involving the G_h family and PLC the following approaches were undertaken: 1) induction of a G_{h7}-PLC complex in bovine liver membranes by incubating the

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[1] The abbreviations use are: PLC, phospholipase C; G_h, a toxin-insensitive guanine nucleotide-binding regulatory protein, first identified by the α_1-adrenergic ternary complex formation, which couples to the α_1-adrenoceptor and activates a PLC; G_{h7}, 78-kDa guanine nucleotide-binding regulatory protein of G_h family; G_s, stimulatory guanine nucleotide-binding protein which activates adenylyl cyclase; G_q, a toxin-insensitive guanine nucleotide-binding protein which couples to various receptors and activates PLC-β1; G_t, a guanine nucleotide-binding protein which is involved in photon-receptor signal transduction; GTPγS, guanosine 5′-*O*-3-(thio)triphosphate; App(NH)p, adenyl-5′-yl β,γ-imidodiphosphate; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-biphosphate; IP_3, inositol 1,3,5-triphosphate; PAGE, polyacrylamide gel electrophoresis.

membranes with (−)-epinephrine and GTP and solubilization of this complex without NaCl and detergent; 2) purification of a 69-kDa PLC by dissociating G_{h7} and PLC in the absence of G-protein activator; 3) reconstitution of purified PLC with the α_1-adrenoceptor and G_h or G_{h7} into phospholipid vesicles, and studies on functional coupling of these proteins. In the present studies, we demonstrate that the purified PLC is stimulated by G_h or G_{h7} and show the functional coupling of the α_1-receptor with G_h or G_{h7} and 69-kDa PLC by the *in vitro* reconstitution. We also furnish, by immunoprecipitation experiments, direct evidence that the α-subunit of G_h and G_{h7} stimulates this effector.

EXPERIMENTAL PROCEDURES

Materials—Sucrose monolaurate (SM-1200) was a gift from Mitsubishi-Kasei Company (Tokyo, Japan). Guanine nucleotides, other nucleotides, and protein A-agarose were obtained from Boehringer Mannheim. All phospholipids were purchased from Sigma, and the column chromatographic resins were from Pharmacia LKB Biotechnology Inc. [α-^{32}P]GTP (3000 Ci/mmol), [^{35}S]GTPγS (~ 1300 Ci/mmol), and [^{3}H]prazosin (76 Ci/mmol) were from DuPont NEN. Phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate (1 Ci/mmol), phosphatidyl[2-^{3}H]inositol phosphate (1 Ci/mmol), and phosphatidyl[2-^{3}H] inositol (10 Ci/mmol) were obtained from Amersham Corp. Other chemicals and biochemical materials were used as described previously (24–26).

Membrane Preparation—Bovine liver was obtained from Pel-Freez (Rogers, AR), and the membranes were prepared by Percoll gradient centrifugation according to the method of Prpic *et al.* (28).

Formation of α_1-Adrenoceptor-mediated G_{h7}-PLC Complex—Bovine liver membranes (10 g of protein) were washed once with HED buffer (20 mM Hepes, pH 7.4, 1 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol) containing protease inhibitors (10 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml bacitracin, 100 μg/ml benzamidine, 2 μg/ml leupeptin, 2 μg/ml pepstatin A, 2 μg/ml soybean trypsin inhibitor, and 20 μg/ml antipain). The pellets were resuspended at 5 mg/ml protein in the same buffer and incubated with 5×10^{-5} M (−)-epinephrine at 4 °C for 2 h. The α_2- and β-adrenoceptor blockers, 10^{-7} M rauwolscine and 10^{-6} M propranolol, respectively, were included to prevent activation of these receptors. G_{h7} and PLC were released from the membranes by addition of 5×10^{-5} M GTP and 2 mM $MgCl_2$ with occasional stirring. The solubilization step was repeated three times at 30 min intervals. The membrane suspension was centrifuged at 45,000 × *g* for 1 h, the clear supernatant was collected, and 10% glycerol (v/v), 100 mM NaCl, and 0.1% sucrose monolaurate (w/v) were added to stabilize the proteins. The protease inhibitors, glycerol, and the detergent were included throughout the purification, and all the procedures were carried out at 4 °C. The amounts of G_{h7} and PLC released from the membranes were quantitated by estimating the PLC activity and measuring the density of the radiolabeled $G_{h7\alpha}$ after SDS-PAGE and autoradiography. For [α-^{32}P]GTP-labeling of G-proteins, an aliquot (100 μl) of the extract was centrifuged on a dry Sephadex G-25 column to remove free GTP/GDP (24–26), pellets and membranes were washed one time with the HED buffer and resuspended to the initial volume in HED buffer. The amount of released $G_{h7\alpha}$ was measured by the density of radiolabeled $G_{h7\alpha}$ in extract, pellets, and membranes after SDS-PAGE and autoradiography.

Isolation of G_{h7}-PLC Complex and Purification of PLC—The extract was loaded on a Q-Sepharose column (50 ml) which was previously equilibrated with HED buffer containing 10% glycerol, 100 mM NaCl, and 0.1% sucrose monolaurate. The column was washed with 3-column volumes of the equilibration buffer, and bound proteins were then eluted at a flow rate of 30–40 ml/h, with a linear gradient of 100–600 mM NaCl in the same buffer. In every third fraction, PLC activity was measured and G-protein was monitored by photoaffinity labeling with 5 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$ and 0.1 mM App(NH)p. Fractions containing both PLC and G_{h7} (fractions, 58–97) were pooled (160 ml), concentrated on Amicon PM-30 membranes, and loaded onto an Ultrogel AcA 34 column (450 ml) pre-equilibrated with the same buffer containing 100 mM NaCl. After elution with the same buffer, the major fractions containing G_{h7} and PLC (fractions 46–70, volume 100 ml) were pooled and applied on a heparin-agarose column (10 ml) pre-equilibrated with HED buffer containing 100 mM NaCl. After washing with 30 ml of this buffer, bound proteins were eluted with a linear gradient of NaCl (100–700 mM) in the same buffer. Fractions were assayed for PLC and G_{h7}. Fractions (50–84, volume 35 ml) of peak II of the PLC activity, which did not contain G_{h7}, were pooled, diluted 1:4, and loaded again on a second heparin-agarose column (3 ml) equilibrated with HED buffer containing 100 mM NaCl. The column was washed with the same buffer containing 300 mM NaCl, and the elution of PLC was achieved with a 250–700 mM NaCl gradient in the same buffer. In each fraction, PLC was assayed and the fractions of the major PLC peak (32–58, volume 13.5 ml) were subjected to SDS-PAGE. The fractions (36–52, volume 8.5 μl), showing a single band on the gels with silver staining, were collected. The results of the purification are representative of several independent experiments. Purified PLC was stable for less than 2 months at −80 °C.

Preparation of α_1-Adrenoceptor, G_h, and G_{h7}—The α_1-adrenoceptor from rat liver membranes was partially purified, but it was free from G-proteins and PLC activity, as previously reported (24). G_h from rat liver membranes was prepared, as described previously (25, 26), and G_{h7} from bovine heart membranes was also purified, as reported in a companion study (41)).

Characterization of the Purified PLC with $G_{h7\alpha}$ Antibody and PLC Antibodies—The G_h- or G_{h7}-PLC complex was re-induced by incubating G_h or G_{h7} with PLC in the presence of 5×10^{-6} M GTPγS or 10^{-4} M GDP or without guanine nucleotides and 2 mM $MgCl_2$ in 0.05% sucrose monolaurate. The G_{h7}-PLC complex from the first peak, obtained from the first heparin-agarose elution, and the induced G_h- or G_{h7}-PLC complex were then treated with $G_{h7\alpha}$-specific antibody for 2 h at 4 °C with shaking. Protein A-agarose beads (10-μl suspension) were then added to the samples and further incubated at 4 °C for 1 h. The samples were centrifuged at 3000 rpm for 10 min, and the residual PLC activity was assayed in supernatant and pellets which were washed three times with 20 mM Hepes (pH 7.2) containing 100 mM NaCl and 0.05% sucrose monolaurate in the presence or absence of GTPγS. As controls, purified PLC alone, nonimmune sera, atrial natriuretic factor antibody, and protein A-agarose were also examined. Immunoprecipitation of the purified 69-kDa PLC by antibodies to PLC-β1 and -β2, -γ1 and -γ2, and -δ1 subtypes was also performed using the same protocol described above. For immunoblotting, we followed the method used by Suh *et al.* (28) with one modification, *i.e.* instead of using 3% bovine serum albumin in the buffer for preventing nonspecific binding, non-fat dry milk (5%) was used. The antibodies of the bovine brain PLC isozymes were kind gifts of Dr. Sue Goo Rhee (National Institutes of Health, Bethesda, MD). It should be noted that all of these antibodies were specific to the respective enzymes, against which they were derived, and neutralized the PLC activities with different efficacies (28).

Phospholipase C Activity Assay—PLC activity was assayed according to the method previously described (26). To assay the PLC activity in the phospholipid vesicles, the PLC preparations in 0.05% sucrose monolaurate were added to the phospholipid mixture (final concentration 0.2 mg/ml) and 500 μM [^{3}H]PIP_2 (specific activity, 700–800 cpm/nmol). The mixture was diluted 1:1 with 20 mM Hepes buffer, pH 7.2, containing 100 mM NaCl and 0.5 mM dithiothreitol or 0.2 mM ascorbic acid. Twenty microliters of this sample/assay were used to measure PLC activity in a final volume of 100 μl unless otherwise specified in the figure legends.

Other Assays—Amounts of the α_1-adrenoceptors were measured using 3 nM [^{3}H]prazosin following the method of Sawutz *et al.* (29), and the amount of G_h or G_{h7} was measured using 1 μM [^{35}S]GTPγS (specific activity, 1000–1500 cpm/pmol) according to the methods previously described (24–26).

Reconstitution—Reconstitution of the isolated proteins was performed in phospholipid vesicles, as described previously (26). Briefly, the phospholipid vesicles were prepared by the dilution method in which the lipid vesicles were formed by decreasing the detergent concentration in the mixtures. The enzymes, which were in 0.05% sucrose monolaurate solution, were mixed with a 5 mg/ml mixture of phosphatidylcholine:phosphatidyl-ethanolamine:phosphatidylserine (3:1:1) (0.2 mg/ml final) and 500 μM [^{3}H]PIP_2 (specific activity, 600–800 cpm/nmol). For the reconstitution of G_{h7} or G_h with PLC, the G-proteins were pre-incubated with 5×10^{-6} M GTPγS, 10^{-4} M GDP, or buffer in the presence of 2 mM $MgCl_2$ in the assay buffer containing 0.05% sucrose monolaurate at 30 °C for 40 min. For the total reconstitution with the α_1-adrenoceptor, G_h or G_{h7}, and PLC, the α_1-receptors were preincubated with 5×10^{-6} M (−)-epinephrine or 5×10^{-6} M (−)-epinephrine and 10^{-4} M phentolamine in the same buffer as described above at 30 °C for 30 min and then chilled in an ice bath. Since PLC alone in the phospholipid vesicles hydrolyzes large

amounts of PIP_2 in <10 min, it was necessary to preactivate the receptor in order to observe the specific response. The proteins were mixed with 0.2 mg/ml (final) phospholipid mixture and then diluted to the appropriate volume with the assay buffer in an ice bath. The ratio of protein in the mixture was obtained by converting the ligand binding to molar concentrations of proteins for the receptor and G-proteins, and molar concentrations of PLC were calculated from the protein concentration of the purified PLC and its molecular mass on SDS-PAGE. Prior to reconstitution, all the samples were passed through dry Sephadex G-25 columns pre-equilibrated with the assay buffer containing 0.05% sucrose monolaurate and 5% glycerol (26). The calcium concentrations in the reaction mixtures were adjusted by the external addition without EGTA, since the calcium chelating agent was found to interrupt the coupling in this system (26). PLC was assayed following the procedure described previously (26).

Protein Determination—The protein concentration was measured by the method of Bradford (30) using a Bio-Rad protein determination kit. Bovine serum albumin was used as a standard.

RESULTS

Formation of G_{h7}-PLC Complex and Purification of PLC from G_{h7}-PLC Complex—The α_1-adrenoceptor-mediated G_{h7}-PLC complex was readily formed and released from the membranes without using salt and detergent. This specific extraction of the $G_{h7(\alpha)}$-PLC complex by GTP resulted in the release of ~90% of membrane-associated $G_{h7(\alpha)}$-protein and ~55% PLC activity. In the absence of GTP or in the presence of GDP, the release of $G_{h7\alpha}$ and PLC was negligible (data not shown), indicating that, only after activation of G_{h7} with GTP, these proteins were released from the membranes. This specific extraction resulted in the release of ~14% of total membrane proteins. However, in the presence of GDP, a significant amount of protein (~7%) was also released.

The sequential chromatographic steps used to isolate PLC from the $G_{h7(\alpha)}$-PLC complex are detailed under "Experimental Procedures." From the Q-Sepharose column, PLC activity was eluted as a single broad peak (Fig. 1*A*). Photolabeling of these fractions with [α-^{32}P]GTP showed the presence of 78-kDa $G_{h7\alpha}$ (Fig. 1*A*, *inset*). The earlier fractions contained <45-kDa GTP-binding proteins (data not shown), but in the later fractions $G_{h7\alpha}$ was the only detectable GTP-binding protein. From the Ultrogel AcA 34 column the PLC activity was again eluted as a single peak along with $G_{h7\alpha}$ (Fig. 1*B*) but $G_{h7\alpha}$ was eluted as a broad peak. $G_{h7\alpha}$ in later fractions, which did not contain PLC, is likely to be $G_{h7\alpha}$ alone which was not associated with either PLC or $G_{h7\beta}$, since the protein eluted later than $G_{h7\alpha}$-PLC and G_h (25) or G_{h7} (see previous paper). Although G-protein activator such as GTPγS or aluminum fluoride (AlF_4^-) was not included throughout the purification, from the gel filtration column the PLC activity was again eluted as a single broad peak. These results suggested that the complex is stable. The dissociation of the G_{h7}-PLC complex was observed only after elution from the first heparin-agarose column (6 days after extraction). Thus, the PLC activity from this heparin-agarose column was eluted into two distinct peaks (peaks I and II), as presented in Fig. 1*C*. $G_{h7\alpha}$ was detected only in the fractions of peak I as well as in some earlier fractions. When the pooled fractions of peak II were applied on a second heparin-agarose column (Fig. 2*A*), a 69-kDa protein was eluted with PLC activity. The intensity of the protein band on the SDS-PAGE gel (Fig. 2*B*) was parallel to the enzyme activity, indicating that the protein is PLC. In order to evaluate the G_{h7}-PLC complex at the different stages of purification, the peak I of the first heparin-agarose column as well as the pooled fractions from Q-Sepharose and the gel filtration columns were analyzed by a molecular sizing column of Sephadex G-200 (superfine). The samples (0.5 ml) were incubated with or without AlF_4^- and then applied onto the column (0.7 × 26 cm) equilibrated with and without AlF_4^- in

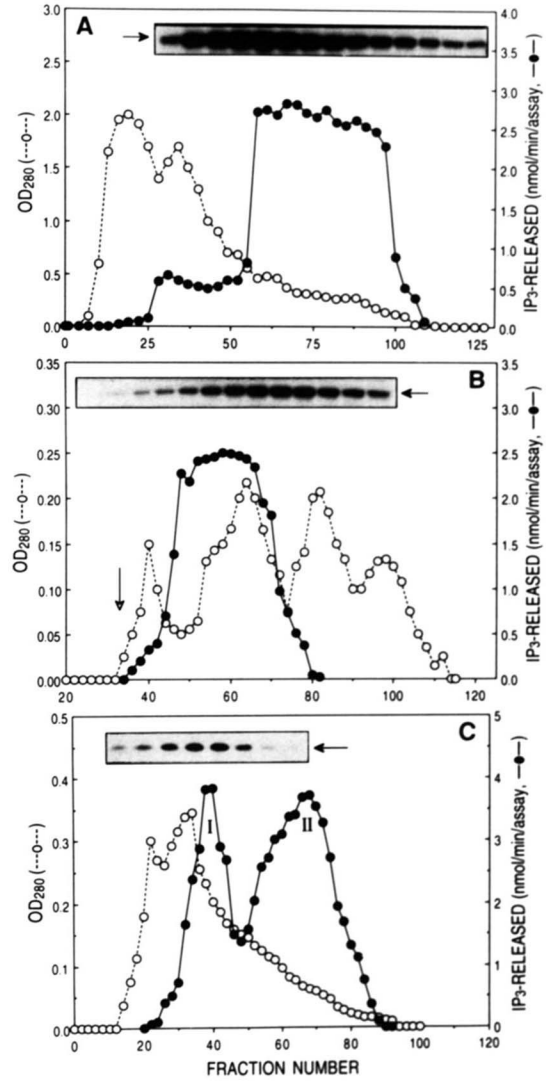

FIG. 1. **Protein elution profile and PLC activity of G_{h7}-PLC complex.** *Panel A*, ion exchange chromatography of G_{h7}-PLC complex on Q-Sepharose. Ten microliters of every third fraction was used to measure PLC activity as described under "Experimental Procedures." *Inset*, an autoradiogram of photolabeled fractions in the region of the PLC activity observed. Fifty microliters of every third fraction (*58–97*) was photolabeled with 5 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$ and 0.1 mM App(NH)p. The *arrow* in the *inset* shows an autoradiogram of the 78-kDa $G_{h7\alpha}$. *Panel B*, gel filtration chromatography on Ultrogel AcA 34. The *open arrow* shows the void volume of the column as measured using blue dextran. After elution, 10 μl of sample from every second fraction were used to measure the PLC activities, and 50 μl of samples from every second fraction were photolabeled for G-proteins as described under "Experimental Procedures." *Inset*, the autoradiogram of photolabeled $G_{h7\alpha}$ in every second fraction (*46–72*) in which PLC activity was obtained. The position of [α-^{32}P]GTP-labeled 78-kDa $G_{h7\alpha}$ is indicated by the *closed arrow*. *Panel C*, the first heparin-agarose chromatography. Photolabeling of G-proteins and assay of PLC were performed in every second fraction. The fractions of *peak II* did not contain G-proteins. *Inset*, radiolabed $G_{h7\alpha}$ in *peak I* coeluted with PLC (*fractions 32–46*).

HED buffer that contained 100 mM NaCl and 0.1% sucrose monolaurate (26). With AlF_4^--activated preparations, the PLC activity and $G_{h7\alpha}$ were eluted as a single peak immediately after the void volume of the column. In non-activated preparations, the PLC activity and $G_{h7\alpha}$ were shifted to later fractions. In fact, these protein activities were found, with partial overlap, near the elution position of bovine serum albumin (data not shown, see Ref. 26). After inserting the selectively

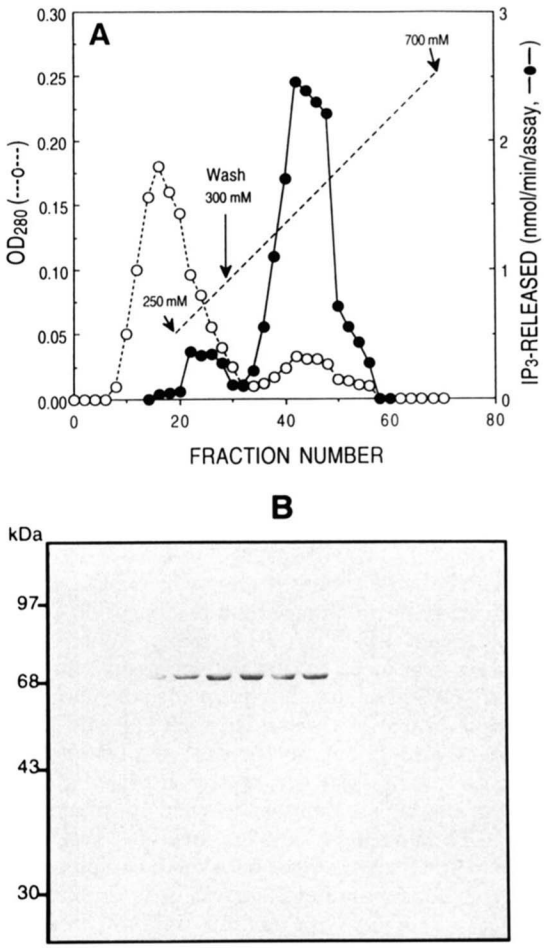

FIG. 2. **Protein elution profile and PLC activity of the fractions eluted from second heparin-agarose chromatography.** *Panel A*, elution profile of protein and PLC activity from the column. Every second fraction (2.5 μl) was used to measure the PLC activities as described under "Experimental Procedures." Although a small protein peak was associated with the major PLC peak, protein could be measured only after 20 times concentrating an aliquot of the pooled samples. *Panel B*, silver-stained gel of PLC peak fractions. Five microliters of every second fraction from 32 to 58 were subjected to SDS-PAGE and silver staining. When a large amount of samples (50 μl) was used for SDS-PAGE and silver staining, trace bands of ~50- and ~200-kDa proteins were visible.

pooled fractions, which were prepared in the presence or absence of AlF_4^-, into phospholipid vesicles, the calcium requirement for the maximal inositol phosphate formation was compared. All the original pooled fractions as well as those which were eluted after the void volume of Sephadex G-200 (in the presence of AlF_4^-) showed a lower calcium requirement (8–10 μM) than PLC alone (20–25 μM), *i.e.* the fractions from Sephadex G-200 in the absence of G-protein activator. These results were very similar to those previously reported (26) and demonstrated that the co-elution of the PLC activity and G_{h7} represented the $G_{h7\alpha}$-PLC complex.

As summarized in Table I, 69-kDa PLC was purified 661-fold, and the recovery was 9.4% with respect to the activity from extraction of the G_{h7}-PLC complex. The specific activity of the purified PLC was 38.5 μmol/min/mg of protein.

Properties of Purified 69-kDa PLC—The biochemical properties of the PLC were characterized in a 20 mM Hepes buffer, pH 7.2, containing 100 mM NaCl, 0.1% sodium deoxycholate, and 0.05% sucrose monolaurate. As shown in Fig. 3*A*, PLC hydrolyzed PIP_2, PIP, and PI. The rate of hydrolysis of these substrates increased with increasing Ca^{2+} concentrations. Over a wide range of calcium concentrations (0–400 μM), the rate of PIP_2 hydrolysis by the enzyme was faster than PIP and PI hydrolysis. Thus, the hydrolysis of PIP_2 reached a maximum at ~100 μM Ca^{2+}, whereas PIP and PI hydrolyses did not reach the maximum up to 400 μM Ca^{2+}. At the same concentration of calcium, the order of substrate hydrolysis by the enzyme was $PIP_2 > PIP > PI$, showing a marked preference for PIP_2 as a substrate. It is of interest to note that the calcium requirement for maximal PIP_2 hydrolysis in phospholipid vesicles was far less (~20 μM) (see below and also Ref. 26) than that in the detergent solution (~100 μM). The PLC activity was then determined using PIP_2 as a function of pH as demonstrated in Fig. 3*B*. The PLC activity elaborated a pH dependence, which exhibited a bell-shaped curve with a pH optimum at 6.0–6.5. In addition, PIP_2 hydrolysis was dependent on enzyme concentration and was linear for up to 20 min at 50 μM calcium (data not shown). All these data showed that the overall properties of the purified PLC, especially the calcium requirement in detergent solution and in phospholipid vesicles, were very similar to those of the partially purified enzyme from rat liver (26).

Studies on 69-kDa PLC with antibodies of various PLC isozymes revealed that the activity of 69-kDa PLC was neither inhibited nor immunoprecipitated. In gist, 69-kDa PLC was incubated with the antibodies of PLC-β1, β2, -γ1, γ2, and -δ1 subtypes for 2 h at 4 °C and the PLC activity was measured in supernatant before and after immunoprecipitation using protein A-agarose. In these experiments, no significant decrease in enzyme activity in the supernatant was observed (5–10% inhibition). Moreover, immunoblotting studies again confirmed that none of these antibodies cross-reacted with 69-kDa PLC (data not shown). These results indicate that probably 69-kDa PLC is immunologically distinct from these PLC(s).

Reconstitution of G_{h7} with 69-kDa PLC—To evaluate the functional coupling ability of 69-kDa PLC to G_{h7}, both proteins were inserted into phospholipid vesicles using a dilution method (26). Fig. 4 demonstrates the G_{h7}-mediated PLC activity in the presence of GTPγS, GDP, or buffer. Stimulation of PLC activity by GTPγS-G_{h7} readily reached the maximal formation of inositol phosphates at 8–10 μM Ca^{2+}, whereas in the presence of GDP or buffer, 20–25 μM Ca^{2+} was required to reach maximal formation of inositol phosphates without changing amounts of inositol phosphates formed. The biphasic PLC activity with the changes in calcium concentrations (0–25 μM) was observed only in the presence of GTPγS. When G_h from rat liver and PLC were reconstituted, similar results were obtained (data not shown). The above results are consistent with the previous observations from reconstitution studies using G_h and partially purified PLC from rat liver (26) or with the G_{h7}-/G_h-PLC complex *versus* PLC alone. It is interesting to note that the shift in the calcium sensitivity of the PLC activity toward the lower concentration by the activation of the G_h family was consistently observed (see also below), suggesting that the biphasic modulation of calcium sensitivity in the PLC activity may be a self-regulation in this system.

Co-immunoprecipitation of PLC with GTPγS-G_{h7} and -G_h by $G_{h7\alpha}$ Antibody—To identify the subunit of G_h and G_{h7} which directly interacts with PLC for stimulation, the G_h- or G_{h7}-PLC complex was induced by incubating purified proteins with 5×10^{-6} M GTPγS, or 10^{-4} M GDP. The samples were immunoprecipitated with $G_{h7\alpha}$ antibody using protein A-agarose. To observe the specificity, PLC alone, nonimmune sera, atrial natriuretic factor antibody and protein A-agarose were used as controls. In these experiments, peak I of the first

TABLE I

Purification schema for the isolation of 69-kDa phospholipase C from bovine liver membranes. The values are representative of several independent experiments

Step	Volume (ml)	Protein (mg)	IP_3-released (nmol/min)	Specific activity (nmol/min/mg)	Recovery (%)	Fold purification
Extract	2,100	1,065.0	61,740	57.9	100.0	0
Q-Sepharose	160	76.8	42,050	547.5	68.1	9.5
Ultrogel AcA34	100	20.5	21,300	1,039.0	34.5	17.9
Heparin (peak I)	25	9.3	4,960	533.3	8.0	9.1
-agarose (peak II)	35	3.8	8,880	2,336.8	14.4	40.6
Heparin-agarose (2nd)	8.5	0.15[a]	5,780	38,533.3	9.4	661.1

[a] The protein concentration was measured after 20 times concentrating an aliquot of the pooled sample.

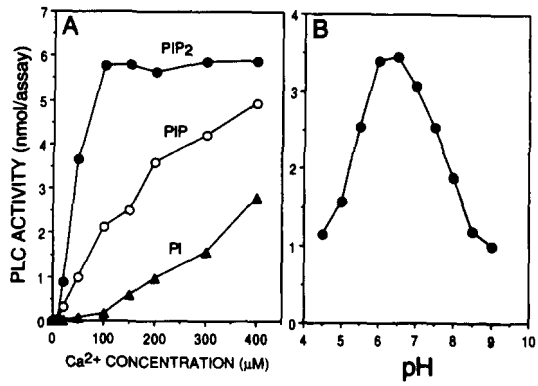

FIG. 3. **Different properties of purified 69-kDa PLC.** *Panel A*, substrate specificity: 69-kDa PLC (20 ng/assay) was used to determine the substrate specificity in response to Ca^{2+} concentrations. As substrate, 50 μM (final) [^{3}H]PIP_2, [^{3}H]PIP, or [^{3}H]PI (specific activity, 400–500 cpm/nmol) was used, and Ca^{2+} concentrations were adjusted using $CaCl_2$ in a 20 mM Hepes (pH 7.2) containing 0.5 mM dithiothreitol and 100 mM NaCl. Reaction was started by the addition of the enzyme. ●——●, PIP_2; ○——○, PIP; ▲——▲, PI. *Panel B*, effect of pH. Activity of 69-kDa PLC (15 ng/assay) was assayed using a series of 10 mM each of sodium acetate (pH 4.5–6.5), Hepes (pH 6.0–7.5), and Tris/HCl (pH 7.0–9.0) buffers to vary the pH. Other conditions were as described under "Experimental Procedures." The data shown here are representative of three independent measurements, and each point is the average of duplicate experiments.

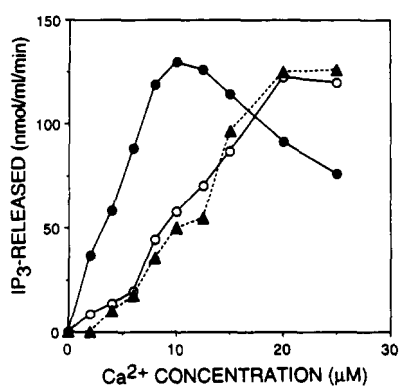

FIG. 4. **Stimulation of the PLC activity by G_{h7} in phospholipid vesicles.** G_{h7} was preincubated with 5×10^{-6} M GTPγS, 10^{-4} M GDP, or buffer in the presence of 2 mM $MgCl_2$ at 30 °C for 40 min. Samples were then mixed with 69-kDa PLC, 0.2 mg/ml phospholipid solution (final), and 500 μM [^{3}H]PIP_2 (specific activity, 600–800 cpm/nmol) and diluted 1:1 with 20 mM Hepes, pH 7.2, containing 100 mM NaCl and 0.5 mM dithiothreitol. Twenty microliters of vesicles containing 50 ng of PLC and 1.5 pmol of G_{h7} were incubated at 30 °C for 10 min with various Ca^{2+} concentrations in a final volume of 100 μl. PLC was assayed as described above. ●——●, GTPγS; ○——○, GDP; ▲- - -▲, buffer. Each point is average of duplicate measurements and representative of three independent experiments using different enzyme preparations.

heparin-agarose elution was also tested. As presented in Table II, substantial co-immunoprecipitation of PLC by $G_{h7\alpha}$ antibody was observed only with the samples in which G_h- or G_{h7}-PLC complex was induced in the presence of GTPγS and which was from peak I of the first heparin-agarose. In these samples, the PLC activities in the supernatants were decreased by ~80–85%. On the other hand, when PLC and G_h or G_{h7} were incubated in the presence of GDP, >90% PLC activity remained in the supernatant. Similarly, $G_{h7\alpha}$ antibody had no significant effect on PLC alone. When the PLC activities were measured in the pellets, significant enzyme activities (45–62% over the activity in the supenatants) were observed in the samples treated with GTPγS and $G_{h7\alpha}$-PLC complex preparations, but not in the samples treated with GDP or buffer. The recovery of the enzyme activities in supernatants and pellets together reached approximately 65–78% with GTPγS-treated samples and $G_{h7\alpha}$-PLC complex preparations. Although the reason for the incomplete recovery of the enzyme activity is not clear yet, it is probably due to protein A-agarose which can not mix well with reaction solution or because a portion of the enyme was lost by washing the pellets. Studies with atrial natriuretic factor antibody and nonimmune sera also resulted in slight decrease (<15%) of the enzyme activity. Such a decrease is probably due to the non-specific interactions between the proteins, because protein A-agarose itself had no effect on the enzyme activity. Since the formation of GTPγS-$G_{h7\alpha}$- or GTPγS-$G_{h\alpha}$-PLC complex was found to be mandatory for co-immunoprecipitation, these results again signify that purified 69-kDa PLC is coupling to the G_h family and re-forming the complex, irrespective of the tissue or the species of origin. Such cross-coupling was also observed in the case of adenylyl cyclase activation with G_s from two different sources (31). Moreover, these results clearly demonstrate that the α-subunit of G_{h7} (and G_h) is the activator of 69-kDa PLC.

Reconstitution of α_1-Adrenoceptor, G_{h7} or G_h, and 69-kDa PLC—Finally, to evaluate the total pathway of the α_1-adrenoceptor-mediated transmembrane signaling, PLC was reconstituted with the α_1-adrenoceptor and either G_{h7} or G_h in phospholipid vesicles. Fig. 5*A* presents the calcium sensitivity of the α_1-agonist-mediated stimulation of the PLC activity with the vesicles containing the α_1-receptor, G_{h7}, and PLC. These combinations were equipotent to decrease the Ca^{2+} requirement (<10 μM) of PLC in the presence of (−)-epinephrine. On the other hand, when the agonist effect was blocked by phentolamine or only buffer was added, the concentration of the metal ion required for maximum PLC activity was >20 μM, as observed with PLC alone and in the presence of GDP. However, at each concentration of Ca^{2+}, the amount of IP_3 released was higher with phentolamine blocking than with buffer. This may either be due to the incomplete blocking of the agonist effect by phentolamine or to some G_{h7}-PLC complex formation in the presence of GTPγS. A lack of complete

TABLE II

Immunoprecipitation of PLC with $G_{h\alpha}$ and $G_{h7\alpha}$ by $G_{h7\alpha}$ antibody

PLC (50 ng) was preincubated with 1 pmol of G_h or G_{h7} with 5×10^{-6} M GTPγS or 10^{-4} M GDP in the presence of 2 mM $MgCl_2$. PLC was precipitated by $G_{h7\alpha}$ antibody using protein A-agarose (10-μl suspension). The PLC activity was measured in the supernatant and pellets after immunoprecipitation as described under "Experimental Procedures." The data shown are representative of three independent experiments and are average of duplicated samples.

Probes	PLC activity					
	PLC alone	G_{h7}-PLC complex	PLC, G_h, GTPγS	PLC, G_h, GDP	PLC, G_{h7}, GTPγS	PLC, G_{h7}, GDP
	%					
None	100.0	100.0	100.0	100.0	100.0	100.0
Protein A-agarose	101.3	99.1	98.3	102.1	100.2	103.5
Nonimmune sera	107.1	101.7	100.6	97.3	99.2	100.6
ANF-antibody[a]	97.4	87.9	98.1	95.3	87.1	85.9
$G_{h7\alpha}$-antibody supernatant	99.5	20.1	16.3	97.7	14.1	93.8
Pellets	0.0	45.3	59.1	0.7	62.5	0.3

[a] ANF, atrial natriuretic factor.

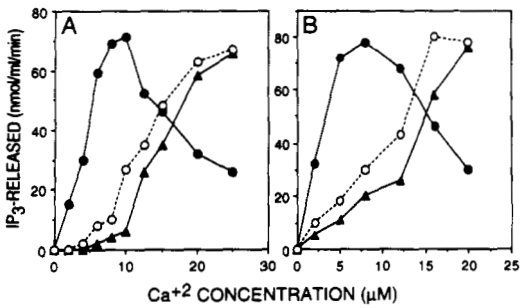

FIG. 5. **Ca^{2+} dependence of α_1-agonist-stimulated PLC activity after reconstitution of α_1-adrenoceptor, G_{h7} or G_h, and 69-kDa PLC.** Partially purified rat liver α_1-adrenoceptor (10 pmol), bovine heart G_{h7} or rat liver G_h (60 pmol), and bovine liver 69-kDa PLC (2 μg) were mixed with 0.2 mg/ml phospholipid mixture and 500 μM [^{3}H]PIP_2 (specific activity, 600–800 cpm/nmol) in a 700-μl final volume. To form vesicles the mixture was diluted as described above and under "Experimental Procedures." Prior to reconstitution of the components, the α_1-receptors were preincubated in the presence of 5×10^{-6} M (−)-epinephrine, 5×10^{-6} M (−)-epinephrine plus 10^{-4} M phentolamine, or buffer at 30 °C for 30 min. The vesicles (20 μl) containing three components were incubated in the presence or absence of 5×10^{-6} M GTPγS and 0.2 mM $MgCl_2$ in a final volume of 100 μl at 30 °C for 10 min. Calcium concentrations in the assay mixtures were adjusted as stated above. The reaction buffer contained 20 mM Hepes, pH 7.2, 100 mM NaCl, and 0.2 mM ascorbic acid. Inositol phosphate formation by PLC was determined as detailed under "Experimental Procedures." *Panel A*, reconstitution of G_{h7} with the α_1-adrenoceptor and PLC. ●——●, (−)-epinephrine + GTPγS; ○- - -○, (−)-epinephrine + phentolamine + GTPγS; ▲——▲, buffer. *Panel B*, reconstitution of G_h with the α_1-adrenoceptor and PLC. ●——●, (−)-epinephrine + GTPγS; ○- - -○, (−)-epinephrine + phentolamine + GTPγS; ▲——▲, buffer. Values shown here are the average of duplicate determinations and representative of five different experiments using different enzyme preparations.

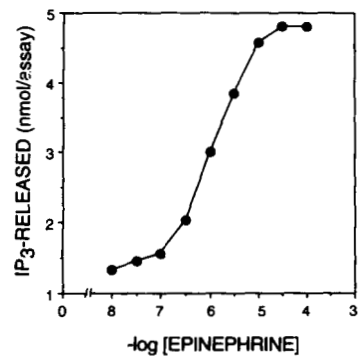

FIG. 6. **(−)-Epinephrine concentration-dependent PLC activity by the vesicles containing α_1-adrenoceptor, G_{h7}, and 69-kDa PLC.** Reconstitution of the α_1-adrenoceptor (5 pmol) with G_{h7} (20 pmol) and PLC (0.3 μg) were performed as described above in legend Fig. 5 and under "Experimental Procedures." The vesicles (10 μl) were preactivated with various concentrations of (−)-epinephrine in 75 μl of 20 mM Hepes (pH 7.2) containing 100 mM NaCl and 0.2 mM ascorbic acid, but in the absence of calcium and GTPγS in a final volume of 100 μl at 30 °C for 30 min. The samples then chilled in an ice bath for 10–15 min. After addition of 5 μM $CaCl_2$, 5×10^{-6} M GTPγS, and 0.2 mM $MgCl_2$, the samples (100 μl, final) were further incubated at 30 °C for 10 min. Inositol phosphate formation was measured as detailed under "Experimental Procedures." Data shown here are representative of three independent experiments, and each point is the average of duplicate determinations.

inhibition by phentolamine was also observed in the case of (−)-epinephrine stimulated GTPase activity of G_h (25). As shown in Fig. 5*B*, when G_h was reconstituted with the α_1-receptor and PLC, a similar pattern of the calcium sensitivity shift was again obtained. In these studies, the most interesting feature of the G_h- or G_{h7}-mediated stimulation of the PLC activity is the calcium sensitivity in response to G-protein activation that was observed above (see Fig. 4) and also previously reported by us (26).

The (−)-epinephrine-concentration dependent induction of PLC activation was also detemined, as shown in Fig 6. The vesicles containing these three components were incubated with various concentrations of the α_1-agonist in the presence of GTPγS. In the presence of 5 μM Ca^{2+}, the PLC activity linearly increased up to 10^{-5} M (−)-epinephrine and reached a plateau at 10^{-4} M. The time course of the PLC stimulation was also measured in response to (−)-epinephrine in the presence of GTPγS, 50 μM [^{3}H]PIP_2, and 8 μM Ca^{2+}, as shown in Fig. 7. The maximal formation of inositol phosphate by the agonist stimulation was achieved within <3 min, whereas in the presence of phentolamine it was observed in ~20 min. All these data clearly demonstrate that these three proteins, the α_1-receptor, G_h or G_{h7}, and PLC, are the key components for transmembrane signaling. It should be noted that the time required for maximal stimulation of PLC in the presence of the agonist was slower than that observed in the cell system (<1 min) (32). This is probably due to the efficacy of the protein-protein interaction in the *in vitro* reconstitution system. These results not only prove that the α_1-adrenoceptor stimulates 69-kDa PLC through the G_h family, but also confirm the cross-coupling between the proteins regardless of their origin.

DISCUSSION

Recently, we have demonstrated that rat liver α_1-adrenoceptor can be isolated in a ternary complex with a high affinity

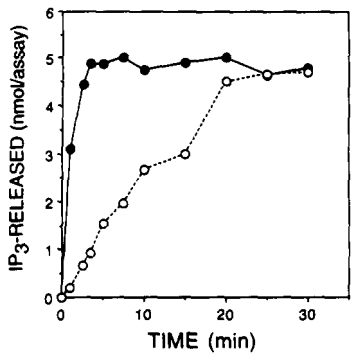

FIG. 7. **Time course of PIP_2 hydrolysis by α_1-receptor activation with vesicles containing α_1-adrenoceptor, G_{h7}, and PLC.** Insertion of the receptor (15 pmol), G_{h7} (30 pmol), and PLC (1 μg) into phospholipid vesicles was performed as described in legend of Fig. 5. The receptors were preactivated with 5×10^{-6} M (−)-epinephrine or 5×10^{-6} M (−)-epinephrine and 10^{-4} M phentolamine for 30 min at 30 °C, prior to reconstitution. The vesicles were then incubated in the presence of 5×10^{-6} M GTPγS, 0.2 mM $MgCl_2$, and 8 μM $CaCl_2$ at 30 °C for 10 min. The final assay volume was 100 μl. At different time intervals, reactions were stopped by adding 0.75 ml of chloroform:methanol:1 N HCl (100:100:0.6) and 0.20 ml 1 N HCl. Formation of inositol phosphate by PLC was determined as detailed under "Experimental Procedures." ●——●, (−)-epinephrine + GTPγS; ○——○, (−)-epinephrine + phentolamine + GTPγS. Points shown here are average of duplicated experiments and representative of three independent measurements.

GTP-binding protein (24) (see also our companion study (41)). Furthermore, we have also demonstrated that a G_h-PLC complex could be induced in the membranes or in the membrane extract, and the formed complex could be isolated without dissociation in the presence of G-protein activator (26). Our preliminary studies also showed that the specific G_h-PLC complex can be released from the membranes by incubating the membranes with the α_1-agonist and GTP (26). Similarly, Kuhn (33) and Baehr *et al.* (34) reported that, under bleaching, photon-activated association of $G_{t\alpha}$ with γ-subunit of cGMP phosphodiesterase was formed in the presence of GTP and released from the membranes. Recently, Ransas *et al.* (35) have also observed the dissociation of $G_{s\alpha}$ from βγ by the incubation of the membranes with GTP through the activation of the β-adrenoceptor and the release of $G_{s\alpha}$ from the membranes.

In this report, we demonstrate the molecular components which are involved in the α_1-adrenoceptor-mediated transmembrane signaling. For the identification of the effector molecule we used the specific extraction method for the G_{h7}-PLC complex which was formed in the presence of the α_1-agonist and GTP. We also demonstrate that the α-subunit of G_h or G_{h7} directly interacts with PLC.

Utilizing the properties of the α_1-agonist-receptor-G-protein ternary complex, membranes were initially incubated with (−)-epinephrine to induce the α_1-agonist-receptor-G-protein ternary complex. Since in the ternary complex G-protein is a primer for further reaction and is in a transient state for the activation, GDP/GTP exchange is fast even at a low temperature (<4 °C) (24, 25, 36), whereas the intrinsic GTPase activity is slow at this temperature. This procedure allows the interaction of G-protein with the corresponding effector for a longer period than at higher temperature, and thus the protein (α- or β-subunit of G-protein) associated with the effector can be monitored by further purification. Based on the above considerations, our studies were designed, *i.e.* the α_1-agonist-receptor-G_{h7} complex was formed, then the $G_{h7(\alpha)}$-PLC complex was induced by incubating with GTP. The $G_{h7(\alpha)}$-PLC complex was then released from the membranes. However, the reason for the release of the complex from the membranes is not understood. Biophysical evidence for the α_1-adrenoceptor-mediated formation of a specific $G_{h7\alpha}$-PLC complex is that the PLC activity was eluted from the columns as a single peak along with $G_{h7\alpha}$, except from the first heparin-agarose column where PLC was partially dissociated from the complex (see Fig. 1*C*). In addition, all of the fractions having both G_{h7} and PLC at each step of purification, as well as the AlF_4^--treated fractions after Sephadex G-200 elutions, showed a shift in Ca^{2+} requirement toward the lower Ca^{2+} concentrations in comparison to PLC alone for the maximal PLC activity (see "Results"), confirming the α_1-adrenoceptor-mediated $G_{h7\alpha}$-PLC complex formation.

This unique approach of inducing specific G_{h7}-PLC complex made the isolation of 69-kDa PLC possible. In rat liver membranes, G_h has been found to activate a 69-kDa PLC.[2] Moreover, other workers have also reported the presence of 68~72-kDa PLCs in rat liver (3, 4). Like other phosphatidylinositol-specific PLC(s), PIP_2 is a preferential substrate for bovine liver 69-kDa PLC at low Ca^{2+} concentrations (Fig. 3*A*). The interaction of this enzyme with various PLC antibodies (β1, β2, γ1, γ2, and δ1 subtypes), which can neutralize the respective enzymatic activity, was evaluated by measuring the catalytic activity of PLC after immunoprecipitation. None of these antibodies precipitated 69-kDa PLC or inhibited its activity. The immunoblotting studies again showed negative cross-reactivity of this 69-kDa PLC with these antibodies. However, since other laboratories reported that a range of 56~70-kDa PLCs are either a proteolytic fragment of PLC-δ (37) or contaminant of PLC with thiol:protein-disulfide oxidoreductase (38), the failure of these antibodies to recognize this PLC cannot exclude a possibility that this enzyme is a degradation product of one of these well characterized PLC isozymes. Our studies, however, showed that a battery of protease inhibitors did not change the immunological reactivity of 69-kDa PLC with antibodies of PLC isozymes nor the apparent molecular mass of the enzyme so far. Additionally, as reported previously (26), PLC-β1, -γ1, and -δ1 were not stimulated by GTPγS-G_h in the reconstituted system. All of these results suggest that this G_{h7}-coupled PLC may not belong to any of those well characterized PLC isozymes.

The purification of 69-kDa PLC enabled us to attempt the total reconstitution of the α_1-adrenoceptor, the G_h family, and this PLC and to study the universality of the system. Our studies demonstrated that the α_1-adrenoceptor-mediated stimulation of the PLC activity was indeed observed by reconstituting the α_1-adrenoceptor, G_{h7} or G_h, and PLC. Thus, co-reconstitution of rat liver α_1-adrenoceptor, G_{h7} or G_h, and 69-kDa PLC into phospholipid vesicles resulted in the stimulation of PLC in the presence of (−)-epinephrine that was blocked by phentolamine. Moreover, by activating the α_1-receptor with the α_1-agonist, a decrease in the calcium requirement and in the activation time for the PLC activity was observed. These changes in calcium sensitivity are consistent with the results of G_{h7}-/G_h-mediated stimulation of the PLC activity in response to calcium as obtained in the presence of GTPγS *versus* GDP or without guanine nucleotides (Fig. 4) as well as with the data of the $G_{h7(\alpha)}$-PLC complex *versus* PLC alone (see "Results"). The profile of calcium response is also in conformity with the results obtained using a membrane preparation when PLC stimulation was measured in the presence of hormone, GTPγS, or Gpp(NH)p (2, 39, 40). It is also of interest to note that, unlike PLC alone, the α_1-receptor- or G_h-/G_{h7}-mediated stimulation of the PLC activity consist-

[2] T. Das, K. J. Baek, C. Gray, and M.-J. Im, unpublished data.

ently showed a decrease not only in the calcium requirement for maximum IP_3 production but also in the activity with increasing concentrations of calcium (>12 μM). The reason for such inhibition by Ca^{2+} is not clear, but this may be one of the mechanisms of regulation of the effector enzyme or the feedback inhibition, since Ca^{2+} is released from the intracellular stores as a result of the α_1-adrenoceptor-mediated formation of second messengers via PLC. Our observations suggested that one of the subunits of the G_h family is likely to be involved in this regulation, since the changes in calcium sensitivity are dependent on the activation of G_h and G_{h7}.

The direct interaction of PLC with the α-subunit of the G_h family was observed by co-immunoprecipitating PLC with the GTPγS-activated G_{h7} or G_h using $G_{h7\alpha}$ specific antibody but not by incubating with GDP or without guanine nucleotide. These experiments not only directly confirm the coupling between 69-kDa PLC and the α-subunits of the G_h family, but also again suggest the universality of coupling because of the capability of bovine liver 69-kDa PLC to cross-couple to a different G_h family. A similar conclusion was made by Feder *et al.* (31), who observed that the interaction domains of the β-adrenoceptor, G_s, and adenylyl cyclase were conserved across species in the course of evolution. Since the liver contains mainly the α_{1b}-subtype, this subtype may regulate the Ca^{2+} efflux via the G_h family-activated 69-kDa PLC.

On the other hand, recent work by Wu *et al.* (18) showed that the coupling of all three subtypes of the α_1-adrenoceptors (α_{1a}, α_{1b}, and α_{1c}) to $G_{\alpha q}$ activated PLC-β1 in transfected cells. Discrepancies between our findings and their results have raised the following multiple possibilities as far as the α_1-adrenoceptor-mediated signal transduction mechanism is concerned. 1) Different G-proteins and PLC isozymes may be the mediators of different activation pathways. 2) Depending on the cell system and the nature and the density of the proteins involved, one or all of the pathways may become active (the observations by Wu *et al.* (18) support this possibility). 3) The discrepancies may be due to the difference between the system used by Wu *et al.* (18), the transfected COS-7 cell lines, and the system used by us, the purification and reconstitution of the components in the phospholipid vesicles. To elucidate the exact reason for such discrepancies as well as to predict the physiological mechanisms, a careful designing of the necessary experiments using all possible combinations is required.

Our novel purification method and the total reconstitution studies as well as the co-immunoprecipitation studies represent two important and confirming sets of experimental work that has provided an understanding of the mechanisms involved in the α_1-adrenoceptor-mediated transmembrane signaling. Moreover, the nature of the G_h family, in which members have different molecular masses in different species, their possible involvement in Ca^{2+} sensitivity of the effector, and the ability of these proteins to cross-couple are also significant findings of our studies.

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