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

I. IDENTIFICATION OF α_1-ADRENOCEPTOR-COUPLED G_h FAMILY AND PURIFICATION OF G_{h7} FROM BOVINE HEART*

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Our previous studies on α_1-adrenoceptor-mediated signaling suggested that G_h is a signal mediator. G_h consists of a 74-kDa GTP-binding α-subunit and a 50-kDa β-subunit. Studies using the α_1-agonist-receptor-G-protein ternary complexes from various tissues and species revealed that the intensity (GTP-binding) of the [α-^{32}P]GTP-labeled proteins resulting from activating the α_1-receptor was significantly attenuated by phentolamine. The molecular masses of GTP-binding proteins were 74 kDa in rat heart and liver, 77 kDa in dog heart, 78 kDa ($G_{h7\alpha}$) in bovine heart and liver, and 80 kDa in human heart. Supporting these observations, a specific antibody to $G_{h7\alpha}$ not only recognized these GTP-binding proteins in the ternary complex preparations, but also co-immunoprecipitated α_1-adrenoceptors, indicating a tight association of these GTP-binding proteins with the α_1-adrenoceptor. These results also demonstrate that functional and structural similarities exist among these GTP-binding proteins. Additionally, one of the identified G-proteins (termed G_{h7}) was purified from bovine heart. G_{h7} consisted of the 78-kDa GTP-binding protein and a 50-kDa protein.

The biochemical responses of epinephrine and norepinephrine are mediated by pharmacologically specific receptors, the α_1-, α_2-, and β-adrenoceptors. Although β-adrenoceptors are classified into three types, the β_1-, β_2-, and β_3-receptors, the transmembrane signaling of these receptors shares a common pathway involving G_s[1] and adenylyl cyclase (1, 2). The α_1- and α_2-adrenoceptor families, however, use multiple signal pathways composed of toxin-sensitive and -insensitive GTP-binding regulatory proteins (G-proteins) and various effectors (3–8). Pharmacological studies of the α_1-adrenoceptors have indicated that at least two subtypes of the α_1-receptors exist; these subtypes are designated α_{1a} and α_{1b}. Based on biochemical studies with various tissues and cell types, two signal pathways of the α_1-receptors can clearly be observed. Stimulation of α_{1b}-receptor leads to the formation of inositol 1,4,5-triphosphate and diacylglycerol via activation of a phospholipase C through a toxin-insensitive G-protein (5, 9). The formation of arachidonic acid via activation of phospholipase A_2, is stimulated by the α_{1a}-receptor through a pertussis toxin-sensitive G-protein (5, 10). Recently, the α_1-receptor subtypes have been cloned including α_{1a}-, α_{1b}-, and α_{1c}-receptors (6, 7, 11, 12), and a new member designated as type α_{1d}, cloned by Perez *et al.* (13). Transfection of cells with cDNAs encoding the α_{1a}-, α_{1b}-, and α_{1c}-receptors stimulated the hydrolysis of phosphatidylinositides via phospholipase C-β1 through the activation of the Gq family (14) (see also Refs. 15 and 16).

We previously reported that in rat liver α_1-receptor (possibly the α_{1b} type) coupled to a 74-kDa GTP-binding protein. This GTP-binding protein was identified by inducing the α_1-agonist-receptor-G-protein ternary complex and by direct photoaffinity labeling of the G-protein in the ternary complex with radiolabeled GTP (17). The isolated G_h consisted of the 74-kDa GTP-binding protein (α-subunit) and a ~50-kDa protein (β-subunit) (18). We have also shown that G_h coupled to an α_1-receptor (18) and stimulated a membrane-bound phospholipase C in an *in vitro* reconstitution system (19).

In this report, we have extended our studies in order to further evaluate the coupling ability of the α_1-receptor with G_h. The following studies have been described. 1) A new high molecular mass GTP-binding regulatory protein (G_{h7})[2] was purified from bovine heart; 2) (−)-epinephrine-α_1-receptor-G_h ternary complexes from various species were induced and isolated; 3) $G_{h7\alpha}$-specific antibody was used to assess the direct interaction of the G_h family with the α_1-receptor. Herein, we have demonstrated that the G_h family which couples to the α_1-adrenoceptor exhibits different molecular masses in various species.

EXPERIMENTAL PROCEDURES

Materials—Sucrose monolaurate (SM-1200) was a gift from the Mitsubishi-Kasei Company (Tokyo, Japan). Lubrol PX and Nonidet

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[1] The abbreviations used are: G_s, stimulatory G-protein; G-protein, a holoprotein consisting of the GTP-binding protein (α-subunit) and other subunits; GTP-binding protein, the α-subunit of G-proteins which bind GTP; G_q, a toxin-insensitive guanine nucleotide-binding protein which couples to various receptors and activates phospholipase C-β1; G_h, a toxin-insensitive guanine nucleotide-binding protein which was first identified as a partner of an α_1-adrenergic ternary complex and couples to the α_1-adrenoceptor and activates phospholipase C; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); App(NH)p, 5′-adenylyl-β,γ-imidodiphosphate; PAGE, polyacrylamide gel electrophoresis; LDB, low detergent blotto; HDB, high detergent blotto.

[2] G_{h7}: The term G_{h7} is used in anticipation of an increase in the number of G_h family proteins from different species, because this G-protein is functionally and structurally similar to G_h. We presented this G-protein as G_v at the 1992 AHA meeting in New Orleans, because earlier data from $G_{h7\alpha}$ antibody experiments and cross-coupling studies were not available.

P-40 were from Sigma. Protein A-agarose, guanine nucleotides, and other nucleotides were obtained from Boehringer Mannheim. The column chromatographic resins were obtained from Pharmacia LKB Biotechnology Inc. [α-^{32}P]GTP (3000 Ci/mmol) and ^{125}I-labeled protein A (30 mCi/mg of protein A) were obtained from Amersham Corp., and [^{35}S]GTPγS (~1300 Ci/mmol) and [^{3}H]prazosin (76 Ci/mmol) were from DuPont NEN. Other chemical and biochemical materials were used as described previously (17–19).

Membrane Preparation from Various Sources—The rat and bovine liver membranes were prepared by Percoll gradient centrifugation using the method of Prpic *et al.* (20). Bovine hearts (4 kg), obtained from Pel-Freez Biologicals (Rogers, AR), were minced, homogenized briefly with a Waring blender, and then with a mechanical homogenizer (Ultra-Turrax, Janke & Kunkel) in 30 liters of 10 mM Hepes buffer (pH 7.5) containing 250 mM sucrose, 5 mM EGTA, and protease inhibitors (bacitracin, 2 μg/ml; benzamidine, 100 μg/ml; leupeptin, 2 μg/ml; pepstatin A, 2 μg/ml; trypsin inhibitor, 2 μg/ml; phenylmethylsulfonyl fluoride, 2 μg/ml; and antipain, 20 μg/ml). The homogenate was filtered through four layers of cheesecloth and centrifuged at 500 × *g* for 5 min. The supernatant was collected and centrifuged at 40,000 × *g* for 1 h. Pellets were harvested and washed three times with 50 mM Hepes buffer (pH 7.5) containing 10 mM $MgCl_2$, 5 mM EGTA, and the protease inhibitors listed above. Crude membranes from various species and various tissues of rat were prepared using essentially the same protocol. The membranes were suspended at 10 mg/ml protein concentration in the same buffer containing 10% glycerol and 1 mM dithiothreitol and stored at −80 °C until use. The heart tissues were obtained from various sources in different lengths of storage periods. Human heart tissue was obtained from the heart transplantation program at the Cleveland Clinic Foundation. Rat tissues were prepared freshly, whereas dog and bovine tissues were obtained from Pel-Freez.

Purification of 78-kDa GTP-binding Protein (G_{h7}) from Bovine Heart—The 78-kDa GTP-binding protein (α-subunit of G_{h7}) was isolated by previously described G_h purification method with some modifications (18) (see also Ref. 19). The following modifications were made. 1) Protein was solubilized without detergent, but the purification of the G_{h7} protein was carried out in the presence of 0.1% sucrose monolaurate, 2) Q-Sepharose ion-exchange resin was used for the first step instead of heparin-agarose, and 3) the isolation of G_{h7} was achieved without using hydrophobic resin. Throughout the purification, the 78-kDa protein was monitored by photoaffinity labeling with [α-^{32}P]GTP as well as by [^{35}S]GTPγS binding. The purifications were carried out at 4 °C, and protease inhibitors were included in the buffers listed above. Glycerol (10%) and 0.1% sucrose monolaurate were included in HED buffer (20 mM Hepes, 1 mM EGTA, and 0.5 mM dithiothreitol, pH 7.5) to stabilize the proteins throughout the purification. To assess the subunit association of the 78-kDa GTP-binding protein, the protein was purified under non-activated conditions. The protocol and results described here are representative of several independent purifications.

The crude membranes (10 g of protein) were washed with ice-cold HED buffer containing protease inhibitors, as described above. After centrifugation at 40,000 × *g* for 40 min, the pellets were collected, resuspended at 5 mg/ml protein in the same buffer (2 liters) supplemented with 250 mM NaCl, then solubilized with gentle agitation for 1 h at 4 °C. After centrifugation at 40,000 × *g* for 1 h, the supernatant (1,120 ml) was collected and diluted 4-fold with HED buffer containing 0.1% sucrose monolaurate and 10% glycerol. The sample was then applied to a Q-Sepharose column (3.6 × 20 cm) which had been equilibrated with HED buffer containing 70 mM NaCl. The column was washed with 600 ml of the equilibration buffer. The retained materials were eluted using 1,000 ml of a linear salt gradient (50–700 mM) in the same buffer, and 9-ml fractions were collected at a flow rate of 30–40 ml/h. The G_{h7}-containing fractions (135 ml, fractions 49–63 of peak II) were pooled and concentrated to 5–7 ml using Amicon PM-30 membranes. The sample was applied to an Ultrogel AcA 34 column (450 ml) that had been equilibrated with HED buffer containing 100 mM NaCl. The column was eluted overnight with the same buffer, and 4.5-ml fractions were collected at a flow rate of 25 ml/h. The pooled fractions (76 ml, fractions 49–65) which contained G_{h7} protein were diluted with 80 ml of HED buffer and loaded onto a Q-Sepharose column (1.4 × 10 cm). The column was washed with 70 ml of HED buffer containing 250 mM NaCl. Elution was achieved using 80 ml of a linear sodium chloride gradient (200–700 mM). Fractions (1.5 ml) were collected at a flow rate of 30 ml/h. The G_{h7}-containing fractions (27 ml) were pooled and diluted 7-fold with HED buffer. The sample was applied to a hydroxylapatite column (3–5 ml) which had been equilibrated with HED buffer containing 50 mM NaCl. After washing the column with the equilibration buffer (15 ml), the bound materials were eluted using 40 ml of a linear phosphate gradient (0–100 mM), and 1 ml fractions were collected.

Purification of G_h—G_h was purified from rat liver membranes isolated by Percoll gradient centrifugation, as described previously (18, 19).

α_1-Agonist-Receptor-G-protein Ternary Complex Formation and Purification—The ternary complex comprised of (−)-epinephrine, α_1-receptor, and G-protein was induced by incubating the membranes with (−)-epinephrine. Thus, the rat liver and heart, bovine liver and heart, and dog and human heart membranes (1 g of protein) were preincubated for 3 h with 5×10^{-5} M (−)-epinephrine, 10^{-6} M (±)propranolol, and 10^{-7} M rauwolscine. The complexes then were solubilized using 0.2% sucrose monolaurate in HED containing 100 mM NaCl and isolated using heparin-agarose and wheat germ agglutinin-agarose by the protocol previously reported (17). For experiments, the ternary complex preparations (100 μl) were used without adjusting the amount of the α_1-receptors and G-proteins.

GTPase Assay—GTPase activity was measured essentially as described previously (18). Briefly, G_h or G_{h7} (20 pmol) was incorporated into phospholipid vesicles using a Sephadex G-50 column (0.6 × 15 cm). The incorporation of G_h and G_{h7} into vesicles was 65–70%. Vesicles (300–400 μl) containing G-proteins were mixed with 0.25 μM GTP and 5 μCi of [γ-^{32}P]GTP, and release of phosphate from [γ-^{32}P]GTP was measured using Norit A charcoal (5% suspension, w/v).

[^{3}H]Prazosin and [^{35}S]GTPγS Binding Assay and [α-^{32}P]GTP Photoaffinity Labeling—These assays were performed essentially as described previously (17, 18). Briefly, the amount of α_1-adrenoceptor was quantitated by specific [^{3}H]prazosin binding after incubation at room temperature for 1 h. The total amount of G_h or G_{h7} in 0.05% sucrose monolaurate solution was determined by means of the binding of [^{35}S]GTPγS in the presence of 1–2 mM $MgCl_2$. The photoaffinity labeling of G-proteins was performed in the presence of 5–20 μCi of [α-^{32}P]GTP and 0.5–2 mM $MgCl_2$ in an ice bath under 254-nm UV irradiation for 6–10 min.

Antibody Experiments—A polyclonal antibody to $G_{h7\alpha}$ was generated in New Zealand White rabbits. $G_{h7\alpha}$ was separated using a Q-Sepharose column as described (19). Seventy micrograms of $G_{h7\alpha}$ (200 μl) were emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into the rabbits. At 3-week intervals, three booster injections were given with 70–100 μg of $G_{h7\alpha}$ and incomplete Freund's adjuvant. Rabbit antisera were characterized by immunoblots using the methods of Harris *et al.* (21) or by immunoprecipitation. Briefly, for Western blots, proteins were separated on 7% gel by SDS-PAGE and then transferred to Immobilon-P (Millipore, Bedford, MA). Immunoblots were incubated in LDB solution (low detergent blotto, 80 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, 0.2% NP-40, 50 mM Tris/HCl, pH 8.0, containing 5% nonfat dry milk) for 2 h at room temperature to block nonspecific binding, then transferred to LDB containing $G_{h7\alpha}$ antibody (1:500 dilution), and incubated for 1 h at room temperature. After washing three times with LDB, the immunoblots were incubated with ^{125}I-labeled protein A (0.2 μCi/ml) in HDB (high detergent blotto, 2% Nonidet P-40 in LDB) for 1 h at room temperature. After washing intensively with HDB and non-detergent blotting buffer, the dried blots were subjected to autoradiography on Kodak XAR-5 x-ray film with intensifying screens for 1–2 days. For immunoprecipitation, G_{h7}, G_h, the α_1-agonist-receptor-G-protein ternary complex preparations and solubilized tissue membranes were photoaffinity-labeled with 10 μCi of [α-^{32}P]GTP in the presence of 0.1 mM App(NH)p and 2 mM $MgCl_2$ (17, 18) and then incubated for 2 h with 5 μl of $G_{h7\alpha}$ antibody at room temperature. The antigen-antibody complexes were precipitated using 10 μl of protein A-agarose (binding capacity, 22 mg of rabbit IgG/ml of agarose gel). The pellets were collected by centrifugation at 3000 rpm and washed three times with 20 mM Hepes buffer (pH 7.4) containing 500 mM NaCl and 0.01% sucrose monolaurate. The samples were denatured by boiling in the presence of Laemmli buffer (22) and subjected to SDS-PAGE (7% gels) and autoradiography for 1–2 days.

Protein Determination—The protein concentration was determined by the method of Bradford (23) using a Bio-Rad protein determination kit and bovine serum albumin as a standard.

RESULTS

Purification of the 78-kDa GTP-binding Protein—For the selective solubilization of the 78-kDa GTP-binding protein,

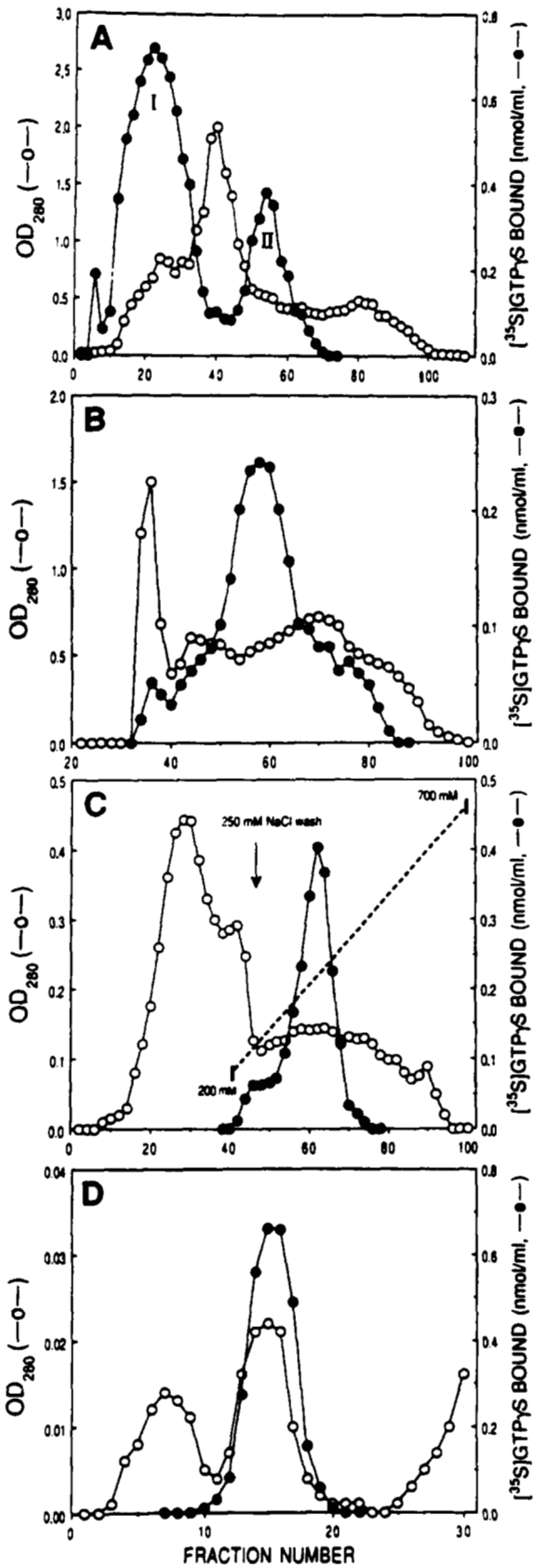

FIG. 1. **Elution profile of protein and GTPγS binding activity.** *Panel A*, the first Q-Sepharose chromatography. The 78-kDa GTP-binding protein from the column was eluted in the range of 350–400 mM salt concentrations (*peak II, fractions 49–63*). The 40-kDa class of GTP-binding proteins were eluted at <200 mM NaCl concentrations (*peak I, fractions 12–38*), as judged by the results of photolabeling with [α-^{32}P]GTP. *Panel B*, Ultrogel AcA 34 chromatography. See "Experimental Procedures" and "Results" for details. *Panel C*, The second Q-Sepharose chromatography. The *arrow* indicates the 250 mM NaCl wash of the column. The *solid line* shows the linear salt gradient of 200–700 mM NaCl. The 78-kDa GTP-binding protein was eluted in a range of 340–380 mM (*fractions 53–70*). *Panel D*, hydroxylapatite chromatography. The 78-kDa GTP-binding protein was eluted at a concentration of 30–50 mM phosphate. See "Experimental Procedures" for details.

TABLE I

Purification of G_{h7} from bovine heart

The values are representative of several independent experiments.

Steps	Volume	Protein	[^{35}S]GTPγS bound	Specific activity	Recovery
	ml	*mg*	*nmol*	*pmol/mg*	*%*
Membranes	1,500	10,000.0	1,124.0	112.4	100.0
Extract	1,120	1,059.0	148.4	258.0	13.4
1st Q-Sepharose	135	85.8	29.3	341.5	2.6
Ultrogel AcA 34	76	24.3	9.7	399.2	0.86
2nd Q-Sepharose	27	3.5	6.4	1,828.6	0.56
Hydroxylapatite	6	0.65	2.97	4,569.2	0.26

we tested various conditions; salt concentrations were critical for increasing the specific solubilization. The optimal concentration of salt was 200–400 mM in HED buffer. An increase in the salt concentration (400–2000 mM) did increase the GTPγS binding, but the specific binding activity was decreased because the amount of total protein extracted from the membranes also increased. At 250 mM NaCl the extraction of G-proteins and other proteins from the membranes reached approximately 13–14% and 10–11%, respectively. When the pellets were resuspended and photolabeled with 10 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$, the 78-kDa GTP-binding protein was not detectable in most experiments, suggesting that the protein was substantially solubilized (data not shown). It should be noted that the 78-kDa GTP-binding protein could be solubilized with salt alone, but in the absence of the detergent, sucrose monolaurate, the protein became easily aggregated and lost the ligand binding activity within less than 1 week. The elution profile of the 78-kDa GTP-binding protein was similar to that of G_h purified from rat liver membranes with Q-Sepharose (Fig. 1*A*). However, the 78-kDa protein from gel filtration column was eluted in somewhat later fractions (at least more than 10 fractions) than G_h (Fig. 1*B*, see Ref. 18). The reasons for this later elution are not clear yet. Washing of the second Q-Sepharose column with high salt (250 mM) resulted in separation of most protein from the 78-kDa GTP-binding protein which was obtained with >25% purity (Fig. 1*C*). Further purification and concentration of the 78-kDa GTP-binding protein were achieved using a hydroxylapatite column, as shown in Fig. 1*D*.

The overall results of the G_{h7} protein purification scheme are summarized in Table I. Based on the specific [^{35}S]GTPγS-binding activity, the $G_{h7\alpha}$ protein obtained after the last purification step was of 80% purity. The yield of $G_{h7\alpha}$ was ~20% compared with that of salt extraction. Based on the final recovery, the amount of $G_{h7\alpha}$ was 0.26% of total G-proteins and 0.0065% of the total protein in the membranes. The 78-kDa GTP-binding protein ($G_{h7\alpha}$) was copurified with a 50-kDa protein ($G_{h7\beta}$) which has the same molecular mass as $G_{h\beta}$ and does not bind GTP (see Fig. 3, *A* and *B*). Differences in the molecular masses between G_h and G_{h7} were seen only with the GTP-binding proteins. The purified protein was stable in this buffer for less than 3 weeks without the addition of a G-protein stabilizer. However, when the aluminum fluoride was included, the protein was stable for a month.

Biochemical Properties of G_{h7}—The specificity of the nucleotide binding was determined by the photolabeling method

in the presence of various nucleotides. The order of inhibition of the photolabeling of G_{h7} with [α-^{32}P]GTP by nucleotides was GTPγS > GDP > ITP >>> ATP $\simeq$ App(NH)p. At 100 nM [α-^{32}P]GTP, photolabeling was completely abolished with 80 nM of GTPγS and 200 nM of GDP, whereas 500 times excess of ATP or App(NH)p did not inhibit the labeling of G_{h7}. With the GTPγS-binding assay using 1 μM [^{35}S]GTPγS, the half-maximal inhibition by the nucleotides was GTP ~15 μM, GDP ~45 μM, ITP ~130 μM. Titrating [^{35}S]GTPγS concentrations, the half-maximal binding of this ligand by G_{h7} was 0.3–0.4 μM and saturation binding was obtained at 1.0–1.2 μM GTPγS. These results demonstrate that G_{h7} is a specific GTP-binding protein and has an affinity for the ligands similar to G_h (18). To further evaluate the properties of G_{h7}, we compared the magnesium ion requirement for GTPγS-binding of G_h and G_{h7}. As shown in Fig. 2, when the GTPγS-binding assays were carried out in 0.05% Lubrol PX solution, the maximal ligand binding was observed at 1–3 mM $MgCl_2$ for G_{h7} and 10–20 mM for G_h which was similar to that previously reported (18). The ligand binding by G_{h7} was subsequently inhibited ~55% of the maximal when the Mg^{2+} concentration was further increased. On the other hand, when the GTPγS-binding experiments were performed in 0.05% sucrose monolaurate solution, the maximal ligand binding by G_{h7} was obtained at <0.2 mM $MgCl_2$ and did not change with any further increases of the metal ion concentration (0.2–20 mM), whereas Mg^{2+} requirement for the maximal GTPγS-binding by G_h was dramatically decreased to 0.8–3 mM in this detergent solution. By further increasing Mg^{2+} concentration (>4 mM), the ligand binding activity was subsequently inhibited ~60% of the maximal ligand binding. However, GTPγS-binding by G_h and G_{h7} was not completely inhibited by further increases of $MgCl_2$ up to 50 mM. In the absence of $MgCl_2$, the GTPγS-binding by G_h and G_{h7} could not be detected in either Lubrol PX or sucrose monolaurate solution. These data indicate that the different amounts of magnesium ion requirement for GTPγS binding by these G-proteins is due rather to the detergent effect than to the specific character of these G-proteins. This property, however, could be an indicator to distinguish between these G-proteins. When intrinsic GTPase activities of G_h and G_{h7} were measured, the turnover of GTPase activity of G_{h7} was slower than that of G_h. Thus, G_h and G_{h7} hydrolyzed GTP; the turnover numbers were 2–3 and ~0.25–0.6 min^{-1}, respectively. It is not yet clear whether the different turnover of GTPase activities is specific for these G-proteins, since the isolated G_{h7} protein was more unstable than G_h, although these proteins are structurally and functionally similar (see below). In addition, G_{h7} was not a toxin substrate to be ADP-ribosylated either by cholera or pertussis toxin, even with the addition of $\beta\gamma$-subunits of the heterotrimeric G-proteins (data not shown).

Immunological Cross-reactivity of $G_{h7\alpha}$ Antibody—As mentioned above, overall biophysical and biochemical properties of G_{h7} are similar to G_h. The distinct difference is in the molecular masses of the GTP-binding α-subunits (Fig. 3, *A* and *B*), whereas the 50-kDa β-subunits of G_{h7} and G_h have the same molecular mass (Fig. 3*A*). To assess whether G_{h7} is distinct from G_h, an antibody raised against the native $G_{h7\alpha}$ protein was used to test the immunological cross-reactivity. As demonstrated in Fig. 3*C*, when G_h and G_{h7} were subjected to immunoprecipitation, the $G_{h\alpha}$ protein was also effectively immunoprecipitated, indicating that G_h and G_{h7} are homologs. The specificity of $G_{h7\alpha}$ antibody was demonstrated in Fig. 4. The [α-^{32}P]GTP-labeled $G_{h7\alpha}$ was incubated with $G_{h7\alpha}$ antibody, nonimmune sera, or the antibody preincubated with unlabeled $G_{h7\alpha}$. The results revealed that the $G_{h7\alpha}$ protein was specifically recognized by the $G_{h7\alpha}$ antibody (Fig. 4*A*, *lane 1*). Thus, [α-^{32}P]GTP-labeled $G_{h7\alpha}$ was not precipitated by nonimmune sera (*lane 2*) or by the antibody pretreated with the unlabeled $G_{h7\alpha}$ (*lane 3*). To further evaluate the homology of the $G_{h\alpha}$ family and other G-proteins, the purified G_h, G_{h7}, and the membrane extracts from rat, dog, bovine, and human heart were tested by immunoblots. As demonstrated in Fig. 4*B*, the antibody recognized $G_{h7\alpha}$, $G_{h\alpha}$, and a 74-kDa protein in rat, a 77-kDa in dog, a 78-kDa in bovine, and an 80-kDa in human heart membranes. The 78-kDa protein in bovine heart is most likely the $G_{h7\alpha}$, and the 74-kDa protein in rat heart is $G_{h\alpha}$ (see below). The 77- and 80-kDa proteins in dog

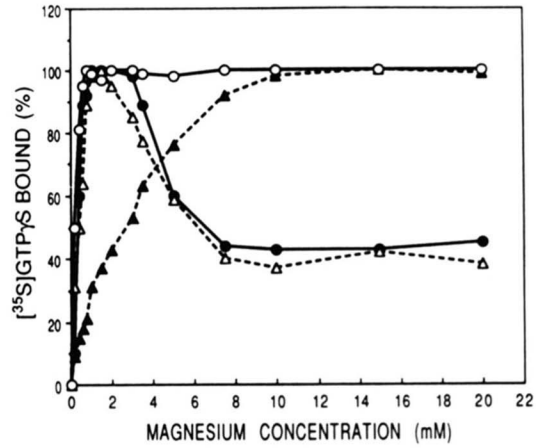

FIG. 2. **Mg^{2+} dependence of GTPγS binding by G_{h7} and G_h in different detergent solutions.** G_{h7} (1.2 pmol/tube) or G_h (1.5 pmol/tube) was incubated with various concentrations of $MgCl_2$ in either 0.05% sucrose monolaurate or 0.05% Lubrol PX at 30 °C for 1 h. The incubation buffer contained 20 mM Hepes, 100 mM NaCl, and 1 μM [^{35}S]GTPγS (specific activity, 1350 cpm/pmol). The amount of bound radiolabeled nucleotide was determined after filtration through BA 85 nitrocellulose filters, as described previously (17–19). Data shown are representative of three independent experiments, and each point is the average of duplicate determinations. GTPγS binding of G_{h7} is in Lubrol PX (●) and in sucrose monolaurate (○). The GTPγS binding of G_h is in Lubrol PX (▲) and in sucrose monolaurate (△).

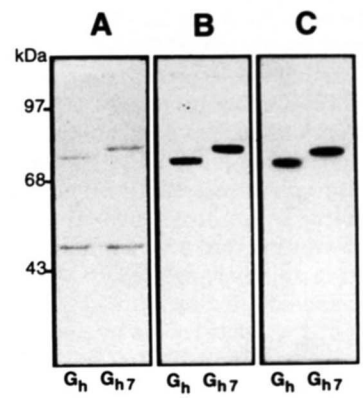

FIG. 3. **Determination of molecular mass and immunoreactivity of G_h and G_{h7}.** Purified G_{h7} was applied to a dried G-25 column to remove phosphate for photolabeling with [α-^{32}P]GTP. The samples were subjected to SDS-PAGE (7% gels). *Panel A*, Coomassie Blue staining of G_h (2 μg) and G_{h7} (2 μg). *Panel B*, an autoradiogram of G_h (0.1 μg/100 μl) and G_{h7} (0.1 μg/100 μl) which were photolabeled with 10 μCi of [α-^{32}P]GTP and 2 mM $MgCl_2$. Photolabeling was performed in an ice bath for 5 min to prevent protein cleavage by UV irradiation. *Panel C*, immunoprecipitation of G_h and G_{h7} by $G_{h7\alpha}$ antibody using protein A-agarose. G-proteins (0.1 μg/30 μl) were incubated with 10 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$ for 10 min and irradiated under 254-nm UV for 8 min, prior to performing the immunoprecipitation experiments (see "Experimental Procedures" for details).

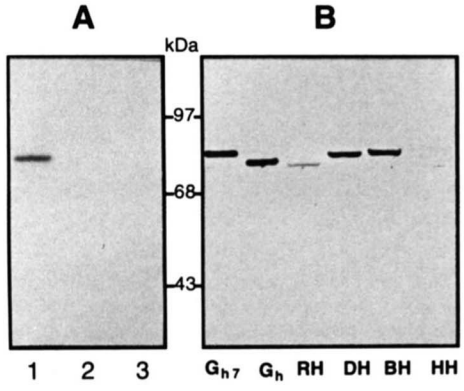

FIG. 4. **Determination of $G_{h7\alpha}$ antibody specificity by immunoprecipitation and immunoblots.** *Panel A*, immunoprecipitation of the purified $G_{h7\alpha}$. Purified $G_{h7\alpha}$ (50 ng/30 μl), labeled with 10 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$, was incubated at room temperature for 2 h with 5 μl of $G_{h7\alpha}$ antibody (*lane 1*) or nonimmune sera (*lane 2*), or with the antibody which was preincubated with unlabeled G_{h7} (*lane 3*). Preincubation with unlabeled G_{h7} and antibody was carried out at room temperature for 2 h prior to incubation with labeled G_{h7}. *Panel B*, immunoblots of the purified G_{h7}, G_h, and heart membranes from various species. Purified G_{h7} (20 ng) and G_h (20 ng) and heart membranes from rat (200 μg, *RH*), dog (200 μg, *DH*), bovine (200 μg, *BH*), and human (300 μg, *HH*) were subjected to SDS-PAGE (7% gel) and transferred to Immobilon-P. Immunoblotting was accomplished using 1:500 dilution of antibody as described under "Experimental Procedures." To evaluate the specificity of antibody, the protein-transferred Immobilon-P was pretreated with nonimmune sera (1:500 dilution) at room temperature for 1 h followed by incubation with unlabeled protein A (5 μg/ml) for 1 h. Each step of washing was carried out as described under "Experimental Procedures." The pretreated blot was subjected to immunoblotting with the antibody and ^{125}I-labeled protein A.

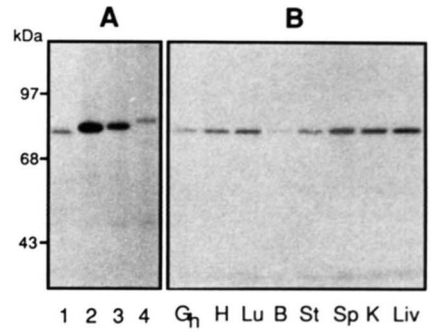

FIG. 5. **Immunoprecipitation of membrane extracts from various species' heart and rat tissue.** The heart membranes (10 mg/ml) from various species were preincubated with 5×10^{-5} M (−)-epinephrine for 30 min at 30 °C to increase the labeling of >74-kDa proteins and then extracted with 0.5% sucrose monolaurate in HED buffer containing 250 mM NaCl at 4 °C for 1 h. After centrifugation at 40,000 × *g* at 4 °C, the extracts (400 μg of protein) were incubated with 20 μCi of [α-^{32}P]GTP in the presence of 0.2 mM App(NH)p and 2 mM $MgCl_2$ for 10 min at 30 °C and then photolabeled in an ice bath for 8 min. *Panel A*, immunoprecipitation of membrane extracts from various species. The labeled samples described above were incubated with 5 μl of antibody at room temperature for 2 h; then precipitation was performed with 10 μl of protein A-agarose (see "Experimental Procedures"). The ~48-kDa bands in *lanes 2*, *3*, and *4* are probably proteolytic fragments of the corresponding G-proteins, since these bands are not observed in Figs. 4, 6, and 7. The lanes indicated are: *1*, rat; *2*, dog; *3*, bovine; *4*, human. *Panel B*, immunoprecipitation of various tissues of rat. Membranes (10 mg/ml) of various tissues of rat were preincubated with 5×10^{-5} M (−)-epinephrine for 40 min at 30 °C and then were solubilized. The extracts (200 μg) were incubated with 10 μCi of [α-^{32}P]GTP and labeled in an ice bath, prior to performing immunoprecipitation experiments, as described above. As a control, the purified G_h (20 ng) was also tested. The ~35-kDa bands are probably photoleolytic fragments, since the intensity of the bands match with of $G_{h\alpha}$. Autoradiography was for 2 days. The lanes are: G_h; *H*, heart; *Lu*, lung; *B*, brain; *St*, stomach; *Sp*, spleen; *K*, kidney; *Liv*, liver.

and human, respectively, might also be homologs of $G_{h\alpha}$ ($G_{h7\alpha}$). These results substantially demonstrate the specificity of $G_{h7\alpha}$ antibody for $G_{h7\alpha}$ and its family and its recognition of the native and denatured $G_{h\alpha}$ family proteins. Moreover, the antibody did not recognize any other proteins, indicating that the $G_{h\alpha}$ family is distinct from other G-proteins. When similar experiments were carried out with nonimmune sera or antibody preincubated with G_h or G_{h7}, the results were negative (data not shown).

To assure whether these proteins recognized by the $G_{h7\alpha}$ antibody are GTP-binding proteins, the membrane extracts from hearts of various species and various tissues of rat were examined for cross-reactivity with $G_{h7\alpha}$ antibody. Prior to immunoprecipitation, the extracts were photolabeled with [α-^{32}P]GTP in the presence of 0.1 mM App(NH)p and 2 mM $MgCl_2$. As shown in Fig. 5*A*, after immunoprecipitation with the antibody using protein A-agarose, only the range of labeled 74–80-kDa proteins were precipitated with $G_{h7\alpha}$ antibody. When extracts of the rat tissues were subjected to immunoprecipitation after labeling with [α-^{32}P]GTP, a 74-kDa molecular mass protein similar to $G_{h\alpha}$ was detected in all tissues tested (Fig. 5*B*). These results clearly indicate that the GTP-binding proteins are the same proteins recognized in the membranes from various species by immunoblots (see Fig. 4*B*) and demonstrate that these GTP-binding proteins are homologous to $G_{h\alpha}$ ($G_{h7\alpha}$) and are species-specific in molecular mass.

G-Proteins Which Couple to α_1-Adrenoceptor in the Ternary Complex Preparations—Utilizing the specific properties of the ternary complex comprised of hormone-receptor-G-protein, the coupling ability of G_h with the α_1-adrenoceptors was examined in various tissues and species. The ternary complex is a result of a sequential process in which the hormone, receptor, and G-protein become associated, forming a heterotrimeric intermediate. As a result of this process, the G-protein in the ternary complex is primed for GTP binding that is fast and occurs at 0–4 °C (17, 24). Therefore, the vesicles containing the complexes were incubated with (−)-epinephrine or (−)-epinephrine plus phentolamine at 30 °C for 30 min and then chilled in an ice bath for 10 min. To observe the specific reaction of the ternary complexes, the samples were immediately subjected to UV irradiation after the addition of 10 μCi of [α-^{32}P]GTP. The results of these studies are demonstrated in Fig. 6. Fig. 6*A* showed the hormone-mediated GTP binding with the vesicles containing the ternary complexes from rat liver and heart and bovine liver and heart. Thus, the apparent labeling of the 74-kDa proteins of either rat liver (*lane 1*) or heart (*lane 3*) obtained in the presence of (−)-epinephrine, was significantly inhibited in the presence of phentolamine (*lanes 2* and *4*). On the other hand, with the ternary complex preparations from bovine liver (*lanes 5* and *6*) and heart membranes (*lanes 7* and *8*), the labeling of 78-kDa proteins in the presence of (−)-epinephrine (*lanes 5* and *7*) was substantially attenuated by phentolamine (*lanes 6* and *8*). These data show that these different molecular mass G-proteins couple to the α_1-adrenoceptor. The residual labeling in the presence of phentolamine is probably due to incomplete blocking of the receptor by the antagonist since the α_1-receptor in the ternary complex is in the state which has high affinity for agonist (17, 18, 24). It is also possible, because of the intrinsic guanine nucleotide exchange of G_h, that it is receptor-independent. The slight differences in molecular masses of 74- and 78-kDa proteins between liver

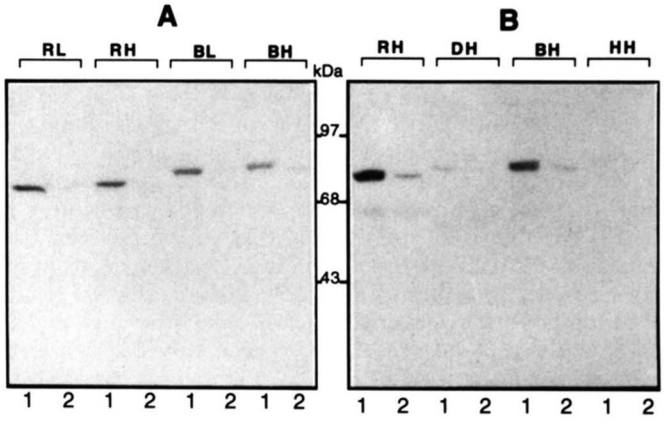

FIG. 6. **G_h family in the reconstituted ternary complex vesicles.** Partially purified ternary complexes from various sources were inserted into phospholipid vesicles (19), and the vesicles were preincubated in the presence of 5×10^{-6} M (−)-epinephrine or 5×10^{-6} M (−)-epinephrine plus 10^{-4} M phentolamine at 30 °C for 30 min. Propranolol (10^{-6} M) and rauwolscine (10^{-7} M) were also included in these reaction mixtures. The original ternary complex preparations isolated under the exact same condition were used for the following experiments without adjusting the protein concentration. *Panel A*, GTP-binding activity of the α_1-agonist-receptor-G_h ternary complexes prepared from rat liver and heart and bovine liver and heart. Vesicles containing the ternary complexes (100 μl) were photolabeled with 10 μCi of [α-^{32}P]GTP for 6 min in an ice bath under 254-nm UV irradiation. The reactions were carried out in the presence of 0.5 mM $MgCl_2$ and 0.1 mM App(NH)p in a 20 mM Hepes buffer (pH 7.4) containing 100 mM NaCl and 0.2 mM ascorbic acid. The reactions were terminated by the addition of Laemmli solution, followed by SDS-PAGE (7% gel) and autoradiography overnight. Each sample (100 μl) contained 150 fmol of α_1-receptors and 153 fmol of G-proteins from rat liver, 120 fmol of α_1-receptor and 145 fmol of G-proteins from rat heart, 137 fmol of α_1-receptor and 145 fmol of G-protein from bovine liver, and 116 fmol of α_1-receptor and 124 fmol of G-protein from bovine heart. *RL*, rat liver; *RH*, rat heart; *BL*, bovine liver; *BH*, bovine heart; *1*, (−)-epinephrine; *2*, (−)-epinephrine + phentolamine. *Panel B*, GTP-binding activity of the α_1-agonist-receptor-G_h ternary complexes from various species. These studies were performed exactly the same way as detailed above. The samples (100 μl) contained 132 fmol of α_1-receptors and 156 fmol of G-proteins from rat, 77 fmol α_1-receptors and 85 fmol G-protein from dog, 110 fmol of α_1-receptors and 148 fmol of G-proteins from bovine, and 45 fmol of α_1-receptor and 47 fmol of G-protein from human. The data shown are representative of five independent experiments. *RH*, rat heart; *DH*, dog heart; *BH*, bovine heart; *HH*, human heart; *1*, (−)-epinephrine; *2*, (−)-epinephrine + phentolamine.

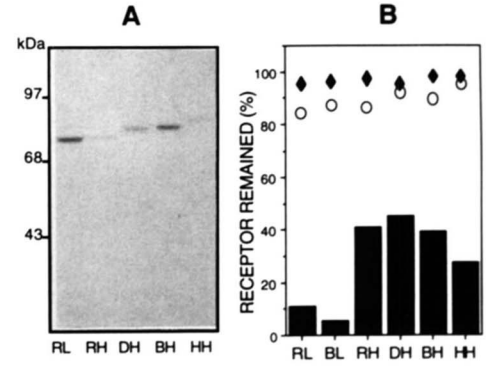

FIG. 7. **Immunological reactivity of the G-proteins in α_1-agonist-receptor-G-protein ternary complex preparation.** *Panel A*, immunoprecipitation of G-proteins in the ternary complexes from various species. The ternary complex preparations in 0.05% sucrose monolaurate were photolabeled with 10 μCi of [α-^{32}P]GTP in the presence of 0.5 mM $MgCl_2$ and 0.1 mM App(NH)p for 6 min in an ice bath. The samples (100 μl) were immunoprecipitated with 5 μl of $G_{h7\alpha}$ antibody, followed by SDS-PAGE (7% gel) and autoradiography overnight. Difference in the intensity of the G-proteins as compared to Fig. 6, is probably due to the experimental conditions, since photolabeling in this experiment was performed in the detergent solution to facilitate immunoprecipitation. The data shown are representative of three independent experiments. *RL*, rat liver; *RH*, rat heart; *DH*, dog heart; *BH*, bovine heart; *HH*, human heart. *Panel B*, co-immunoprecipitation of the α_1-adrenoceptors in the ternary complex preparations with $G_{h7\alpha}$ antibody. The density of the α_1-receptors in the ternary complex preparations was measured in the supernatant using 3 nM [^{3}H]prazosin before and after immunoprecipitation with $G_{h7\alpha}$ antibody. For these studies $G_{h7\alpha}$ antibody which was purified using protein A-agarose was further purified by hydroxylapatite column. The loaded antibody in the column was eluted using a phosphate gradient (0–300 mM). The inorganic phosphate in the eluate was removed through a dry Sephadex G-25 column (17–19). For the measurement of the amount of the receptors in the samples, (−)-epinephrine was removed by a dry Sephadex G-25 column (3 ml) equilibrated with a 20 mM Hepes buffer (pH 7.4) containing 100 mM NaCl, 1 mM EDTA, and 0.05% sucrose monolaurate. Samples were also incubated with protein A-agarose (♦) or nonimmune sera (○) to determine the specificity of the $G_{h7\alpha}$ antibody. Treatment of the ternary complex preparations with these probes did not significantly change the amount of the receptors in the supernatants. The data shown are representative of three independent experiments. *RL*, rat liver; *BL*, bovine liver; *RH*, rat heart; *DH*, dog heart; *BH*, bovine heart; *HH*, human heart.

and heart tissues are probably due to the purity of the complex preparations since it is evident that in the same species the same molecular mass of $G_{h\alpha}$ exists, as shown in Figs. 5*B* and 7*A*.

To further assess the α_1-receptor-coupled G_h family in various species preparations, the α_1-agonist-receptor-G-protein ternary complexes from rat, dog, bovine, and human heart membranes were also examined. As presented in Fig. 6*B*, (−)-epinephrine-stimulated [α-^{32}P]GTP labeling of the different molecular mass GTP-binding proteins was significantly decreased by the α_1-receptor antagonist, phentolamine. Thus, the molecular masses of GTP-binding proteins which coupled to the α_1-receptors were 74 kDa in rat heart, 77 kDa in dog heart, 78 kDa in bovine heart, and 80 kDa in human heart. To ensure that these GTP-binding proteins were the same proteins recognized by $G_{h7\alpha}$ antibody (see Figs. 4 and 5), the ternary complex preparations were photoaffinity-labeled with [α-^{32}P]GTP, incubated with $G_{h7\alpha}$ antibody, and precipitated using protein A-agarose. As shown in Fig. 7*A*, the GTP-binding proteins which coupled to the α_1-receptors were the same proteins observed above by immunoblots and immunoprecipitation. Supporting this notion, when the amounts of the receptors in the ternary complex preparations were measured in the supernatants after immunoprecipitation, the receptors remaining were 13% in rat liver and 5% in bovine liver. The preparations from rat, dog, bovine, and human heart showed a range of 28–45% of the α_1-receptors remaining (Fig. 7*B*). The samples treated with nonimmune sera or protein-A agarose did not significantly decrease the receptor density, remaining ~82–93% and ~94–100% in the supernatants, respectively (Fig. 7*B*). These data clearly demonstrate the tight association of the α_1-receptor with the G_h family proteins and the specificity of the α_1-receptor coupling to the G_h family. The reason is not clear why less precipitation of the α_1-receptor by $G_{h7\alpha}$ antibody occurs in the ternary complex preparations from the heart tissues. However, it is probably due to the instability of the ternary complex from heart tissues or because of the proteolytic fragments of the related proteins as seen in rat and dog hearts (Fig. 6*B*). It is also possible that other α_1-receptor subtypes present in this tissue are not coupling to G_h (5, 25).

DISCUSSION

To further understand the α_1-receptor-G_h coupling mechanism, we have made three different determinations. 1) A new

high molecular mass GTP-binding regulatory protein (G_{h7}) was purified from bovine heart membranes. Its properties and the associated protein (β-subunit) were compared with those of the previously characterized G_h (18). 2) The G-protein which coupled to the α_1-adrenoceptor was further characterized in different species and tissues. 3) Using $G_{h7\alpha}$ antibody raised against $G_{h7\alpha}$, the coupling ability of the α_1-receptor with the G_h family was evaluated.

We have previously used well established procedures for the purification of G_h to compare the biophysical properties with well characterized heterotrimeric G-proteins (G_s, G_i, and G_o). For the purification of the 78-kDa GTP-binding protein ($G_{h7\alpha}$), the change in extraction procedure and the GTP-photolabeling of GTP-binding proteins made it possible to purify this protein. As shown in Table I, $G_{h7\alpha}$ is one of the rare GTP proteins. The amount of this protein present, based on the final yield, was estimated to be 0.26% of the GTP-binding proteins and 0.0065% of membrane proteins. The simple salt extraction procedure resulted in the increase of $G_{h7\alpha}$ protein to 20% of the GTP-binding proteins and 0.065% of proteins in solution. In the case of the 74-kDa $G_{h\alpha}$, $G_{h\alpha}$ protein was 0.42% of GTP-binding proteins and 0.022% of membrane proteins (18). The existence of greater amount of $G_{h\alpha}$ in the rat liver membranes is probably due to the purity of the membrane preparations since we used the rat liver membranes purified by Percoll gradient centrifugation for $G_{h\alpha}$ and crude bovine heart membranes for $G_{h7\alpha}$. Extraction of $G_{h7\alpha}$ with a rather low concentration of salt also suggested that this protein is not tightly associated with membranes. $G_{h7\alpha}$ purified from bovine heart was associated with a 50-kDa protein which showed the same molecular mass as $G_{h\beta}$ (Fig. 3*A*) and did not bind GTP (Fig. 3*B*). The tight association of 50-kDa protein with $G_{h7\alpha}$ or $G_{h\alpha}$ leads to the suggestion that >70-kDa GTP-binding proteins probably associate with a distinct ~50-kDa protein(s) which is different from ~40–50-kDa GTP-binding proteins and ras-like GTP-binding proteins. Biochemical studies[3] with the 50-kDa protein from both sources on GTPase and GTPγS-binding activities of $G_{h\alpha}$ and $G_{h7\alpha}$ revealed that these enzyme activities of $G_{h7\alpha}$ and $G_{h\alpha}$ were modulated biphasically by both 50-kDa proteins and that these proteins can cross-talk, resulting in the same biphasic modulation by changing the affinity of the α-subunits for GTP *versus* GDP.

Overall biophysical and biochemical properties of G_{h7} were very similar to those of G_h, except for their magnesium ion requirement in different detergent solutions (Fig. 2) and the molecular masses of their GTP-binding α-subunits (Fig. 3). The homology between G_h and G_{h7} was evaluated using $G_{h7\alpha}$ antibody which recognized the native (Figs. 3*C* and 4*A*) and denatured $G_{h\alpha}$ and $G_{h7\alpha}$ (Fig. 4*B*). The $G_{h7\alpha}$ antibody cross-reacted with $G_{h\alpha}$ with similar affinity, indicating that these proteins are homologous (Fig. 3*C*). As expected, this antibody recognized different molecular mass GTP-binding proteins in different species: 74 kDa in rat, 77 kDa in dog, 78 kDa in bovine, and 80 kDa in human (Figs. 4*B*, 5*A*, and 7*A*). On the other hand, various tissues from the same species contained the same molecular mass proteins (Figs. 4*B* and 5*B*). Since these proteins are homologous to $G_{h\alpha}$, they 1) would be GTP-binding proteins and 2) may couple to the α_1-adrenoceptor. These postulations were tested in two different ways. First, the immunoprecipitation studies with [α-^{32}P]GTP-labeling in tissue extracts from these species clearly showed the presence of ≥74-kDa radiolabeled proteins whose molecular masses matched with those mentioned above. Second, the studies with the α_1-agonist-receptor-G-protein ternary complexes from various species revealed that the agonist-activated [α-^{32}P]GTP-labeling of a group of ≥74-kDa GTP-binding proteins was substantially inhibited by the α_1-antagonist, indicating that these G-proteins couple to α_1-receptors (Fig. 6). Furthermore, the $G_{h7\alpha}$ antibody recognized all α_1-adrenoceptor-coupled high molecular mass GTP-binding proteins in the ternary complex preparations from different species and tissues (Fig. 7, see also Fig. 6). Coupling of the α_1-receptors to these G-proteins was further confirmed by the co-immunoprecipitation of the α_1-receptor in the ternary complex preparations with $G_{h7\alpha}$ antibody (Fig. 7*B*). These consistent results clearly support the notion that G_h, G_{h7}, and other high molecular mass G-proteins of different species from rat to human are homologous as well as analogous and again confirm our previous observations (17–19) that the G_h family is the signal mediator of the α_1-receptor to the effector. Furthermore, the purified G_{h7} effectively stimulated purified phospholipase C through activation of the α_1-receptor purified from rat liver (see our companion study (26)).

Our observations showed the existence of a high molecular mass G-protein family (G_h family) which has a species-specific molecular mass and which couples to the α_1-adrenoceptor, probably the α_{1b} subtype, since the α_{1b}-receptor exists homogeneously in liver and in a significant amount in heart (5). The reason for species-specific differences should be further studied. However, the possibility of changes in the DNA level, such as by alternative splicing, may be excluded, because the same species contains the same molecular mass G_h, regardless of tissue origin. Thus, post-transcriptional modification and differences in amino acid alignment are the more probable explanations. Additionally, although our data clearly demonstrate that the G_h family is a signal mediator of the α_1-adrenoceptor, a possibility that other G-proteins, such as Gq or the G_q family, also couple to the α_1-receptors has been reported (14–16), suggesting that coupling ability of the α_1-receptors may differ depending upon reconstitution systems (*in vivo versus in vitro*), the source of protein, or external stimuli concentration (in this regard, see our companion study (26) for detailed discussion).

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