Evidence That the \( G_h \) Protein Is a Signal Mediator from \( \alpha_1 \)-Adrenoceptor to a Phospholipase C

I. IDENTIFICATION OF \( \alpha_1 \)-ADRENOCEPTOR-COUPLED \( G_h \) FAMILY AND PURIFICATION OF \( G_{\alpha_7} \) FROM BOVINE HEART*

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Our previous studies on \( \alpha_1 \)-adrenoceptor-mediated signaling suggested that \( G_h \) is a signal mediator. \( G_h \) consists of a 74-kDa GTP-binding \( \alpha \)-subunit and a 50-kDa \( \beta \)-subunit. Studies using the \( \alpha \)-agonist-receptor-G-protein ternary complexes from various tissues and species revealed that the intensity (GTP-binding) of the [\( \alpha \]GTP]GTP-labeled proteins resulting from activating the \( \alpha_1 \)-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 (\( G_{\alpha_7h} \)) in bovine heart and liver, and 80 kDa in human heart. Supporting these observations, a specific antibody to \( G_{\alpha_7h} \), not only recognized these GTP-binding proteins in the ternary complex preparations, but also co-immunoprecipitated \( \alpha_1 \)-adrenoceptors, indicating a tight association of these GTP-binding proteins with the \( \alpha_1 \)-adrenoceptor. These results also demonstrate that functional and structural similarities exist among these GTP-binding proteins. Additionally, one of the identified G-proteins (termed \( G_{\alpha_7} \)) was purified from bovine heart. \( G_{\alpha_7} \) 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 \( \alpha_1 \)-, \( \alpha_2 \)-, and \( \beta \)-adrenoceptors. Although \( \beta \)-adrenoceptors are classified into three types, the \( \beta_1 \)-, \( \beta_2 \)-, and \( \beta_3 \)-receptors, the transmembrane signaling of these receptors shares a common pathway involving \( G_h \) and adenyl cyclase (1, 2). The \( \alpha_1 \)- and \( \alpha_2 \)-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 \( \alpha_1 \)-adrenoceptors have indicated that at least two subtypes of the \( \alpha_1 \)-receptors exist; these subtypes are designated \( \alpha_{1a} \) and \( \alpha_{1b} \). Based on biochemical studies with various tissues and cell types, two signal pathways of the \( \alpha_1 \)-receptors can clearly be observed. Stimulation of \( \alpha_{1b} \)-receptors 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 A2 is stimulated by the \( \alpha_{1a} \)-receptor through a pertussis toxin-sensitive G-protein (5, 10). Recently, the \( \alpha_1 \)-receptor subtypes have been cloned including \( \alpha_{1a} \)–, \( \alpha_{1b} \)-, and \( \alpha_{1c} \)-receptors (6, 7, 11, 12), and a new member designated as type \( \alpha_{1d} \) cloned by Perez et al. (13). Transfection of cells with cDNAs encoding the \( \alpha_{1a} \)-, \( \alpha_{1b} \)-, and \( \alpha_2 \)-receptors stimulated the hydrolysis of phosphatidylinositol 4,5-biphosphate via phospholipase C-\( \beta_1 \) through the activation of the Gq family (14) (see also Refs. 15 and 16).

We previously reported that in rat liver \( \alpha_1 \)-receptor (possibly the \( \alpha_{1a} \) type) coupled to a 74-kDa GTP-binding protein. This GTP-binding protein was identified by inducing the \( \alpha_1 \)-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 \( G_{\alpha_7} \) consisted of the 74-kDa GTP-binding protein (\( \alpha \)-subunit) and a \(~50-kDa protein (\( \beta \)-subunit) (18). We have also shown that \( G_{\alpha_7} \) could be purified from bovine heart; 2) (\( \pm \))-epinephrine-\( \alpha_1 \)-receptor-G, ternary complexes from various species were induced and isolated; 3) \( G_{\alpha_7} \)-specific antibody was used to assess the direct interaction of the \( G_{\alpha_7} \) family with the \( \alpha_1 \)-receptor. Herein, we have demonstrated that the \( G_{\alpha_7} \) family which couples to the \( \alpha_1 \)-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 P-40 were from NP Biochemicals, Inc. (Cleveland, OH). bovine heart phosphatidylinositol 4,5-biphosphate was a gift from Dr. H. Niehaus, University of California, Los Angeles, CA. [\( \alpha \]GTP]GTP was purchased from New England Nuclear, Boston, MA. [\( \alpha \]GTP]GTP-AlexaFluor488 was purchased from Molecular Probes, Eugene, OR.

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other nucleotides were obtained from Boehringer Mannheim. The protein A (30 mCi/mg of protein A) were obtained from Amersham. Column chromatographic resins were obtained from Pharmacia LKB. Liver membranes were prepared by Percoll gradient centrifugation (1,120 ml) was collected and diluted 4-fold 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 alpha-4 was purified from rat liver membranes isolated by Percoll gradient centrifugation, as described previously (17, 18). 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 incorporation of 0.1 mM ATP and 2 mM MgCl2 into vesicles was 65-70%.

**RESULTS**

Purification of the 78-kDa GTP-binding Protein (G80) from Bovine Heart—The 78-kDa GTP-binding protein (a-subunit of G80) was isolated by previously described G80 purification method with some modifications (18) (see also Ref. 19). The purification modifications were made: 1) Protein was solubilized without detergent, but the purification of the G1 alpha 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 G1 alpha was achieved without using hydrophobic resin. Throughout the purification, the 78-kDa protein was monitored by photoaffinity labeling with [32P]GTP as well as by [35S]GTPγS binding. The purification was 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-denaturing conditions. The samples were then transferred to LDB containing antibody (1:500 dilution), and the antigen-antibody complexes were precipitated 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 CaCl2, 0.02% NaN3, 0.2% NP-40, 50 mM Tris/HC1, pH 8.0, containing 5% nonfat dry milk) for 2 h at room temperature. The blotted proteins 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 x 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 x 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 x 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 G80-containing fractions (135 ml, fractions 49–63 of peak 11) 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 9-ml fractions were collected at a flow rate of 25 ml/h. The G80-containing fractions (78 ml, fractions 27–65) which were used for the following experiments were diluted with 80 ml of HED buffer and loaded onto a Q-Sepharose column (1.4 x 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). Proteins were collected at 1 ml/step with a flow rate of 30 ml/h. 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.

**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-32P]GTP. Panel B, Ultrogel AcA 34 chromatography. The 78-kDa GTP-binding protein was eluted in a range of 340–380 mM (fractions 53–70). 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 350–400 mM salt concentrations (peak II, fractions 49–63). Panel D, hydroxyapatite chromatography. The 78-kDa GTP-binding protein was eluted at a concentration of 30–50 mM phosphate. See “Experimental Procedures” for details.

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-32P]GTP in the presence of 2 mM MgCl2, 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 Gs purified from rat liver membranes with Q-Sepharose (Fig. 1A). However, the 78-kDa protein from gel filtration column was eluted somewhat later fractions (at least more than 10 fractions) than Gs (Fig. 1B, 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. 1C). Further purification and concentration of the 78-kDa GTP-binding protein were achieved using a hydroxyapatite column, as shown in Fig. 1D.

The overall results of the Gs protein purification scheme are summarized in Table I. Based on the specific [35S]GTPγS-binding activity, the Gs protein obtained after the last purification step was of 80% purity. The yield of Gs was ~20% compared with that of salt extraction. Based on the final recovery, the amount of Gs was 0.26% of total G-proteins and 0.0065% of the total protein in the membranes. The 78-kDa GTP-binding protein (Gs) was copurified with a 50-kDa protein (G50), which has the same molecular mass as Gs and does not bind GTP (see Fig. 3, A and B). Differences in the molecular masses between Gs and G50 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.

Table I

| Purification of Gs from bovine heart |
|-------------------------------------|
| Steps            | Volume | Protein | [35S]GTPγS Bound | Specific Activity | Recovery |
|------------------|--------|---------|-----------------|------------------|---------|
| 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            | 0.8%    |
| 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      | 0.85    | 2.97            | 4,569.2          | 0.26    |

Biochemical Properties of Gs—The specificity of the nucleotide binding was determined by the photolabeling method. See “Experimental Procedures” and “Results” for details.
in the presence of various nucleotides. The order of inhibition of the photolabeling of Gh7 with \([\alpha-\text{32P}]\text{GTP}\) by nucleotides was GTPyS > GDP > GTP >> ATP = App(NH)p. At 100 nM \([\alpha-\text{32P}]\text{GTP}\), photolabeling was completely abolished with 80 nM of GTPyS and 200 nM of GDP, whereas 500 times excess of ATP or App(NH)p did not inhibit the labeling of Gh7. With the GTPyS-binding assay using 1 \(\mu\)M \([\text{32S}]\text{GTP}\), the half-maximal inhibition by the nucleotides was GTP \approx 15 \(\mu\)M, GDP \approx 45 \(\mu\)M, ITP \approx 130 \(\mu\)M. Titrating \([\text{32S}]\text{GTP}\) concentrations, the half-maximal binding of this ligand by Gh7 was 0.3-0.4 \(\mu\)M and saturation binding was obtained at 1.0-1.2 \(\mu\)M GTPyS. These results demonstrate that Gh7 is a specific GTP-binding protein and has an affinity for the ligands similar to Gh.

With the GTPyS-binding assay using specific GTP-binding protein and has an affinity for the ligands similar to Gh7, we compared the magnesium ion requirement for GTPyS-binding of Gh7 and Gh7. As shown in Fig. 2, when the GTPyS-binding assays were carried out in 0.05% Lubrol PX solution, the maximal ligand binding was observed at 1-3 mM MgCl₂ for Gh7 and 10-20 mM for Gh7, which was similar to that previously reported (18). The ligand binding by Gh7 was subsequently inhibited \(\approx 55\%\) of the maximal when the Mg\(^{2+}\) concentration was further increased. On the other hand, when the GTPyS-binding experiments were performed in 0.05% sucrose monolaurate solution, the maximal ligand binding by Gh7 was obtained at \(\approx 0.2 \text{ mM MgCl}_2\) and did not change with any further increases of the metal ion concentration (0.2-20 mM), whereas Mg\(^{2+}\) requirement for the maximal GTPyS-binding by Gh7 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 \(\approx 60\%\) of the maximal ligand binding. However, GTPyS-binding by Gh7 was not completely inhibited by further increases of MgCl₂ up to 50 mM. In the absence of MgCl₂, the GTPyS-binding by Gh7 could not be detected in either Lubrol PX or sucrose monolaurate solution. These data indicate that the different amounts of magnesium ion requirement for GTPyS 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 Gh7 and Gh7 were measured, the turnover of GTPase activity of Gh7 was slower than that of Gh7. Thus, Gh7 and Gh7 hydrolyzed GTP; the turnover numbers were 2-3 and \(\approx 0.25-0.6\) min\(^{-1}\), respectively. It is not yet clear whether the different turnover of GTPase activities is specific for these G-proteins, since the isolated Gh7 protein was more unstable than Gh7, although these proteins are structurally and functionally similar (see below). In addition, Gh7 was not a toxin substrate to be ADP-ribosylated either by cholera or pertussis toxin, even with the addition of \(\gamma\)-subunits of the heterotrimeric G-proteins (data not shown).

**Immunological Cross-reactivity of Gh7, Antibody**—As mentioned above, overall biophysical and biochemical properties of Gh7 are similar to Gh. The distinct difference is in the molecular masses of the GTP-binding \(\alpha\)-subunits (Fig. 3, A and B), whereas the 50-kDa \(\beta\)-subunits of Gh7 and Gh have the same molecular mass (Fig. 3A). To assess whether Gh7 is distinct from Gh, an antibody raised against the native Gh7 protein was used to test the immunological cross-reactivity. As demonstrated in Fig. 3C, when Gh and Gh7 were subjected to immunoprecipitation, the Gh7 protein was also effectively immunoprecipitated, indicating that Gh and Gh7 are homologs. The specificity of Gh7, antibody was demonstrated in Fig. 4. The \([\alpha-\text{32P}]\text{GTP}\)-labeled Gh7 was incubated with gh7, antibody, nonimmune sera, or the antibody preincubated with unlabeled Gh7. The results revealed that the Gh7 protein was specifically recognized by the Gh7, antibody (Fig. 4A, lane 1). Thus, \([\alpha-\text{32P}]\text{GTP}\)-labeled Gh7, was not precipitated by nonimmune sera (lane 2) or by the antibody pretreated with the unlabeled Gh7 (lane 3). To further evaluate the homology of the Gh7 family and other G-proteins, the purified Gh7, and the membrane extracts from rat, dog, bovine, and human heart were tested by immunoblots. As demonstrated in Fig. 4B, the antibody recognized Gh7, Ghar, 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 Gh7, and the 74-kDa protein in rat heart is Ghar (see below). The 77- and 80-kDa proteins in dog

**Fig. 2.** Mg\(^{2+}\) dependence of GTPyS binding by Gh7 and Gh in different detergent solutions. Gh7 (1.2 pmol/tube) or Gh (1.5 pmol/tube) was incubated with various concentrations of MgCl₂ 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 \(\mu\)M \([32S]\text{GTPyS}\) (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. GTPyS binding of Gh7, is in Lubrol PX (○) and in sucrose monolaurate (○). The GTPyS binding of Gh is in Lubrol PX (■) and in sucrose monolaurate (Δ).

**Fig. 3.** Determination of molecular mass and immunoreactivity of Gh7 and Gh7. Purified Gh7 was applied to a dried G-25 column to remove phosphate for photolabeling with \([\alpha-\text{32P}]\text{GTP}\). The samples were subjected to SDS-PAGE (7% gels). Panel A, Coomassie Blue staining of Gh (2 pg) and Gh (2 pg). Panel B, an autoradiogram of Gh (0.1 pg/100 \(\mu\)l) and Gh (0.1 pg/100 \(\mu\)l) which were photolabeled with 10 \(\mu\)Ci of \([\alpha-\text{32P}]\text{GTP}\) and 2 mM MgCl₂. Photolabeling was performed in an ice bath for 5 min to prevent protein cleavage by UV irradiation. Panel C, immunoprecipitation of Gh and Gh7 by Gh7, antibody using protein A-agarose. G-proteins (0.1 pg/30 \(\mu\)l) were incubated with 10 \(\mu\)Ci of \([\alpha-\text{32P}]\text{GTP}\) in the presence of 2 mM MgCl₂ for 10 min and irradiated under 254-nm UV for 8 min, prior to performing the immunoprecipitation experiments (see "Experimental Procedures" for details).
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**Fig. 4. Determination of G\textsubscript{7}\text{a} antibody specificity by immunoprecipitation and immunoblots.** Panel A, immunoprecipitation of the purified G\textsubscript{7}\text{a}. Purified G\textsubscript{7}\text{a} (50 ng/20 \mu l), labeled with 10 \( \mu \)Ci of \( [\alpha-32P] \)GTP in the presence of 2 mM MgCl\textsubscript{2}, was incubated at room temperature for 2 h with 5 \( \mu l \) of G\textsubscript{7}\text{a} antibody (lane 1) or nonimmune sera (lane 2), or with the antibody which was preincubated with unlabeled G\textsubscript{7} (lane 3). Preincubation with unlabeled G\textsubscript{7} and antibody was carried out at room temperature for 2 h prior to incubation with labeled G\textsubscript{7}. Panel B, immunoblots of the purified G\textsubscript{7}, G\textsubscript{a}, and heart membranes from various species. Purified G\textsubscript{7} (20 ng) and G\textsubscript{a} (20 ng) and heart membranes from rat (200 \( \mu \)g, RH), dog (200 \( \mu \)g, DH), bovine (200 \( \mu \)g, BH), and human (300 \( \mu \)g, 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 \textquotedblleft Experimental Procedures.” To evaluate the specificity of antibody, the protein-transferred Immobilon-P was pre-treated with nonimmune sera (1:500 dilution) at room temperature for 1 h followed by incubation with unlabeled protein A (5 \( \mu g/ml \)) for 1 h. Each step of washing was carried out as described under \textquotedblleft Experimental Procedures.” The pre-treated blot was subjected to immunoblotting with the antibody and \( [\alpha-32P] \)-labeled protein A.

and human, respectively, might also be homologs of G\textsubscript{b} (G\textsubscript{b}\text{a}). These results substantially demonstrate the specificity of G\textsubscript{b}\text{a} antibody for G\textsubscript{b} and its family and its recognition of the native and denatured G\textsubscript{b} family proteins. Moreover, the antibody did not recognize any other proteins, indicating that the G\textsubscript{b} family is distinct from other G-proteins. When similar experiments were carried out with nonimmune sera or antibody preincubated with G\textsubscript{b} or G\textsubscript{b7}, the results were negative (data not shown).

To assure whether these proteins recognized by the G\textsubscript{b}\text{a} 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\textsubscript{b}\text{a} antibody. Prior to immunoprecipitation, the extracts were photolabeled with \( [\alpha-32P] \)GTP in the presence of 0.1 mM App(NH)p and 2 mM MgCl\textsubscript{2}. As shown in Fig. 5A, after immunoprecipitation with the antibody using protein A-agarose, only the range of labeled 74-80-kDa proteins were precipitated with G\textsubscript{b}\text{a} antibody. When extracts of the rat tissues were submitted to immunoprecipitation after labeling with \( [\alpha-32P] \)GTP, a 74-kDa molecular mass protein similar to G\textsubscript{b} was detected in all tissues tested (Fig. 5B). These results clearly indicate that the GTP-binding proteins are the same proteins recognized in the membranes from various species by immunoblots (see Fig. 4B) and demonstrate that these GTP-binding proteins are homologous to G\textsubscript{b} (G\textsubscript{b}\text{a}) and are species-specific in molecular mass.

**G-Proteins Which Coupled to \( \alpha \)-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\textsubscript{a} with the \( \alpha \)-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 phenolamine 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 \( \mu \)Ci of \( [\alpha-32P] \)GTP. The results of these studies are demonstrated in Fig. 6. Fig. 6A 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 phenolamine (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 phenolamine (lanes 6 and 8). These data show that these different molecular mass G-proteins couple to the \( \alpha \)-adrenoceptor. The residual labeling in the presence of phenolamine is probably due to incomplete blocking of the receptor by the antagonist since the \( \alpha \)-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\textsubscript{a}, that it is receptor-independent. The slight differences in molecular masses of 74- and 78-kDa proteins between liver...
cubated in the presence of preparations since it is evident that in the same species the shown are representative of five independent experiments.

protein from bovine heart.

inserted into phospholipid vesicles with heart; these reaction mixtures. The original ternary complex preparations from rat, dog, bovine, and heart tissues are probably due to the purity of the complex fmol of al-receptor and heart membranes were also examined. As presented in Fig. 6B, (−)-epinephrine-stimulated [α-32P]GTP labeling of the different molecular mass GTP-binding proteins was significantly decreased by the al-receptor antagonist, phentolamine. Thus, the molecular masses of GTP-binding proteins which coupled to the al-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投资人 antibody (see Figs. 4 and 5), the ternary complex preparations were photoaffinity-labeled with [α-32P]GTP, incubated with G投资人 antibody, and precipitated using protein A-agarose. As shown in Fig. 7A, the GTP-binding proteins which coupled to the al-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 samples treated with nonimmune sera or protein-A agarose did not significantly change the amount of the receptors in the supernatants. The data shown are representative of three independent experiments. RL, rat liver; RH, rat heart; DH, dog heart; BH, bovine heart; HH, human heart.

DISCUSSION

To further understand the al-receptor-G投资人 coupling mechanism, we have made three different determinations. 1) A new
high molecular mass GTP-binding regulatory protein (G_\text{z4}) was purified from bovine heart membranes. Its properties and the associated protein (\alpha-subunit) were compared with those of a previously characterized G_\text{z4} (18). 2) The G-protein which coupled to the \alpha_1-adrenergic receptor was further characterized in different species and tissues. 3) Using G_\text{z7a} antibody raised against G_\text{z7a}, the coupling ability of the \alpha_1-receptor with the G_\text{z4} family was evaluated.

We have previously used well established procedures for the purification of G_\text{z4} to compare the biophysical properties with well characterized heterotrimeric G-proteins (G_\text{z4}, G_\text{z6}, and G_\text{z7a}). For the purification of the 78-kDa GTP-binding protein (G_\text{z7a}), 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_\text{z7a} 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_\text{z7a} protein to 20% of the GTP-binding proteins and 0.065% of proteins in solution. In the case of the 74-kDa G_\text{z7b}, G_\text{z7b} protein was 0.42% of GTP-binding proteins and 0.022% of membrane proteins (19). The existence of greater amount of G_\text{z7b} 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_\text{z7b}, and crude bovine heart membranes for G_\text{z7b}. Extraction of G_\text{z7b} with a rather low concentration of salt also suggested that this protein is not tightly associated with membranes. G_\text{z7b} purified from bovine heart was associated with a 50-kDa protein which showed the same molecular mass as G_\text{z7b} (Fig. 3A) and did not bind GTP (Fig. 3B). The tight association of 50-kDa protein with G_\text{z7b}, or G_\text{z7a}, 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 with the 50-kDa protein from both sources on GTPase and GTP\text{S-binding activities of G_\text{z7b} and G_\text{z7a}} revealed that these enzyme activities of G_\text{z7b} and G_\text{z7a} were modulated biphasically by both 50-kDa proteins and that these proteins can cross-talk, resulting in the same molecular masses of their GTP-binding \alpha-subunits (Fig. 3).

The homology between G_\text{z4} and G_\text{z7a} was evaluated using G_\text{z7a} antibody which recognized the native (Figs. 3C and 4A) and denatured G_\text{z4} and G_\text{z7a} (Fig. 4B). The G_\text{z7a} antibody cross-reacted with G_\text{z4} with similar affinity, indicating that these proteins are homologous (Fig. 3C). 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. 4B, 5A, and 7A). On the other hand, various tissues from the same species contained the same molecular mass proteins (Figs. 4B and 5B). Since these proteins are homologous to G_\text{z7a}, they 1) would be GTP-binding proteins and 2) may couple to the \alpha_1-adrenergic receptor. These postulations were tested in two different ways. First, the immunoprecipitation studies with [\alpha-\text{\text{\textsuperscript{32}}P}]GTP-labeling in tissues extracts from these species clearly showed the presence of \text{>74-kDa radiolabeled proteins whose molecular masses matched with those mentioned above. Second, the studies with the \alpha_1-agonist-receptor-G-protein ternary complexes from various species revealed that the agonist-activated [\alpha-\text{\text{\textsuperscript{32}}P}]GTP-labeling of a group of \text{>74-kDa GTP-binding proteins was substantially inhibited by the \alpha_1-antagonist, indicating that these G-proteins couple to \alpha_1-receptors (Fig. 6). Furthermore, the G_\text{z7a} antibody recognized all \alpha_1-adrenergic receptor-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 \alpha_1-receptors to these G-proteins was further confirmed by the co-immunoprecipitation of the \alpha_1-receptor in the ternary complex preparations with G_\text{z7a} antibody (Fig. 7B). These consistent results clearly support the notion that G_\text{z4}, G_\text{z7b}, 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_\text{z4} family is the signal mediator of the \alpha_1-receptor to the effector. Furthermore, the purified G_\text{z4} effectively stimulated purified phospholipase C through activation of the \alpha_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_\text{z4} family) which has a species-specific molecular mass and which couples to the \alpha_1-adrenergic receptor, probably the \alpha_1 subtype, since the \alpha_1-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_\text{z4} 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_\text{z4} family is a signal mediator of the \alpha_1-adrenergic receptor, a possibility that other G-proteins, such as G_\text{z4} or the G_\text{z7a} family, also couple to the \alpha_1-receptors has been reported (14–16), suggesting that coupling ability of the \alpha_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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