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Distinct pH Modulation for Dual Function of $G\alpha_h$ (Transglutaminase II)

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$G\alpha_h$, also known as transglutaminase II, has GTPase as well as transglutaminase activities. To better understand the factors affecting these dual enzymatic activities, we examined the optimal pH (at 25°C) and thermal stability (at 37°C) of the activities using membranous $G\alpha_h$ from mouse heart. The optimum pH for the GTPase activity of $G\alpha_h$ is ~7.0. As well, the GTP binding activity of $G\alpha_h$ is more thermostable at pH 7.0 than that at pH 9.0. Consistent with these observations on the GTPase function of $G\alpha_h$, both the phospholipase C-δ1 activity and the yield of co-immunoprecipitation of $G\alpha_h$-coupled phospholipase C-δ1 in α_1-adrenoceptor/$G\alpha_h$/phospholipase C-δ1 complex preparations were enhanced by incubation with an α_1-agonist, phenylephrine, at pH 7.0. On the other hand, the transglutaminase activity of $G\alpha_h$ is higher in the basic pH range with an optimum activity at pH ~9.0. Also, the transglutaminase activity of $G\alpha_h$ is more thermostable at pH 9.0 than that at pH 7.0. These results indicate not only pH as a modulator for the dual functions of $G\alpha_h$, but also provide direct evidence for the involvement of pH in the $G\alpha_h$-mediated α_1-adrenoceptor signaling system *in vitro*.

Key words: α_1-adrenoceptor, G-protein, GTPase, pH, transglutaminase.

$G\alpha_h$ is a bifunctional enzyme with GTPase and transglutaminase (TGase) activities (*1*). The GTPase reaction of $G\alpha_h$, which binds and hydrolyzes GTP to GDP and P_i, transfers the signals from receptors to effectors, and the subsequent association of a GDP-bound $G\alpha_h$ with calreticulin completes one cycle of signaling (*2*). $G\alpha_h$, known as TGase II (tissue TGase), is also a member of the TGase family, which catalyzes the formation of ε-(γ-glutamyl) lysine isopepetide bonds between peptide-bound glutamyl residues and the lysyl group of polypeptidse in a Ca^{2+}-dependent manner (*3*, *4*). The physiological role of the TGase function of $G\alpha_h$ remains unclear, and the regulation of isopeptide formation is completely unknown. The isopeptide-specific protease(s) has been identified. However, irreversible isopepetide formation from various proteins has been observed in diverse cellular processes, including cell differentiation, cell adhesion, cross-linking of interacting extracellular matrix and induction of apoptosis (*5*, *6*). The GTPase function of $G\alpha_h$ has been shown to be involved in the regulation of cell cycle progression (*7*) and receptor-mediated signaling. The $G\alpha_h$-coupled receptors include the $\alpha_{1B/D}$-adrenoceptor (AR) (*1*, *8*), oxytocin receptor (*9*) and thromboxaneA_2 receptor (*10*). $G\alpha_h$ selectively interacts with these receptors in a subtype-specific manner (*8*, *10*). In receptor signal transduction, $G\alpha_h$ associates with calreticulin as a β-subunit of G-protein (*2*), and the known effector for $G\alpha_h$ is phospholipase C (PLC)-δ1 (*11*, *12*). $G\alpha_h$ is involved in the activation of the large conductance Ca^{2+}-activated K^+ channel in vascular smooth muscle cells (*13*). Moreover, $G\alpha_h$ modulates intracellular Ca^{2+} *via* α_{1B}-AR signaling (*14*, *15*) as well as cell motility *via* the association of GTP-bound $G\alpha_h$ with the GFFKR motif located in integrin α subunits (*16*).

$G\alpha_h$ is distributed ubiquitously, and expressed highly in certain cells and tissues such as liver and heart (*17*). It has been found in the nucleus, cytosol, cell membrane and on the cell surface (*4*). The molecular masses of $G\alpha_h$ from various species show distinct differences (*17*). Translocation of $G\alpha_h$ from the cytosol to the membrane is observed in some pathological conditions (*18*). Recently, it was also reported that the GTP binding activity of $G\alpha_h$ is higher in the membrane than in the cytosol. On the other hand, the TGase activity of cytosolic $G\alpha_h$ is higher than that of membranous $G\alpha_h$. These reports indicate that the two distinct functions of $G\alpha_h$ are regulated by its localization, which can reflect the cellular functions of $G\alpha_h$ (*18*, *19*). Upon GTP binding to $G\alpha_h$, the TGase activity is down-regulated. On the other hand, the GTPase activity is down-regulated by Ca^{2+} binding to $G\alpha_h$ (*20*, *21*). These findings indicate that $G\alpha_h$ can adjust and regulate environmental conditions by switching between bifunctional enzyme activities, TGase and GTPase.

Understanding the factors affecting the bifunctional properties of $G\alpha_h$ is important for defining its cellular and extracellular functions. In this study, to find out factors affecting the properties of $G\alpha_h$, we examined the optimal pH and stabilities of both the GTPase and TGase activities of $G\alpha_h$, and also investigated the stimulation of PLC-δ1 at appropriate pH using α_1-AR/$G\alpha_h$/PLC-δ1 complex preparations. The results show pH as a modulator of the dual functions of $G\alpha_h$. This study also demonstrates, for the first time, direct evidence for the involvement of pH in the $G\alpha_h$-mediated α_1-AR signaling system *in vitro*.

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MATERIALS AND METHODS

This experiment was approved by the Ethical Committee for the Protection of Animals in Biomedical Research of the Institute of Medical Science, Chung-Ang University, Seoul, Republic of Korea.

Membrane Preparations—Membrane extracts were prepared from ICR mouse heart using the method of Baek *et al.* (*9*, *17*). All procedures were carried out at 0–4°C. Homogenates of mouse heart were prepared mechanically (Ultra-Turrax, Janke & Kunkel) at a ratio of 1:10 (w/v) in 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 homogenates were filtered through four layers of cheesecloth and centrifuged at 500 × *g* for 5 min. The supernatants were centrifuged again at 40,000 × *g* for 1 h. The pellets were rehomogenized and recentrifuged three times with 50 mM Hepes buffer, pH 7.5, containing 10 mM $MgCl_2$, 5 mM EGTA and the protease inhibitors listed above, and resuspended at 10 mg protein/ml in HEDG buffer (20 mM Hepes, 1 mM EGTA, 0.5 mM dithiothreitol, 10% glycerol, pH 7.5) containing 100 mM NaCl and protease inhibitors. The membrane suspensions were stored at –80°C until use. The 72-kDa $G\alpha_h$ family protein (specific activity, 24.4 nmol/mg protein) from mouse heart membrane (*19*) was purified by sequential column chromatographies using DEAE-Cellulose, Q-Sepharose and GTP-agarose (*17*, *22*).

Assay of $G\alpha_h$—$G\alpha_h$ assays were performed essentially as described previously (*9*, *17*, *20*). The TGase activity was determined by evaluating the incorporation of [^{3}H]putrescine (0.1 mM, 44.4 Ci/mmol, DuPont NEN) into *N*,*N*′-dimethylated casein (1%) in the presence of 100 μM $CaCl_2$ at 25°C for 20 min in HSD buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM DTT) (100 μl final volume). After incubation, samples were transferred to an ice-water bath, and the reaction was stopped by the addition of ice-cold 50% trichloroacetic acid (100 μl). The precipitates were trapped on GF/F glass fiber filters (Whatman).

For GTPγS binding, samples in HSD buffer containing 20 mM $MgCl_2$ and 100 μM App(NH)p were incubated with 1 μM GTPγS plus 1 μCi [^{35}S]GTPγS (1,300 Ci/mmol, DuPont NEN) in a final volume of 50 μl for 20 min at 25°C. Unlabeled GTP (1 mM) was used to determine nonspecific binding. The reaction was stopped by the addition of 4 ml of ice-cold filtration buffer (20 mM Hepes, pH 7.5, containing 100 mM NaCl and 20 mM $MgCl_2$). Bound [^{35}S]GTPγS was determined using a liquid scintillation counter (Beckman LS3200) after filtration through nitrocellulose filters (Whatman).

For photoaffinity labeling, samples were incubated with 5–10 μCi [α-^{32}P]GTP (3,000 Ci/mmol, DuPont NEN) in the presence of 2 mM $MgCl_2$ in HSD buffer. The samples were first incubated at room temperature for 20 min, and then subjected to 254 nm UV irradiation in an ice-water bath for 10 min. The GTP binding activity was then analyzed by SDS-PAGE and autoradiography. The pH of the buffer was adjusted using appropriate buffering salts, 20 mM acetate (pH 5.0–6.0), 20 mM Hepes (pH 6.5–7.5), 20 mM Tris-HCl (pH 8.0–8.5) and 20 mM Borate buffer (pH 9.0–10.0).

Assay for α_1-Adrenoceptor—The α_1-AR was purified from mouse heart membrane. Mouse heart membranes were solubilized with HEDG buffer containing 100 mM NaCl and CHAPS (final detergent concentration of 0.3%) for 1 h at 4°C. The α_1-AR was isolated using heparin-agarose and wheat germ agglutinin-agarose. The α_1-AR preparation showed no GTPγS binding or PLC activity, thus confirming its purity. The amount of α_1-AR was determined by measuring [^{3}H]prazosin (76 Ci/mmol, DuPont NEN) binding after incubation at 25°C for 20 min (*17*).

Assay for Phospholipase C-δ1—PLC-δ1 was expressed in *E. coli* DH5α cells and purified as described (*11*). The purity of the PLC-δ1 preparation was ≥90% as judged by silver staining, and neither GTPγS nor α_1-AR binding was observed. PLC activity was measured using phosphatidyl[2-^{3}H]inositol 4,5-bisphosphate ([^{3}H]PIP_2,1 Ci/mmol, DuPont NEN) as a substrate (*12*). The reaction mixture contained 20 mM Hepes (pH 7.5), 0.05% CHAPS, 10 μM $CaCl_2$, 100 mM NaCl and 50 μM [^{3}H]PIP_2 (specific activity, 700–800 cpm/nmol) in a total volume of 100 μl. The reaction was stopped by adding 0.2 ml of 1 M HCl followed by 0.75 ml of chloroform:methanol:1 M HCl (100:100:0.6). The mixtures were centrifuged at 500 × *g* for 10 min. The radioactivity in an aliquot (300 μl) of the upper phase was counted (Beckman LS3200).

Reconstitution of Proteins—Phospholipid vesicles were used to reconstitute the isolated proteins, as described previously (*9*). Phospholipid vesicles were prepared by the dilution method in which the lipid vesicles are formed by decreasing the detergent concentration in the mixture. The proteins, 15 pmol of α_1-AR, 20 ng of purified $G\alpha_h$ and 100 ng of PLC-δ1 in 0.05% sucrose monolaurate solution, were mixed with a 5 mg/ml phospholipid mixture of phosphatidylcholine:phosphatidylethanolamine:phosphatidylserine (3:1:1; final, 0.2 mg/ml) at 4°C for 30 min. Prior to reconstitution, all samples were passed through dry Sephadex G-25 columns pre-equilibrated with the assay buffer containing 0.05% sucrose monolaurate and 5% glycerol.

Immunological Experiments—Immunological cross-reaction experiments were performed essentially as described previously (*12*). Protein A–agarose (binding capacity, 25 mg human IgG/ml of agarose) was used for the co-immunoprecipitation of PLC-δ1. The anti–TGase II antibody (clone CUB 7402, NeoMarkers) was mixed with the same amount of protein A–agarose suspended in 10 ml of 0.1 mM borate buffer (pH 8.2) for 45 min at room temperature. The α_1-AR/$G\alpha_h$/PLC-δ_1 complex preparations were incubated with TGase II antibody–protein A–agarose (50 μl) with gentle rotation at 4°C for 2 h. The pellets were washed with HSD buffer and subjected to immunoblotting.

For immunoblots, proteins in pellets were separated in a 10% gel by SDS-PAGE. The resolved proteins were then transferred to an Immobilon-P membrane (Millipore). The membrane was incubated in blocking buffer (50 mM Tris-HCl, pH 8.0, 2 mM $CaCl_2$, 80 mM NaCl and 0.02% sodium azide) containing 0.2% Nonidet P-40 and 5% nonfat dry milk. After 2 h incubation at room temperature, the membranes were further incubated in the same buffer containing specific anti–TGase II or PLC-δ1 anti-

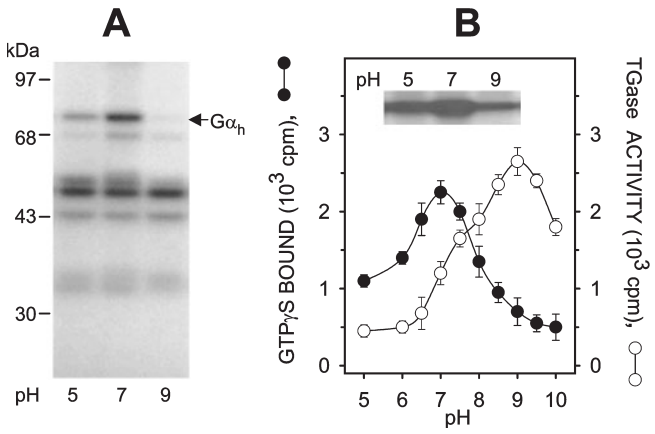

Fig. 1. **Determination of optimum pH for the TGase and GTPase activities of Gα$_h$.** (A) The optimum pH for the GTP binding activity of Gα$_h$ in solubilized membrane extracts from mouse heart. Membrane fractions of mouse heart (10 mg protein/ml) were solubilized with 0.3% CHAPS in 20 mM Hepes buffer (pH 7.5) containing 0.5 mM DTT, 10% glycerol and 100 mM NaCl at 4°C for 1 h. After solubilization, the membrane extracts (100 μg protein) were incubated in an appropriate buffer (pH 5.0, 7.0 or 9.0) with 5 μCi [α-^{32}P]GTP and 2 mM $MgCl_2$, and photolabeled with UV light (254 nm). The proteins were analyzed by SDS-PAGE (10% gel) and autoradiography. The data shown are representative of five independent experiments. Arrows show the ~72-kDa Gα$_h$. (B) pH optima for the bifunctional activities of purified Gα$_h$ from mouse heart. To determine the optimum pH for GTPγS binding by purified Gα$_h$, purified 72-kDa Gα$_h$ (20 ng) was incubated in various pH buffers containing 0.5 mM DTT, 10% glycerol, 100 mM NaCl, 2 mM Mg^{2+} and 1 μCi [^{35}S]GTPγS at 25°C for 20 min. To confirm the optimal pH for TGase activity by purified Gα$_h$, purified 72-kDa Gα$_h$ (20 ng) was incubated in various pH buffers containing 0.5 mM DTT, 10% glycerol, 100 mM NaCl, 1 mg/ml dimethyl casein, 100 μM Ca^{2+} and 1 μCi [^{3}H]putrescine at 25°C for 20 min. The results shown are means ± SD of three independent experiments performed in triplicate. Inset: pH-dependent [α-^{32}P]GTP photoaffinity labeling of purified 72-kDa Gα$_h$ from mouse heart. Photoaffinity labeling of purified Gα$_h$ (20 ng) was performed in an appropriate buffer (pH 5.0, 7.0 or 9.0) with 5 μCi [α-^{32}P]GTP and 2 mM $MgCl_2$ by UV irradiation.

body (Upstate Biotechnology Inc.) for 1 h at room temperature. The membranes were incubated with the secondary antibody (peroxidase-linked species-specific antibody, Amersham) for 1 h at room temperature. The membranes were treated with Enhanced Chemiluminescence reagent. The dried membranes were subjected to Kodak XAR-5 film.

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

RESULTS AND DISCUSSION

Optimum pH for the GTPase and Transglutaminase Activities of Gα$_h$—The pH-dependence of the GTPase activity of Gα$_h$ has not been studied, but the reported optimal pHs for TGases from different species vary between 6.0 to 9.0 (*24*, *25*). The 72-kDa GTP-binding protein from mouse heart was identified as one of the Gα$_h$ family of proteins that play roles as signal mediators of α_1-AR, but also have bifunctional enzyme activities (*19*). To evaluate the pH-dependence of the GTPase activity of Gα$_h$, the GTP binding activity of solubilized mouse heart membranes was examined by incubation in an appropriate buffer (pH 5.0, 7.0 or 9.0) in the presence of [α-^{32}P]GTP and $MgCl_2$. As shown in Fig. 1A, the GTP binding activity of 72-kDa Gα$_h$, assessed by photoaffinity labeling, was markedly more enhanced at pH 7.0 than that at either pH 5.0 or 9.0, even though the same amount of solubilized membranes were loaded. To confirm whether the amount of Gα$_h$ was same in solubilized membranes, the experimental group in Fig. 1A was subjected to immunoblotting using anti–TGase II antibody. The 72-kDa proteins showed cross-reactivity with the antibody, indicating that the proteins are homologous to Gα$_h$. Furthermore, the band density in the immunoblots was almost same (results not shown).

To ascertain whether the optimum pH for the GTPase activity is distinct from that for the TGase activity of Gα$_h$, we performed dual enzymatic assays in buffers of various pH using Gα$_h$ purified from membrane fractions of mouse heart. During purification, Gα$_h$ was detected by photochemically modifying G-proteins with [α-^{32}P]GTP and then assessing their molecular mass by SDS-PAGE and autoradiography. In addition, G-proteins were tracked throughout the purification procedures by their ability to bind irreversibly to GTPγS using [^{35}S]GTPγS as a tracer. The optimal pH for the dual enzymatic activities of Gα$_h$ differed significantly. As demonstrated in Fig. 1B, the TGase of Gα$_h$ was active in the basic pH range with an optimum activity at pH ~9.0. On the other hand, the optimum pH for the GTP binding activity was ~7.0. At pH 7.0, the GTP binding activity increased ~3-fold relative to the activity measured at pH 9.0. Consistent with these observations on GTP binding as shown in Fig. 1, A an B, the density of [α-^{32}P]GTP photoaffinity labeling of purified Gα$_h$ was higher at pH 7.0 than at pH 5.0 or 9.0 (inset in Fig. 1B). These results clearly demonstrate the differential pH optima for the dual functional enzymatic activities of Gα$_h$, GTPase and TGase. These results also suggest the possibility that pH may be one of the factors modulating these bifunctional properties.

Recently, Fleckenstein *et al.* (*26*) reported that lowering the pH decreases the reaction rate of Gα$_h$-catalyzed transamidation, whereas the rate of the deamidation reaction increases considerably. This suggests that the deamidation by Gα$_h$ more likely takes place in slightly acidic environments, indicating that pH is a factor affecting on function of Gα$_h$, as suggested by the results of Fig. 1A. We have previously reported that Gα$_h$ consistently co-purifies with a 50-kDa protein that inhibits GTP binding to Gα$_h$. The identity of this protein has been revealed to be calreticulin, a calcium binding protein. Calreticulin inhibits GTP binding to Gα$_h$ and the TGase activity of GDP-bound Gα$_h$ in an allosteric fashion (*2*). Recently, we have also shown that PLC-δ1 has a role as a modulator of the dual functions of Gα$_h$ (*14*). To confirm the associated molecules in the purified Gα$_h$ used in these experiments, we performed silver staining and immunoblotting with antibodies to PLC-δ1 and calreticulin. The purified Gα$_h$ from membrane fractions of mouse heart was not associated with any other proteins, as reported earlier (*22*).

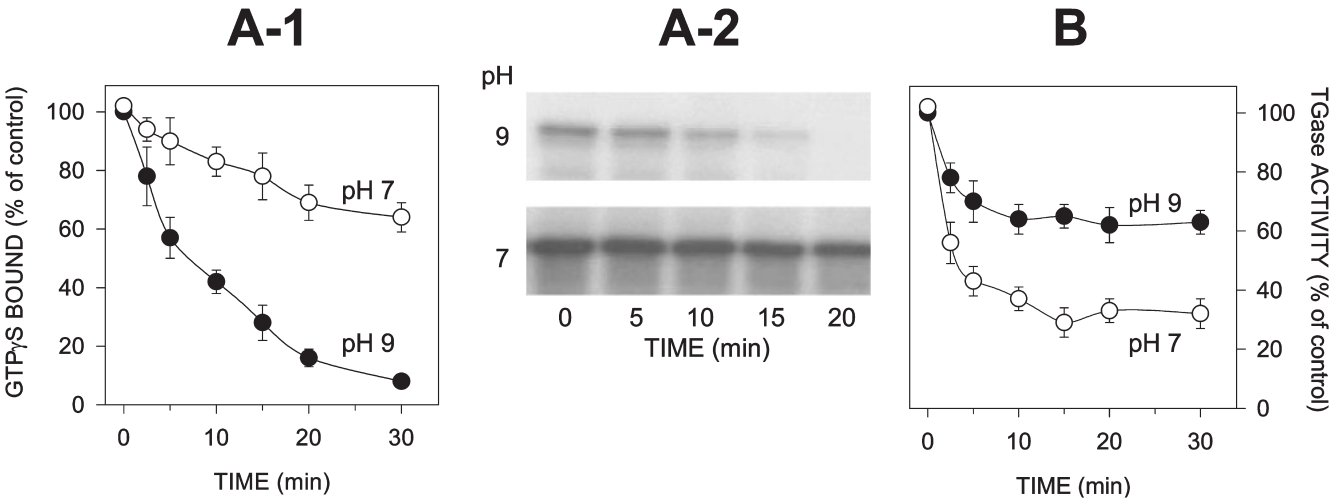

Fig. 2. **pH-Dependent thermal stability of the GTPase and TGase activities of Gα_h.** (A) Stability of GTP binding to Gα_h. Thermal stability of purified Gα_h was determined by preincubation in an appropriate buffer (pH 7.0 or 9.0) at 37°C. At the indicated time points, the GTP binding activity of preincubated-Gα_h (20 ng) was measured using the same pH buffer (pH 7.0 or 9.0) by [^{35}S]GTPγS binding (A-1) or [α-^{32}P]GTP photoaffinity labeling (A-2) in the presence of 2 mM $MgCl_2$. GTPγS binding is expressed as the percentage of GTPγS binding in an appropriate buffer (pH 7.0 or 9.0) compared with purified Gα_h without incubation at 37°C (control). The results shown in A-1 are means ± SD of three independent experiments performed in triplicate. The data shown in A-2 are representative of five independent experiments. (B) TGase stability of Gα_h. TGase stability of purified Gα_h was determined by preincubation in an appropriate buffer (pH 7.0 or 9.0) containing 0.5 mM Ca^{2+} at 37°C. After different incubation times, the TGase activity of the Gα_h (20 ng) was measured in the presence of 100 μM Ca^{2+} at 25°C for 20 min. TGase activity is expressed as a percentage of TGase activity in an appropriate buffer (pH 7.0 or 9.0) compared with purified Gα_h without incubation at 37°C (control). The results shown are means ± SD of three independent experiments performed in triplicate.

pH-Dependent Thermal Stability of the GTPase and Transglutaminase Activities of Gα_h—To further characterize the bifunctional properties of Gα_h, we investigated the pH-dependent thermal stability. The thermal stabilities of the dual enzymatic activities of Gα_h were determined by preincubation in an appropriate buffer (pH 7.0 or 9.0) at 37°C. We also included Ca^{2+} in the preincubation buffer for TGase stability, because it has been reported that Ca^{2+} has a positive effect on the thermal stability of the TGase activity (*21*). The thermal stability of the dual enzymatic activities of Gα_h differed significantly at different pHs. As shown in Fig. 2A, GTP binding to Gα_h was thermally stable at pH 7.0, but not at pH 9.0 where it decreased by ~50% within 10 min (Fig. 2A-1) and almost disappeared by 20 min (Fig. 2A-2). Calcium is an important regulator of Gα_h functions (*20–21*). When Gα_h was preincubated in the presence of Ca^{2+} at 37°C, Ca^{2+} did not protect the GTP binding of Gα_h against thermal inactivation at pH 9.0; even GTP binding at both pH 7.0 and 9.0 was more potently inhibited by Ca^{2+} (results not shown).

On the other hand, ~40% of the TGase activity of Gα_h remained after 5 min at pH 7.0, and decreased further to ~30% during prolonged preincubation at 37°C. However, the TGase activity of Gα_h at pH 9.0 remained greater than 60% (Fig. 2B). When Gα_h was preincubated in the absence of Ca^{2+} at 37°C, the TGase activity at pH 9.0 was also more stable than that at pH 7.0. A similar pattern was observed in the presence of Ca^{2+}. However, in the absence of Ca^{2+}, the remaining TGase activity decreased further with incubation time, and the time scale of activity loss was also much shorter at pH 7.0 and 9.0 (results not shown). Taken together, the results indicate that the thermal stabilities of the dual enzymatic functions of Gα_h are dependent on their optimal pH, either at pH 7.0 or 9.0.

Bergamini *et al.* (*27*) reported that human erythrocyte TGase is irreversibly inactivated by heat. Further, the rate of inactivation increases much more at alkaline pH (9.0–9.5), suggesting that specific effects take place in the alkaline pH range, possibly in relation to decreased stability of the transition-state intermediate as pH is raised above 9.0. But, no posttranslational modification of Gα_h has been observed so far, and molecular genetic analyses indicate that Gα_h is derived from a single gene, and mutated Gα_h genes have not been found. Moreover, Signorini *et al.* (*28*) observed similar molecular masses under denaturing conditions, catalytic and immunological properties for the membrane and the cytosolic Gα_h, and identity of limited proteolytic maps, and suggested that Gα_h isoforms are the same protein capable of translocation. Conformational differences in Gα_h were demonstrated by the observation that the GTP-bound Gα_h is resistant to tryptic digestion and inhibits the TGase activity, whereas the guanine nucleotide-unbound form is ineffective (*29*). This finding is also consistent with our observation in this study that GTP protects the thermal inactivation for GTPase function of Gα_h at the optimal pH 7.0, which is a maximum GTP binding state (Figs. 1B and 2A-2). The occurrence of multiple conformations of Gα_h has been also suggested by various studies (*30*). When the Gα_h from guinea pig liver was heated at 40°C, the loss of TGase activity progressed in two steps mechanism, one irreversible, and the other reversible by Ca^{2+}. Our findings in this study, as well as others, indicate that Gα_h is a flexible enzyme that adapts readily to surround-

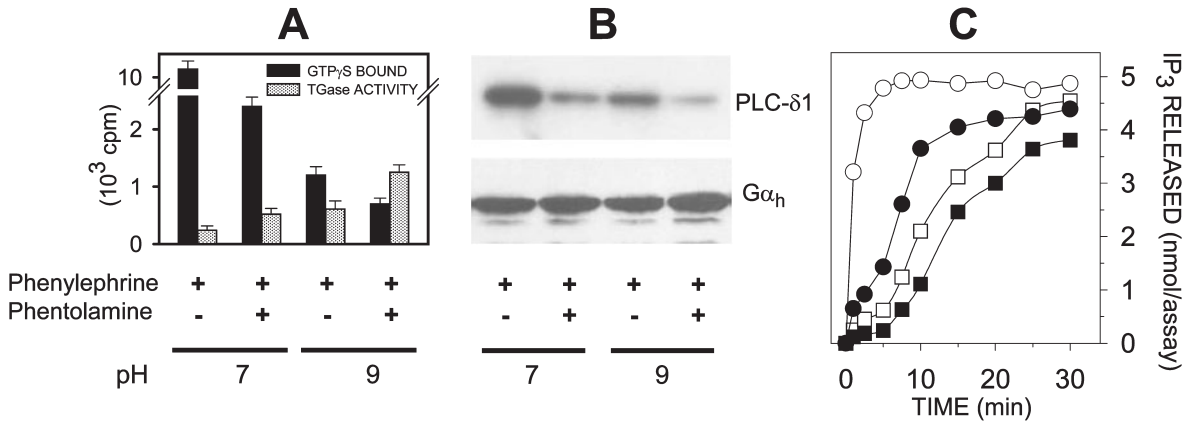

Fig. 3. **Effects of pH on α$_1$-adrenoceptor signaling in preparations of the α$_1$-adrenoceptor/Gα$_h$/PLC-δ1 complex.** Insertion of the α$_1$-adrenoceptor (10 pmol), purified Gα$_h$ (20 ng) from mouse heart membrane and PLC-δ1 (100 ng) into phospholipid vesicles was performed as described in "MATERIALS AND METHODS." The receptors were preactivated with 5 μM phenylephrine or 5 μM phenylephrine and 100 μM phentolamine at 30°C for 30 min and then chilled in an ice bath. The vesicles were then incubated in an appropriate buffer (pH 7.0 or 9.0). (A) Bifunctional activities of Gα$_h$ in reconstituted vesicles. The results shown are means ± SD of three independent experiments performed in triplicate. (B) Coupling of purified Gα$_h$ to PLC-δ1. For the total reconstitution with α$_1$-adrenoceptor, purified Gα$_h$ and PLC-δ1, the vesicles were incubated in the presence of 5 μM GTPγS, 0.5 mM $MgCl_2$ and 10 μM $CaCl_2$ at 30°C for 30 min. The reaction mixtures containing vesicles were then immunoprecipitated with anti-TGII antibody. Immunoblotting was performed using antibodies to TGII or PLC-δ1. (C) Time course of purified Gα$_h$-mediated PLC-δ1 activation. To confirm the activation of PLC-δ1, reaction mixtures including vesicles were incubated with 50 μM [^{3}H]PIP_2 at 30°C. After different incubation times, the formation of inositol phosphate by PLC-δ1 was measured. The results are representative of three independent measurements, and each point is the average of triplicate determinations. Open cirlcle, pH 7.0 + phenylephrine; solid circles, pH 9.0 + phenylephrine; open squares, pH 7.0 + phenylephrine + phentolamine; solid squares, pH 9.0 + phenylephrine + phentolamine.

ing environments and switches its intrinsic enzyme activities by conformational differences.

Effects of pH on Gα$_h$-Mediated α$_1$-Adrenoceptor Signaling—Recently PLC-δ1 was identified as an effector molecule in Gα$_h$-mediated signaling in experiments with α$_1$-AR and oxytocin receptor (*11*, *12*). Elucidation of the crystal structure of mammalian PLC-δ1 has provided a mechanism for membrane attachment and the Ca^{2+}-dependent hydrolysis of phosphoinositide (*31*). We made the reconstituted preparations with purified Gα$_h$, PLC-δ1 and α$_1$-AR to confirm the α$_1$-AR signaling system at different pHs *in vitro*. To determine whether α$_1$-AR and PLC-δ1 modulate the dual functions of Gα$_h$, we measured TGase activity and GTPγS binding at both pH 7.0 and 9.0. As shown in Fig. 3A, GTPγS binding was increased by activated α$_1$-AR and PLC-δ1 in the presence of phenylephrine at pH 7.0. Moreover, this stimulation of GTPγS binding at pH 7.0 was attenuated in the presence of phentolamine. When the above experiment was performed at pH 9.0, the results showed the same tendency as at pH 7.0, but the binding activity was much lower than that observed at pH 7.0. TGase activity in presence of phentolamine at pH 9.0 was higher than any other experimental groups. But the activity was much lower than for Gα$_h$ alone at pH 9.0. These results are consistent with Figure 1B as well as our previous report that PLC-δ1 stimulates the GTP-binding of Gα$_h$ through the action of its guanine nucleotide exchanging factor and inhibited the TGase activity of Gα$_h$ (*14*).

To evaluate the functional coupling of Gα$_h$ and PLC-δ1 in the appropriate buffers (either pH 7 or 9, the reconstituted vesicles in the presence of phenylephrine and GTPγS were immunoprecipitated with anti–TGase II antibody. The pellets were then subjected to immunoblot analysis with antibodies to TGase II and PLC-δ1. As demonstrated in Fig. 3B, PLC-δ1 coupled to Gα$_h$ which was activated by phenylephrine and GTPγS at both pH 7.0 and 9.0. However, the coupling ability between Gα$_h$ and PLC-δ1 in reconstituted preparations was distinctly higher at pH 7.0 than at pH 9.0. When a selective α$_1$-antagonist, phentolamine, was included in the reconstituted preparations, the coupling of Gα$_h$ and PLC-δ1 was attenuated. These results indicate that phentolamine effectively blocks as well as PLC-δ1 could couple to Gα$_h$ at both pH 7.0 and 9.0 in this α$_1$-AR signaling system *in vitro*. We also confirmed the presence of Gα$_h$ in the immunoprecipitated pellets by anti-TGase II antibody, and the amount of Gα$_h$ in immunoprecipitated pellets was the same.

Finally, to further investigate the possibility of the differential activation of PLC-δ1 *via* Gα$_h$ by pH, the time course of PLC-δ1 activity in response to Gα$_h$ at pH 7.0 or pH 9.0 was measured in this reconstituted preparation. PLC-δ1 activation stimulated by activated Gα$_h$ in the presence of phenylephrine at pH 7.0 was much faster than that observed at pH 9.0. Further, this stimulation of PLC-δ1 by Gα$_h$ at both pH 7.0 and 9.0 was attenuated in the presence of phentolamine (Fig. 3C). These results again not only demonstrate that activated Gα$_h$ can couple to PLC-δ1 in the α$_1$-AR signaling at both pH 7.0 and 9.0, but also show that the coupling ability and the activity of PLC-δ1 is higher at pH 7.0 than that at pH 9.0 in these reconstituted preparations. Consistent with the observations in Figs. 1 and 2, these results also show that the GTPase function of Gα$_h$ is higher at pH 7.0 than that at pH 9.0. The time required for the stimulation of PLC in the presence of agonist was slower than that observed in a cultured cell system (<1 min) (*32*). This may also be caused by an altered efficacy of the protein-protein inter-

action in this system. PLC-δ1 in the reconstituted preparations was not stimulated by GDP, even in the presence of phenylephrine (results not shown), indicating that the TGase function of $G\alpha_h$ is not involved in PLC-δ1 activation.

Supporting the results of this study, the effects of pH on other receptor signaling has been reported (*33–36*). In rat spinal cord, treatment of membranes at low pH had no effect on μ- and κ-opioid binding characteristics, but significantly reduced the NaF-stimulated adenylyl cyclase activity. This report demonstrates that low pH causes selective changes in the functional coupling of Gs-proteins to adenylyl cyclase without affecting opioid receptor binding in the spinal cord (*33*). Recently, various experiments on G-protein coupled receptor rhodopsin have shown that active photointermediates are pH-dependent and decrease with increasing pH (*35*), demonstrating that pH may be one of the factors regulating receptor signaling states (*34*, *36*). We have previously reported that the dual functions of $G\alpha_h$ are regulated by its membrane or cytosolic localization (*19*). The GTP binding activity of $G\alpha_h$ is higher in membranes than in the cytosol. On the other hand, the TGase activity of cytosolic $G\alpha_h$ is higher than that of membranous $G\alpha_h$. Therefore, it is also important to know whether pH affects distinct dual functions between membrane and cytosol. When the above experiments were performed with cytosolic $G\alpha_h$ from mouse heart, the results were the same as described above. Namely, the GTPase function of cytosolic $G\alpha_h$ is more active and thermostable at pH 7.0, while TGase activity is higher at 9.0; nevertheless the enzymatic activities are not exactly same as for membranous $G\alpha_h$ (results not shown).

Changes in intracellular pH are mediated by various stimuli and are linked to cardiac hypertrophy (*37*). As a consequence, increased fibril formation can be observed as a typical marker of hypertrophy. $G\alpha_h$ has been implicated in the polymerization of extramatrix and cross-linking a number of cytoskeletal proteins (*38*, *39*). Therefore, it is feasible to postulate that alkalization changes cells from the $G\alpha_h$ to TGase conformation. Supporting this idea, studies using hearts from transgenic mice over-expressing $G\alpha_h$ demonstrated increased fibril formation (*40*). Intracellular alkalization is also considered to play a role in apoptosis (*41*, *42*). The TGase activity of $G\alpha_h$ is stable under alkaline conditions (Figs. 1B and 2B), and increases during apoptosis without changing the level of $G\alpha_h$ expression (*6*). These observations suggest that the biochemical parameters may change so that it becomes physiological or pathological optimum for the activation of the TGase activity.

Over the years, various aspects of the cellular functions of $G\alpha_h$, including cell adhesion, growth and differentiation, apoptosis and signaling, have been studied (*2*, *4–6*). However, the exact cellular functions of $G\alpha_h$ remain unclear, allowing suspicion that other parameters may be involved. In this regard, the findings in this study of differential $G\alpha_h$ activities by pH may lead not only to better insight into cellular functions of $G\alpha_h$, but also to careful design for $G\alpha_h$-related experiments.

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