Oxytocin receptor couples to the 80 kDa $G_{h\alpha}$ family protein in human myometrium

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One of the primary functions of the oxytocin receptor is to modulate intracellular calcium levels in myometrium. The oxytocin receptor has been purified and cloned. Although it has been suggested that oxytocin receptor couples with a GTP-binding regulatory protein (G-protein), the identity of this G-protein remains unclear. To elucidate the mechanism of oxytocin receptor signalling, we used the oxytocin–receptor–G-protein ternary complex preparation from human myometrium, and evaluated oxytocin-mediated activation of [^{35}S]guanosine 5′-[γ-thio]triphosphate ([^{35}S]GTP[S]) binding and [α-^{32}P]GTP photoaffinity labelling to a G-protein. Binding of [^{35}S]GTP[S] and the intensity of the [α-^{32}P]GTP photoaffinity labelled protein resulting from activation of the oxytocin receptor were significantly attenuated by the selective oxytocin antagonist, desGlyNH_2d$(CH_2)_5$[Tyr(Me)^2,Thr^4]OVT. Furthermore, the molecular mass of the specific GTP-binding protein was ~80 kDa; homologous with the $G_{h\alpha}$ family, the new class of GTP-binding proteins first identified in rat liver that couples to the α_{1B}-adrenoceptor. Consistent with these observations, in co-immunoprecipitation and co-immunoadsorption of the oxytocin receptor in the ternary complex preparation by anti-$G_{h7\alpha}$ antibody, the $G_{h\alpha}$ family protein tightly coupled to the oxytocin receptor. These findings demonstrate that oxytocin receptor couples with ~80 kDa $G_{h\alpha}$ in signal mediation.

INTRODUCTION

Oxytocin, a neurohypophysial nonapeptide hormone, is involved in a variety of physiological responses, such as control of milk ejection, uterine contraction, penile erection, and yawning [1,2]. The ability to contract myometrium has been widely used to induce labour during parturition. However, the mechanism of oxytocin-dependent uterine contraction has not been fully elucidated. In myometrium, oxytocin binding to its receptor leads to hydrolysis of membrane-bound inositol phospholipids via activation of a phospholipase C (PLC) [3–5]. Inositol 1,4,5-trisphosphate (IP_3) generated from this hydrolysis initiates intracellular calcium mobilization, thus resulting in muscle contraction [6,7].

The oxytocin receptor contains seven transmembrane domains, a common property of receptors coupled with GTP-binding regulatory proteins (G-proteins) [8]. Furthermore, pertussis toxin-sensitive or -insensitive oxytocin-induced formation of IP_3 suggested participation of G-proteins in the signal transduction pathway of oxytocin receptor in myometrium [9]. Supporting this notion, recent studies using antibody against G_q- and G_{11}-proteins showed possible involvement of these G-proteins in the oxytocin-induced process in rat myometrium [10,11].

G_q family proteins shown to couple to the α_{1A}-, α_{1B}- and α_{1C}-adrenoceptors stimulated hydrolysis of phosphoinositides via PLC-β1 [12]. We demonstrated that the species-specific G_h family also coupled to the α_{1B}-adrenoceptor [13–15], and activated a membrane-bound 69 kDa PLC [16,17]. These findings suggested that the same receptor may couple to different G-proteins, and in particular the α_1-adrenoceptor may activate PLC not only through coupling with G_q, but also through G_h [18]. In the present study to elucidate the mechanism of oxytocin receptor signalling, we demonstrated functional coupling of oxytocin receptor to an ~80 kDa $G_{h\alpha}$ family protein in human myometrium by purifying the oxytocin–receptor–G-protein ternary complex, and assessing the direct interaction of the G_h family with oxytocin receptor using anti-$G_{h7\alpha}$ antibody.

EXPERIMENTAL

Materials

Oxytocin, heparin–agarose, protease inhibitors, dithiothreitol, and *N*-acetylglucosamine were obtained from Sigma. The selective oxytocin antagonist, desGly-NH_2d$(CH_2)_5$[Tyr(Me)^2, Thr^4]OVT (SS-1-7) was a gift from Dr. Maurice Manning, Department of Biochemistry and Molecular Biology, Medical College of Ohio, OH, U.S.A. [19]. Sucrose monolaurate (SM-1200) was a gift from the Mitsubishi-Kasei Company (Tokyo, Japan). Wheat germ agglutinin (WGA)–agarose was from Pharmacia LKB Biotechnology Inc. Protein A–agarose, guanine nucleotides, and other nucleotides were obtained from Boehringer Mannheim. [α-^{32}P]GTP (3000 Ci/mmol) , [^{3}H]oxytocin (48.5 Ci/mmol), and [^{35}S]guanine 5′-[γ-thio]triphosphate ([^{35}S]GTP[S]) (1300 Ci/mmol) were from DuPont–NEN. Other chemical and biochemical materials were commercial preparations of the highest available purity. Non-pregnant human myometrium was obtained from cycling women undergoing total hysterectomy for gynaecological reasons such as fibromyoma (provided by Department of Pathology at the Chung-Ang

Abbreviations used: PLC, phospholipase C; G-protein, GTP-binding regulatory protein consisting of the GTP-binding protein (α-subunit), β-, and γ-subunit; GTP-binding protein, α-subunit of G-proteins which bind GTP; G_q, a toxin-insensitive guanine nucleotide-binding protein which couples to various receptors and activates PLC-β1; G_{11}, a guanine nucleotide-binding protein of the G_q family; G_o, a guanine nucleotide-binding protein first found in brain, and with high levels in brain; G_s, stimulatory guanine nucleotide-binding protein of adenylate cyclase; G_h, a toxin-insensitive guanine nucleotide-binding regulatory protein with transglutaminase activity, first identified by the α_1-adrenergic ternary complex formation, which couples to the α_1-adrenoceptor and activates a 69 kDa PLC; G_{h7}, 78 kDa guanine nucleotide-binding regulatory protein of G_h family; GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppA, adenosine 5′-[β,γ-imido]triphosphate; IP_3, inositol 1,4,5-trisphosphate; WGA, wheat germ agglutinin; LDB, low detergent blotto; HDB, high detergent blotto; SS-1-7, selective oxytocin antagonist, desGly-NH_2d$(CH_2)_5$[Tyr(Me)^2,Thr^4]OVT; NP-40, Nonidet P-40.

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University Medical Center). This study was approved by the ethical committee for the protection of persons in biomedical research of the Institute of Medical Science, Chung-Ang University, Seoul, Korea.

Membrane preparations

Human myometrium membranes were prepared using the method of Baek et al. [15]. All procedures were carried out at 0–4 °C. Homogenates of human myometrium were prepared mechanically (Ultra-Turrax, Janke and 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; PMSF, 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 collected and centrifuged at 40000 ***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 as 10 mg of protein/ml in HEDG buffer [20 mM Hepes, 1 mM EGTA, 0.5 mM dithiothreitol, 10 % (v/v) glycerol, pH 7.5] containing 100 mM NaCl and protease inhibitors. The membrane suspensions were stored at −80 °C until use.

Ternary complex formation and purification

The ternary complex composed of oxytocin, oxytocin receptor, and G-protein was induced by incubating the membranes with oxytocin. The oxytocin and protease inhibitors were included throughout the procedure. Human myometrial membranes (500 mg of membrane protein) in HEDG buffer were preincubated for 3 h at 4 °C with 5 μM oxytocin, and then solubilized for 1 h at 4 °C with HEDG buffer containing 100 mM NaCl and sucrose monolaurate (final detergent concentration of 0.2 %). The preparations were centrifuged at 45000 ***g*** for 1 h at 4 °C, and the ternary complex was isolated using heparin–agarose and WGA–agarose by the method of Im and Graham [13]. The resulting partially purified ternary complex was stored at −80 °C until use.

Photoaffinity labelling of G-protein

Photoaffinity labelling of G-protein with [α-^{32}P]GTP was carried out by the method of Linse and Mandelkow [20], with minor modifications [13,15]. The samples were photolabelled with 5–10 μCi of [α-^{32}P]GTP and 2 mM $MgCl_2$ in an ice bath under 254 nm UV irradiation for 5–10 min. After irradiation, the samples were mixed with Laemmli stopping solution [21] and allowed to stand at room temperature for 1 h. The samples were subjected to SDS/PAGE using 7.5–12 % gels, and the gels were dried and exposed on Kodak X-OMAT XAR-5 film using DuPont image-intensifying screens.

Binding assays

The oxytocin-binding activity of oxytocin receptors was determined using the method of Klein and Fahrenholz [22]. Briefly, the samples were incubated in a total volume of 250 μl of binding buffer (50 mM Hepes, pH 7.5, 10 mM $MnCl_2$, 0.05 % BSA) with [^{3}H]oxytocin (final concentration of 40 nM) at 30 °C. After 30 min, the samples were diluted with 1 ml of filtration buffer (10 mM Hepes, pH 7.5, 0.05 % BSA). Bound radioactivity was separated from free by rapid vacuum filtration over Whatman GF/F glass filters. Unlabelled oxytocin (10 μM) was used to determine non-specific binding.

[^{35}S]GTP[S] binding by G-proteins was determined using the method of Asano and Ross [23]. The samples in 20 mM Hepes buffer, pH 7.5, containing 100 mM NaCl, 20 mM $MgCl_2$, 100 μM adenosine 5′-[β,γ-imido]triphosphate (p[NH]ppA), and 0.05 % sucrose monolaurate were incubated with 1 μM GTP[S] plus 1 μCi of [^{35}S]GTP[S] in a final volume of 50 μl for 30 min at 30 °C. Unlabelled GTP (1 mM) was used to determine non-specific binding. The reaction was stopped by 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]GTP[S] was determined using a liquid scintillation counter (Beckman LS100) after filtration through BA85 nitrocellulose filters (Scheicher & Schuell).

Antibody experiments

An anti-$G_{h7\alpha}$ polyclonal antibody was prepared and characterized as previously described [15]. $G_{h7\alpha}$ (70 μg/200 μl) was emulsified with an equal volume of Freund's complete adjuvant and injected subcutaneously into the New Zealand White rabbit. At 3-week intervals, three booster injections were given with 70–100 μg of $G_{h7\alpha}$ and adjuvant. Rabbit antisera were characterized by immunoblots or by immunoprecipitation. Immunoblots were performed using the methods of Harris et al. [25]. Briefly, proteins were separated on 7.5–12 % gels by SDS/PAGE, and then transferred to Immobilon-P (Millipore). The membranes were blocked for 1 h with LDB [low detergent blotto; 80 mM NaCl, 2 mM $CaCl_2$, 0.02 % NaN_3, 0.2 % Nonidet P-40 (NP-40), 50 mM Tris/HCl, pH 8.0, containing 5 % (w/v) non-fat dry milk] at room temperature, then incubated with LDB containing anti-$G_{h7\alpha}$ antibody (1:500 dilution) for 1 h at room temperature. After washing three times with LDB, the membranes were incubated with anti-(rabbit immunoglobulin), peroxidase-linked species-specific antibody (1:1000 dilution) in HDB (high detergent blotto; 2 % NP-40 in LDB) for 1 h at room temperature. After washing three times, the membranes were subjected to procedures for enhanced chemiluminescence (Amersham, U.K.). Immunoprecipitation of solubilized membranes using anti-$G_{h7\alpha}$ antibody were performed essentially as described previously [15].

For co-immunoprecipitation of the oxytocin receptor, the oxytocin–receptor–G-protein ternary complex preparations (containing 250 fmol of receptor/tube) were incubated for 2 h with 5 μl of anti-$G_{h7\alpha}$ antibody at 4 °C. The antigen–antibody complexes were precipitated using 10 μl of Protein A–agarose (binding capacity, 22 mg of rabbit IgG/ml of agarose). The supernatants were collected by centrifugation at 500 ***g*** for 10 min and the receptor binding assay was performed as described above. For co-immunoadsorption, the antibody-affinity column (50 μl) was prepared by incubation of the Protein A–agarose gel with anti-$G_{h7\alpha}$ antibody [26]. Briefly, the Protein A–agarose gel (50 μl) was mixed with the same amount of anti-$G_{h7\alpha}$ antibody in 1 ml of 0.1 M borate buffer, pH 8.2, for 30 min at room temperature. The antibody-bound agarose beads were washed with borate buffer, and further washed three times with 0.2 M triethanolamine (pH 8.2). The resins were incubated with freshly made 20 mM dimethyl pimelimidate in 0.2 M triethanolamine (1 ml) for 30 min at room temperature. The beads were washed with 50 mM ethanolamine (pH 8.2) and then 0.1 M borate buffer containing 0.02 % sodium azide. The covalently linked antibody-affinity column was stored at 4 °C until required for use. The anti-$G_{h7\alpha}$ antibody-affinity gel was then incubated with the oxytocin–receptor–G-protein ternary complex including 250 fmol of receptor for 2 h at 4 °C. After the resin was washed

with 20 mM Hepes (pH 7.4) containing 100 mM NaCl, 0.5 mM dithiothreitol and 0.05% sucrose monolaurate, the receptor density of both unbound and bound materials was measured. For these studies, anti-$G_{h7\alpha}$ antibody was purified using a hydroxyapatite column. The bound antibody was eluted using a phosphate gradient (0–300 mM). The inorganic phosphate in the eluate was removed through a dry Sephadex G-25 column [13–17].

Protein determination

The amount of protein was estimated by the method of Bradford [27] using a Bio-Rad protein determination kit and BSA as a standard.

RESULTS

Identification of the oxytocin receptor-coupled G-protein

To determine the possibility of G-protein mediation in oxytocin receptor signalling, stimulation of [^{35}S]GTP[S] binding by the activation of the receptor with the agonist was evaluated using solubilized human myometrial membranes. As shown in Figure 1, in the presence of oxytocin, binding of [^{35}S]GTP[S] was much higher than in the absence of oxytocin. Furthermore, when the oxytocin receptor antagonist, SS-1-7, was included in the pre-incubation mixture, GTP[S] binding was markedly attenuated. Upon activation of the oxytocin receptor with the agonist, the rate of GTP[S] binding was much faster than that observed in SS-1-7 or without oxytocin (inset in Figure 1). These results suggested that oxytocin receptor mediated signals through a G-protein.

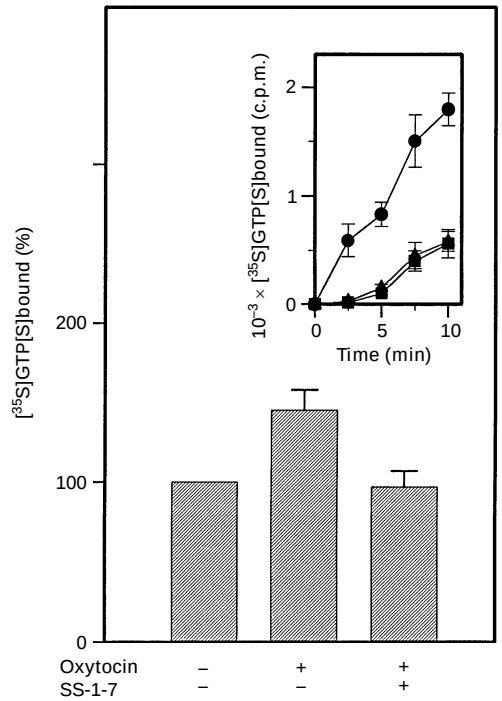

Figure 1 Oxytocin receptor-stimulated GTP[S]-binding activity in solubilized fractions of human myometrial membranes

Purified human myometrial membranes (10 mg of protein/ml) were solubilized with 0.2% sucrose monolaurate in HEDG buffer containing 100 mM NaCl at 4 °C for 1 h. The solubilized membranes (0.5 mg of protein/ml) were preincubated in the presence of 5 μM oxytocin or 5 μM oxytocin plus 100 μM oxytocin antagonist, SS-1-7, for 2 h at 4 °C, and GTP[S] binding assay was then carried out as detailed in the Experimental section. The GTP[S] bound was expressed as a percentage of GTP[S]-binding activity in the solubilized membranes preincubated without ligand. The data shown are means ± S.D. of three independent experiments performed in duplicate. Inset: incubation time course of GTP[S]-binding activity. After preincubation with oxytocin (●), oxytocin plus SS-1-7 (▲), or without ligand (■) for 2 h, GTP[S] binding was performed in a time-dependent manner. The results shown are means ± S.D. of three independent experiments performed in duplicate.

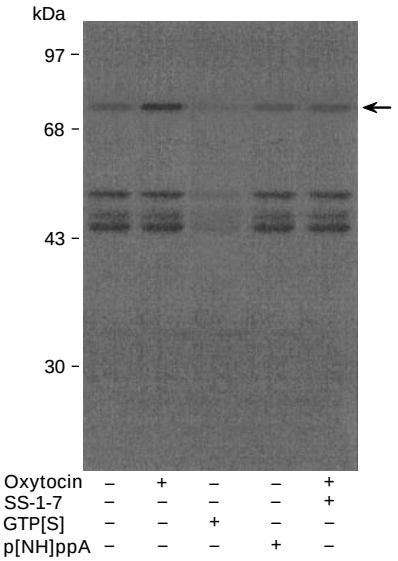

Figure 2 Oxytocin receptor-stimulated photoaffinity-labelling in solubilized fractions of human myometrial membranes

After solubilization, the membrane extracts were preincubated with 5 μM oxytocin or 5 μM oxytocin plus 100 μM SS-1-7 for 2 h at 4 °C. The preincubated extracts (50 μl) were further incubated with 5 μCi of [α-^{32}P]GTP, 5 μCi of [α-^{32}P]GTP plus 0.5 mM unlabelled GTP[S], or 5 μCi of [α-^{32}P]GTP plus 0.5 mM p(NH)ppA in the presence of 2 mM $MgCl_2$, and photolabelled with UV light (254 nm). The proteins were analysed by SDS/PAGE (10% gel) and autoradiography, as described in the Experimental section. The data shown are representative of five independent experiments. Arrows show the ~80 kDa GTP-binding protein.

In order to examine the nature of the G-protein involved in the oxytocin receptor signal transduction, the solubilized membranes were incubated with [α-^{32}P]GTP in the presence of oxytocin, SS-1-7, unlabelled GTP[S], or p(NH)ppA. The samples were then subjected to UV irradiation, SDS/PAGE and autoradiography. As demonstrated in Figure 2, labelling of ~80 kDa protein was markedly enhanced by the presence of oxytocin. This increased labelling of ~80 kDa protein was completely abolished by SS-1-7, whereas the labelling of ~55, ~50 and ~45 kDa bands remained unchanged. The labelling of all four protein bands was specific for guanine nucleotides, since all these bands were blocked by unlabelled GTP[S], but not by p(NH)ppA which is a non-hydrolysable ATP analogue. These results revealed that the ~80 kDa GTP-binding protein is probably the signal mediator in the oxytocin receptor signalling.

Species-specific $G_{h\alpha}$ family proteins are the only known examples of G-proteins with such high molecular masses [15]. Therefore, we tested the homology of the ~80 kDa GTP-binding protein and high-molecular-mass G_{h7} [15] by immunoblot analysis using polyclonal antibody against $G_{h7\alpha}$, a 78 kDa GTP-binding protein purified from bovine heart [15]. As shown in Figure 3(a), the antibody recognized only an ~80 kDa protein in human myometrium. The molecular mass was the same as the oxytocin receptor-associated GTP-binding protein in Figure 2. Moreover, the antibody did not recognize any other proteins,

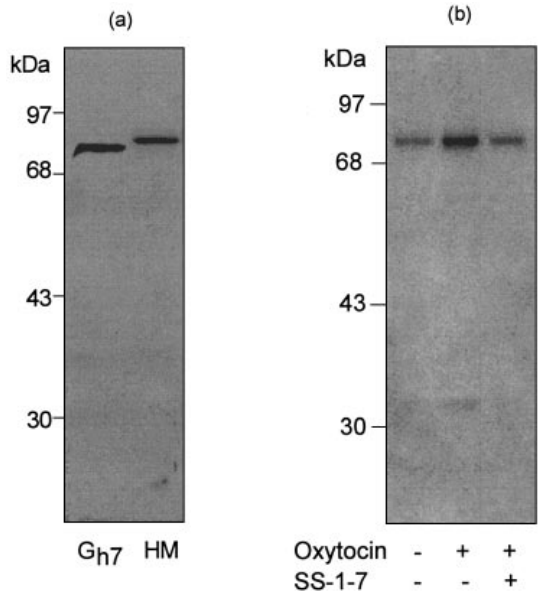

Figure 3 Immunological cross-reactivity of solubilized membrane extracts from human myometrium

(**a**) Immunoblots of the purified G_{h7} from bovine heart and human myometrial membranes. Purified G_{h7} (20 ng, G_{h7}) and solubilized human myometrial membranes (100 μg, HM) were subjected to SDS/PAGE (10% gel) and transferred to Immobilon-P. Immunoblotting was carried out using a 1:500 dilution of anti-$G_{h7\alpha}$ antibody as described in the Experimental section. (**b**) Immunoprecipitation of $G_{h\alpha}$ family protein in human myometrial membranes with anti-$G_{h7\alpha}$ antibody. After incubation with 5 μM oxytocin or 5 μM oxytocin plus 100 μM SS-1-7, solubilized membranes (75 μl) were photolabelled with 10 μCi of [α-^{32}P]GTP as described in the Experimental section. The samples were immunoprecipitated with anti-$G_{h7\alpha}$ antibody and Protein A–agarose. Immunoprecipitates were then analysed by SDS/PAGE (10% gel) and autoradiography.

and these GTP-binding proteins again showed species specificity in molecular mass, as observed previously [15]. To evaluate the specificity of anti-$G_{h7\alpha}$ antibody, when similar experiments were carried out with non-immune sera the results were negative (results not shown). To ensure that the oxytocin receptor-associated ~80 kDa GTP-binding protein was the same protein recognized by anti-$G_{h7\alpha}$ antibody (see Figures 2 and 3a), the solubilized membranes were photoaffinity-labelled with [α-^{32}P]GTP in the presence of oxytocin or SS-1-7, incubated with anti-$G_{h7\alpha}$ antibody, and precipitated using Protein A–agarose. As demonstrated in Figure 3(b), the ~80 kDa GTP-binding protein was effectively immunoprecipitated, indicating that the ~80 kDa GTP-binding protein and $G_{h7\alpha}$ are homologues. Furthermore, the increased labelling of ~80 kDa protein in the presence of oxytocin was substantially attenuated by SS-1-7, as observed in Figure 2. The results strongly suggested that the ~80 kDa GTP-binding protein that coupled to the oxytocin receptor was homologous to the $G_{h\alpha}$ family.

Immunological cross-reactivity of oxytocin receptor with G_h family protein by anti-$G_{h7\alpha}$ antibody

In general, formation of a hormone–receptor–G-protein ternary complex can be induced by the binding of the hormone to the receptors in the absence of GTP, after which the activated receptors interact with their specific G-proteins [28]. As a result of this sequential process, hormone, receptor, and G-protein become associated, forming a heterotrimeric intermediate, in which the G-protein is primed for guanine nucleotide binding

Table 1 Co-immunoprecipitation of the oxytocin receptors in the ternary complex preparations with anti-$G_{h7\alpha}$ antibody

The ternary complex contained 250 fmol of oxytocin receptor as described in the Experimental section. The density of the oxytocin receptor in the ternary complex was measured in the supernatants using 40 nM [^{3}H]oxytocin after immunoprecipitation with anti-$G_{h7\alpha}$ antibody. For the measurement of the receptors in the samples, oxytocin 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 non-immune sera or Protein A–agarose to determine the specificity of the anti-$G_{h7\alpha}$ antibody. The receptor density was expressed as a percentage of the 250 fmol of oxytocin receptor (Basal) in the ternary complex before immunoprecipitation. The data shown are means ± S.D. of three independent experiments performed in triplicate.

	Receptor density (% of basal)
Anti-$G_{h7\alpha}$ antibody	36 ± 11
Protein A–agarose	98 ± 4
Non-immune sera	95 ± 8

[28a]. Based on the above considerations, we prepared the oxytocin–receptor–G-protein complex from human myometrial membranes, and the protein components were co-purified by ion-exchange and then lectin affinity chromatography using heparin–agarose and WGA–agarose [13,29]. After purification of the ternary complex, we confirmed the existence of oxytocin receptors and ~80 kDa GTP-binding proteins in the ternary complex by the receptor binding assay and [α-^{32}P]GTP photoaffinity labelling (results not shown). In order to confirm that the oxytocin receptor-coupled ~80 kDa GTP-binding protein was a member of the $G_{h\alpha}$ family, co-immunoprecipitation of the oxytocin receptor with the $G_{h\alpha}$ family was assessed by anti-$G_{h7\alpha}$ antibody. After co-immunoprecipitation, the unbound receptor to the antibody was measured. As presented in Table 1, the remaining receptor was 36% in the supernatant, which indicates that most of the receptor (63%) was bound to the antibody. On the other hand, in the samples treated with non-immune sera or Protein A–agarose, most receptor was found in the supernatants. These data clearly demonstrated the tight association and coupling of the oxytocin receptor with the ~80 kDa $G_{h\alpha}$ family proteins.

A functional interaction between the oxytocin receptor and $G_{h\alpha}$ family was further examined by immunoadsorption of oxytocin receptor-containing ternary complex to a $G_{h7\alpha}$–Protein A–agarose antibody-affinity resin. As shown in Table 2, co-immunoadsorption of oxytocin receptors did not occur if the ternary complex was dissociated by treatment with either oxytocin and GTP[S], or oxytocin and SS-1-7. When the complex was incubated only with oxytocin, sample eluted from the antibody-affinity column demonstrated the highest receptor binding activity (56%), indicating that the oxytocin receptors were effectively bound to the antibody-affinity resin through the $G_{h\alpha}$ family. Consistent with this observation, the lowest binding (26%) was observed in the flow-through samples. In the presence of oxytocin and GTP[S], or the oxytocin antagonist SS-1-7, the receptor density in the eluates was less than 20% of the initial receptor density applied. In these experiments, 61 and 71% of the receptor (dissociated from $G_{h\alpha}$ family) was found in the flow-through fractions. As a control, when we incubated non-immune sera–Protein A–agarose with the ternary complex in the presence of oxytocin, most of the receptors were found in the flow-through fractions, with residual binding (< 5%) to the antibody-affinity resins. By analogy with G-protein-coupled receptors [28,30], if oxytocin promotes the formation of an oxytocin–

Table 2 Co-immunoadsorption of the oxytocin receptors in the ternary complex preparations by anti-$G_{h7\alpha}$ antibody-affinity resins

The anti-$G_{h7\alpha}$ antibody-affinity column (50 μl) was prepared by incubation of Protein A–agarose with antibodies to $G_{h7\alpha}$. The ternary complex including 250 fmol of oxytocin receptor was incubated with the resin at 4 °C for 2 h in the presence of 1 mM $MgCl_2$, and either 5 μM oxytocin, 5 μM oxytocin plus 5 μM GTP[S], or 5 μM oxytocin plus 100 μM SS-1-7. Unbound material was eluted from the column by applying gentle pressure until the column was dry. After the resin was washed without the ligand, adsorbed oxytocin receptor was eluted by incubating the gel with 2 μM oxytocin, 5 μM GTP[S] and 1 mM $MgCl_2$ for 1 h at 4 °C. Receptor density in the flow-through and eluted fractions was determined after removing excess ligand on a dried Sephadex G-25 column as described above. The receptor density was expressed as a percentage of the 250 fmol of oxytocin receptor (basal) in the ternary complex before co-immunoadsorption. The data shown are means ± S.D. of three independent experiments performed in triplicate.

	Receptor density (% of basal)	
	Flow-through	Eluates
Oxytocin	26 ± 5	56 ± 12
Oxytocin + GTP[S]	71 ± 6	20 ± 6
Oxytocin + SS-1-7	61 ± 10	18 ± 10

receptor–G-protein ternary complex and addition of GTP causes a dissociation of the G-protein from the receptor, these observations provide evidence for the involvement of a G-protein in oxytocin receptor signalling. These data strongly suggested that the ~80 kDa GTP-binding protein is a member of the $G_{h\alpha}$ family, and couples to oxytocin receptor in human myometrium, supporting the above observations (Figures 1–3 and Table 1).

DISCUSSION

In the present study, we showed faster and increased GTP[S] binding in the presence of oxytocin than in the presence of SS-1-7 or in the absence of oxytocin. Increased binding of GTP[S] was caused by an ~80 kDa GTP-binding protein which was homologous to the $G_{h\alpha}$ family. Consistent with this observation, immunological cross-reactivity by anti-$G_{h7\alpha}$ antibody demonstrated tight coupling of oxytocin receptor with the G_h family protein.

In a reconstituted system, a 69 kDa PLC was shown to couple to the G_h family proteins that is a tissue-type transglutaminase [16,17,31]. The known isoenzymes, PLC-β1, -γ1 and -δ1 were not stimulated by GTP[S]–G_h in the reconstituted system. Additionally, the 69 kDa PLC was found in human myometrial membrane by immunoblotting with anti-(69 kDa PLC) antibody (K. J. Baek, N. S. Kwon, H. S. Lee and M.-J. Im, unpublished work). These observations suggested that 69 kDa PLC could be coupled to $G_{h\alpha}$ family protein activated by oxytocin receptor.

The first suggestion for involvement of a G-protein in the action of oxytocin on myometrium was presented by Ruzycky and Crankshaw [32] and Marc et al. [33]. Marc et al. [33] showed that oxytocin receptors coupled to a PLC through a pertussis toxin-insensitive G-protein in guinea-pig myometrium, whereas Crankshaw et al. [4] could not reproduce an influence of GTP on oxytocin binding to myometrial cell membranes. In human myometrium, Zumbihl et al. [34] demonstrated an increase in the expression of both isoforms of the $G_o\alpha$ subunits in late pregnancy, and Europe-Finner et al. [35] showed a substantial increase in the level of G_s during pregnancy, suggesting a role for these G-proteins throughout gestation and/or near term for parturition. Phaneuf et al. [9] suggested that the oxytocin-induced production of inositol phosphates and increase in intracellular free Ca^{2+} are mediated by a pertussis toxin-sensitive G-protein in human myometrial cells. However, a significant fraction of the oxytocin response appeared to be mediated by a pertussis toxin-insensitive G-protein, possibly a member of the G_q family [9]. Arnaudeau et al. [10] showed that the oxytocin stimulation of intracellular free Ca^{2+} was insensitive to incubation with pertussis toxin, and was blocked by anti-$G_q\alpha/_{11}\alpha$ antibody in single myometrial cells from pregnant rats under voltage-clamp at a holding potential of −50 mV. Ku et al. [11] demonstrated that oxytocin stimulated both GTPase and PLC activities, and both stimulations were attenuated by incubation with an antibody directed against the C-termini of $G_q\alpha$ and $G_{11}\alpha$ in rat and human myometrial membranes. Neutralization of the antibody by preincubation with antigenic peptide reversed this inhibition. These reports provided evidence for the stimulation of PLC (probably PLC-β) by oxytocin receptor through the G_q/G_{11}-protein.

Our results clearly demonstrate that the G_h family protein is also a signal mediator of oxytocin receptor. The discrepancies between our findings and other results [9–11] have raised the following possibilities as far as the mechanism of oxytocin receptor-mediated signalling is concerned. First, diverse regulatory mechanisms imply that different G-proteins and PLC isoenzymes may have specific roles and be preferentially activated in response to various external stimuli [18], such that α_1-adrenoceptor couples to G_h and G_q family proteins [12–15]; endothelin receptor couples to G_q, G_{11}, G_s and G_{i2} [36]; κ-opioid receptor couples to G_i and G_z [37]; and μ-opioid receptor couples to G_{i2}, G_{o2}, G_{i3} and an unknown G-protein [38]. Secondly, the system used by Arnaudeau et al. [10], the single myometrial cell, and the system used by us, ternary complex formation of the components, might have been different. The mechanism of the cross-talk of the receptor to various G-proteins is not clear. To elucidate the exact reason for such discrepancies, as well as to predict the physiological and biochemical mechanisms, careful design of the necessary experiments using all possible combinations is required. Since the same receptor uses multiple G-proteins and effectors to transmit the signal [39], these experiments could help to elucidate mechanisms for oxytocin receptor signalling and diverse regulatory mechanisms of G-protein-mediated signalling.

We thank Dr. Maurice Manning of the Medical College of Ohio for the gift of oxytocin antagonist, Dr. Sang Chul Park of Seoul National University for the use of his radioisotope-handling facilities, Dr. Ki Jeong Kim of Chung-Ang University for help on graphic work, Dr. Dae Young Uhm of Chung-Ang University for comments on the manuscript, and Caroline Gray of The Cleveland Clinic Foundation for editorial assistance. This work was supported by a research grant from the Korea Science and Engineering Foundation (K.J.B.), a grant for basic medical science from the Ministry of Education, Republic of Korea (K.J.B.) and National Institutes of Health Grant, USA (M.-J.I.).

REFERENCES

1 Higuchi, T., Honda, K., Fukuoka, T., Negoro, H. and Wakabayashi, K. (1985) J. Endocrinol. **105**, 339–346
2 Argiolas, A., Melis, M. R. and Gessa, G. L. (1986) Eur. J. Pharmacol. **130**, 265–272
3 Marc, S., Leiber, D. and Harbon, S. (1986) FEBS Lett. **201**, 9–14
4 Crankshaw, D., Gaspar, V. and Pliska, V. (1990) J. Receptor Res. **10**, 269–285
5 Schrey, M. D., Conford, P. A., Read, A. M. and Steer, P. J. (1988) Am. J. Obstet. Gynecol. **159**, 964–970
6 Carsten, M. E. and Miller, J. D. (1985) Biochem. Biophys. Res. Commun. **130**, 1027–1031
7 Anwer, K., Hovington, J. A. and Sanborn, B. M. (1989) Endocrinology **124**, 2995–3002
8 Kimura, T., Tanizawa, O., Mori, K., Brownstein, M. J., and Okayama, H. (1992) Nature (London) **356**, 526–529
9 Phaneuf, S., Europe-Finner, G. N., Varney, M., MacKenzie, I. Z., Watson, S. P. and Lopez Bernal, A. (1993) J. Endocrinol. **136**, 497–509

10 Arnaudeau, S., Lepretre, N. and Mironneau, J. (1994) Eur. J. Physiol. **428**, 51–59
11 Ku, C.-Y., Qian, A., Wen, Y., Anwer, K. and Sanborn, B. M. (1995) Endocrinology **136**, 1509–1515
12 Wu, D., Katz, A., Lee, C.-H. and Simon, M. I. (1992) J. Biol. Chem. **267**, 25798–25802
13 Im, M.-J. and Graham, R. M. (1990) J. Biol. Chem. **265**, 18944–18951
14 Im, M.-J., Riek, R. P. and Graham, R. M. (1990) J. Biol. Chem. **265**, 18952–18960
15 Baek, K. J., Das, T., Gray, C., Antar, S., Murugasan, G. and Im, M.-J. (1993) J. Biol. Chem. **268**, 27390–27397
16 Im, M.-J., Gray, C. D. and Rim, A. J.-W (1992) J. Biol. Chem. **267**, 8887–8894
17 Das, T., Baek, K. J., Gray, C. and Im, M.-J. (1993) J. Biol. Chem. **268**, 27398–27405
18 Bristol, J. A. and Rhee, S. G. (1994) Trends Endocrinol. Metab. **5**, 402–406
19 Manning, M., Kruszynski, M., Bankowski, K., Olma, A., Lammek, B., Cheng, L. L., Klis, W. A., Seto, J. and Sawyer, W. H. (1989) J. Med. Chem. **32**, 382–391
20 Linse, K. and Mandelkow, E.-M. (1988) J. Biol. Chem. **263**, 15205–15210
21 Laemmli, U. K. (1970) Nature (London) **227**, 680–685
22 Klein, U. and Fahrenholz, F. (1994) Eur. J. Biochem. **220**, 559–567
23 Asano, T. and Ross, E. M. (1984) Biochemistry **23**, 5467–5471
24 Reference deleted
25 Harris, B. A., Robinshaw, J. D., Mumby, S. M. and Gilman, A. G. (1985) Science **229**, 1274–1277
26 Schneider, C., Newman, R. A., Sutherland, D. R., Asser, U. and Greaves, M. F. (1982) J. Biol. Chem. **257**, 10766–10769
27 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
28 Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
28a May, D. C. and Ross, E. M. (1988) Biochemistry **27**, 4888–4893
29 Sawutz, D. G., Lanier, S. M., Warren, C. D. and Graham, R. M. (1987) Mol. Pharmacol. **32**, 565–571
30 Uhing, R. J., Prpic, V., Jiang, H. and Exton, J. H. (1986) J. Biol. Chem. **261**, 2140–2146
31 Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Misono, K., Im, M.-J. and Graham, R. M. (1994) Science **264**, 1593–1596
32 Ruzycky, A. L. and Crankshaw, D. J. (1987) Can. J. Physiol. Pharmacol. **66**, 10–17
33 Marc, S., Leiber, D. and Harbon, S. (1988) Biochem. J. **255**, 705–713
34 Zumbihl, R., Breuiller-Fouche, M., Carrette, J., Dufour, M.-N., Ferre, F., Bockaert, J. and Rouot, B. (1994) Eur. J. Pharmacol. **288**, 9–15
35 Europe-Finner, G. N., Phaneuf, S. P., Watson, S. P. and Lopez Bernal, A. (1993) Endocrinology **132**, 2484–2490
36 Takigawa, M., Sakurai, T., Kasuya, Y., Abe, Y., Masaki, T. and Goto, K. (1995) Eur. J. Biochem. **228**, 102–108
37 Lai, H. W. L., Minami, M., Sato, M. and Wong, Y. H. (1995) FEBS Lett. **360**, 97–99
38 Chakrabarti, S., Prather, P. L., Yu, L., Law, P. Y. and Loh, H. H. (1995) J. Neurochem. **64**, 2534–2543
39 Boege, F., Neumann, E. and Helmreich, E. J. M. (1991) Eur. J. Biochem. **199**, 1–15

Received 17 October 1995/11 December 1995; accepted 18 December 1995