Phospholipase C- δ 1 and oxytocin receptor signalling: evidence of its role as an effector

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Although the oxytocin receptor modulates intracellular Ca^{2+} ion levels in myometrium, the identities of signal molecules have not been clearly clarified. Our previous studies on oxytocin receptor signalling demonstrated that 80 kDa $G_{h\alpha}$ is a signal mediator [Baek, Kwon, Lee, Kim, Muralidhar and Im (1996) *Biochem. J.* **315**, 739–744]. To elucidate the effector in the oxytocin receptor signalling pathway, we evaluated the oxytocin-mediated activation of phospholipase C (PLC) by using solubilized membranes from human myometrium and a three-component preparation containing the oxytocin receptor– $G_{h\alpha}$ –PLC- δ 1 complex. PLC- δ 1 activity in the three-component preparation, as well as PLC activity in solubilized membranes, was increased by

oxytocin in the presence of Ca^{2+} and activated $G_{h\alpha}$ (GTP-bound $G_{h\alpha}$). Furthermore the stimulated PLC- δ 1 activity resulting from activation of $G_{h\alpha}$ via the oxytocin receptor was significantly attenuated by the selective oxytocin antagonist desGly-NH₂d(CH₂)₅[Tyr(Me)²,Thr⁴]ornithine vasotocin or GDP. Consistent with these observations, co-immunoprecipitation and co-immunoabsorption of PLC- δ 1 in the three-component preparation by anti- $G_{h\alpha}$ antibody resulted in the PLC- δ 1 being tightly coupled to activated $G_{h\alpha}$ on stimulation of the oxytocin receptor. These results indicate that PLC- δ 1 is the effector for $G_{h\alpha}$ -mediated oxytocin receptor signalling.

INTRODUCTION

The nonapeptide hormone oxytocin has several major physiological functions including milk ejection, penile erection, yawning and the induction of uterine contraction at term [1,2]. The physiological responses to oxytocin are mediated by a specific oxytocin receptor, a 388-residue polypeptide with seven transmembrane domains, a typical structure for a GTP-binding regulatory protein (G-protein)-coupled receptor [3]. In myometrium, binding of oxytocin to its receptor can lead to intracellular Ca^{2+} mobilization by activating phosphoinositide-specific phospholipase C (PLC) via stimulation of a G-protein [4–7].

The hydrolysis of membrane-bound inositol phospholipids by a specific PLC that generates the second messengers Ins(1,4,5) P_3 and diacylglycerol is a widespread signalling pathway triggering the release of free Ca^{2+} ions and activation of protein kinase C [8]. Phosphoinositide-specific PLC is a family of isoenzymes consisting of three major groups (designated PLC- β , PLC- γ , and PLC- δ) and classified by molecular mass, deduced amino acid sequence and immunological cross-reactivity [9]. PLC- β is stimulated by the G_q family, which mediates the actions of a variety of transmembrane receptors [10,11]. PLC- γ is thought to be mainly a cytosolic isoenzyme that is phosphorylated by activated tyrosine kinase-containing receptors [12,13]. This phosphorylation induces the translocation of PLC- γ from the cytosol to the membrane to enhance interaction with its substrates [14,15]. However, little information is available on the regulation of PLC- δ , although it is widespread in its distribution [10]. PLC- δ 1 has been shown to be modulated not only by $G_{h\alpha}$ (trans-

glutaminase II)-mediated signalling [16,17] but also by thrombin-mediated signalling via pertussis toxin-sensitive G-protein(s) [18]. We have shown previously that the α_{1B} -adrenoceptor stimulates a 69 kDa PLC through activation of the $G_{h\alpha}$ family of proteins [19,20], indicating that the 69 kDa PLC might be an effector in $G_{h\alpha}$ -mediated signalling. Recently, Feng et al. [17] demonstrated that the 69 kDa PLC is a proteolytic fragment of PLC- δ 1 and that PLC- δ 1 might be the effector of $G_{h\alpha}$ signalling.

G-proteins might have a role as signal mediators of the oxytocin receptor [3]. In human myometrial cells, oxytocin-induced production of Ins(1,4,5) P_3 and the subsequent increase of free Ca^{2+} concentration were shown to be mediated by G-proteins sensitive or insensitive to pertussis toxin [21]. Furthermore these oxytocin-induced effects are attenuated by an anti- $G_{q/11\alpha}$ antibody, indicating that these G-proteins can take part in oxytocin receptor signalling [5,6]. Previously we have shown that an 80 kDa $G_{h\alpha}$ family protein is stimulated by oxytocin-bound receptor in human myometrium, suggesting that the oxytocin receptor couples to the $G_{h\alpha}$ family of proteins in signal mediation [22]. These findings suggest that the oxytocin receptor might couple to different G-proteins, and that likewise that α_1 -adrenoceptor can activate PLC via either G_q or G_h [23]. To clarify the mechanism of oxytocin receptor signalling further, we have here examined the stimulation of PLC (especially PLC- δ 1) by oxytocin with solubilized human myometrial membranes and an oxytocin receptor and $G_{h\alpha}$ –PLC- δ 1 complex preparation. We have also investigated the direct interaction of $G_{h\alpha}$ with PLC- δ 1 in the oxytocin receptor signalling pathway by using an anti- $G_{h\alpha}$ antibody-affinity gel. These results demonstrate that PLC- δ 1 is an effector of oxytocin receptor signalling via activation

Abbreviations used: G-protein, GTP-binding regulatory protein; PLC, phospholipase C; G_q , a toxin-insensitive G-protein that activates PLC- β 1; $G_{h\alpha}$, a toxin-insensitive G-protein with transglutaminase activity; anti- $G_{h\alpha}$ antibody, a polyclonal antibody against 78 kDa $G_{h\alpha}$ family in bovine heart and previously designated as $G_{h7\alpha}$; SS-1–7, a selective oxytocin antagonist, desGly-NH₂d(CH₂)₅[Tyr(Me)²,Thr⁴]ornithine vasotocin; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppA, adenosine 5'-[β , γ -imido]triphosphate; WGA, wheatgerm agglutinin; RG, oxytocin receptor– $G_{h\alpha}$ complex; LDB, low-detergent blotto; HDB, high-detergent blotto; DTT, dithiothreitol.

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of $G_{\text{h}\alpha}$ in human myometrium. Our study also demonstrates, for the first time, direct isolation of the three key components involved in the oxytocin receptor signalling system.

EXPERIMENTAL

Materials

Oxytocin, PtdIns(4,5) P_2 , protease inhibitors, dithiothreitol (DTT) and CHAPS were obtained from Sigma. Wheatgerm agglutinin (WGA)-agarose was from Pharmacia LKB Biotechnology. Protein A-agarose, guanine nucleotides and other nucleotides were obtained from Boehringer Mannheim. [α - ^{32}P]GTP (3000 Ci/mmol), [^3H]oxytocin (48.5 Ci/mmol), [^{35}S]guanosine 5'-[γ -thio]triphosphate (GTP[S]) (1300 Ci/mmol) and phosphatidyl[2- ^3H]inositol 4,5-bisphosphate ([^3H]PtdIns(4,5) P_2 , 1 Ci/mmol) were from DuPont-NEN. Other chemical and biochemical materials were commercial preparations of the highest available purity. The selective oxytocin antagonist, desGly-NH $_2$ d(CH $_2$) $_5$ [Tyr(Me) 2 , Thr 4]ornithine vasotocin (SS-1-7) was a gift from Dr. Maurice Manning (Department of Biochemistry and Molecular Biology, Medical College of Ohio, Toledo, OH, U.S.A.) [24]. Non-pregnant human myometrium was obtained from cycling women undergoing total hysterectomy for gynaecological reasons such as fibromyoma (provided by the Department of Pathology at the Chung-Ang University Medical Center, Seoul, Korea). 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

The membrane extracts were prepared from human myometrium by the method of Baek et al. [19]. All procedures were performed at 0–4 °C. Homogenates of human myometrium 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 (2 $\mu\text{g}/\text{ml}$ bacitracin, 100 $\mu\text{g}/\text{ml}$ benzamidine, 2 $\mu\text{g}/\text{ml}$ leupeptin, 2 $\mu\text{g}/\text{ml}$ pepstatin A, 2 $\mu\text{g}/\text{ml}$ trypsin inhibitor, 2 $\mu\text{g}/\text{ml}$ PMSF and 20 $\mu\text{g}/\text{ml}$ antipain). The homogenates were filtered through four layers of cheesecloth and centrifuged at 500 g for 5 min. The supernatants were centrifuged again 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 at 10 mg/ml protein in HEDG buffer [20 mM Hepes/1 mM EGTA/0.5 mM dithiothreitol (DTT)/10% (v/v) glycerol (pH 7.5)] containing 100 mM NaCl and protease inhibitors. The membrane suspensions were stored at –80 °C until use.

Oxytocin receptor- $G_{\text{h}\alpha}$ complex (RG) preparation

RG was induced by incubating the membranes with oxytocin by the method of Im and Graham [25]. Oxytocin and protease inhibitors were included throughout the procedure. Human myometrial membranes (500 mg of membrane protein) in HEDG buffer were preincubated with 5 μM oxytocin for 3 h at 4 °C, and then solubilized with HDG buffer (HEDG buffer lacking 1 mM EGTA) containing 100 mM NaCl and 0.3% CHAPS for 1 h at 4 °C. The preparation was centrifuged at 45000 g for 1 h at 4 °C, and the supernatant fraction was collected. The supernatant was diluted with HEDG buffer containing 100 mM NaCl to obtain a final concentration of 0.1% CHAPS. The diluted preparation was then applied to a heparin-agarose column that had been pre-equilibrated with the same buffer containing 0.1% CHAPS. The

bound material was eluted with HEDG buffer containing 0.1% CHAPS and 700 mM NaCl. The eluted fraction was incubated overnight in batches at 4 °C with WGA-agarose that had been pre-equilibrated with HEDG buffer containing 300 mM NaCl and 0.1% CHAPS. Then the material was eluted with HEDG buffer containing 100 mM NaCl, 0.1% CHAPS and 300 mM *N*-acetylglucosamine. The resulting partly purified RG was stored at –80 °C until use.

To confirm RG formation and purification, [α - ^{32}P]GTP photoaffinity labelling and [^{35}S]GTP[S] binding of G-protein in partly purified RG were performed by the methods of Linse and Mandelkow [26] and Asano and Ross [27]. The oxytocin-binding activity of oxytocin receptors in partly purified RG was also determined by the method of Klein and Fahrenholz [28]. 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 [^3H]oxytocin (final concentration 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 and free radioactivity were separated by rapid vacuum filtration over Whatman GF/F glass fibre filters. Unlabelled oxytocin (10 μM) was used to determine non-specific binding.

Overexpression and purification of PLC- δ 1

Construction and purification of recombinant vaccinia virus for PLC- δ 1 were accomplished by the method of Cochran et al. [29]. Suspension-cultured HeLa cells were infected with purified recombinant virus at a multiplicity of infection of 20 plaque-forming units per cell. After 24 h the infected cells were centrifuged at 1000 g for 10 min. The pellets were suspended gently in 20 mM Tris/HCl, pH 7.6, containing 1 mM EDTA, 1 mM EGTA, 0.1 mM DTT and 1 mM PMSF. The suspension was sonicated at 4 °C for 1 min (Branson sonifier Model 450) and the lysate was then centrifuged at 100000 g for 1 h. The cytosolic fraction was loaded on a TSKgel DEAE-5PW column that had been pre-equilibrated with 20 mM Tris/HCl, pH 7.6, containing 1 mM EDTA, 1 mM EGTA and 0.1 mM DTT. The bound materials were eluted with an increasing KCl gradient from 0 to 0.3 M over 40 min. The eluted fractions containing PLC activity were applied to TSKgel Heparin-5PW that had been pre-equilibrated with 20 mM Hepes, pH 7.0, containing 1 mM EDTA, 1 mM EGTA and 0.1 mM DTT. The material was then eluted with a KCl gradient from 0 to 1 M. The purified PLC- δ 1 was stored in the presence of 10% (v/v) glycerol at –80 °C until use. The 69 kDa PLC was purified from bovine liver by using Q-Sepharose, Ultrogel AcA34 and heparin-agarose, by the method described previously [20]. The purities of PLC- δ 1 and 69 kDa PLC used in these experiments were approx. 80% and 70% respectively.

Phospholipase C activity assay

PLC activity was measured with [^3H]PtdIns(4,5) P_2 as a substrate [30]. The reaction mixture contained 20 mM Hepes, pH 7.4, 0.05% CHAPS, 0–175 μM CaCl_2 , 100 mM NaCl and 50 μM [^3H]PtdIns(4,5) P_2 (specific radioactivity 700–800 c.p.m./nmol) in a total volume of 100 μl . The reaction was initiated by adding substrate, then the mixture was incubated at 30 °C for 10 min. The reaction was stopped by adding 0.2 ml of 1 M HCl followed by 0.75 ml of chloroform/methanol/HCl (100:100:0.6, by vol.). The mixtures were centrifuged at 500 g for 10 min. An aliquot (300 μl) of the upper phase was counted for radioactivity (Beckman LS3200).

Reconstitution of RG with PLC- $\delta 1$

Phospholipid vesicles were used for reconstitution of the isolated proteins, as described previously [20]. The lipid vesicles were formed by decreasing the detergent concentration in the mixture. The partly purified RG containing 30 pmol of $G_{h7\alpha}$ and 300 ng of PLC- $\delta 1$ was mixed with a 5 mg/ml phospholipid mixture of phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (3:1:1) (final concentration 0.2 mg/ml) at 4 °C for 30 min. The PLC activity was then measured with various combinations of ligands.

Antibody experiments

Polyclonal antibodies against $G_{h7\alpha}$ or 69 kDa PLC were prepared and characterized as described previously [17,19]. Purified proteins (40–70 μ g/200 μ l) were emulsified with an equal volume of complete Freund's adjuvant and injected subcutaneously into New Zealand White rabbits. Rabbit antisera were characterized by immunoblots or immunoprecipitation. Immunoblots were performed by the methods of Harris et al. [31]. Briefly, proteins were separated on 7.5–12% (w/v) gels by SDS/PAGE and then transferred to Immobilon-P (Millipore). The membranes were blocked for 1 h with low-detergent blotting [LDB; 80 mM NaCl/2 mM $CaCl_2$ /0.02% NaN_3 /0.2% (v/v) NP40/50 mM Tris/HCl (pH 8.0) containing 5% (w/v) non-fat dry milk] at room temperature, then incubated with LDB containing monoclonal antibody against PLC- $\delta 1$ (1:1000 dilution) for 1 h at room temperature. After being washed with LDB, the membranes were incubated with anti-mouse immunoglobulin, peroxidase-linked species-specific antibody (1:1000 dilution) in high-detergent blotting [HDB; 2% (v/v) NP40 in LDB] for 1 h at room temperature. After being washed three times, the membranes were subjected to procedures for enhanced chemiluminescence (Amersham, Little Chalfont, Bucks., U.K.). Immunoprecipitation of solubilized membranes with anti- $G_{h7\alpha}$ antibody was performed essentially as described previously [19].

For co-immunoprecipitation of PLC- $\delta 1$, the partly purified RG preparation (containing 2 pmol of $G_{h7\alpha}$) was preincubated with 20 ng of PLC- $\delta 1$ and 5 μ M GTP[S], 0.1 mM GDP or 0.5 mM adenosine 5'-[β,γ -imido]triphosphate (p[NH]ppA) in the presence of 5 μ M oxytocin and 2 mM $MgCl_2$ at 4 °C for 1 h. The oxytocin receptor- $G_{h7\alpha}$ -PLC- $\delta 1$ complex preparation was then incubated for 2 h with 5 μ l of anti- $G_{h7\alpha}$ antibody at 4 °C. The antigen-antibody complexes were precipitated with 10 μ l of Protein A-agarose (binding capacity 22 mg of rabbit IgG/ml of agarose). The supernatant was collected by centrifugation at 500 *g* for 10 min and the PLC activity was then measured as described above. For co-immunoadsorption, the antibody-affinity column (50 μ l) was prepared by incubation of Protein A-agarose gel with anti- $G_{h7\alpha}$ antibody [32]. Briefly, the Protein A-agarose gel (50 μ l) 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, then further washed three times with 0.2 M triethanolamine, pH 8.2. The beads 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 use. The anti- $G_{h7\alpha}$ antibody-affinity gel was then incubated with the oxytocin receptor-G-protein-PLC- $\delta 1$ complex, which contained 250 fmol of receptor and 20 ng of PLC- $\delta 1$, for 2 h at 4 °C. After this time the gel was washed with 20 mM Hepes, pH 7.4, containing 100 mM NaCl, 0.5 mM DTT and 0.05%

CHAPS, and the receptor density and PLC activity of both unbound and bound material were measured. For these studies anti- $G_{h7\alpha}$ antibody was purified by using Protein A-Sepharose CL-4B.

Protein determination

The amount of protein was estimated by the method of Bradford [33], with the Bio-Rad protein determination kit and BSA as a standard.

RESULTS

Stimulation of PLC activity in solubilized membranes from human myometrium

To evaluate the possibility of functional activation of PLC in oxytocin receptor-mediated transmembrane signalling, PLC activation with the solubilized membranes from human myometrium was examined in the presence of guanine nucleotides and oxytocin. As shown in Figure 1, stimulation of PLC activity by GTP[S] reached a maximal formation of inositol phosphates at 50–75 μ M Ca^{2+} , whereas in the presence of GDP or buffer approx. 150 μ M Ca^{2+} was required to achieve maximal PLC activity without changing the maximal amount of inositol phosphates formed. Furthermore when the oxytocin receptor antagonist SS-1-7 was included in the preincubation mixture, PLC activity was markedly attenuated; more than twice the concentration of Ca^{2+} was required for maximal activity. These results demonstrate that the oxytocin receptor activates PLC through a G-protein in the solubilized membranes. The biphasic PLC activity with the changes in Ca^{2+} concentration (0–175 μ M) was observed only in the presence of oxytocin and GTP[S]. The

Figure 1 Stimulation of PLC activity by GTP[S] in solubilized membrane extracts from human myometrium

Purified human myometrial membranes (10 mg/ml protein) were solubilized with 0.3% CHAPS in HDG buffer containing 100 mM NaCl at 4 °C for 1 h. Membranes (0.5 mg/ml protein) were preincubated with 5 μ M GTP[S], 0.1 mM GDP or HDG buffer in the presence of 5 μ M oxytocin, or 5 μ M oxytocin plus 100 μ M SS-1-7 and 2 mM $MgCl_2$ at 30 °C for 30 min. Samples were then incubated with 20 mM Hepes, pH 7.4, containing 100 mM NaCl, 0.5 mM DTT and various concentrations of Ca^{2+} in a final volume of 100 μ l at 30 °C for 10 min. Inositol phosphate formation by PLC was measured as detailed in the Experimental section. Symbols: ●, oxytocin plus GTP[S]; ▲, oxytocin plus SS-1-7 plus GTP[S]; ■, oxytocin plus GDP; ▼, oxytocin plus HDG buffer. Each point is the average of triplicate measurements and is representative of three independent experiments.

Table 1 $G_{h\alpha}$ -mediated PLC activation in solubilized membranes of human myometrium

After solubilization, the membrane extracts (0.5 mg/ml protein) were immunoprecipitated with anti- $G_{h\alpha}$ antibody conjugated to Protein A-agarose as described in the Experimental section. The PLC activity was measured in the supernatants after incubation with 5 μ M GTP[S] in the presence of 5 μ M oxytocin and 2 mM $MgCl_2$ at 4 °C for 1 h. Samples were also treated with preimmune sera or Protein A-agarose to determine the specificity of the anti- $G_{h\alpha}$ antibody. PLC activity is expressed as a percentage of inositol phosphate formation in the solubilized membranes without immunoprecipitation (Control). The results shown are means $\pm$ S.D. for three independent experiments performed in triplicate.

Treatment	PLC activity (% of control)
Protein A-agarose	102 $\pm$ 3
Preimmune sera	93 $\pm$ 7
Anti- $G_{h\alpha}$ antibody	45 $\pm$ 9

G-protein-mediated stimulation of the PLC activity decreased as the concentration of Ca^{2+} increased (more than 100 μ M). We have previously shown the same type of PLC activation by G_h in α_1 -adrenoceptor-mediated signalling [16,20]. The reason for such inhibition by high concentrations of calcium is not clear, but it may be a self-regulatory system.

Our previous observation that the oxytocin receptor couples to the 80 kDa $G_{h\alpha}$ protein for signal mediation in human myometrium [22] and the above results indicating activation of PLC via a G-protein led us to examine the role of $G_{h\alpha}$ on PLC activation in oxytocin receptor signalling. We therefore performed immunoprecipitations on the membrane extracts with anti- $G_{h\alpha}$ antibody to remove $G_{h\alpha}$, and confirmed the decrease of $G_{h\alpha}$ in the supernatants by immunoblotting with anti- $G_{h\alpha}$ antibody (results not shown). The PLC activity was then measured in the supernatants after incubation with GTP[S] and oxytocin. As shown in Table 1, the PLC activity in the supernatant was decreased to 45% of the control in spite of receptor activation. Thus 55% of the PLC activity in the supernatant was attenuated by the precipitation of $G_{h\alpha}$ by anti- $G_{h\alpha}$ antibody. The remaining PLC activity, (i.e. that activity not affected by the absence of $G_{h\alpha}$), might come from another signal pathway mediated by the G_q family [5,6], from an unidentified mechanism of PLC activation or from $G_{h\alpha}$ remaining in the supernatant owing to incomplete immunoprecipitation of $G_{h\alpha}$. In contrast, nearly 100% of control PLC activity was found in supernatants when the samples were treated with preimmune sera or Protein A-agarose. These results clearly demonstrate that PLC functions as an effector of oxytocin receptor signalling stimulated by activated- $G_{h\alpha}$.

Coupling of activated $G_{h\alpha}$ and PLC- $\delta 1$ in solubilized membranes from human myometrium

Recently PLC- $\delta 1$ has been implicated in G_h -mediated signalling in experiments with synthetic peptides derived from human heart $G_{h\alpha}$ [17], and elucidation of the crystal structure of mammalian PLC- $\delta 1$ has provided a mechanism for membrane attachment and Ca^{2+} -dependent hydrolysis of phosphoinositides [34]. We have previously shown that the oxytocin receptor couples to the 80 kDa $G_{h\alpha}$ protein for transmembrane signalling in human myometrium [22]. The present results imply strongly that PLC- $\delta 1$ might also be an effector of oxytocin receptor signalling. To examine the nature of the PLC involved in oxytocin receptor signal transduction, we first tested for the existence of PLC- $\delta 1$ in human myometrium by immunoblot analysis with a monoclonal

Figure 2 Activated $G_{h\alpha}$ couples to PLC- $\delta 1$ in solubilized membranes of human myometrium

(a) Identification of PLC- $\delta 1$ and 69 kDa PLC in human myometrial membranes. Purified PLC- $\delta 1$ (50 ng), solubilized human myometrial membranes (100 μ g; HM) or purified 69 kDa PLC (50 ng) were subjected to SDS/PAGE (10% gel) and transferred to Immobilon-P. Immunoblotting was performed with a monoclonal antibody to PLC- $\delta 1$ as described in the Experimental section. (b) Coupling of activated $G_{h\alpha}$ and PLC- $\delta 1$. After solubilization in the presence of 5 μ M GTP[S], 2 mM $MgCl_2$ and 5 μ M oxytocin, or 5 μ M oxytocin plus 100 μ M SS-1-7, the membrane extracts (0.5 mg/ml protein) were immunoprecipitated with anti- $G_{h\alpha}$ antibody. Immunoblotting was then performed in the pellets by using the monoclonal antibody to PLC- $\delta 1$. Samples were also treated with preimmune sera or Protein A-agarose. Lane 1, oxytocin plus Protein A-agarose; lane 2, oxytocin plus preimmune sera; lane 3, oxytocin plus anti- $G_{h\alpha}$ antibody; lane 4, oxytocin plus SS-1-7 plus anti- $G_{h\alpha}$ antibody.

antibody to PLC- $\delta 1$. As shown in Figure 2(a), the antibody recognized 69 and 85 kDa proteins in human myometrium as well as purified PLC- $\delta 1$ and 69 kDa PLC. The cross-reactivity of the antibody against 69 kDa PLC was the same as that reported by Feng et al. [17]. Moreover, when similar experiments were performed with a polyclonal antibody to 69 kDa PLC, the results were essentially the same as above, and the preimmune sera showed completely negative results (results not shown). These results suggested the presence of common epitopes in 85 and 69 kDa proteins from human myometrial membranes. To evaluate the coupling of $G_{h\alpha}$ and PLC- $\delta 1$ in oxytocin receptor signalling from human myometrium, solubilized membranes in the presence of oxytocin and GTP[S] were immunoprecipitated with anti- $G_{h\alpha}$ antibody and then the pellets were subjected to immunoblot analysis with monoclonal antibody to PLC- $\delta 1$. As demonstrated in Figure 2(b), PLC- $\delta 1$ coupled to $G_{h\alpha}$, which was activated by oxytocin and GTP[S] (lane 3). When SS-1-7 was included in the solubilized mixture, the coupling ability of $G_{h\alpha}$ and PLC- $\delta 1$ was attenuated (Figure 2b, lane 4). Furthermore, preimmune sera and Protein A-agarose did not give the above results (Figure 2b, lanes 1 and 2). We also confirmed the presence of $G_{h\alpha}$ in the pellets shown in Figure 2(b), lanes 3 and 4, by immunoblot analysis with anti- $G_{h\alpha}$ antibody (results not shown). These results showed that activated- $G_{h\alpha}$ couples to PLC- $\delta 1$ in oxytocin receptor signalling in this human myometrium preparation.

Reconstitution of an oxytocin receptor- $G_{h\alpha}$ -PLC- $\delta 1$ complex preparation

To further investigate whether PLC- $\delta 1$ is activated in the presence of the oxytocin receptor, the purified RG from human myometrial membranes and PLC- $\delta 1$ were mixed with phospholipids. The presence of the 80 kDa $G_{h\alpha}$ and the oxytocin receptor in purified RG was confirmed by photoaffinity labelling with [α - ^{32}P]GTP and binding assays. As we have found previously [22], despite

Figure 3 Oxytocin-mediated PLC- $\delta 1$ activation in the preparation of oxytocin receptor- $G_{h7\alpha}$ -PLC- $\delta 1$ complex

RG containing 30 pmol of $G_{h7\alpha}$ was purified from human myometrial membranes. The partly purified RG and 300 ng of PLC- $\delta 1$ were mixed with a phospholipid mixture (5 mg/ml) to a final concentration of 0.2 mg/ml in 500 μ l of 20 mM Hepes, pH 7.4, containing 100 mM NaCl and 1 mM DTT. (a) Oxytocin-mediated PLC- $\delta 1$ activation. The phospholipid vesicles (20 μ l) containing the three components were preactivated with various concentrations of oxytocin in 75 μ l of 20 mM Hepes (pH 7.4) containing 100 mM NaCl, but in the absence of Ca^{2+} and GTP[S], at 30 °C for 30 min. The mixtures were then chilled in an ice bath for 10 min. After addition of 5 μ M $CaCl_2$, 5 μ M GTP[S] and 0.2 mM $MgCl_2$, the mixtures were further incubated in a final volume of 100 μ l at 30 °C for 10 min. The PLC activity was measured as detailed in the Experimental section. (b) Time course of oxytocin-mediated PLC- $\delta 1$ activation. The vesicles (20 μ l) were preincubated with 5 μ M oxytocin, 5 μ M oxytocin plus 100 μ M SS-1-7, or without ligand at 30 °C for 30 min. The samples were then incubated with 5 μ M $CaCl_2$, 0.2 mM $MgCl_2$ and 5 μ M GTP[S] in a final volume of 100 μ l at 30 °C. After different durations, reactions were stopped by adding 0.75 ml of chloroform/methanol/1 M HCl (100:100:0.6, by vol.). To confirm that oxytocin-stimulated PLC activity occurred through a G-protein, the oxytocin-preincubated samples were incubated with 5 μ M GDP instead of GTP[S], and the PLC activity assay was performed as described in the Experimental section. Symbols: ●, oxytocin plus GTP[S]; ▲, oxytocin plus SS-1-7 plus GTP[S]; ■, without ligand plus GTP[S]; ▼, oxytocin plus GDP. The results are representative of three independent experiments, and each point is the average of triplicate determinations.

contamination of the partly purified RG by other proteins, it did not contain PLC or other G-proteins (results not shown). Figure 3(a) shows oxytocin-mediated PLC- $\delta 1$ stimulation. The reconstituted samples containing RG and PLC- $\delta 1$ were incubated with various concentrations of oxytocin in the presence of GTP[S] and Ca^{2+} . The PLC- $\delta 1$ activity increased linearly up to 50 μ M oxytocin; the EC_{50} for oxytocin was approx. 3 μ M, with a maximal response at 100 μ M in the presence of 5 μ M Ca^{2+} . When similar experiments were performed with solubilized membranes from human myometrium, the EC_{50} for oxytocin was approx. 10 nM with a maximal response at 1 μ M (results not shown). This difference is probably due to an altered efficacy of the protein-protein interactions in the system *in vitro* with phospholipid reconstitution.

The time course of PLC- $\delta 1$ stimulation in response to oxytocin was also measured in the presence of GTP[S], GDP or SS-1-7. PLC- $\delta 1$ activation stimulated by oxytocin in the presence of GTP[S] was much faster than that observed in the presence of SS-1-7, GDP or in the absence of oxytocin (Figure 3b). These results not only confirm that PLC- $\delta 1$ is a key component in oxytocin receptor signalling but also suggest that oxytocin receptor-coupled $G_{h7\alpha}$ is involved in oxytocin-induced PLC- $\delta 1$ activation. The time required for stimulation of PLC in the presence of agonist was longer than that observed in a cultured

Table 2 Co-immunoprecipitation of PLC- $\delta 1$ in the oxytocin receptor/ $G_{h7\alpha}$ -PLC- $\delta 1$ complex preparation by the anti- $G_{h7\alpha}$ antibody

RG containing 2 pmol of $G_{h7\alpha}$ was purified from human myometrial membranes. The partly purified RG was incubated with 20 ng of PLC- $\delta 1$ and 5 μ M GTP[S], 0.1 mM GDP or 0.5 mM p[NH₂]ppA in the presence of 5 μ M oxytocin and 2 mM $MgCl_2$ at 4 °C for 1 h. PLC activity was measured in the supernatants after immunoprecipitation with anti- $G_{h7\alpha}$ antibody as described in the Experimental section. Samples were also incubated with preimmune sera or Protein A-agarose to determine the specificity of the anti- $G_{h7\alpha}$ antibody. PLC activity is expressed as a percentage of inositol phosphate formation in each experimental condition without immunoprecipitation (none). The results shown are means $\pm$ S.D. for three independent experiments performed in triplicate.

Treatment	PLC activity (%)			
	PLC- $\delta 1$ alone	PLC- $\delta 1$ + RG + GTP[S]	PLC- $\delta 1$ + RG + GDP	PLC- $\delta 1$ + RG + p[NH]ppA
None	100	100	100	100
Protein A-agarose	97 $\pm$ 7	98 $\pm$ 4	102 $\pm$ 5	97 $\pm$ 4
Preimmune sera	101 $\pm$ 6	105 $\pm$ 12	95 $\pm$ 13	102 $\pm$ 10
Anti- $G_{h7\alpha}$ antibody	96 $\pm$ 13	37 $\pm$ 10	98 $\pm$ 11	93 $\pm$ 14

cell system (less than 1 min) [35]. This might also be caused by an altered efficacy of the protein-protein interactions in this system.

To investigate the role of 69 kDa PLC in oxytocin receptor signalling, we have also replaced PLC- $\delta 1$ with purified 69 kDa PLC under the same experimental conditions. Results with 69 kDa PLC (results not shown) were similar to those with PLC- $\delta 1$, as shown in Figure 3, suggesting that the 69 kDa PLC is functionally similar to PLC- $\delta 1$. Recently the 69 kDa PLC has been suggested to be a proteolytic fragment of PLC- $\delta 1$ [17]. Our results further support this notion in that these two PLC isoforms of the enzyme share a common structural feature, thus exhibiting similarities in their functional response as well as immunological properties.

Co-immunological reactivity of PLC- $\delta 1$ and $G_{h7\alpha}$ complex with an anti- $G_{h7\alpha}$ antibody

The formation of the G-protein-PLC complex might be induced by the binding of agonist to the receptor in the presence of GTP. The activated receptors stimulate specific G-protein(s), and activated G-proteins then couple with their specific effector(s), such as PLC [36]. As a result of this sequential process, hormone, receptor, G-protein and effector become associated, forming an intermediate in which the G-protein is primed for guanine nucleotide binding [37]. On the basis of the above considerations, we prepared the three-component mixture containing RG and PLC- $\delta 1$ to identify the direct interaction between $G_{h7\alpha}$ and PLC- $\delta 1$. The three-component mixture was incubated with GTP[S], GDP or p(NH)ppA in the presence of oxytocin. The PLC activity was measured in supernatants after immunoprecipitation with anti- $G_{h7\alpha}$ antibody conjugated to Protein A-agarose. As shown in Table 2, decreased-PLC- $\delta 1$ activity was observed only in the sample in which the $G_{h7\alpha}$ -PLC- $\delta 1$ complex was induced in the presence of GTP[S]. In this sample, the PLC- $\delta 1$ activity was decreased by 63 %, indicating that most PLC- $\delta 1$ was co-immunoprecipitated by the antibody. In contrast, when the samples were incubated with GDP or p(NH)ppA, most PLC- $\delta 1$ activity remained in the supernatant. Studies with preimmune sera also resulted in most of the enzyme activity remaining in the supernatant. Similarly, anti- $G_{h7\alpha}$ antibody had no effect on PLC- $\delta 1$ alone. These results demonstrate the tight association of PLC- $\delta 1$ with activated $G_{h7\alpha}$ in oxytocin receptor signalling.

Table 3 Co-immunoabsorption of the oxytocin receptor- $G_{h\alpha}$ -PLC- $\delta 1$ complex preparation by anti- $G_{h\alpha}$ antibody-affinity gel

The anti- $G_{h\alpha}$ antibody-affinity gel (50 μ l) was prepared by incubating Protein A-agarose with antibodies raised against $G_{h\alpha}$. RG containing 250 fmol of oxytocin receptor was purified from human myometrial membranes. The partly purified RG was incubated with 20 ng of PLC- $\delta 1$ and the affinity gel at 4 °C for 2 h in the presence of 1 mM $MgCl_2$, and either 5 μ M oxytocin alone, 5 μ M oxytocin plus 50 μ M GTP, or 5 μ M oxytocin plus 50 μ M GTP and 100 μ M SS-1-7. Unbound materials were eluted from the column by applying gentle pressure until the column was dry. For the co-immunoabsorption of oxytocin receptors, co-adsorbed components were eluted by incubating the gel with 2 μ M oxytocin, 5 μ M GTP[S] and 1 mM $MgCl_2$ for 1 h at 4 °C. The receptor density is expressed as a percentage of the 250 fmol of oxytocin receptor in the RG before co-immunoabsorption (control). For co-immunoabsorption of PLC- $\delta 1$, co-adsorbed components were eluted by incubating the gel with 2 μ M oxytocin, 0.1 mM guanosine 5'-[β -thio]diphosphate and 1 mM $MgCl_2$ for 1 h at 4 °C. PLC activity is expressed as a percentage of inositol phosphate formation in the 20 ng of PLC- $\delta 1$ before co-immunoabsorption (control). The PLC activity and receptor density in the flow-through and eluted fractions were determined after removing excess ligands on a dried Sephadex G-25 column. The results are means $\pm$ S.D. for three independent experiments performed in triplicate.

Treatment	Receptor density (% of control)		PLC activity (% of control)	
	Flow-through	Eluate	Flow-through	Eluate
Oxytocin	21 $\pm$ 4	73 $\pm$ 7	87 $\pm$ 4	12 $\pm$ 2
Oxytocin + GTP	65 $\pm$ 5	18 $\pm$ 8	31 $\pm$ 6	71 $\pm$ 8
Oxytocin + GTP + SS-1-7	79 $\pm$ 6	15 $\pm$ 11	75 $\pm$ 7	18 $\pm$ 3

Finally, to determine the functional interaction between the $G_{h\alpha}$ and PLC- $\delta 1$ as well as the oxytocin receptor, the three-component mixture was treated with an anti- $G_{h\alpha}$ antibody-affinity gel in the presence of oxytocin, with either GTP, or GTP plus SS-1-7. The PLC- $\delta 1$ activity and oxytocin receptor binding activity were then measured in the flow-through and eluted fractions after removing excess ligands. As shown in Table 3, co-immunoabsorption of oxytocin receptors did not occur if RG was dissociated by treatment with either oxytocin plus GTP[S] or oxytocin plus SS-1-7. When the complex was incubated only with oxytocin, sample eluted from the antibody-affinity column demonstrated the highest receptor binding activity (73 %), indicating that the oxytocin receptors were effectively bound to the antibody-affinity gel through $G_{h\alpha}$. Consistent with this observation, the lowest binding (21 %) was observed in the flow-through samples. In the presence of oxytocin and GTP or the oxytocin antagonist SS-1-7, the receptor binding activity in the eluates was less than 20 % of that initially applied. In these experiments, 65 % and 79 % of the receptor (dissociated from $G_{h\alpha}$) was found in the flow-through fractions. As a control, when the samples were incubated with preimmune sera-Protein A-agarose, most of the receptors were found in the flow-through fractions with residual binding (less than 7 %) to the antibody-affinity gel. These results were the same as co-immunoabsorption of oxytocin receptor with RG preparations in our previous studies [22]. In contrast, co-immunoabsorption of PLC- $\delta 1$ did not occur if $G_{h\alpha}$ was not activated by the addition of oxytocin either in the absence of GTP or in the presence of GTP and SS-1-7. When incubated with oxytocin and GTP, the eluate showed the highest PLC activity (71 %), whereas the lowest binding (31 %) was observed in the flow-through samples, indicating that PLC- $\delta 1$ was effectively bound to the antibody-affinity gel through $G_{h\alpha}$. When $G_{h\alpha}$ was not activated, the PLC activity in the eluates was less than 20 % of the initial PLC applied. In these experiments, 87 % and 75 % of PLC- $\delta 1$ were found in the flow-through fractions. When preimmune sera-Protein A-agarose was used in all of the above experiments, most of the PLC- $\delta 1$ activity was

found in the flow-through fractions, with residual binding (less than 5 %) to the antibody affinity gel.

To confirm the role of 69 kDa PLC in this signalling, we have also undertaken experiments with the purified 69 kDa PLC instead of PLC- $\delta 1$. The 69 kDa PLC again showed similar results to those described for PLC- $\delta 1$ (results not shown), suggesting that the 69 kDa PLC is functionally similar to PLC- $\delta 1$. By analogy with other G-protein-coupled receptors [36,38], if oxytocin promotes the formation of an oxytocin-receptor- $G_{h\alpha}$ ternary complex, the addition of GTP causes a dissociation of the $G_{h\alpha}$ from the receptor, and activated (GTP-bound) $G_{h\alpha}$ then couples to PLC- $\delta 1$. These observations strongly suggest that PLC- $\delta 1$ couples to the activated $G_{h\alpha}$ and has a role as an effector in oxytocin receptor signalling in the human myometrium.

DISCUSSION

Despite the potential physiological significance, the mechanism by which oxytocin stimulates Ca^{2+} mobilization has not been elucidated. The three classes of phosphoinositide-specific PLC, PLC- β , PLC- γ and PLC- δ , are products of separate genes and can exist in both soluble and membrane-bound forms [12]. Among these known isoenzymes, PLC- $\beta 1$ and PLC- $\gamma 1$ are not stimulated by activated $G_{h\alpha}$ in reconstituted systems, but a 69 kDa PLC was coupled to the $G_{h\alpha}$ family proteins [16,20]. Recently it has been suggested that 69 kDa PLC is the proteolytic fragment of PLC- $\delta 1$, and PLC- $\delta 1$ might couple to the activated $G_{h\alpha}$ as an effector of $G_{h\alpha}$ signalling [17].

The first suggestion for involvement of a G-protein in the action of oxytocin on myometrium was presented by Marc et al. [4]. They showed that oxytocin receptors were coupled to PLC through a pertussis toxin-insensitive G-protein in guinea pig myometrium [4]. Phaneuf et al. [21] suggested that, in human myometrial cells, the oxytocin-induced production of inositol phosphates and increase in intracellular free Ca^{2+} are mediated by a pertussis toxin-sensitive G-protein. However, a significant fraction of the oxytocin response seemed to be mediated by a pertussis toxin-insensitive G-protein, possibly a member of the G_q family. Recently Phaneuf et al. [7] again demonstrated that oxytocin activates human myometrium by interacting with at least two G-proteins and possibly three PLC- β isoforms, but $G_{h\alpha}$ and PLC- $\delta 1$ were not discussed in that paper. Arnaudeau et al. [5] showed that oxytocin stimulation of intracellular free Ca^{2+} was insensitive to incubation with pertussis toxin, and could be blocked by an anti- $G_{q/11\alpha}$ antibody in single myometrial cells from pregnant rats. Ku et al. [6] have demonstrated that oxytocin stimulates both GTPase and PLC activities, and both these stimulations are attenuated by an antibody directed against the C-termini of $G_{q\alpha}$ and $G_{11\alpha}$ in rat and human myometrial membranes. These reports provided evidence for the stimulation of PLC (probably PLC- β) by the oxytocin receptor through $G_{q/11}$.

In contrast, our previous results showed that the oxytocin receptor couples to the $G_{h\alpha}$ family of proteins in human myometrium [22]. Moreover, our present experiments showed that PLC- $\delta 1$ activity seemed to be activated more rapidly and achieved higher activities in the presence of oxytocin than in the presence of SS-1-7 or in the absence of oxytocin. Increased PLC- $\delta 1$ activity was caused by activated $G_{h\alpha}$. Consistent with these observations, immunological cross-reactivity by anti- $G_{h\alpha}$ antibody demonstrated a tight coupling of PLC- $\delta 1$ with activated $G_{h\alpha}$ by stimulation of oxytocin receptor.

The mechanism of cross-talk of the oxytocin receptor to various G-proteins and effectors in cellular signal transduction is

not clear. The discrepancies between our findings and the other results have the following multiple possibilities as far as the oxytocin receptor-mediated signal transduction mechanism is concerned. First, different G-proteins and PLC isoenzymes might be the mediators of different activation pathways [23], just as the thrombin receptor simultaneously and directly couples to G_{i2} and $G_{q/11}$ [39] and the endothelin receptor couples to modulate PLC and adenylate cyclase activities [40]. Secondly, subtypes of the oxytocin receptor might exist in human myometrium, such that the presence of two distinct subtypes has been suggested in rat myometrium and decidua [41] and kidney [42]. Thirdly, depending on the cell system and the nature and the density of the protein involved, one or all of the pathways may become active. Recently, Vincan et al. [43] reported that isolation and culture of neonatal cardiomyocytes decreased the expression of G_h without reducing the content of G_q or G_i . But G_h was easily detected in extracts from intact heart tissue from newborns and adults. The expression of G_h thus seems to be a feature of intact cardiac tissue. Fourthly, the discrepancies might be due to the difference between the system used by Arnaudeau et al. [5], single myometrial cells, and that used by us, the purification and reconstitution of the components in the phospholipid vesicles. Because the same receptor can use multiple G-proteins and effectors to transmit its signal [44,45], careful design of experiments with many possible combinations of receptor, G-proteins and effectors is required. These experiments could help not only to elucidate the exact reason for discrepancies, but also to predict the diverse regulatory mechanisms in G-protein-mediated receptor signalling.

We thank Dr. Maurice Manning (Medical College of Ohio, Toledo, OH, U.S.A.) for the gift of oxytocin antagonist; Dr. Mie-Jae Im (The Cleveland Clinic Foundation, Cleveland, OH, U.S.A.) and Dr. Sangduk Kim (Korea University, Seoul, Korea) for valuable suggestions; Caroline Gray (Hiram College, Hiram, OH, U.S.A.) for editorial assistance on the manuscript; and Mr. Hyunguk Park (Chung-Ang University, Seoul, Korea) for technical assistance. This work was supported by research grants from the Korea Science and Engineering Foundation (961-0713-089-2 and 95-0403-08-02-3), a basic medical science grant from the Korean Ministry of Education (1995–1996), and a Chung-Ang University research grant in 1996.

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