α₁-Adrenergic Receptor Signaling via Gₖ Is Subtype Specific and Independent of Its Transglutaminase Activity*

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Songhai Chen, Fang Lin, Siiri Iismaa, Kyung N. Lee‡§, Paul J. Birckbichler¶, and Robert M. Graham

From the Victor Chang Cardiac Research Institute, St. Vincent’s Hospital, Darlinghurst, New South Wales 2010, Australia and The Samuel Roberts Noble Foundation, Ardmore, Oklahoma 73401

Tissue transglutaminase (TGase II) is a Ca²⁺- and thiol-dependent enzyme that catalyzes the post-translational modification of proteins via the formation of ε(γ-glu-tamyl) lysine bonds. We have shown previously that the GTP-binding protein, Gₖ, is a TGase II that mediates intracellular signaling by the α₁B-adrenergic receptor (AR) (Nakaoka, H., Perez, D. M., Baek, K. J., Das, T., Husain, A., Mison, K., Im, M.-J., and Graham, R. M. (1994) Science 263, 1592–1596). Here, we evaluated the ability of Gₖ as compared with Gₛ to mediate receptor-stimulated inositol phosphate turnover by the three α₁-subtypes (α₁A, α₁B, and α₁D). In addition, we questioned if the transglutaminase function of Gₖ is involved in its receptor signaling activity. A mutant form of a human TGase II cDNA in which the codon for the active site cysteine (Cys²⁷⁷) was replaced by serine was cloned into the mammalian expression vector pMT2. Compared with wild-type TGase II, no transglutaminase activity was observed with transient transfection of this Cys→Ser mutant in COS-1 cells. However, like wild-type TGase, the Cys→Ser mutant mediated receptor-stimulated inositol phosphate turnover when cotransfected with an α₁B-AR cDNA. Gₖ supported α₁B-AR-mediated inositol phosphate turnover by all three receptor subtypes. By contrast, although both the wild-type and Cys→Ser construct mediated receptor signaling by the α₁B AR and α₁D AR, the α₁A-AR was unable to interact with Gₖ. However, α₁D-AR-dependent signaling phenotype could be rescued by a chimeric α₁A construct in which the third intracellular loop of the α₁A-AR was replaced by that of the α₁B-AR. Thus, the signaling function of Gₖ is independent of its transglutaminase activity and is α₁-AR subtype specific. This subtype specificity of the interaction between α₁ ARs and Gₖ involves important determinants in their third intracellular loops.

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‡ Present address: Dept. of Urology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190.

§ Present address: Dept. of Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190.

¶ Present address: Dept. of Urology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73190.

To whom correspondence should be addressed: Victor Chang Cardiac Research Inst., 376 Victoria St., Darlinghurst 2010, New South Wales, Australia. Fax: 61-2-295-8501; E-mail: b.graham@victorchang.unsw.edu.au.

The abbreviations used are: AR(s), adrenergic receptor(s); G-protein, guanine nucleotide-binding regulatory proteins; EC₅₀, concentration of agonist causing 50% of the maximal response; PI-PLC, phosphoinositide-specific phospholipase C; IP, inositol phosphate(s); GTPγS, guanosine 5’-O-(3-thiotriphosphate).
levels were low or absent could transglutaminase activity be observed. Along the same lines, there is recent evidence that a role of TGase II in the regulation of cell cycle progression is independent of its transglutaminase activity (15).

Based on these considerations, this study was designed to obtain more definitive evidence that the receptor signaling function of Gh is independent of its transglutaminase activity. This was achieved by evaluating the receptor-signaling activity of a mutant form of TGase II that retains its ability to bind and hydrolyze GTP, but lacks transglutaminase activity. In addition, we investigated the ability of Gh, as compared with the heterotrimeric G-protein Gq, to interact not only with the a subunit, but also with other a subtypes.

The results of these studies indicate that the receptor signaling function of Gh is dependent on its protein cross-linking activity. Moreover, while Gh supports PI-PLC activation by all three a subtypes, the interaction of Gh with a subunit is subtype specific, since it is observed with the a1B and a1D subtypes but not with the a1A-AR. This subtype specificity involves determinants in the third intracellular loop, since Gh-dependent signaling can be restored with a chimeric construct in which the third intracellular loop of the a1A-AR is replaced with that of the a1B-AR.

EXPERIMENTAL PROCEDURES

MATERIALS—(1) Epinephrine, phenolamine hydrochloride, lithium chloride, N,N'-dimethylcasein, and m-propanol were purchased from Sigma. AG 1-XS (100–200 mesh, formate form) was from Bio-Rad, and fetal calf serum and culture media were from Trace Biosciences (Australia). myo-[3H]Insitol (80 Ci/mmol), [7-methoxy-[3H]proprazin (77.9 Ci/mmol), [1,6,7,9]H]putrescine, and chemiluminescent reagents were obtained from Amersham Corp. U73122 (1-6-[17β-3-methoxyestra-1,3,5(10)-trien-17-ylaminio]-1H-pyrrole-2,3-dione) and U73443 (1-6-[17β-3-methoxyestra-1,3,5(10)-trien-17-yl] aminoxylyl]-2,5-pyridyldione-dime) were from Calbiochem. Other chemicals were of the highest grade available commercially.

Vectors and DNA Constructs—The a1-AR and rat TGase II (Gq) constructs used were the hamster a1B-AR cDNA, the rat a1A-AR cDNA, and the rat a1D-AR cDNA, and rat TGase II cDNA cloned into the modified eukaryotic expression vector, pMT2, as described previously (17–20). All of the following cDNAs were also subcloned into pMT2 between the EcoRI (5) and NotI (3’) sites in its polylinker: a1B-AR, originally isolated by Moser (21); human TGase II, kindly provided in pGEM by Dr. V. Gentile (University of Naples); and the mutant form of human TGase II containing a serine codon instead of the codon for the active site cysteine (Cys577), originally provided in the prokaryotic expression vector, pET-Sc (22). This mutant is designated here as mTG. Although the studies reported here utilized the wild-type rat TGase II cDNA, the rat and human cDNAs show 90% amino acid homology, and both support a1-AR-mediated PI-PLC activation (data not shown). In all cases, the nucleotide sequences in the cloning site regions were confirmed after subcloning. For transfection, plasmid DNAs were purified by CaCl2-gradient centrifugation followed by Bio-gel A-50m (Bio-Rad) column chromatography, as described (1, 23). Cell Culture and Transfection—COS-1 cells (American Type Culture Collection) were cultured and transiently transfected with the indicated constructs using the DEAE-dextran method, as described previously (1, 18). This method provides a reproducible transfection efficiency of 30–40%, as determined by in situ staining of cells transfected with pSV-Neo2, a plasmid encoding the reporter, β-galactosidase, and treatment of the cells with 0.2% 5-bromo-4-chloro-3-indol β-D-galactoside. Cells were harvested post-transfection.

Membrane Preparation—Membranes were prepared from transfected COS-1 cells, as described previously (17). The membranes were resuspended in HEM buffer (20 mM HEPES, pH 7.5, 1.5 mM EDTA, 12.5 mM MgCl2) containing 10% (w/v) glycerol and stored at −70°C. Protein concentration was determined by the Bradford method (24). Western Blotting—Membranes (20 µg of protein) were dissolved in SDS sample buffer, followed by boiling for 5 min and then subjected to SDS-polyacrylamide gel electrophoresis, as described previously (1, 7). The resolved proteins were electroblotted onto Immobilon-P membranes and then immunostained with detection using the ECL chemiluminescence system (Amersham Corp.), as described previously (7). Gh was detected using a monoclonal antibody to guinea pig liver TGase II (25), and Gh was detected using a polyclonal antisera that was kindly provided by Dr. Michael Crochat (John Curtin School of Medicine, Australian National University). The relative levels of protein expression were determined by densitometric scanning of the bands obtained by immunoblotting.

Transglutaminase Activity—TGase activity was determined as described previously (7). Briefly, 10 µg of membrane protein in 50 µl of 40 mM Tris-HCl (pH 7.4) containing 1 mM MgCl2, 20 mM dithiothreitol, and 20% (v/v) glycerol were incubated with 0.4% (w/v) N,N’-dimethylcaseinin 1 µl of [3H]putrescine, and the various additions indicated for 30 min at 37°C. The reactions were stopped and the incorporated radioactivity determined as described previously (7). Phosphatidylinositol Hydrolysis in Intact Cells—PI hydrolysis by intact, transfected COS-1 cells was determined largely as described previously (4, 18, 19). Briefly, 1 day after transfection, the cells were seeded onto 12-well plates and labeled for 20–24 h with myo-[3H]inositol at 10 µCi/ml in RPMI 1640 inositol-free medium containing 5% fetal calf serum. After labeling, the cells were washed and incubated in serum-free Dulbecco’s modified Eagle’s medium for 4 h, followed by a 20-min incubation with 10 mM LiCl plus 0.1 mM ascorbic acid and 5 x 10−5 M m-propanol. Various drugs were added, as indicated, and the reactions were then stopped by the addition of 20 mM formic acid. Cells were sonicated and the supernatants fractionated to 1-ml packed AG 1-XS columns. The columns were washed with 20 ml of 5 mM inositol, and then total IPs were eluted with 2 ml of 1 M ammonium formate, 0.1 M formic acid. Radioactivity in the eluted fractions was determined by β-spectrometry as described (4). Differences in the number of cells/dish were normalized, based on the basal accumulation of inositol phosphates or the total counts incorporated into the labeled inositol, which were roughly proportional to cell number/dish, as also reported by Wong and Ross (2). The accumulation of total (IP1 and IP3, plus IP7) [3H]inositol phosphates in different experiments is thus given either as counts/minute compared with basal release or as the increase over basal levels. To avoid interassay differences, all treatments e.g. a1B alone, a1B plus SG, and a1B plus Gh were always evaluated together following the transfection of a single batch of cells with the respective cDNAs. Results are expressed as the mean ± S.E.M. (bars). An analysis of variance and Student’s t test were used to determine significant differences (p < 0.05).

Ligand Binding—The ligand binding characteristics of the expressed receptors were determined in a series of radioligand binding studies performed exactly as described previously (18, 19), except using [3H]putrescine, an α1-specific antagonist, as the radioligand. Binding data were analyzed using the iterative non-linear, curve-fitting program LIGAND.

Construction of the a1B (i3-B)-AR chimera—To construct an a1B-AR chimera in which the native third intracellular loop (i3) was replaced with i3 of the a1B-AR, designated a1B (i3-B)-AR, a Phoenix mutagenesis strategy (28) was employed using the hapaxoterminal restriction enzyme, BstXI (Fig. 1). This approach was necessitated by the lack of convenient restriction sites to allow construction of the chimera by a conventional mutagenesis strategy. Briefly, the plasmid containing the cDNA for the hamster i3 was used as a template to amplify a DNA fragment by PCR, which corresponded to the region encoding its i3 as well as nine residues at the C-terminal end of the fifth transmembrane segment that are identical in both the a1B-AR and a1D-AR. The 5’ (sense) 5′-CCATCATTGGCCTTTTGCGGCTTTTTTCCCCTGGAAC-3′, 47 mer) and the 3’ (antisense) 5′-CCAGCGTCTTTGGGCACGCGGTTCGCTTTTTTCCCTGGAAC-3′, 47 mer) primers used for PCR amplification each contained two BstXI recognition sites (underlined) such that with subsequent digestion of the resulting PCR fragment with BstXI, the liberated 5’ and 3’ termini would be complementary to the unique 5’ and 3’ termini generated by digestion of pMPT2 a1B-AR at the BstXI sites (BstXa and BstXb, Fig. 1) flanking the i3 region of the a1B-AR cDNA. pMPT2 a1B-AR was then digested with BstXI to yield four fragments each containing unique 5’ and 3’ termini, which cannot self-hybridize. The a1B-AR cDNA-amplified PCR fragment was digested with BstXI and isolated after gel purification. The four BstXI-generated a1B-AR cDNA fragments were ligated with T4 DNA ligase in the presence of a 10-fold molar excess of the digested and gel-purified a1B-AR cDNA PCR fragment (i3-B) and used to transform Escherichia coli (DH10a). Resulting ampicillin-resistant colonies (due to the expression of an ampicillin resistance gene in pMPT2) containing i3-B incorporated into the a1B-AR cDNA-backbone were identified by colony hybridization using i3-B as a probe. A positive colony was then used to isolate plasmid containing the chimeric construct, pMT2 a1D (i3-B)-AR, and the sequence of the chimeric construct was confirmed.
As shown in Fig. 2, membranes prepared from COS-1 cells transiently transfected with the \( \alpha_{1B} \)-AR cDNA alone showed a low level of endogenously expressed TGase II that was evident as Ca\(^{2+} \)-sensitive TGase activity. As reported previously (7), this Ca\(^{2+} \)-stimulated TGase activity was sensitive to inhibition by monodansylcadaverine or GTP\( \gamma \)S.

The intrinsically COS-1 cell TGase activity was unaltered by cotransfection of \( \alpha_{1B} \) plus mTG, or \( \alpha_{1B} \) plus Gq, although in the cells transfected with \( \alpha_{1B} \) plus mTG the membrane expression of the mutant TGase II was readily apparent (Fig. 2). This confirms that although, as previously shown, the TGase II mutant still binds and hydrolyzes GTP (22), it lacks TGase activity because of replacement of its active site cysteine with a serine residue. COS-1 cells cotransfected with \( \alpha_{1B} \) plus the wild-type TGase II cDNA showed markedly increased membrane expression of TGase II that was evident by immunoblot analysis. Although basal TGase activity (i.e. TGase activity in the absence of Ca\(^{2+} \)) was unaltered in these membranes, TGase activity was markedly increased with Ca\(^{2+} \) activation, and this increase could be inhibited by both monodansylcadaverine and GTP\( \gamma \)S (Fig. 2).

In intact COS-1 cells transiently transfected with \( \alpha_{1B} \)-AR cDNA, stimulation with the adrenergic agonist, (-)epinephrine (in the presence of the \( \beta \)-AR antagonist, dl-propranolol), caused a significant increase in IP accumulation (Fig. 3). This response was due to expression of \( \alpha_{1B} \)-ARs and interaction with endogenously expressed G-proteins and PLC, since it could be inhibited by the \( \alpha \)-antagonist, phenotolamine. Native COS-1 cells do not express \( \alpha_1 \) ARs and stimulation with \( \alpha_1 \) agonists does not increase IP accumulation (data not shown). Both the wild-type TGase II and the mutant TGase II, as well as Gq, significantly enhanced (-)epinephrine-stimulated IP accumulation when cotransfected with the \( \alpha_{1B} \)-AR cDNA, and these (-)epinephrine-mediated responses could be inhibited with phenotolamine. These findings indicate that the mutant TGase II, although lacking TGase activity, can still support \( \alpha_{1B} \)-AR-mediated PLC activation. This was also evident from the time- and dose-dependent activation of PLC observed in COS-1 cells cotransfected with \( \alpha_{1B} \) plus mTG versus \( \alpha_{1B} \) alone (Fig. 4, A and B). As shown in Fig. 4A, the time-dependent increase in IP accumulation with \( \alpha_{1B} \) plus mTG, and also \( \alpha_{1B} \) plus Gq, was significantly greater than with \( \alpha_{1B} \) alone. The EC\(_{50} \) for (-)epinephrine-stimulated IP accumulation decreased from 0.1 ± 0.02 \( \mu \)M in cells transfected with \( \alpha_{1B} \) alone to 0.03 ± 0.00 \( \mu \)M (p < 0.05) in \( \alpha_{1B} \) plus mTG cells, and in these latter cells maximal IP accumulation was significantly greater (Fig. 4B). Since \( \alpha_{1B} \)-AR density was similar in these studies (11.9 ± 1.2 or 8.7 ± 0.7 pmol/mg membrane protein in cells transfected with \( \alpha_{1B} \) or \( \alpha_{1B} \) plus mTG, respectively), these findings indicate that the mutant TG was expressed more abundantly and interacted more efficiently with the \( \alpha_{1B} \)-AR than endogenously expressed G-proteins mediating PLC activation. In cells transfected with \( \alpha_{1B} \) plus Gq, the potency of (-)epinephrine (EC\(_{50} \) 0.4 ± 0.2 \( \mu \)M) for IP accumulation was similar to that observed with \( \alpha_{1B} \) alone. However, consistent with the overexpression of Gq, and despite similar levels of receptor expression in the cells cotransfected with \( \alpha_{1B} \) plus Gq (10.5 ± 1.3 pmol/mg membrane protein), maximal IP accumulation was significantly greater in the \( \alpha_{1B} \) plus Gq cells than in the cells transfected with \( \alpha_{1B} \) alone.

To demonstrate that the (-)epinephrine-stimulated increase in IP accumulation was due to activation of a PI-PLC, we evaluated the effects of the aminosteroid PI-PLC inhibitor...
Species difference (7). In cells transfected with a mutant TGase II, or with Gq. Although, as shown to mediate PLC-activation via an interaction with either the wild-type or mutant TGase II, or Gq. By contrast, U73343 had no effect.

We next evaluated the ability of the α1D and α1A ARs to mediate PLC-activation via an interaction with either the wild-type or mutant TGase II, or with Gq. Although, as shown previously, the α1D-AR receptor was expressed at much lower levels than the α1B-AR, the increase in IP accumulation in COS-1 cells cotransfected with the G-proteins was significantly greater than in cells transfected with the α1B-AR cDNA alone (Fig. 6). In contrast, although able to mediate PLC activation through an interaction with Gq, the α1A-AR receptor did not interact with either TGase II or the mutant TGase II (Fig. 7A).

This was also evident from dose-response studies, which despite similar levels of receptor expression (4.4 ± 0.2, 4.3 ± 0.2, and 4.1 ± 0.1 pmol/mg membrane protein for α1A, α1A + TG, or α1A + mTG, respectively) showed no change in (−)-epinephrine potency (EC50 = 0.8 ± 0.4, 1.0 ± 0.2, or 1.3 ± 0.7 μM for α1A, α1A + TG, or α1A + mTG) or in maximal IP accumulation in cells co-expressing the α1A-AR and either TGase II or the mutant TGase II, as compared with the α1A AR alone (Fig. 8A). The EC50 for (−)-epinephrine-stimulated IP accumulation was also not significantly different in cells cotransfected with the α1A-AR plus Gq (0.5 ± 0.2 μM). However, consistent with over-expression of Gq, in these cells, maximal IP accumulation was significantly increased (Fig. 8A). In these studies, the levels of α1A-AR expression were similar for α1A plus Gq versus α1A alone (4.4 ± 0.2 and 4.0 ± 0.2 pmol/mg protein, respectively). An inability of the α1A-AR to interact with TGase II or the mutant TGase II was also evident when receptor expression was altered by transfecting COS-1 cells with increased amounts of α1A-AR cDNA (Fig. 8B). In these studies, receptor expression ranged from 2 to 15 pmol/mg membrane protein. Failure of the α-AR to mediate PLC activation through an interaction with Gq, was not due to differential expression of TG or mTG, since the levels of TG or mTG expression were not significantly different with cotransfection of cDNAs for any of the α1-AR subtypes (data not shown).

Since the third intracellular loop (i3) of G-protein-coupled receptors appears to be importantly involved in the activation of their coupled G-protein, we questioned whether these loops, which differ markedly in their primary structure among the various α1-ARs, could account for the observed subtype difference in PLC activation via Gq (TGase II). To this end, we constructed a chimeric α1A-AR in which its i3 was replaced by that of the α1B-AR. This α1A (i3-B)-AR chimera was expressed at similar levels to the wild-type α1A-AR, and its binding of the radioligand [3H]prazosin and of (−)-epinephrine was unaltered (data not shown). In addition, its ability to mediate (−)-epinephrine-stimulated IP accumulation via Gq was similar to that of the wild-type α1A-AR (Fig. 7B). However, by contrast with the wild-type α1A-AR, the chimera supported (−)-epinephrine stimulated PLC activation via both TGase II and the mutant TGase II (Fig. 7B).

**DISCUSSION**

The results of this study provide strong support for our initial contention that the receptor-signaling function of TGase II/Gq, is independent of its transglutaminase activity. Thus, receptor-mediated IP turnover was unaltered when tested with a mutant form of TGase II that lacks protein cross-linking activity. Together with the finding that intracellular transglutaminase activity is absent in the presence of physiological cytosolic concentrations of nucleotides and Ca2+ (16), this suggests that the major biological role of TGase II may be as a

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**Fig. 2.** Transglutaminase activity in membranes from COS-1 cells transfected with the hamster α1B-AR cDNA (α1B) or α1A plus cDNAs for rat TGase II (TG), the mutant human TGase II (mTG), or Gq (Gq). Cells were transfected with α1B (2 μg) alone or with the various G-proteins (10 μg each). Upper panel, membranes prepared from the transfected cells were subjected to immunoblotting after SDS-polyacrylamide gel electrophoresis resolution, as described under “Experimental Procedures,” in the absence or presence of 0.2 mM CaCl2 (open bars) or 0.4 mM monodansylcadaverine (MDC), a TGase inhibitor, or 0.1 mM GTP[S], as indicated. Results are from three independent experiments.

U73122 and its inactive analogue U73343. As shown in Fig. 5, U73122 inhibited both the activation of PI-PLC mediated by interaction of the α1B-AR with endogenous G-proteins and the activation of PI-PLC mediated by the interaction of the α1B-AR with either the mutant TGase II or Gq. By contrast, U73343 had no effect.

We next evaluated the ability of the α1D and α1A ARs to mediate PLC-activation via an interaction with either the wild-type or mutant TGase II, or with Gq. Although, as shown previously, the α1D-AR receptor was expressed at much lower levels than the α1B-AR, the increase in IP accumulation in COS-1 cells cotransfected with the G-proteins was significantly greater than in cells transfected with the α1B-AR cDNA alone (Fig. 6). In contrast, although able to mediate PLC activation through an interaction with Gq, the α1A-AR receptor did not interact with either TGase II or the mutant TGase II (Fig. 7A). This was also evident from dose-response studies, which despite similar levels of receptor expression (4.4 ± 0.2, 4.3 ± 0.2, and 4.1 ± 0.1 pmol/mg membrane protein for α1A, α1A + TG, or α1A + mTG, respectively) showed no change in (−)-epinephrine potency (EC50 = 0.8 ± 0.4, 1.0 ± 0.2, or 1.3 ± 0.7 μM for α1A, α1A + TG, or α1A + mTG) or in maximal IP accumulation in cells co-expressing the α1A-AR and either TGase II or the mutant TGase II, as compared with the α1A AR alone (Fig. 8A). The EC50 for (−)-epinephrine-stimulated IP accumulation was also not significantly different in cells cotransfected with the α1A-AR plus Gq (0.5 ± 0.2 μM). However, consistent with over-expression of Gq, in these cells, maximal IP accumulation was significantly increased (Fig. 8A). In these studies, the levels of α1A-AR expression were similar for α1A plus Gq versus α1A alone (4.4 ± 0.2 and 4.0 ± 0.2 pmol/mg protein, respectively). An inability of the α1A-AR to interact with TGase II or the mutant TGase II was also evident when receptor expression was altered by transfecting COS-1 cells with increased amounts of α1A-AR cDNA (Fig. 8B). In these studies, receptor expression ranged from 2 to 15 pmol/mg membrane protein. Failure of the α-AR to mediate PLC activation through an interaction with Gq, was not due to differential expression of TG or mTG, since the levels of TG or mTG expression were not significantly different with cotransfection of cDNAs for any of the α1-AR subtypes (data not shown).

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Fig. 4. Time-dependent and dose-related increase in IP accumulation with (−)epinephrine stimulation of COS-1 cells. Transfected COS-1 cells were stimulated with (−)epinephrine (1 μM) for the times indicated (A) or for 30 min with the doses indicated (B), and IP accumulation was determined as described in Fig. 3. Results are from three or four independent experiments performed in duplicate. Asterisks indicate significant differences (*, p < 0.05; **, p < 0.01) versus the response in cells transfected with α<sub>1B</sub> alone.

As demonstrated here the α<sub>1D</sub>-AR also interacts with both TGase II and mutant TGase II in activating the endogenous PI-PLC in COS-1 cells. However, we were not able to demonstrate ligand-induced inositol phosphate release when cells were cotransfected with TG or mTG and the α<sub>1D</sub>-AR cDNA. Since it has been demonstrated previously that the α<sub>1A</sub>-AR family have been demonstrated by Wu et al. (5). In those studies it was shown that while the α<sub>1B</sub>-AR interacted with G<sub>q</sub>, G<sub>a11</sub>, and G<sub>a14</sub>, the α<sub>1D</sub>-AR did not interact with G<sub>a16</sub>, and the α<sub>1D</sub>-AR did not interact with G<sub>a14</sub> or G<sub>a16</sub>. Consistent with these studies, we also found that G<sub>q</sub> could mediate ligand-induced inositol phosphate release by all three α<sub>1</sub>-AR receptor subtypes. For the α<sub>1B</sub> and α<sub>1D</sub> Rs, the EC<sub>50</sub> values for (−)epinephrine-induced IP accumulation were not markedly different whether these receptors were cotransfected with TGase II/G<sub>q</sub> or G<sub>q</sub>. Since it has been demonstrated pre-

rise in intracellular Ca<sup>2+</sup> associated with receptor stimulation modulates receptor input by limiting TGase-mediated effector activation. Such a mechanism would be analogous to, but distinct from, the limitation of receptor input that results from the activation of the GTPase activity of G<sub>q</sub> or transducin following interaction with their effectors, PLCβ or cGMP-dependent phosphodiesterase, respectively (27, 28).

mediator of membrane signaling rather than as an enzyme involved in the cross-linking of intracellular proteins. Nevertheless, it is possible that the transglutaminase activity of TGase II is relevant to processes such as programmed cell death, in which nucleotide generation is likely to be impaired and intracellular Ca<sup>2+</sup> levels are increased. Whether the multifunctional nature of TGase II/G<sub>q</sub> also has a role in the physiological functioning of cells remains unclear. Since GTP binding by TGase II is inhibited by Ca<sup>2+</sup> (9), it is possible that the

Fig. 5. Sensitivity of (−)epinephrine-stimulated IP accumulation to the PLC inhibitor, U73122 and its inactive analogue, U73343. COS-1 cells were transfected with the various plasmids indicated, and (−)epinephrine (1 μM for 30 min)-stimulated IP accumulation was determined as described in Fig. 3, in the absence (solid bars) or presence of U73122 (10<sup>−4</sup> M) or U73343 (10<sup>−4</sup> M) in duplicate. Asterisks indicate significant differences (*, p < 0.05; **, p < 0.01) versus the respective response in cells transfected with α<sub>1B</sub> alone. In all cases, IP accumulation in the presence of U73122 was significantly (p < 0.001) less than in the absence of U73122. Results are from five independent experiments performed in duplicate.

Fig. 6. α<sub>1D</sub>-AR-mediated IP accumulation. COS-1 cells were transfected with the rat α<sub>1D</sub>-AR eDNA (α<sub>1D</sub>) (10 μg) or α<sub>1D</sub> plus TG, mTG, or G<sub>q</sub> (10 μg each), and the increase in IP accumulation after 30 min of stimulation with either (−)epinephrine (1 μM) or (−)epinephrine 1 μM plus phentolamine (10<sup>−4</sup> M) was determined as described in Fig. 3. The receptor densities were 0.54 ± 0.1, 0.49 ± 0.14, 0.36 ± 0.13, and 0.58 ± 0.19 pmol/mg for α<sub>1D</sub>, α<sub>1D</sub> + TG, α<sub>1D</sub> + mTG, and α<sub>1D</sub> + G<sub>q</sub> respectively. Results are from four independent experiments performed in duplicate. Asterisks indicate significant differences (*, p < 0.05; **, p < 0.01) versus the response in cells transfected with α<sub>1D</sub> alone.
vously that differences in receptor-G-protein interactions can be discriminated despite overexpression following transient transfection in COS-1 cells (29), it is likely that the α₁B-or α₁D-AR recognizes both TGase II/G₉h and G₀q with similar efficacy (whether the slightly lower EC₅₀ for (−)-epinephrine-stimulated IP accumulation observed in the cells transfected with α₁B + mTG (0.03 ± 0.01 μM) compared with the cells transfected with α₁B + Gq (0.4 ± 0.2 μM) is physiologically significant remains unclear). Further, since TGase II/G₉h is expressed ubiquitously in mammalian tissues, and G₀q is also expressed in a wide variety of cell types, except for some hematopoietic cells (5), it is likely that α₁B- and α₁D-AR signaling combined input from both TGase II/G₉h and members of the G₀q family. Indeed, such dual coupling to distinct G-proteins may explain why transfection of a dominantly active G₀q mutant failed to induce transformation in Rat-1 and 3T3 fibroblast cells, whereas transformation of these cells was readily achieved when transfected with a constitutively active α₁B-AR (5).

Since G-protein activation by the G-protein-coupled receptor superfamily involves important determinants in the third intracellular loop, we questioned whether such determinants could also be involved in the subtype-selective interaction of α₁-ARs with TGase II/G₉h. As demonstrated here, this indeed appears to be the case, since exchange of the third intracellular loop of the α₁B-AR onto the backbone of the α₁A-AR receptor allowed rescue of a TGase II/G₉h-dependent signaling phenotype.

The 69-kDa PI-PLC activated by TGase II/G₉h is not a member of the PLCβ family (7, 30). Recently, based on in vitro studies, we demonstrated that this 69-kDa PI-PLC, in contrast to PLCβ, is resistant to the aminosteroid PI-PLC inhibitor, U73122 (31). Moreover, in intact heart tissue, which expresses TGase II/G₉h abundantly, α₁A-AR activation of inositol phosphate release is resistant to U73122. With culture of cardiac myocytes, expression of TGase/G₉h falls markedly (32), whereas expression of G₀q remains unaltered, and α₁A-AR activation of inositol phosphate release is now U73122-sensitive. This suggests that in vivo myocardial α₁-ARs activate the U73122-resistant 69-kDa PI-PLC via interaction with TGase II/G₉h, but with culture there is a switch to a U73122-sensitive PLC that is activated by G₀q. The finding, therefore, that α₁B-AR-mediated IP accumulation is U73122-sensitive (Fig. 5) suggests that either COS-1 cells do not express the U73122-insensitive 69-kDa IP-PLC, or with overexpression of TGase II/G₉h it promiscuously interacts with an endogenous U73122-sensitive PI-PLC.

Resolution of this issue will require further studies including the full identification and characterization of the 69-kDa PI-PLC.

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