Novel bimodal effects of the G-protein tissue transglutaminase on adrenoceptor signalling

Jianwen ZHANG, Janusz TUCHOLSKI¹, Mathieu LESORT¹, Richard S. JOPE and Gail V. W. JOHNSON²

Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham, Birmingham, AL 35294-0017, U.S.A.

Tissue transglutaminase (tTG) is a novel G-protein that previous studies showed can couple ligand-bound activated α_{1B} adrenoceptors to phospholipase C- δ , resulting in phosphoinositide (PI) hydrolysis. In human neuroblastoma SH-SY5Y cells we found that although endogenous tTG can facilitate α_{1B} adrenoceptor-stimulated PI hydrolysis, its contribution is minor compared with the classical heterotrimeric G-protein $G_{q/11}$. Further, we show that the α_{1B} adrenoceptor recruits tTG to the membrane and that this recruitment is enhanced by agonist

occupancy of the receptor. In addition, the effects of tTG on signalling are bimodal. At low expression levels, tTG enhanced α_{1B} adrenoceptor-stimulated PI hydrolysis, whereas at higher expression levels tTG attenuated significantly this response. These findings are the first to demonstrate that a protein can both facilitate and attenuate receptor-stimulated PI hydrolysis.

Key words: α_{1B} adrenoceptors, phosphoinositide hydrolysis, phospholipase C, SH-SY5Y cells.

INTRODUCTION

The importance of the functioning of the seven-transmembrane G-protein-coupled receptors (GPCRs) was recently underscored by the revelation that 5% of all *Caenorhabditis elegans* genes code for this class of proteins [1]. This prominence is indicative of the widespread utility and cruciality of these proteins. According to current understanding, GPCRs require G-proteins to convey messages to intracellular targets. Two classes of G-proteins have been identified that fulfil this function. One class consists of the highly investigated classical heterotrimeric G-proteins [2,3], such as $G_{q/11}$ that links receptors to the intracellular enzyme phospholipase C (PLC), which cleaves phosphoinositides (PIs). The second class, which includes tissue transglutaminase (tTG) [4], has received relatively little attention. Thus basic critical questions remain unresolved, such as how GPCRs interact with tTG and how this interaction affects signalling activity.

tTG is a unique multifunctional protein that exhibits three distinct enzymic activities; a calcium-dependent transamidating activity that crosslinks proteins or incorporates polyamines into substrates [5,6], GTPase activity [7–9] and ATPase activity, with separate binding sites for Mg-ATP and Mg-GTP [9]. Substantial evidence indicates that each of these activities is distinct; however, there are interactions. For example, GTP inhibits the transamidating activity of tTG activity [7] and Mg-ATP inhibits the GTPase activity but has no effect on transamidation [9]. In addition, the transamidating activity of tTG apparently does not effect the signal-transducing function of tTG. A Cys $\rightarrow$ Ala mutation within the active site for transamidation eliminated this activity [10], but did not alter the ability of tTG to functionally couple to the α_{1B} adrenoceptors [11].

The GTP-binding and GTPase activities were found to be critical properties, allowing tTG to function as a signalling intermediary coupling cell-surface receptors to intracellular effectors [4,11–13]. Based on this, tTG has been referred to as

$G\alpha_n$ [4], by analogy to the nomenclature of heterotrimeric G-proteins, although tTG does not bind $\beta\gamma$ units as do classical heterotrimeric G-proteins [13]. In reconstitution studies in COS1 cells, tTG was capable of coupling the α_{1B} and α_{1D} adrenoceptors (but not α_{1A} adrenoceptors) to PLC δ , and thus function as a signal-transducing G-protein [4,11–13]. There is also evidence that tTG functionally couples the oxytocin receptor to PLC δ in myometrium [14]. However, the α_1 adrenoceptor and oxytocin receptor also effectively couple to the classical heterotrimeric G-protein, $G_{q/11}$ [11,15,16]. Further, co-transfection of the α_{1B} adrenoceptor and $G_{q/11}$ into COS1 cells resulted in greater (–)jepinephrine (adrenaline)-stimulated PI hydrolysis than in cells co-transfected with the receptor and tTG [11]. These findings indicate clearly that further investigations are required to clarify the putative role of tTG in signal transduction.

Although these previous studies indicate clearly that tTG can mediate receptor-coupled signal-transduction activity, tTG differs substantially from the ‘classical’ α -subunit of heterotrimeric G-proteins. tTG does not have GTP-binding motifs common to heterotrimeric G-protein α -subunits [2,13]. tTG does not activate PLC β , which is the PLC isozyme activated by the heterotrimeric G-protein α -subunits [17,18], but rather has an eight amino acid stretch in the C-terminal region that is essential for binding and activating PLC δ [12,19]. In addition, no ‘ $\beta\gamma$ -like’ subunits are apparently required for tTG to function in its signal-transducing capacity, although a 50-kDa protein was found to co-purify with tTG and to decrease GTP binding and GTPase activity [20]. However the 50-kDa protein does not modulate receptor–tTG interactions [13,20]. Thus it is clear that tTG is not a member of the classical heterotrimeric G-protein family.

Based on these previous findings, this study was designed to examine the novel role of tTG in mediating the activation of PLC in response to stimulation of the α_{1B} adrenoceptor in a

Abbreviations used: GPCR, G-protein-coupled receptor; PLC, phospholipase C; PI, phosphoinositide; tTG, tissue transglutaminase.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (e-mail gwj@uab.edu).

neuronal-like cell line. All previous investigations of the role of tTG in GPCR signalling have been carried out *in vitro* or in non-neuronal cells. Human neuroblastoma SH-SY5Y cells do not contain α_1 adrenoreceptors [21], and the levels of tTG expressed in the cells can be regulated by treating cells with drugs such as retinoic acid [6] and azacytidine [22]. Therefore stable transfection of these cells with the α_{1B} adrenoreceptor yielded a suitable neuronal-like cell model for evaluating the signal-transduction role of tTG through just the α_{1B} adrenoreceptor subtype.

The results of these studies demonstrate that expression of the α_{1B} adrenoreceptor selectively recruits both tTG and PLC δ to the membrane, but not G $_{q/11}$ or PLC β . Further, activation of the α_{1B} adrenoreceptor enhanced the translocation of tTG to the membrane. In addition, the effects of tTG on signalling through the α_{1B} adrenoreceptor are bimodal. At low expression levels, tTG enhanced α_{1B} adrenoreceptor-stimulated PI hydrolysis, whereas at higher expression levels, tTG significantly attenuated signalling through the α_{1B} adrenoreceptor. These findings are the first to demonstrate that a protein can both facilitate and attenuate GPCR-stimulated PI hydrolysis. Further evidence is provided that even at optimal facilitatory levels, the direct contribution of tTG to α_{1B} adrenoreceptor-stimulated PI hydrolysis is relatively small compared with G $_{q/11}$, and therefore the primary role of tTG in GPCR signalling is likely to be more modulatory rather than in the capacity of a classical signal-transducing G-protein. These and other findings clearly indicate that tTG does not function in the capacity of a classical heterotrimeric G-protein, but rather plays a novel role in modulating α_{1B} adrenoreceptor-coupled signalling activity.

EXPERIMENTAL

Materials

5-Azacytidine, BSA, carbachol, GTP, GTP γ S, genistein, guinea pig liver tTG, L-phenylephrine, phentolamine, prazosin and retinoic acid were purchased from Sigma (St. Louis, MO, U.S.A.). 2-Fluoropalmitic acid was from Alexis (San Diego, CA, U.S.A.); PMSF, SDS and FuGENE[®] 6 were purchased from Roche (Indianapolis, IN, U.S.A.). The enhanced chemiluminescence (ECL) reagents and L-3-phosphatidyl [2-³H]inositol (18 Ci/mmol) were purchased from Amersham (Boston, MA, U.S.A.). [2-³H(n)]myo-inositol (20 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO, U.S.A.). [7-methoxy-³H]Prazosin (77.2 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). BCA protein-assay reagents were purchased from Pierce (Rockford, IL, U.S.A.). Nonidet P-40 and rabbit non-immune IgG were purchased from Calbiochem (La Jolla, CA, U.S.A.). RPMI 1640 was purchased from Cellgro (Herndon, VA, U.S.A.); penicillin/streptomycin and horse serum, geneticin (G418) and lipofectamine were from Gibco BRL (Gaithersburg, MD, U.S.A.). Fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA, U.S.A.). Fetal Clone II was purchased from Hyclone (Logan, UT, U.S.A.). The tTG monoclonal antibody 4C1 was produced by the Hybridoma Core Facility at University of Alabama at Birmingham [23]. The tTG monoclonal antibodies TG100 and CUB7402 were from NeoMarkers (Union City, CA, U.S.A.). The tTG polyclonal antibody was purchased from UBI (Lake Placid, NY, U.S.A.). The PLC δ 1 monoclonal antibody was from Transduction Laboratories (Lexington, KY, U.S.A.), and PLC β 1 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Horseradish peroxidase-conjugated goat anti-mouse IgG was purchased from Bio-Rad (Hercules, CA, U.S.A.). The expression vector pcDNA3.1+ was from Invitrogen

(Carlsbad, CA, U.S.A.), and the remaining molecular-biology reagents were purchased from Promega (Madison, WI, U.S.A.).

Vectors and DNA constructs

The cDNA for α_{1B} adrenoreceptor in the vector PMT2' [11,24,25] was provided kindly by Dr. M. J. Im, and the cDNA for human tTG in the vector pSG5 [26,27] was a generous gift from Dr. P. Davies. The cDNA for the α_{1B} adrenoreceptor was cut out of PMT2' with *EcoRI*/*NotI* and ligated into the same sites of pcDNA3.1+ (α_{1B} /pcDNA). The cDNA for human tTG was cut out of pSG5 with *EcoRI* and inserted into the same site of pcDNA3.1+ (TG/pcDNA).

Cell culture and transfection of cells

Human neuroblastoma SH-SY5Y cells were maintained on Corning dishes in continuous culture media (RPMI 1640 medium supplemented with 20 mM glutamine, 10 units/ml penicillin, 100 μ g/ml streptomycin, 5% Fetal Clone II serum and 10% horse serum). Stably transfected cells were maintained with transfection-maintenance medium that contained 100 μ g/ml G418 (geneticin) and 5% fetal bovine serum instead of Fetal Clone II; other components were the same as continuous culture media.

For stable transfection, SH-SY5Y cells were replated 1 day before electroporation. The cells were removed from the dish with RPMI 1640 medium containing 0.05% of trypsin and 0.053 mM EDTA, diluted with continuous culture medium and centrifuged for 5 min at 250 g. Cells (3×10^6) resuspended in 1 ml of Hepes buffer (0.14 M NaCl, 25 mM Hepes, 0.075 mM NaHPO $_4$, pH adjusted to 7.05 with 10 M NaOH), were mixed with 10 μ g of linearized α_{1B} /pcDNA and kept on ice for 10 min. Electroporation was carried out with a Bio-Rad Pulse II set at 0.25 kV and 960 μ F as described previously [28]. After electroporation cells were incubated on ice for 10 min and then mixed with 10 ml of continuous culture medium and plated on to a 100-mm Corning dish. After 48 h, the cells were collected and reseeded on to 10 100-mm Corning dishes at very low densities ($\approx$ 1000 cells/dish) in transfection-maintenance medium. Cells were maintained in transfection-maintenance medium for $\approx$ 1 month until individual cells, which contained the plasmid and thus were resistant to geneticin, formed small colonies. Individual colonies were collected and transferred into each well of a 24-well Corning plate. The cells were grown and the cultures expanded prior to screening for positive clones by prazosin binding and the PI hydrolysis assay (see below).

For transient transfection of the α_{1B} adrenoreceptor or tTG, the constructs were introduced into the cells either with lipofectamine or FuGENE[®] 6 using the manufacturer's protocol. To induce tTG expression with retinoic acid or azacytidine, the cells were incubated with the indicated concentrations for 3–6 days. Treatment with 10 μ M retinoic acid for 3 days resulted in a small decrease in viability as determined by lactate dehydrogenase release [6]. In control cells there was $7.4 \pm 0.5\%$ lactate dehydrogenase released, while in the retinoic acid-treated cells the values were $10.0 \pm 0.5\%$ ($n = 6$ separate determinations). All experiments were carried out on subconfluent cultures.

Ligand binding

To screen for clones expressing the α_{1B} adrenoreceptor, a previously described binding assay was used [21] with some modifications. Transfected cells were harvested by trypsin digestion and rinsed twice with a Krebs buffer (122 mM NaCl,

4.9 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 1.3 mM CaCl₂, 3.6 mM NaHCO₃, 11 mM glucose and 30 mM Hepes, pH 7.35). The cells were resuspended in Krebs buffer and aliquoted [(1–2) × 10⁶ cells/aliquot]. The total binding-reaction volume was 500 µl per assay including 0.4 mg/ml BSA and [³H]prazosin (0.12 µCi/ml). To measure non-specific binding, 1.6 µM phentolamine, another α₁ adrenoceptor antagonist, was also included. The reactions were incubated at 4 °C for 1 h with continuous shaking, and stopped by filtering on to GF/B filters and rinsing with 5 ml of washing buffer (140 mM NaCl, 5 mM MgCl₂, 1 mM EGTA, 50 mM Tris/HCl, pH 7.2). The filters were then subjected to scintillation counting. To determine the binding constant of the expressed α_{1B} adrenoceptor, the cells were incubated with [³H]prazosin in the presence of increasing concentrations of unlabelled prazosin and the binding constant (K_d) was determined by fitting data with a linear-regression program as described by Lin and Riggs [29]. All values were obtained from at least three independent experiments measured in triplicate.

PI hydrolysis assay

Measurements of PI hydrolysis were carried out as described previously [30]. Cells were pre-labelled with [³H]myo-inositol (75 µCi/100 mm plate) for 48 h. The labelled cells were harvested, rinsed twice with Krebs buffer and resuspended in Krebs buffer containing 10 mM LiCl. Aliquots (1 × 10⁶ cells) were pre-incubated at 37 °C for 5 min, 50 µM phenylephrine, or other drugs as indicated, was added and the incubation continued for 1 h at 37 °C. The reactions were terminated by adding 1.7 ml of methanol/chloroform/HCl (2:1:0.01, by vol.). Samples were fractionated and [³H]inositol monophosphate measured as described previously [30]. All values were obtained from triplicate measurements from at least three independent experiments, and data are expressed as means ± S.E.M.

Plasma-membrane preparation

Cells were suspended and sonicated in lysis buffer (0.32 M sucrose, 100 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 0.5 mM dithiothreitol, 0.1 mM PMSF, 10 µg/ml each of aprotinin, leupeptin and pepstatin, and 50 mM Tris/HCl, pH 7.3), and the lysates were spun at 700 g for 10 min. The postnuclear supernatant was spun at 100 000 g for 1 h to separate the membrane and cytosol fractions, and the resulting membrane pellet was washed three times with lysis buffer before use in the assays. In some cases, 2 mM GTP, 50 µM phenylephrine or 1 µM prazosin was also included during the preparation.

Membrane PI hydrolysis

Membranes were resuspended in membrane-assay buffer containing 5 mM Trizma[®]-maleate, pH 7.0, 8 mM LiCl, 5 mM MgCl₂, 3.0 mM EGTA and 1.36 mM CaCl₂ (these concentrations of EGTA and CaCl₂ yielded a free calcium concentration of 300 nM). Aliquots (75 µg of protein/100 µl of total reaction volume) were incubated in the presence of [³H]PI (0.005 µCi/100 µM) for 1 h at 37 °C either with or without 25 µM GTPγS, 50 µM phenylephrine and different concentrations of tTG as indicated. The reaction was terminated by adding chloroform/methanol (1:2, v/v), and the labelled products were extracted as described previously [31].

PI hydrolysis in streptolysin-O-permeabilized cells

To examine the rate *in situ* of PI hydrolysis after selectively blocking either G_{q/11} or tTG function, cells were permeabilized

with streptolysin-O prior to incubation with antibodies against these proteins using a modification of a protocol described previously [32]. Cells prelabelled with [³H]inositol for 48 h were harvested, rinsed twice with Ca²⁺-free Krebs buffer and resuspended in Ca²⁺-free Krebs buffer containing 1 mM dithiothreitol. The cell suspension was kept on ice for 10 min. Streptolysin-O (final concentration 100 haemolytic units/ml) was added to the cell suspension, and samples were incubated for 20 min at 4 °C. The cells were spun, resuspended in incubation buffer (calcium-free Krebs buffer supplemented with 300 nM CaCl₂, 1 mM dithiothreitol, 2 mM GTP and 5 mM magnesium acetate), aliquoted [(1–2) × 10⁶ cells/500 µl] and pre-incubated with selected antibodies or non-immune-IgG (all at 10 µg/ml) at 30 °C for 1 h. Phenylephrine was then added to the cell suspension at a final concentration of 50 µM, and the incubation was continued for another 1 h at 30 °C. PI hydrolysis was measured as described above.

Immunoblotting

To evaluate levels of tTG, G_{q/11}, PLCδ1 or PLCβ1, cell extracts or membranes were prepared and quantitatively immunoblotted as described previously [6]. The blots were probed with the anti-tTG monoclonal antibodies 4C1 (1–2 µg/ml) or TG100 (0.1 µg/ml), the anti-G_{q/11} polyclonal antibody Q253 (0.05 µg/ml), the anti-PLCδ1 monoclonal antibody (0.4 µg/ml) or the anti-PLCβ1 polyclonal antibody (0.4 µg/ml) in 5% milk/borate saline buffer (100 mM boric acid, 20 mM sodium borate, 80 mM NaCl and 0.05% Nonidet P-40). The immunoblots were developed with ECL, and analysed using a Bio-Rad GS-670 Imaging Densitometer. To quantitatively measure the amounts of tTG in the membrane fraction, 3 ng of guinea pig liver tTG was run on each gel as an internal standard. Samples (20 µg/lane) and the standard were electrophoresed, immunoblotted and the blots developed with ECL. The blots were quantified by densitometry and the values normalized to the guinea pig liver tTG standard. The normalized data from both whole homogenate and membrane were then compared.

Statistical analysis

Statistical significance was determined using analysis of variance, with a Tukey–Kramer multiple-comparisons test where appropriate. The values were considered significantly different if the *P* value was < 0.05.

RESULTS

Selection of α_{1B} adrenoceptor-expressing clone

In initial studies, over 20 geneticin-resistant clones were screened for prazosin binding, and seven of these clones showed binding activity. The seven clones that showed prazosin-binding activity were assayed to determine if the expressed α_{1B} adrenoceptors showed specific agonist stimulation of PI hydrolysis. Out of the seven clones, three showed phenylephrine-stimulated PI hydrolysis. The clone that showed the highest level of α_{1B} adrenoceptor expression and greatest PI hydrolysis in response to phenylephrine was chosen for full characterization and subsequent studies.

Characterization of α_{1B} adrenoceptor expression and function

α_{1B} Adrenoceptor density was measured in untransfected wild-type human neuroblastoma SH-SY5Y cells and in cells either transiently or stably transfected with the α_{1B} adrenoceptor. As

Figure 1 Characterization of the function the α_{1B} adrenoreceptor transfected into SH-SY5Y cells

PI hydrolysis in response to stimulation with 300 μ M carbachol (CARB) or 50 μ M phenylephrine (PE) in wild-type SH-SY5Y cells (SH/WT) and cells stably transfected with the α_{1B} adrenoreceptor (α_{1B} /ST). Stimulation of muscarinic acetylcholine receptors with carbachol resulted in equivalent increases in PI hydrolysis in both SH/WT and α_{1B} /ST cells. However, only cells transfected with the α_{1B} adrenoreceptor showed increased PI hydrolysis in response to phenylephrine; $n = 3$ separate experiments.

reported previously, no α_1 adrenoreceptors were detected in human neuroblastoma SH-SY5Y cells [21]. Stable transfection resulted in significantly higher levels of α_{1B} adrenoreceptor expression (2048 ± 50 fmol/mg) than transient transfection (1090 ± 30 fmol/mg); therefore, the majority of experiments were carried out with stably transfected cells. The binding constant (K_d) of the α_{1B} adrenoreceptor for the selective antagonist prazosin in stably transfected cells was 0.12 ± 0.03 nM, which is similar to previously reported values [33].

To determine whether the α_{1B} adrenoreceptor in the stably transfected cells was functional, receptor-coupled activation of PI hydrolysis was measured using phenylephrine, a selective α_1 adrenoreceptor agonist. Phenylephrine (50 μ M) stimulated PI hydrolysis in transfected cells, but not in wild-type cells (Figure 1), and as expected this stimulation was potently inhibited by prazosin ($IC_{50} = 2.5 \pm 0.2$ nM; results not shown). SH-SY5Y cells express cholinergic muscarinic M3 receptors coupled to PLC β by $G_{q/11}$ [34,35]. Stimulation with the cholinergic agonist carbachol induced equivalent activation of PI hydrolysis in wild-type and α_{1B} adrenoreceptor-transfected cells (Figure 1). Atropine (10 nM) completely blocked the carbachol-induced increase in PI hydrolysis, but as expected had no effect on the phenylephrine-stimulated increases in PI hydrolysis (results not shown).

The α_{1B} adrenoreceptor selectively recruits tTG to the membrane

To investigate the interactions of the α_{1B} adrenoreceptor with tTG and $G_{q/11}$, the localization of these G-proteins was examined in wild-type and α_{1B} adrenoreceptor-transfected cells. Immunoblot analysis revealed that, in wild-type cells, relatively little tTG was membrane-bound, but expression of the α_{1B} adrenoreceptor resulted in a significant increase in the amount of tTG in the membrane fraction (Figure 2). The α_{1B} adrenoreceptor-induced membrane translocation of tTG was evident in both undifferentiated cells and cells differentiated with retinoic acid (1 μ M for 3 days) to increase tTG levels (Figure 2). The amount of membrane-bound tTG was increased further when α_{1B} adrenoreceptor-expressing cells were treated with phenylephrine before and during the membrane preparation, and was slightly decreased when the antagonist prazosin was

included (Figure 3). $G_{q/11}$ is predominantly membrane-bound, and equivalent amounts of $G_{q/11}$ were present in the membrane fractions from wild-type and α_{1B} adrenoreceptor-transfected SH-SY5Y cells, and unaffected by the presence of phenylephrine or prazosin (Figure 3). Transient transfection of tTG into wild-type SH-SY5Y cells or cells stably transfected with the α_{1B} adrenoreceptor yielded similar results: tTG was recruited to the membrane by the α_{1B} adrenoreceptor (Figures 4A and 4B). Further, PLC δ was also recruited to the membrane by the α_{1B} adrenoreceptor (Figures 4C and 4D), presumably due to its interaction with tTG [12,13]. The presence of the α_{1B} adrenoreceptor had no effect on the distribution of PLC β between the membrane and cytosol (results not shown). Increasing the levels of tTG either by treating with retinoic acid or azacytidine had no effect on the expression levels of PLC δ or the α_{1B} adrenoreceptor. Transient transfection of the cells with tTG also did not alter the expression levels of PLC δ or the α_{1B} adrenoreceptor (results not shown).

tTG bimodally modulates phenylephrine-stimulated PI hydrolysis

To test if receptor-coupled stimulation of PI hydrolysis is modulated by the level of tTG, three methods were used to increase tTG levels, followed by measurement of phenylephrine-induced PI hydrolysis. Because retinoic acid increases tTG expression in SH-SY5Y cells [6], treatment with retinoic acid was used to increase tTG expression in cells transfected with the α_{1B} adrenoreceptor. Cells were treated with 0.1–10 μ M retinoic acid for 3 days, and then tTG levels and phenylephrine-stimulated PI hydrolysis were measured. Retinoic acid induced a concentration-dependent increase in tTG levels, with 10 μ M retinoic acid causing a maximal increase that was 650% of control levels (Figure 5A). Associated with retinoic acid-induced increases in tTG levels, there was a biphasic change in phenylephrine-stimulated PI hydrolysis. PI hydrolysis in response to phenylephrine increased with increasing levels of tTG up to a retinoic acid concentration of 1 μ M. However, further increases in the tTG levels attenuated the increase in phenylephrine-stimulated PI (Figure 5A).

Azacytidine has been shown to increase tTG expression by demethylating the promoter region of the tTG gene [22]. Treatment of the cells with 5 μ M of azacytidine for 3 days increased tTG levels and significantly increased phenylephrine-stimulated PI hydrolysis (Figure 5B). However, 6 days of treatment of azacytidine, which resulted in a further increase in tTG, slightly decreased phenylephrine-stimulated PI hydrolysis compared with 3 days of treatment (Figure 5B).

Because retinoic acid and azacytidine regulate the expression of other proteins besides tTG and thus could indirectly alter PI hydrolysis, α_{1B} adrenoreceptor-expressing cells were transiently transfected with human tTG, and PI hydrolysis in response to phenylephrine was examined. In these experiments tTG/pcDNA was transfected into the cells and phenylephrine-stimulated PI hydrolysis was measured before (time 0) and at 12, 24 and 48 h after transfection. As found with the other models, a moderate increase in tTG expression resulted in an increase in phenylephrine-stimulated PI hydrolysis, but higher expression levels of tTG attenuated the response (Figure 5C). Sham transfection of the cells with vector alone had no effect on phenylephrine-stimulated PI hydrolysis (results not shown). Thus the results from three different experimental protocols indicated that tTG bimodally regulates signalling through the α_{1B} adrenoreceptor. To demonstrate further that high levels of tTG expression inhibit phenylephrine-stimulated PI hydrolysis, SH-SY5Y cells that were stably transfected with tTG (TG/pcDNA)

Figure 2 The α_{1B} adrenoceptor recruits tTG to the plasma membrane

The representative immunoblots in (A) and (B) show the distribution of tTG in cell homogenates (A) and membrane preparations (B) from undifferentiated, wild-type SH-SY5Y cells (SH/U), undifferentiated cells stably expressing the α_{1B} adrenoceptor (α_{1B}/U), differentiated wild-type SH-SY5Y cells (SH/D) and differentiated cells stably expressing the α_{1B} adrenoceptor (α_{1B}/D). To increase tTG expression in either wild-type SH-SY5Y cells or cells stably expressing the α_{1B} adrenoceptor, the cells were differentiated with 1 μ M retinoic acid for 3 days. (A) Although endogenous tTG expression levels in α_{1B}/U cells were slightly higher than in SH/U cells, there were no differences between wild-type cells and cells expressing the α_{1B} adrenoceptor after treatment with 1 μ M retinoic acid (compare SH/D and α_{1B}/D). (B) Representative immunoblots showing that tTG is selectively recruited to the plasma membrane by the presence of the α_{1B} adrenoceptor. (C) Quantitative analysis of the relative amount of tTG recruited to the membrane by the presence of the α_{1B} adrenoceptor. Data are expressed as the relative amounts of tTG in the membrane compared with the homogenate. All values were normalized to an internal tTG standard prior to analysis. Data are means $\pm$ S.E.M. from four independent experiments. The amount of tTG in the membrane of the cells expressing the α_{1B} adrenoceptor was significantly greater for both undifferentiated (U) and differentiated (D) cells (* $P < 0.005$).

or vector only [pcDNA3.1(+)], were transiently transfected with the α_{1B} adrenoceptor and 48 h later phenylephrine-stimulated PI hydrolysis was measured. The cells stably transfected with tTG expressed high levels of the protein, ≈ 6 –8 fold over the levels of tTG in cells stably transfected with vector only [36]. In these experiments phenylephrine-stimulated PI hydrolysis in the cells overexpressing tTG was 79 ± 3 % less than in the cells with vector alone. These findings demonstrate clearly that high levels of tTG expression can significantly attenuate phenylephrine-stimulated PI hydrolysis.

Figure 3 Phenylephrine selectively increases the recruitment of tTG to the membrane

tTG and $G_{q/11}$ immunoblots of membranes from undifferentiated cells stably transfected with the α_{1B} adrenoceptor in the absence (—) or presence of phenylephrine (+PE) or prazosin (+PR). Prior to membrane preparation the undifferentiated cells were harvested with Krebs buffer and incubated at 37 °C for 60 min in presence or absence of phenylephrine (50 μ M) and/or prazosin (1 μ M). These data demonstrate that the agonist binding of the α_{1B} adrenoceptor increases the presence of tTG at the plasma membrane, but that antagonist prohibits this process. In contrast, both phenylephrine and prazosin do not alter the association of $G_{q/11}$ on the membrane. Immunoblots are representative of data from three independent experiments.

Figure 4 The α_{1B} adrenoceptor recruits both tTG and PLC δ to the plasma membrane

Wild-type untransfected SH-SY5Y cells (SH) and cells stably transfected with the α_{1B} adrenoceptor (α_{1B}) were transiently transfected with human tTG. Homogenates (A and C) and membranes (B and D) were immunoblotted for tTG (A and B) or PLC δ (C and D). These results demonstrate that, in cells expressing the α_{1B} adrenoceptor, there is significantly more tTG and PLC δ at the membrane than in wild-type cells. Immunoblots are representative of data from three independent experiments.

In contrast to these findings, increasing tTG levels by treatment with retinoic acid or azacytidine had no effect on carbachol-stimulated PI hydrolysis (Figure 5D).

tTG modulates PI hydrolysis in membranes

To examine further the role of tTG in regulating phenylephrine-stimulated PI hydrolysis, membrane preparations from cells

Figure 5 tTG bimodally modulates PI hydrolysis in response to stimulation of the α_{1B} adrenoreceptor

(A) α_{1B} Adrenoreceptor-expressing cells were differentiated by treating with the indicated concentrations of retinoic acid for 3 days, and then PI hydrolysis in response to phenylephrine (50 μ M; $\blacktriangle$), and the levels of tTG ($\bullet$) in the corresponding groups, were measured quantitatively. Up to a concentration of 1 μ M retinoic acid, phenylephrine-stimulated PI hydrolysis increased as tTG levels increased. However, at higher concentrations of retinoic acid and further increases in tTG levels, agonist-stimulated PI hydrolysis was attenuated. A representative immunoblot showing the tTG levels at the retinoic acid concentrations indicated is shown above the graph in (A). (B) α_{1B} Adrenoreceptor-expressing cells were treated with (+) or without (-) 5 μ M azacytidine (AZ) for 3 or 6 days, and then PI hydrolysis in response to phenylephrine (50 μ M), and the levels of tTG in the corresponding groups, were measured quantitatively. There was a significant increase in α_{1B} adrenoreceptor-stimulated PI hydrolysis corresponding to increased tTG levels at day 3, but the higher expression of tTG that occurred after 6 days of treatment with azacytidine resulted in a slight decrease in phenylephrine-stimulated PI hydrolysis compared with day 3. (C) α_{1B} Adrenoreceptor-expressing cells were transiently transfected with TG/pcDNA, and then PI hydrolysis in response to phenylephrine (50 μ M), and the levels of tTG in the corresponding groups, were measured quantitatively at the time periods indicated after transfection. A slight increase in tTG levels (12 h) resulted in an increase in phenylephrine-stimulated PI hydrolysis, whereas higher levels of tTG expression (24 and 48 h) significantly attenuated PI hydrolysis in response to phenylephrine compared with 12 h. The inset in (C) shows a representative immunoblot of tTG levels. In all panels PI hydrolysis activity was expressed as percentage of control (CTL), and results were considered significantly different when $P < 0.05$ ($n = 3$ –5 independent experiments, each in triplicate). All immunoblots for detecting tTG expression were probed with the tTG monoclonal antibody 4C1 instead of TG100. (D) α_{1B} Adrenoreceptor-expressing cells were differentiated with 1 or 10 μ M retinoic acid for 3 days or with 5 μ M azacytidine for 6 days, and PI hydrolysis in response to the stimulation by carbachol (1 mM) was measured quantitatively. The data are expressed as means $\pm$ S.E.M. ($n = 3$). The data reveal that carbachol-stimulated PI hydrolysis was not affected by treating the cells with the drugs indicated.

stably transfected with the α_{1B} adrenoreceptor were used to allow precise control of the amount of tTG present. In these experiments PI hydrolysis was measured in the presence of varying concentrations of exogenously added tTG. Because this is a cell-free system, addition of exogenous GTP γ S, a stable analogue of GTP, is necessary to support receptor-coupled, G-protein-mediated, PI hydrolysis. At the lower concentrations of tTG (2.4 and 6.1 nM) phenylephrine-stimulated PI hydrolysis was not significantly increased compared with hydrolysis with no tTG added, but higher concentrations of tTG significantly inhibited PI hydrolysis (Figure 6). As a control, 24 nM BSA (the highest

concentration of tTG used) was added, and this treatment had no effect on the PI hydrolysis (Figure 6).

G $_{q/11}$ and tTG both contribute to α_{1B} adrenoreceptor signalling

To determine the relative contributions of tTG and G $_{q/11}$ to signalling through the α_{1B} adrenoreceptor, α_{1B} adrenoreceptor-expressing cells were permeabilized and incubated with polyclonal antibodies to either the α -subunit of G $_{q/11}$ or tTG (10 μ g/ml) prior to measurements of PI hydrolysis. Control cells

Figure 6 Exogenously added tTG modulates PI hydrolysis

Purified membranes prepared from α_{1B} adrenoceptor cells were used to measure PI hydrolysis in the presence of increasing concentrations of tTG as described in the Experimental section. PI hydrolysis is expressed as c.p.m., and the data presented are the means $\pm$ S.E.M. from three separate experiments done in triplicate. Although tTG did not significantly increase phenylephrine (PE)-stimulated PI hydrolysis, higher concentrations of tTG (12.2 and 24.4 nM) significantly inhibited PI hydrolysis. As a control, 24 nM BSA was included in the reaction (the highest concentration of tTG used), and had no effect on the PI hydrolysis. *, $P < 0.05$, significantly different from GTP γ S and PE alone; ★, $P < 0.05$, significantly different from GTP γ S alone.

were incubated with an equivalent amount of non-immune rabbit IgG. Pre-incubation with the G $\alpha_{q/11}$ antibody inhibited phenylephrine-stimulated PI hydrolysis by more than 85%; however, pre-incubation with the tTG antibody only inhibited phenylephrine-stimulated PI hydrolysis by $\approx 25\%$. Pre-treatment with both antibodies completely inhibited phenylephrine-stimulated PI hydrolysis. Non-immune rabbit IgG had no effect on PI hydrolysis (Figure 7). These data suggest that endogenous tTG facilitates phenylephrine-stimulated PI hydrolysis, but the contribution of tTG to α_{1B} adrenoceptor-stimulated PI hydrolysis is significantly less than G $\alpha_{q/11}$.

Inactivation of G $\alpha_{q/11}$ activity by inhibition of palmitoylation and tyrosine phosphorylation

In order to assess further the relative contributions of G $\alpha_{q/11}$ and tTG in mediating receptor-coupled PI hydrolysis, post-translational modifications of G $\alpha_{q/11}$ were targeted. tTG is neither palmitoylated [37] nor tyrosine phosphorylated (J. Zhang and J. Tucholski, unpublished work), whereas palmitoylation [38] and tyrosine phosphorylation [39] have been reported to be crucial modifications for regulating the optimal functioning of G $\alpha_{q/11}$. Therefore the difference in the extent of inhibition of carbachol- and phenylephrine-stimulated PI hydrolysis in response to inhibition of palmitoylation or tyrosine phosphorylation was assessed to obtain an estimate of the relative contribution of tTG to signalling through the α_{1B} adrenoceptor. For these studies two drugs were used. Fluoropalmitic acid has been shown previously to inhibit the palmitoylation and function of the α -subunit of heterotrimeric G-proteins [40]. G $\alpha_{q/11}$ is activated by tyrosine phosphorylation and genistein, a tyrosine kinase inhibitor, inhibits this response [39]. Therefore, in these studies cells were pretreated for 30 min with 300 μ M 2-fluoropalmitic

Figure 7 Inhibition of phenylephrine (PE)-stimulated PI hydrolysis by antibodies to G $\alpha_{q/11}$ and tTG in streptolysin-O-permeabilized cells

Addition of G $\alpha_{q/11}$ antibody inhibited PI hydrolysis more than 85%; however, addition of the tTG antibody only inhibited phenylephrine-stimulated PI hydrolysis by $\approx 25\%$. If both antibodies were added together, phenylephrine-stimulated PI hydrolysis was completely inhibited. As a control, an equivalent amount of non-immune rabbit IgG was added, and had no effect on PI hydrolysis. All antibodies were added at a final concentration of 10 μ g/ml. The data presented are the means $\pm$ S.E.M. from three separate experiments done in triplicate; *, $P < 0.05$ values were significantly different when compared with cells treated with phenylephrine alone.

Table 1 Inactivation of G $\alpha_{q/11}$ activity by inhibition of palmitoylation or tyrosine phosphorylation significantly decreases phenylephrine-stimulated PI hydrolysis

α_{1B} Adrenoceptor-expressing cells were labelled with [3 H]inositol, and then pretreated with no additions, 100 μ M genistein for 10 min to inhibit tyrosine phosphorylation or 300 μ M 2-fluoropalmitic acid for 30 min to inhibit palmitoylation prior to measurement of carbachol or phenylephrine-stimulated PI hydrolysis. Significantly less inhibition ($P < 0.0001$) of phenylephrine-stimulated PI hydrolysis occurred in response to both 2-fluoropalmitic acid and genistein compared with carbachol-stimulated PI hydrolysis. Results are the means from five independent experiments performed in triplicate.

	PI hydrolysis (% of control, mean $\pm$ S.E.M.)		Difference between inhibition of carbachol and phenylephrine (%)
	With carbachol	With phenylephrine	
2-Fluoropalmitic acid	25 $\pm$ 3	41 $\pm$ 5	16
Genistein	35 $\pm$ 4	50 $\pm$ 2	15

acid to inhibit palmitoylation [40] or 10 min with 100 μ M genistein to inhibit tyrosine phosphorylation [39] prior to measurement of carbachol- or phenylephrine-stimulated PI hydrolysis. The results of these experiments are summarized in Table 1. As predicted from their inhibitory effects on the function of G $\alpha_{q/11}$, both 2-fluoropalmitic acid and genistein substantially inhibited carbachol-stimulated PI hydrolysis. However, significantly less inhibition of phenylephrine-stimulated PI hydrolysis occurred with both 2-fluoropalmitic acid and genistein compared with carbachol-stimulated PI hydrolysis ($P < 0.0001$, Table 1). The contribution of tTG to the phenylephrine-stimulated signalling can be estimated from the difference between the amount of inhibition observed for carbachol- and phenylephrine-

stimulated PI hydrolysis, which was 15–16%, similar to the estimation obtained by inhibiting PI hydrolysis with tTG antibodies. This indicates further that tTG contributes to α_{1B} adrenoreceptor-stimulated PI hydrolysis, but that $G_{q/11}$ plays the predominant role in mediating this process.

DISCUSSION

This study revealed two unique characteristics about the interaction of tTG with the α_{1B} adrenoreceptor. First, tTG, as opposed to the heterotrimeric G-proteins, bimodally regulates α_{1B} adrenoreceptor-mediated PI hydrolysis, with low levels of tTG being facilitatory and high levels being inhibitory. The second characteristic identified that is unique to tTG is that the receptors contribute to the recruitment of tTG to the membrane, its probable site of activity when functioning as a G-protein. Furthermore, agonist-induced activation of α_{1B} adrenoreceptors enhanced the translocation of tTG to the membrane. These findings suggest that GPCRs play an active role in regulating the activity of tTG by orienting tTG to allow it to assume its role as a G-protein.

Up-regulation by tTG of α_{1B} adrenoreceptor-stimulated PI hydrolysis extends previous reports that tTG can couple to the α_{1B} and α_{1D} adrenoreceptors [4,11–13] or the oxytocin receptor in myometrium [14] to mediate activation of PLC. In agreement with those results, phenylephrine-stimulated PI hydrolysis was facilitated by modest increases in tTG induced by retinoic acid, azacytidine or by transient transfection with tTG, and was inhibited by the presence of a tTG antibody. However, this study also revealed that relatively high levels of tTG inhibited GPCR-stimulated PI hydrolysis. Indeed, an earlier study provided preliminary evidence that tTG may bimodally regulate α_{1B} adrenoreceptor-stimulated PI hydrolysis. Reconstitution studies using phospholipid vesicles indicated that calcium biphasically regulated α_{1B} adrenoreceptor–tTG-coupled PI hydrolysis, and that longer times of incubation resulted in significantly less α_{1B} adrenoreceptor-stimulated PI hydrolysis [41]. The findings presented in this present study provide additional evidence that tTG is a unique GTP-binding protein that regulates GPCR signalling in a manner that is distinct from the α -subunit of the heterotrimeric G-proteins [13,42]. Indeed, tTG shows no homology with the α -subunits of the heterotrimeric G-proteins [2,13,26], and the nucleotide-binding and hydrolytic characteristics of tTG are distinctly different from $G_{q/11}$. For example, the rate of hydrolysis of bound GTP is 0.8 min^{-1} for $G_{q/11}$ [43], whereas it is approximately 10-fold lower (0.06 min^{-1}) for tTG [9]. Furthermore, tTG has a much lower affinity for GTP than $G_{q/11}$. tTG binds GTP with a K_m of $\approx 130 \mu\text{M}$, whereas $G_{q/11}$ binds GTP with a K_m of $0.3 \mu\text{M}$ [9,42]. Additionally, heterotrimeric G-protein α -subunits do not show significant ATP binding [2,44,45], whereas tTG has a higher affinity for ATP ($K_m \approx 38 \mu\text{M}$) than GTP, and the binding of ATP potently inhibits the GTPase activity of tTG ($\text{IC}_{50} = 24 \mu\text{M}$) [9]. Given that the concentration of ATP in the cell is estimated to be at least 1 mM [46], it is likely that under physiological conditions the GTPase activity of free tTG is inhibited, and thus tTG would be predominantly in a constitutively GTP-bound state. These results suggest that, unlike heterotrimeric G-proteins, which are activated by receptor-induced binding of GTP, GTP-bound tTG is recruited to specific receptors, and ligand binding may enhance this translocation of tTG to the membrane. Further, the results from this study indicate that the contribution of tTG to α_{1B} adrenoreceptor-stimulated PI hydrolysis is significantly less than that of $G_{q/11}$, indicating that tTG is likely to have a more modulatory role in regulating signalling through GPCRs.

Presently, the mechanism by which tTG can both facilitate and diminish α_{1B} adrenoreceptor-stimulated PI hydrolysis depending on the cellular level of tTG is not clear. However, given the results, one plausible hypothesis is that the α_{1B} adrenoreceptor–tTG–PLC δ signalling module is less efficient at stimulating PI hydrolysis than the α_{1B} adrenoreceptor– $G_{q/11}$ –PLC β signalling complex. For example, this was evident when $G_{q/11}$ was inactivated by introduction of a $G_{q/11}$ α -subunit antibody, which greatly reduced α_{1B} adrenoreceptor-stimulated PI hydrolysis, whereas exposure to a tTG antibody caused a significantly smaller reduction in receptor-activated PI hydrolysis. Hence, at lower concentrations tTG may contribute to α_{1B} adrenoreceptor-stimulated PI hydrolysis, albeit with lower efficiency than the heterotrimeric G-protein, but at higher concentrations more tTG may be bound to receptor and thus decrease the efficiency of α_{1B} adrenoreceptor– $G_{q/11}$ coupling, resulting in less PI hydrolysis. Thus these results indicate that tTG plays a unique modulatory role in regulating α_{1B} adrenoreceptor-activated PI hydrolysis.

tTG displays the unique characteristic of being selectively recruited to the plasma membrane by the α_{1B} adrenoreceptor and this recruitment is enhanced by activation of the receptor with agonist. Previous studies have demonstrated that a complex of agonist, α_1 adrenoreceptor and tTG can be isolated from rat liver membranes [4,47]. However, to our knowledge, this study is the first to demonstrate that recruitment of tTG from the cytoplasm to the membrane is dependent upon the α_{1B} adrenoreceptor, and that this translocation is enhanced by agonist stimulation. In addition, PLC δ is also recruited to the membrane by the presence of the receptor, presumably through its association with tTG [12]. In contrast, heterotrimeric G-proteins have not been shown to be recruited by receptors to the plasma membrane, although GPCRs that share a common α -subunit can interact by regulating the redistribution of their G-proteins [48]. Additionally, $G_{q/11}$ activates PLC β , not PLC δ , and does not selectively recruit the enzyme to the membrane [18]. Further, tTG apparently does not require auxiliary proteins to interact with the receptor [13,20]. In contrast, the α -subunit without GTP bound interacts with the GPCR as part of the $\alpha\beta\gamma$ heterotrimeric G-protein complex, and there is good evidence for receptor-contact surfaces on all three subunits [49,50]. It is also of interest that tTG shows significant selectivity in its interactions with GPCRs, and only modulates the activity of the α_{1B} and α_{1D} adrenoreceptors and the oxytocin receptor [11,14]. In contrast, $G_{q/11}$ shows a high degree of promiscuity, effectively coupling to numerous GPCRs, including all the α_1 adrenoreceptor subtypes, muscarinic receptors, the bombesin receptor and many others [16,51]. Given the lack of sequence homology between $G_{q/11}$ and tTG [26,52], as well as the findings cited above and the data from this study, it is likely that $G_{q/11}$ and tTG interact differentially with the α_{1B} adrenoreceptor. Although the third intracellular loop of the α_{1B} adrenoreceptor contains interaction sites for both $G_{q/11}$ and tTG [11], these proteins are likely to bind differentially to specific sites in this region on the receptor, and to be activated by different mechanisms.

In conclusion, these studies demonstrate that tTG is a novel signal transducing G-protein that bimodally regulates agonist-stimulated PI hydrolysis through a limited number of GPCRs. This is the first identification of a protein having the capacity to both facilitate and to attenuate GPCR-stimulated PI hydrolysis in a concentration-dependent manner. This type of regulation may allow the cell to 'fine tune' its response to specific signals. Indeed the recent discovery of the RGS (regulator of G-protein signalling) proteins, which also regulate GPCR signalling [53], further underscores the complexity of these signalling processes. Continuing investigations into the role of tTG in modulating

GPCR signalling is clearly required to fully elucidate the function of this protein in its capacity as a novel G-protein.

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