Enhanced bradykinin-stimulated phospholipase C activity in murine embryonic stem cells lacking the G-protein α_q -subunit

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The gene coding for the G-protein α_q subunit was interrupted by homologous recombination in murine embryonic stem cells (α_q -null ES cells) as detected by Southern analysis and reverse-transcriptase PCR. The bradykinin (BK) B2 receptor was stably transfected into wild-type (WT) $\alpha_{i,2}$ -null and α_q -null ES cells. The B2 receptor bound BK with high affinity and mobilized Ca^{2+} . BK also activated phospholipase C (PLC), as determined by total inositol phosphate (IP) accumulation in a *Bordetella pertussis* toxin- and genistein-insensitive manner. In WT and $\alpha_{i,2}$ -null ES cells, BK increased IP levels approx. 4-fold above

baseline. Most interestingly, in α_q -null ES cells, BK increased IP accumulation approx. 9-fold above baseline. Re-expression of α_q in α_q -null ES cells resulted in normalization of the BK-stimulated IP accumulation (4-fold above baseline). These results suggest that the B2 receptor activates PLC through more than one member of the G_q family. Additionally, the absence of α_q alters the kinetics of IP generation, which may reflect intrinsic characteristics of individual members of the G_q family or a decreased susceptibility to heterologous regulation in the α_q -null ES cells, thus allowing for a more sustained generation of IP.

INTRODUCTION

Bradykinin (BK) is a pleiotrophic effector that is involved in such processes as inflammation, generation of pain and regulation of blood pressure and electrolyte fluxes [1–5]. The BK² receptor family consists of the B1 and B2 receptor subtypes [6–9], with the B2 receptor apparently mediating many of these physiological effects [10]. The B2 receptor has been reported to signal via the heterotrimeric G-proteins of the G_i and G_q families, to activate a number of target systems, including phospholipase C (PLC), phospholipase D and phospholipase A₂ [11–13]. The B2 receptor has been heterologously expressed in both hamster fibroblasts CCL39 and Rat-1 cells, and demonstrated to couple to phospholipase A₂ and PLC [14].

Three classes of PLC isoenzymes (β , γ and δ) have been identified [15]. The PLC γ isoenzymes are activated by tyrosine kinases and the PLC δ isoenzymes are activated by unknown mechanism(s) [16]. The various PLC β isoenzymes are differentially activated by the α subunit of the G_q family and by the G-protein $\beta\gamma$ dimers presumably released from *Bordetella pertussis* toxin (PTX)-sensitive G-proteins [17–19].

The murine G_q family consists of four members (α_q , α_{11} , α_{14} and α_{15}), none of which are ADP-ribosylated by PTX [20,21]. At the amino acid level, α_q and α_{11} are 88% homologous and are expressed in nearly all cells [21]. The α_{14} subunit, 81% homologous to α_q , is expressed in a limited number of tissues, including murine embryonic stem (ES) cells; and α_{15} , 57% homologous to α_q , is expressed in specific haematopoietic cell types [20]. In phospholipid vesicles, purified α_q and α_{11} indistinguishably activate PLC β isoforms when stimulated with the

non-hydrolysable GTP analogue, GTP[S] [22,23]. However, the *in vivo* function of α_q as opposed to other G_q α subunits has not been determined.

As an experimental model to investigate the biochemical properties of α_q , ES cells were developed with both alleles of the α_q gene inactivated (α_q -null ES cells) by homologous recombination. Previously our laboratory produced ES cells with both alleles of the G-protein subunit $\alpha_{i,2}$ inactivated ($\alpha_{i,2}$ -null ES cells), in which α_2 -adrenergic-receptor-mediated regulation of adenylate cyclase was investigated [24–26]. We now report that the BK B2 receptor, stably transfected into wild-type (WT) $\alpha_{i,2}$ -null and α_q -null ES cells, activates PLC by a PTX-insensitive and genistein-insensitive mechanism, and that, in α_q -null ES cells, the BK-stimulated inositol phosphate (IP) accumulation was enhanced. Re-expression of α_q in α_q -null ES cells resulted in normalization of the BK-stimulated PLC activity. Our data suggest that the B2 receptor interacts with α_q , resulting in a limited activation of PLC, and that in α_q -null ES cells, the BK-stimulated activation of PLC is mediated through other members of the G_q family.

MATERIALS AND METHODS

Materials

G418 (Gibco), puromycin and hygromycin (Calbiochem, San Diego, CA, U.S.A.), proteinase K (Boehringer Mannheim, Mannheim, Germany), UK-14,304 (Research Biochemical International, Natick, MA, U.S.A.), restriction enzymes (New England Biolabs, Beverly, MA, U.S.A.), oligonucleotides (Great

Abbreviations used: BK, bradykinin; DMEM, Dulbecco's Modified Eagle's Medium; HBS, Hepes-buffered saline; IP, inositol phosphate; LPA, lysophosphatidic acid; ES cells, murine embryonic stem cells; PGK, phosphoglycerate kinase; PLC, phospholipase C; RT, reverse transcriptase; PTX, *Bordetella pertussis* toxin; TRH, thyrotropin-releasing hormone; WT, wild-type.

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American Gene Company, Ranson Hill, CA, U.S.A.), tissue-culture plasticware (Costar, Cambridge, MA, U.S.A.), tritiated BK ($[^3\text{H}]\text{BK}$, 90 Ci/mmol) and tritiated *myo*-inositol ($[^3\text{H}]\text{inositol}$, 46.2 Ci/mmol; New England Nuclear, Boston, MA, U.S.A.) were obtained as indicated. Unless otherwise stated, all other chemicals and reagents are from Sigma, St. Louis, MO, U.S.A.

Culture of ES cells

D3 ES cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% (v/v) heat-inactivated fetal-bovine serum (Hyclone, Logan, UT, U.S.A.), 0.1 mM β -mercaptoethanol, 2.0 mM L-glutamine, 20 mM glucose and 25 mM Hepes in a humidified air/ CO_2 (19:1) atmosphere. ES cells were maintained in an undifferentiated state by the addition of conditioned medium from leukaemia-inhibitory-factor-expressing Chinese hamster ovarian fibroblasts (1:500–1:1000 dilution). The cells are routinely grown in tissue-culture-grade dishes coated with 0.1% (w/v) gelatin to promote attachment.

Expression vectors

Neomycin resistance gene, puromycin resistance gene, hygromycin resistance gene, thymidine kinase gene, B2 receptor cDNA and α_q cDNA were expressed from the phosphoglycerate kinase (PGK) promoter [27] with a PGK 3' polyadenylation signal (PGK-NEO, PGK-PURO, PGK-HYGRO, PGK-TK, PGK-B2 and PGK- α_q respectively). The B2 expression vector consisted of PGK-B2 with a collinear PGK-PURO. The α_q expression vector consisted of PGK- α_q with a collinear PGK-HYGRO. (α_q cDNA was generously given by Dr. M. I. Simon.)

Production of α_q -null ES cells

ES cells (5×10^6) were transfected by electroporation using 25 μg of linearized targeting construct and subjected to positive and negative selection with G418 (0.2 mg/ml) and gancyclovir (0.2 mg/ml) [28]. Cells with both α_q alleles interrupted were identified after two rounds of antibiotic selection, as described previously [25,26].

Southern analysis

Genomic DNA was prepared by incubation of ES cells (10^6) with lysis buffer [20 mM Tris (pH 8.0)/10 mM NaCl/10 mM EDTA/0.5% (w/v) SDS/proteinase K (1 mg/ml)] at 55 °C for 16 h, followed by precipitation with 2 vol. of 95% (v/v) ethanol. The genomic DNA (10–20 μg) was digested for 4 h in a 10-fold excess of the restriction enzyme *Xba*I, separated by electrophoresis on a 1.0% (w/v) agarose gel, and transferred to nylon membranes (Genescreen Plus; New England Nuclear, Boston, MA, U.S.A.) in alkaline transfer solution (0.5 M NaOH/1.5 M NaCl). Filters were hybridized overnight at 42 °C with ^{32}P -labelled probes prepared by the random hexamer priming method. After washing for 1 h at 65 °C in $0.2 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl/0.015 M sodium citrate) with 1% SDS, specific hybridization was revealed by autoradiography or phosphorimaging (Molecular Dynamic, Sunnyvale, CA, U.S.A.).

Northern analysis

Total RNA (20 μg) isolated by the guanidine thiocyanate method [29] was separated on 1.4% (w/v) formaldehyde/agarose gel, then transferred to nylon membranes (Genescreen; New England Nuclear) in $20 \times \text{SSC}$ [30]. The membranes were hybridized and washed as described for Southern analysis.

Reverse-transcriptase (RT) PCR

RT PCR was performed on a Perkin–Elmer 9800 instrument using 1 μg of total RNA from WT or α_q -null ES cells as template for reverse transcription with random hexamer primers at 37 °C for 15 min. The PCR primers at a concentration of 50 nM were for α_q 5'-GACCCTTCCTATCTGCCTACACAAC-3' and 5'-GTTTCCGCAGAAATACAGTCCC-3', and for α_{11} 5'-TCATCTTCAGGATGGTGATGTG-3' and 5'-TGAGGAAGAGG-GACAGGGACAGGA-3'. PCR had 35 cycles of 95 °C for 15 s and 65 °C for 30 s.

Cell transfection

Cultured ES cells were transfected with liposomal agents according to the manufacturer's instructions (DOTAP, Boehringer Mannheim). Transfected cells were selected with either puromycin (2 mg/ml) or hygromycin (0.2 mg/ml).

$[^3\text{H}]\text{BK}$ binding assay

Receptor number and binding affinity were determined by binding of $[^3\text{H}]\text{BK}$ to intact ES cells, as described previously [31]. ES cells (5×10^5) in 24-well plates were washed twice with 1 ml of binding buffer [0.05% (w/v) BSA/2 mM bacitracin/10 mM Hepes (pH 7.3)/120 mM *N*-methyl-D-glucamine (replacing NaCl)/0.65 mM CaCl_2 /0.25 mM MgCl_2 /0.25 mM MgSO_4], followed by a 15 min equilibration with 0.5 ml of cold binding buffer. The binding buffer was replaced with binding buffer containing 0.16–20 nM $[^3\text{H}]\text{BK}$. Non-specific binding was determined in the presence of 4.5 μM unlabelled BK. After 2 h at 4 °C, the cells were washed three times with 1 ml of wash buffer (binding buffer supplemented with 0.15% BSA). The cells were solubilized with 0.25 ml of 0.2% SDS and scintillation counted (Ecoscint A; National Diagnostics, Atlanta, GA, U.S.A.). The binding data were modelled using the computer program LIGAND [32,33].

PtdIns(4,5) P_2 hydrolysis

ES cells (5×10^5) in 6-well plates were labelled with $[^3\text{H}]\text{inositol}$ at 1 $\mu\text{Ci}/\text{ml}$ for 24 h in medium 199, supplemented with 1% fetal-bovine serum. The cells were washed with PBS, incubated in DMEM, with 20 mM LiCl/20 mM Hepes, pH 7.4, for 10 min at 37 °C, then stimulated with 50 nM BK for 30 min at 37 °C. The cells were lysed and total $[^3\text{H}]\text{inositol}$ phosphates ($[^3\text{H}]\text{IP}$) were isolated by ion-exchange chromatography on a Bio-Rad Dowex AGI-X8 and eluted with 2 M ammonium formate (pH 5.0) as described previously [34]. Radioactivity was determined by scintillation counting.

Intracellular Ca^{2+} concentration determination

Trypsinized ES cells were washed twice in Hepes-buffered saline [HBS; 140 mM NaCl/5 mM KCl/1 mM MgCl_2 /10 mM glucose/0.9 mM CaCl_2 /0.1% BSA/15 mM Hepes (pH 7.4)]. ES cells (10^7 cells/ml) were loaded with 1 μM fura 2 AM (Molecular Probes, Eugene, OR, U.S.A.) for 30 min in the dark at room temperature, then diluted with 10 vol. of HBS, and incubated for an additional 30 min in the dark. The cells were pelleted and resuspended in HBS at a concentration of 4×10^6 cells/ml. Fluorescence was determined using a computer-driven Perkin–Elmer LS3B fluorescence spectrometer. ES cells (2×10^6) in a total vol. of 2 ml of HBS were continuously stirred with a magnetic stir bar at 37 °C. The excitation monochromator alternated between 340 nm and 380 nm and the emission was

measured at 550 nm. Intracellular Ca^{2+} concentrations were determined according to the following equation: $[\text{Ca}^{2+}] = K_d / (R - R_{\min}) / (R_{\max} - R) / (S_{f2}/S_{b2})$, with the K_d value for the fura-2- Ca^{2+} complex at 37 °C at 225 nM. $R_{\max}$ is the fluorescence ratio determined after addition of 10 μM ionomycin. $R_{\min}$ is the minimal ratio determined by the subsequent addition of 8 mM EDTA. S_{f2}/S_{b2} is the fluorescence value at 380 nm excitation determined at $R_{\min}$ and $R_{\max}$ [35].

RESULTS

The α_q targeting construct was engineered from a 7 kb 129SV/J genomic fragment containing α_q exon 6, which was interrupted at the *Bcl*I site (Asp-237) by the insertion of PGK-NEO, as illustrated in Figure 1. With the *Bcl*I site adjacent to the switch II region (amino acids 205–210), which is involved in nucleotide binding and the activating conformational changes, any protein (either truncated or α_q -Neo fusion) produced from the interrupted alleles should be inactive. Included in the targeting construct, outside the region of homology, was the negative selector PGK-TK. When probed with a fragment not contained in the targeting construct (1.5 kb *Xba*I/*Sac*I fragment), exon 6 is revealed on a Southern blot as an 11 kb band in WT genomic DNA digested with the restriction enzyme *Xba*I (+ / +) (Figure 2). Clones with

Figure 1 α_q Inactivation

Wild type: structure of WT genomic DNA containing exon 6 of α_q with restriction enzyme sites. Digestion of WT ES cell genomic DNA with *Xba*I yields an 11 kb fragment when probed with the 1.5 kb *Xba*I/*Sac*I fragment as indicated. Targeting construct: plasmid targeting construct of genomic DNA (8.5 kb *Sac*I/*Sac*I fragment) with PGK-NEO (NEO) inserted into the *Bcl*I site of exon 6 and PGK-TK (TK) outside the region of homology. Recombinant: digestion of recombinant genomic DNA with *Xba*I yields a 12.5 kb fragment when probed with the indicated fragment. Restriction-enzyme sites are denoted as: X, *Xba*I; B, *Bcl*I; S, *Sac*I.

Figure 2 Identification of interrupted alleles

Southern blot of genomic DNA digested with *Xba*I is probed with a 1.5 kb *Xba*I/*Sac*I fragment. Lane (+ / +), genomic DNA from WT ES cells; lane (+ / -), genomic DNA from ES cells with one α_q allele interrupted; lanes (- / -), genomic DNA from ES cells with both α_q alleles interrupted with PGK-NEO (clones A and B). Endogenous band is 11 kb. Recombinant band is 12.5 kb.

Figure 3 RT PCR with RNA from WT or α_q -null ES cells

The PCR template was synthesized by reverse transcription of 1 μg of total RNA with random hexamer primers. PCR with α_q primers produced a 655 bp product and with α_{11} primers a 589 bp product. PCR products were separated on a 1 % (w/v) agarose gel. Lane 1, 500 bp DNA marker; lane 2, PCR product using α_q primers with RNA from WT ES cells; lane 3, PCR product using α_q primers with RNA from α_q -null ES cells; lane 4, PCR product using α_{11} primers with RNA from WT ES cells; lane 5, PCR product using α_{11} primers with RNA from α_q -null ES cells.

Table 1 Summary of Scatchard plots

Scatchard plots generated from the binding of [^3H]BK (0.16–20 nM) to WT ES cells, α_{i2} -null ES cells and two clones of α_q -null ES cells expressing 25 000 or 140 000 receptors per cell were used to determine receptor number and affinity. Data are means of indicated number (in parentheses) of experiments $\pm$ S.E.M.

Clone	$10^{-3} \times \text{Receptors/cell}$	K_d (nM)
WT	60 ± 8.2	4.77 ± 0.74 (4)
α_{i2} -null	60 ± 3.6	4.56 ± 0.38 (3)
α_q -null (25 000)	25 ± 7.7	7.32 ± 1.65 (4)
α_q -null (140 000)	140 ± 19.4	11.02 ± 2.13 (3)

both alleles interrupted (- / -) do not have the endogenous 11 kb band and exhibit only the 12.5 kb band, representing the insertion of PGK-NEO into exon 6. RT PCR confirmed the absence of transcript from the targeted gene (Figure 3). RT PCR with α_q primers generates a 655 bp product, and with α_{11} primers a 589 bp product. With RNA from WT ES cells, RT PCR produced the appropriate size fragments for the α_q and α_{11} primers. With RNA from α_q -null ES cells, RT PCR produced the appropriate size fragment for the α_{11} primers but failed to produce any product for the α_q primers.

As ES cells do not bind BK, nor activate PLC in response to BK, the BK B2 receptor was stably transfected into WT, α_{i2} -null and α_q -null ES cells. The binding of [^3H]BK (0.16–20 nM) for the three cell lines is saturable, and the data best fit a model for one independent binding site (results not shown). Analysis of the Scatchard plots is summarized in Table 1. WT, α_{i2} -null and α_q -null ES cells exhibit high-affinity BK binding with no statistical differences between the K_d values (Student's *t* test). The K_d values of α_q -null ES cells expressing 140 000 receptors per cell are also not significantly different from the K_d values of α_q -null ES cells expressing 25 000 receptors per cell.

In WT, α_{i2} -null and α_q -null (clones A and B) ES cells, the BK-stimulated activation of PLC was assayed by quantification of IP accumulation. The basal PLC activities are similar in the four cell lines (Table 2A). In WT and α_{i2} -null ES cells, the BK-stimulated IP accumulations were comparable and the amount of increase above basal was not significantly different ($P > 0.05$). In α_q -null ES cells (clone A), BK stimulated an 8.9-fold increase in IP accumulation above basal ($P < 0.01$). To rule out that this increased BK-stimulated IP accumulation is due to a non-targeted mutation unique to this α_q -null clone, a second, independently developed, α_q -null ES cell line (clone B) was trans-

Table 2 BK-stimulated and LPA-stimulated IP accumulation

ES cells labelled with [3 H]inositol in 6-well plates were stimulated with 50 nM BK for 30 min in DMEM containing 20 mM LiCl. Data (c.p.m. released per 10^6 cells) are means of triplicates from the indicated number of experiments (in parentheses) $\pm$ S.E.M. Fold increase is the ratio of the BK-stimulated IP accumulation compared with the basal level of IP accumulation. Statistical analysis was performed with Student's *t* test comparing the fold increase of the WT ES cells with experimental values. *Not significant compared with WT ($P > 0.05$); ** $P < 0.01$ compared with WT. (A) WT ES cells expressing 60 000 receptors per cell (WT), α_{i2} -null ES cells expressing 60 000 receptors per cell (α_{i2} -null), α_q -null ES cells expressing 140 000 receptors per cell (α_q -null A) and a second independently developed α_q -null ES cell strain expressing 140 000 receptors per cell (α_q -null B) were untreated (basal) or stimulated with BK (+BK). (B) Effect of receptor number on the BK-stimulated IP accumulation. Basal and BK-stimulated IP accumulations are reported for WT ES cells expressing 60 000 B2 receptors per cell and two α_q -null ES cell clones expressing either 25 000 or 140 000 receptors per cell. (C) Stimulation by LPA. IP accumulations are reported for WT ES cells and α_q -null ES cells that were untreated (Basal) or stimulated with 10 μ M LPA (+LPA).

(A) IP accumulation			
Clone	Basal (c.p.m./ 10^6 cells)	+ BK (c.p.m./ 10^6 cells)	Fold increase
WT	722 $\pm$ 128	2340 $\pm$ 431	3.40 $\pm$ 0.47(5)
α_{i2} -null	810 $\pm$ 77	2247 $\pm$ 421	2.73 $\pm$ 0.36*(5)
α_q -null A	961 $\pm$ 123	8296 $\pm$ 888	8.86 $\pm$ 0.70**(6)
α_q -null B	918 $\pm$ 81	6603 $\pm$ 469	7.45 $\pm$ 0.85**(6)

(B) IP accumulation			
Clone	Basal (c.p.m./ 10^6 cells)	+ BK (c.p.m./ 10^6 cells)	Fold increase
WT	842 $\pm$ 15	2889 $\pm$ 309	3.41 $\pm$ 0.34(6)
α_q -null (25 000)	928 $\pm$ 79	6467 $\pm$ 322	7.15 $\pm$ 0.51**(6)
α_q -null (140 000)	1097 $\pm$ 63	8464 $\pm$ 490	7.79 $\pm$ 0.50**(5)

(C) IP accumulation			
Clone	Basal (c.p.m./ 10^6 cells)	+ LPA (c.p.m./ 10^6 cells)	Fold increase
WT	582 $\pm$ 23	1499 $\pm$ 79	2.53 $\pm$ 0.12(4)
α_q -null	848 $\pm$ 24	1679 $\pm$ 288	1.97 $\pm$ 0.37*(4)

fectured with the B2 receptor and found to exhibit an enhanced response to BK (7.5-fold increase above baseline).

The ES cell clones reported above expressed the B2 receptor ranging from 60 000 to 140 000 receptors per cell. We tested if this difference in receptor expression could account for the observed enhanced PLC activity. As illustrated in Table 2(B), two α_q -null ES cell clones expressing either 25 000 or 140 000 receptors per cell were assayed for BK-stimulated IP accumulation. In both clones, BK induced comparable IP accumulations (7.2- or 7.8-fold increase above baseline). Thus the level of expression of the B2 receptor, ranging from 25 000 to 140 000 receptors per cell, does not affect the BK-stimulated accumulation of IP in α_q -null ES cells.

To demonstrate that the signalling cascade of G_i -coupled receptors is not disrupted in α_q -null ES cells, the PLC activity of WT and α_q -null ES cells was assayed after stimulation with lysophosphatidic acid (LPA). The LPA-induced IP accumulations (2.5- and 2.0-fold increase above baseline respectively; Table 2C) were not statistically different ($P > 0.05$).

Other factors that may account for the increased PLC activity are an altered time course or a shift in the dose-response curves.

Figure 4 Time course for the BK-stimulated IP accumulation

ES cells labelled with [3 H]inositol in 6-well plates were stimulated with 50 nM BK in DMEM containing 20 mM LiCl for the indicated time periods. IP accumulations for (○) WT ES cells, and (□) α_q -null ES cells, are presented as the percentage of maximum response per 10^6 cells (with the maximum set at the c.p.m. released from α_q -null ES cells after 40 min stimulation). Data are means of triplicates of four experiments $\pm$ S.E.M.

Figure 5 BK dose-response curves

ES cells labelled with [3 H]inositol in 6-well plates were stimulated with BK (0.001–100 nM) for 30 min in DMEM containing 20 mM LiCl. IP accumulations for (○) WT ES cells, and (□) α_q -null ES cells, are presented as the percentage of maximum response per 10^6 cells (with the maximum set at the c.p.m. released from α_q -null ES cells by 100 nM BK). Data are means of triplicates of three experiments $\pm$ S.E.M.

The time-course studies (5–40 min) for the BK-stimulated IP accumulation for WT and α_q -null ES cells (Figure 4) indicate linear IP accumulations for both cell types, with the PLC assay endpoint (30 min) on the linear portion of the curve. BK (0.001–100 nM) stimulates IP accumulation in a dose-dependent manner for the WT and α_q -null ES cells (Figure 5). The BK response is first observed at 0.01 nM for both cell strains. Only the magnitude of the response appears to differ.

Table 3 Effect of PTX and genistein on BK-stimulated IP accumulation

ES cells labelled with [3 H]inositol in 6-well plates were stimulated with 50 nM BK for 30 min in DMEM containing 20 mM LiCl. Data (c.p.m. released per 10^6 cells) are means of triplicates $\pm$ S.D. from two experiments. Fold increase is the ratio of the BK-stimulated IP accumulation compared with the basal level of IP accumulation. (A) Effect of PTX on BK-stimulated IP accumulation. ES cells incubated with or without PTX (100 ng/ml, 4 h) in 6-well plates were stimulated with 50 nM BK for 30 min in DMEM containing 20 mM LiCl. Basal and BK-stimulated (+ BK) IP accumulations for WT ES cells and α_q -null ES cells are presented. (B) Effect of genistein on BK-stimulated PLC activity. Basal and BK-stimulated (+BK) IP accumulations are reported for WT ES cells and α_q -null ES cell clones pretreated with vehicle (0.25% DMSO) or 50 μ M genistein for 10 min.

(A)			
IP accumulation			
Clone	Basal (c.p.m./ 10^6 cells)	+ BK (c.p.m./ 10^6 cells)	Fold increase
Experiment 1			
WT	495 $\pm$ 9	2432 $\pm$ 18	4.9 $\pm$ 0.03
WT + PTX	463 $\pm$ 23	1954 $\pm$ 212	4.2 $\pm$ 0.3
α_q -null	570 $\pm$ 109	3644 $\pm$ 294	6.4 $\pm$ 0.5
α_q -null + PTX	511 $\pm$ 31	2905 $\pm$ 260	5.7 $\pm$ 0.6
Experiment 2			
WT	481 $\pm$ 7	1513 $\pm$ 209	3.1 $\pm$ 0.4
WT + PTX	448 $\pm$ 9	1155 $\pm$ 88	2.6 $\pm$ 0.1
α_q -null	422 $\pm$ 23	2741 $\pm$ 57	6.5 $\pm$ 0.1
α_q -null + PTX	410 $\pm$ 86	2198 $\pm$ 340	6.4 $\pm$ 0.7
(B)			
IP accumulation			
Clone	Basal (c.p.m./ 10^6 cells)	+ BK (c.p.m./ 10^6 cells)	Fold increase
Experiment 1			
WT + DMSO	934 $\pm$ 68	3163 $\pm$ 120	3.4 $\pm$ 0.1
WT + genistein	839 $\pm$ 53	3440 $\pm$ 276	4.1 $\pm$ 0.3
α_q -null + DMSO	637 $\pm$ 174	4159 $\pm$ 52	6.5 $\pm$ 0.1
α_q -null + genistein	538 $\pm$ 122	4419 $\pm$ 454	8.2 $\pm$ 0.8
Experiment 2			
WT + DMSO	991 $\pm$ 121	3240 $\pm$ 151	3.3 $\pm$ 0.2
WT + genistein	936 $\pm$ 89	3324 $\pm$ 479	3.6 $\pm$ 0.5
α_q -null + DMSO	628 $\pm$ 111	4133 $\pm$ 323	6.6 $\pm$ 0.5
α_q -null + genistein	612 $\pm$ 67	4467 $\pm$ 240	7.3 $\pm$ 0.4

Mechanisms such as PTX-sensitive G-protein activation of PLC, or activation of PLC γ isoenzymes, may also account for the increased PLC activity of α_q -null ES cells. The contribution of PTX-sensitive G-proteins to the BK-activation of PLC in WT and α_q -null ES cells is demonstrated in Table 3(A). The basal and the BK-stimulated IP accumulations are unaffected by pretreatment with PTX. In parallel experiments with CCE ES cells expressing the α_2 C10 adrenergic receptor [24], the α_2 -adrenergic agonist (UK14,304)-stimulated IP accumulation was completely blocked by PTX treatment (results not shown).

The PLC γ isoenzymes, which are activated by tyrosine phosphorylation, do not appear to be activated by BK in WT and α_q -null ES cells. The isoflavone genistein (4',5,7-trihydroxyisoflavone), which selectively inhibits tyrosine kinases, as opposed to serine/threonine kinases [36,37], has no effect on basal or BK-stimulated PLC activity in both WT and α_q -null ES cells (Table 3B).

If the enhanced PLC activity of α_q -null ES cells is due to the absence of α_q , then re-expression of α_q should restore the WT phenotype. The α_q -null ES cells, stably transfected with α_q cDNA (α_q rescue ES cells), were identified by Northern blot

Figure 6 Northern blot probed with α_q cDNA

RNA (20 μ g) from: lane 1, WT ES cells; lane 2, α_q -null ES cells; and lanes 3 and 4, two α_q rescue clones; are separated on a 1.1% agarose gel, transferred to Genescreen, and probed with full-length α_q cDNA. Positions of 28 S and 18 S markers are shown.

Table 4 Restoration of WT phenotype in α_q -null ES cells

ES cells labelled with [3 H]inositol in 6-well plates were stimulated with 50 nM BK for 30 min in DMEM containing 20 mM LiCl. Basal and BK-stimulated (+ BK) IP accumulations for WT ES cells, α_q -null ES cells and α_q -rescue clones are presented. Data (c.p.m. released per 10^6 cells) are means of triplicates from the indicated number of experiments (in parentheses) $\pm$ S.E.M. Fold increase is the ratio of the BK-stimulated IP accumulation compared with the basal level of IP accumulation. Statistical analysis was performed with Student's *t* test, comparing the fold increase of the WT ES cells with experimental values. *Not significant compared with WT ($P > 0.05$); ** $P < 0.01$ compared with WT.

IP accumulation			
Clone	Basal (c.p.m./ 10^6 cells)	+ BK (c.p.m./ 10^6 cells)	Fold increase
WT	663 $\pm$ 16	2547 $\pm$ 317	3.79 $\pm$ 0.48(5)
α_q -null	701 $\pm$ 48	7223 $\pm$ 883	9.95 $\pm$ 0.68**(5)
α_q -null rescue	825 $\pm$ 44	2984 $\pm$ 666	4.40 $\pm$ 2.2*(6)

(Figure 6). When probed with full-length α_q cDNA, the endogenous α_q mRNA is not seen in a Northern blot of WT ES-cell RNA (lane 1) (probably due to low levels of endogenous transcript) and the transfected α_q cDNA appears as an 1.6 kb band (lanes 3 and 4).

In the α_q -rescue ES cell clones, BK stimulated a 4.4-fold increase in IP accumulation; in WT and α_q -null ES cells, BK stimulated a 3.8- and a 10.0-fold increase in IP accumulation respectively (Table 4). The increased BK-stimulated PLC activity appears to be dependent on the absence of α_q .

We also examined the ability of BK to stimulate an increase in intracellular Ca^{2+} (Figure 7). In WT ES cells, BK induced a typical intracellular Ca^{2+} profile with an initial sharp peak probably due to release of intracellular stores by $\text{Ins}(1,4,5)\text{P}_3$, followed by a sustained increase in intracellular Ca^{2+} levels. In α_q -null ES cells, the baseline level of intracellular Ca^{2+} is unchanged from the baseline seen in WT ES cells, but the initial spike of intracellular Ca^{2+} is of greater magnitude than the spike observed in BK-stimulated WT ES cells. This is consistent with our data showing increased PLC activity in α_q -null ES cells.

Figure 7 BK-stimulated Ca^{2+} mobilization

Intracellular calcium levels of fura-2-loaded WT ES cells (dotted line) and α_q -null ES cells (broken line) stimulated with 100 nM BK at 280 s. Representative of three experiments.

DISCUSSION

To investigate the role of the G-protein α_q subunit in the BK-stimulated PLC activation, the gene coding for the α_q - or α_{i-2} subunit was interrupted by homologous recombination. In WT, α_{i-2} -null and α_q -null ES cells, the B2 receptor coupling to PLC was PTX-insensitive and genistein-insensitive with the α_q -null ES cells displaying increased BK-stimulated PLC activity. Our results establish that α_q is not required for BK-stimulated activation of PLC, and it appears that the presence of α_q limits BK-stimulated PLC activity.

Studies with G_q α subunits have demonstrated that GTP[S]-stimulated α_q and α_{11} activate PLC with nearly identical kinetics [22,23]. Other studies, using $\alpha_{q/11}$ -specific antibodies, have demonstrated that the B2 receptor activates PtdIns(4,5)*P* hydrolysis through α_q and/or α_{11} [38]. But the role of α_q , as opposed to other G_q α subunits, in the activation of PLC in mammalian cells has not been reported.

One explanation for the enhanced PLC activity observed in α_q -null ES cells is that, in the absence of α_q , other G_q α subunits couple to the B2 receptor and activate PLC more efficiently. The putative receptor and effector binding sequences of α_q and α_{11} suggest that these subunits bind the same receptors and activate the same effectors [39–41]. The α_{14} subunit, also expressed in ES cells, shares the identical effector binding sequences as α_q , but the N-terminus (one of the putative receptor-binding domains and the $\beta\gamma$ binding domain) is completely divergent [20]. These structural features may allow the G_q α subunits to selectively bind to the B2 receptor.

The homology that exists among the G_q family members (α_q and α_{11} are 88 % homologous; α_q and α_{14} are 81 % homologous) is comparable with the homology among G_i family members (α_{i-1} and α_{i-3} are 94 % homologous; α_{i-2} is 88 % homologous with α_{i-1} and α_{i-3}). Characterization of the G_i family members reveals that α_{i-1} , α_{i-2} and α_{i-3} display distinct rates of GDP release, GTP binding and GTP hydrolysis [19,42–46]. The structural variance among the G_q family members may result in analogous kinetic diversity, which may account for the increased PLC activity reported in α_q -null ES cells.

Studies with *Xenopus* oocytes injected with RNA coding for the thyrotropin-releasing hormone (TRH) receptor, α_q or α_{11} , has provided qualitatively similar results [47]. Lipinsky and co-workers [47] found that oocytes injected with RNA coding for the TRH receptor activate PLC when stimulated with TRH. When RNA coding for α_{11} was injected, along with the TRH-receptor RNA, the TRH-stimulated PLC activity increased above the level observed in oocytes injected with TRH-receptor RNA only. When RNA coding for α_q was injected into oocytes, along with the TRH-receptor RNA, the TRH-stimulated PLC activity decreased below the level observed for oocytes injected with TRH-receptor RNA only. It appears that α_{11} and α_q expressed in oocytes differentially activate *Xenopus* PLC.

An equally plausible hypothesis is that decreased desensitization of the signal transduction pathway may account for the enhanced PLC activity of the α_q -null ES cells. The RGS (regulators of G-protein signal) family currently consists of approx. 15 members. RGS 1, RGS 4, RGS 10 and GAIP have been reported to interact with the α_{i-2} subunit to increase the GTPase activity [48–52]. RGS 3 has been shown recently to bind the α_q subunit and limit the PLC activation by α_q [53]. RGS 4 and GAIP have also been shown to attenuate PTX-insensitive PLC activity [54]. The signal-transduction pathway of the α_q -null ES cells may be less susceptible to desensitization than WT ES cells.

Other mechanisms that could account for the enhanced BK-stimulated PLC activity of α_q -null ES cells are increased PLC isoenzyme expression, activation of PLC isoenzymes not activated in WT ES cells, or removal of a negative PLC modulator. Inactivation of the α_q gene or the α_{i-2} gene does not affect the basal PLC activity, suggesting that expression of PLC isoenzymes is not altered. PLC β isoenzymes are activated by the α subunits of the G_q family and $\beta\gamma$ dimers presumably released from PTX-sensitive G-proteins; and PLC γ isoenzymes are activated by tyrosine phosphorylation [19,38,42–44]. Since neither PTX treatment nor the absence of α_{i-2} alters the BK-stimulated PLC activation, it appears that the BK-stimulated PLC activation does not involve activation of PLC isoenzymes by PTX-sensitive G-proteins. The lack of effect by genistein, a tyrosine kinase inhibitor shown to inhibit ATP-stimulated PLC γ activation in cardiomyocytes [55], in both WT and α_q -null ES cells, suggests that BK does not activate PLC γ isoenzymes.

An increasing number of reports suggest that PLC activity can be negatively modulated by PTX-sensitive G-proteins [56–60]. Watkins et al. report that elimination of α_{i-2} by antisense RNA increased PLC activity under both basal and thrombin-stimulated conditions [61]. In our system, elimination of α_{i-2} has no statistically significant effect on basal or BK-stimulated PLC activity. The PTX-insensitive manner in which BK stimulates PLC activity confirms that the α_{i-2} subunit does not negatively modulate PLC activity in ES cells. These data are consistent with members of the G_q family mediating the stimulation of PLC β isoenzymes.

An inherent problem with manipulation of gene expression is that expression of non-targeted genes may be altered. Gene inactivation by homologous recombination has been shown in a number of developmentally regulated multi-gene families to affect gene transcription of non-targeted neighbouring genes [62–64]. Similarly, recent reports in which expression of the α_{i-2} subunit has been eliminated by homologous recombination or antisense RNA indicate that expression of other G-protein subunits may or may not be affected. Rudolph et al., using embryonic fibroblasts from α_{i-2} -deficient mice, report a 30–50 % increase in α_{i-3} levels [65]. On the other hand, Gao and Malbon, using α_{i-2} antisense RNA, report no increase in α_{i-3} expression in F9 teratocarcinoma cells and rat osteosarcoma 17/2.8 cells [66].

The enhanced BK-stimulated PLC activity observed in α_q -null ES cells could result from increased expression of a number of proteins, such as enzymes involved in PtdIns(4,5) P_2 synthesis, PLC isoenzymes, G-protein subunits and phosphatidylinositol transfer proteins [67]. But in our system, the basal PLC activity was unchanged in the WT, α_{i-2} -null and α_q -null ES cells; inactivation of α_{i-2} had no effect on the BK-stimulated PLC activity. The LPA response was unchanged in both WT and α_q -null ES cells, and WT-like BK-stimulated PLC activity was restored by re-expression of α_q in α_q -null ES cells. These results indicate that the process resulting in the inactivation of the α_q gene does not alter expression of other genes to account for the increased BK-stimulated PLC activity in α_q -null cells.

Our results suggest that, in α_q -null ES cells, the increased BK-stimulated PLC activity is due to activation of PLC by other members of the G_q family and that these putative G_q α subunits activate PLC more efficiently than α_q .

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