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Biochemical and Biophysical Research Communications 331 (2005) 1239–1244
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Constitutive activity of endogenous receptors by inducible G_q overexpression

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Received 7 April 2005
Available online 19 April 2005

Abstract

We have developed an inducible cell line that transiently expresses $G_q\alpha$ G protein subunits in response to doxycycline. HEK293/Tet-On pBI($G_q\alpha$) cells worked consistently, achieving high and tightly regulated levels of $G_q\alpha$ overexpression (38-fold increase compared with non-induced cells). We investigated the possibility of using an inducible system to increase the proportion of constitutively active endogenously expressed G protein-coupled receptors (GPCRs) by overexpressing $G_q\alpha$. Not only did we observe an increase in basal activity following doxycycline treatment, but also increased intrinsic activity of agonists such as carbachol, endothelin, lysophosphatidic acid (LPA), and bradykinin. Furthermore, carbachol and LPA potency increased following $G_q\alpha$ overexpression, as did the intrinsic activity of the partial agonist pilocarpine, observations indicative of constitutive activity. An inducible cell line, transiently expressing G proteins, can therefore be employed to induce constitutive activity of endogenously expressed GPCRs. This model system could be used to identify clinically important inverse agonists.

Keywords: G protein; G_q; Constitutive activity; HEK293; Tet-On; Inducible; Overexpression

Receptors which display increased agonist-independent (basal) activity, compared to the wild type receptor, are said to be 'constitutively active.' The first artificially created constitutively active mutant (CAM) G protein-coupled receptor (GPCR) was described in 1990 by Cotecchia et al. [1]. A significant number of CAM receptors have since been described in the literature by many groups (for review see [2,3] and references therein). In the framework of the extended ternary complex model (eTCM), the proportion of receptors adopting the active (R^*) conformation can also be increased by either overexpressing the wild-type receptor itself [4], or by overexpressing the compatible G protein [5–7]. Theoretically, assuming that G proteins, like agonists, preferentially interact with the active (R^*) conformation of the receptor, raising G protein concentration should shift receptor equilibrium to favour the formation and stabilisation of R^*. The net result is increased receptor-G protein coupling (R^*G) and effector activity [8]. This can be explained by the following simplified model of the extended TCM:

$$R \leftrightarrow R^* + G \leftrightarrow R^*G \rightarrow \text{Response}$$

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We therefore decided to investigate the possibility of constitutively activating endogenously expressed wild-type receptors in HEK293 cells by overexpressing the $G_q\alpha$ G protein. Unfortunately, the traditional approach of transiently transfecting mammalian cells with the desired cDNA had to be ruled out for two main reasons: (1) the potential lack of transfection consistency from experiment to experiment and (2) the overall low efficiencies in our laboratory (i.e., the non-guarantee that 100% of the cells) would be successfully

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doi:10.1016/j.bbrc.2005.04.037

transfected. Furthermore, it was concluded that a cell line permanently overexpressing $G_q\alpha$ G protein subunits could not be created, as cells would undergo morphological changes possibly resulting in rapid cell death. An inducible system was therefore considered the most suitable alternative. This system has the potential to provide a source of cells in which ALL cells transiently express $G_q\alpha$ G proteins. As a result, we created a robust and tightly regulated inducible HEK293/Tet-On expression system (parent HEK293/Tet-On cell line commercially available from Clontech Laboratories UK, Basingstoke, UK) that transiently expresses $G_q\alpha$ subunits, only in response to doxycycline (Dox).

The aim of this project was not only to test the feasibility of using an inducible system to express G-proteins but also to increase the proportion of constitutively active endogenously expressed wild-type GPCRs by overexpressing the $G_q\alpha$ G protein. Theoretically, transiently expressed $G_q\alpha$ subunits, together with endogenously expressed $G_q\alpha$, will therefore induce constitutive activity of the endogenously expressed GPCRs by driving receptor equilibrium from R to R^*.

Materials and methods

cDNA encoding the hamster $G_q\alpha$ subunit of the G_q protein, kindly donated by Prof. G. Milligan (Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, UK), was subcloned into multiple cloning site II (MCSII) of the pBI bidirectional Tet vector (Clontech).

HEK293/Tet-On cells were cultured in growth medium comprising MEM Alpha medium (Alpha-MEM) without ribonucleosides and deoxyribonucleotides, supplemented with 10%(v/v) Tetracycline-free foetal calf serum (Clontech), 200 μg/ml G-418, 50 μg/ml gentamicin, 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin in a humidified atmosphere of air/CO_2 (19:1) at 37 °C. All cell culture reagents were purchased from Gibco-BRL (Paisley, UK) unless otherwise stated. To permit generation of a double-stable HEK293/Tet-On cell line, cells were co-transfected with both the pBI($G_q\alpha$) 'Response plasmid' and pTK-Hyg 'Selection vector' (9:1, Clontech) using the Superfect transfection reagent (Qiagen, Hybaid, Teddington, UK) according to manufacturer's instructions. Stable HEK293/Tet-On pBI($G_q\alpha$) cell lines were achieved by antibiotic selection following addition of hygromycin B (200 μg/ml, Calbiochem, Nottingham, UK) to the medium 3 days after transfection and for all subsequent passages of the cells. Only those stable clones expressing significantly enhanced levels of $G_q\alpha$ upon Dox (2 μg/ml) exposure were retained for further examination. Experiments were performed on cells grown in six-well tissue culture plates until confluent (7 days). Forty-eight hours prior to assay, cells were cultured in standard growth medium (unless stated otherwise) in the absence (Control cells, using water as a placebo) or presence of 2 μg/ml Dox (unless stated otherwise) in order to induce $G_q\alpha$ expression.

The level of $G_q\alpha$ expression in HEK293/Tet-On pBI($G_q\alpha$) cells was measured by immunoblotting whole-cell homogenates, using the method described previously [9], sequentially probing with anti-$G_{q/11}\alpha$ (C-terminal, QLNLKEYNLV, diluted 1:4000, Calbiochem) and the HRP-conjugated donkey anti-rabbit secondary antibody (diluted 1:2000, Amersham Life Science, Little Chalfont, UK). Immunolabelled proteins were detected using the SuperSignal enhanced chemiluminescence kit (Pierce & Warriner, Chester, UK) and autoradiography using Hyperfilm-ECL (Amersham Life Science). All autoradiographs shown are representative of at least three separate experiments. Densitometric analysis of autoradiographs was performed using the MagiScan system and Gemini computer program (Applied Imaging, Sunderland, UK). All SDS–polyacrylamide gel electrophoresis (SDS–PAGE) reagents were obtained from Bio-Rad (Hemel Hempstead, UK).

To assess the effect of Dox-induced $G_q\alpha$ overexpression on total inositol phosphate (IP) production following endogenous G_q-coupled receptor activation in HEK293/Tet-On pBI($G_q\alpha$) cells, 48 h prior to assay the standard growth medium was replaced with 2 ml of inositol-free MEM with Earle's salts, supplemented with 10%(v/v) dialysed Tetracycline-free foetal calf serum (Clontech), 1% L-glutamine, 1% L-cysteine, 1% L-arginine, 1% L-leucine, 1% L-methionine, 1% glucose, 50 μg/ml gentamicin, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin, and 6 μCi [^{3}H]-myo-inositol (Amersham Life Science). Again, cells were exposed to either 2 μg/ml Dox or placebo during this 48 h period. Immediately prior to assay, cells were washed with pre-warmed (37 °C) Krebs–Ringer buffer (145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 1.3 mM $CaCl_2$, 10 mM glucose, and 20 mM Hepes, pH 7.4) containing 10 mM lithium chloride (LiCl). IP formation was then stimulated by the addition of agonists in 1 ml Krebs–Ringer buffer containing 0.25%(w/v) BSA and 10 mM LiCl (drug solution) for 12 min at 37 °C (5% CO_2/95% air). The assay was terminated by the addition of 0.5 ml ice-cold 1.5 M perchloric acid to the medium and the cells were incubated on ice for a further 20 min. An aliquot was removed (1.4 ml) and neutralised with 2 M KOH in 1 M Tris–base. The precise amount of 2 M KOH in 1 M Tris–base added, was calculated as 10% of the volume of the same solution needed to change the pH of 10 ml of agonist solution supplemented with 5 ml of the 1.5 M perchloric acid to 7.4. The solution was then vortexed for 10 s and centrifuged (5500 rpm) for 10 min (at 4 °C). 1.3 ml of the supernatant was removed, diluted with 3.7 ml of 5 mM disodium tetraborate containing 0.5 M EDTA, and total IPs were isolated by ion-exchange chromatography [10] on Dowex columns containing a 2 ml suspension of 1 g/ml AG1X8 resin (mesh, 100–200 formate form, Bio-Rad). Total IPs were eluted with 10 ml of 1 M ammonium formate in 0.1 M formic acid. After mixing, a 3 ml aliquot of this eluate was combined with 20 ml of scintillation fluid and the [^{3}H] content was determined using a Packard 2000CA scintillation counter. All chemicals were obtained from Sigma–Aldrich (Poole, UK) unless otherwise stated.

All IP and $G_q\alpha$ expression data were computer-analysed using GraphPAD Prism software (GraphPAD Software, San Diego, USA). The EC_{50} value for each dose–response curve was determined using a non-linear regression program and a fixed Hill coefficient. All results are quoted as means ± SEM of three experiments, unless stated otherwise.

Results and discussion

Western blot analysis of HEK293/Tet-On pBI($G_q\alpha$) cells revealed a concentration-dependent increase in $G_q\alpha$ expression (ranging from 0 to 2.5 μg/ml Dox), which reached maximum levels when cultured for 48 h (recommended by Clontech to achieve maximum levels of induction) in medium containing between 2 and 2.5 μg/ml of Dox ($n = 3$, Fig. 1A). In all further experiments, maximal induction of $G_q\alpha$ was achieved using 2 μg/ml Dox. The actual level of $G_q\alpha$ subunit (42 kDa) expression in control (non-induced) cells, quantified by densometric analysis of the autoradiographs, was $2.6 \pm 0.2\%$ ($n = 8$, Fig. 1B) of that in Dox-treated cells. This equates to an approximate 38-fold increase in $G_q\alpha$ subunit expression following Dox induction. This significant increase ($n = 8$, $P < 0.001$) was confirmed by seri-