ELSEVIER
Biochimica et Biophysica Acta 1592 (2002) 185–192
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
www.bba-direct.com

Differential coupling of the extreme C-terminus of G protein α subunits to the G protein-coupled melatonin receptors

Janice E. Drew [a,*], Perry Barrett [b], Shaun Conway [c], Philippe Delagrange [d], Peter J. Morgan [b]

[a] *Phytochemicals and Genomic Stability, Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, Scotland, UK*
[b] *Molecular Neuroendocrinology, Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, Scotland, UK*
[c] *Gut Immunology, Rowett Research Institute, Bucksburn, Aberdeen, AB21 9SB, Scotland, UK*
[d] *Institut de Recherches Internationales Servier, 6 Place des Pleiades, F-92415 Courbevoie cedex, France*

Received 31 January 2002; received in revised form 1 August 2002; accepted 3 September 2002

Abstract

Melatonin receptors interact with pertussis toxin-sensitive G proteins to inhibit adenylate cyclase. However, the G protein coupling profiles of melatonin receptor subtypes have not been fully characterised and alternative G protein coupling is evident. The five C-terminal residues of Gα subunits confer coupling specificity to G protein-coupled receptors. This report outlines the use of Gαs chimaeras to alter the signal output of human melatonin receptors and investigate their interaction with the C-termini of Gα subunits. The Gαs portion of the chimaeras confers the ability to activate adenylate cyclase leading to cyclic AMP production. Co-transfection of HEK293 cells expressing MT_1 or MT_2 melatonin receptors with Gαs chimaeras and a cyclic AMP activated luciferase construct provided a convenient and sensitive assay system for identification of receptor recognition of Gα C-termini. Luciferase assay sensitivity was compared with measurement of cyclic AMP elevations by radioimmunoassay. Differential interactions of the melatonin receptor subtypes with Gα chimaeras were observed. Temporal and kinetic parameters of cyclic AMP responses measured by cyclic AMP radioimmunoassay varied depending on the Gαs chimaeras coupled. Recognition of the C-terminal five amino acids of the Gα subunit is a requisite for coupling to a receptor, but it is not the sole determinant.

Keywords: Melatonin receptor; G protein-coupled receptor; Gα chimaera

1. Introduction

G protein-coupled receptors conduct intracellular signalling through coupling to the Gα subunits of heterotrimeric G proteins in cell membranes. There are at least 17 Gα subunits that have been grouped into distinct classes based on sequence homology. Each has the ability to activate a downstream effector molecule with subsequent activation of a cascade of intracellular signalling events [1]. Identification of the G protein coupling specificity of a G protein-coupled receptor is a necessary requisite to identifying signalling events regulated by a particular receptor and allows an insight into the possibilities for receptor regulation of signalling pathways in complex cell environments.

The G protein-coupled melatonin receptors have been identified in a number of different tissues [2–5]. They are activated by the hormone melatonin, synthesised primarily by the pineal gland during the hours of darkness and regulate seasonal and circadian rhythms [6]. The signalling systems regulated by the melatonin receptors are poorly understood, particularly in fetal tissue and peripheral sites. Based on sequence similarities, three melatonin receptor subtypes have been identified: MT_1, MT_2 and mel1c [3,7,8]. Only MT_1 and MT_2 are expressed in mammals and all three subtypes are found in chicken, *Xenopus* and zebrafish. In native cells, the coupling of melatonin receptors and their interaction with different signalling systems is poorly defined. Melatonin has the ability to inhibit forskolin-stimulated cyclic AMP production in a number of native tissues [9]. In some cases, this event is sensitive to pertussis toxin (PTX) and this suggests that the melatonin receptors are coupled to Gi or Go proteins [10–14]. Since Gi

Abbreviations: PTX, pertussis toxin; AMP, adenosine monophosphate; NAD, nicotinamide adenine dinucleotide; EGTA, ethylene glycol-bis(B-aminoethyl ether)-*N*,*N*; IBMX, 3-isobutyl-1-methylxanthine

* Corresponding author. Tel.: +44-1224-712751x2510; fax: +44-1224-716653.
E-mail address: jed@rri.sari.ac.uk (J.E. Drew).

0167-4889/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0167-4889(02)00312-9

mediates the inhibition of cyclic AMP it has been presumed that the G protein involved is Gαi1, Gαi2 or Gαi3. In addition, PTX-insensitive adenylyl cyclase inhibition of cyclic AMP has been observed in the sheep pars tuberalis [15,16]. Coupling of *Xenopus* mel1c subtypes to Gz [17] and coupling of MT_1 to Gq [18] have been reported. Melatonin receptor activation of cyclic AMP through coupling to Gαs is unknown. However, the involvement of G protein coupling and activation of PTX-insensitive pathways has not undergone thorough investigation.

There is considerable evidence to substantiate the importance of the extreme C-terminus of the Gα subunits in mediating contact and coupling specificity with G protein-coupled receptors [19–21]. Subsequently, researchers have reported that the C-terminal five amino acid residues can confer G protein-coupled receptor coupling specificity within cell membranes.

Using the ability of the C-terminal five amino acid residues of Gα to confer G protein coupling specificity, G protein coupling profiles were obtained for the human melatonin receptors, MT_1 and MT_2. Chimeric Gαs subunits were generated that allow reversal of melatonin inhibition of cyclic AMP to activation of cyclic AMP. The Gαs subunit contact effector site is located at the N-terminal hence cyclic AMP stimulation by this subunit is unaffected by C-terminal changes [22,23]. Gαs subunit activation of cyclic AMP generates a robust signal that is more easily measured since measurement of cyclic AMP inhibition often requires pre-stimulation with forskolin. The paradoxical cyclic AMP stimulation of Gαs–αx chimaeras (where αx is the C-terminal five residues of the known Gα subunits) by cyclic AMP inhibitory melatonin receptors also provides an opportunity to obtain incontrovertible evidence of Gαs–αx chimaera coupling. Cell lines stably expressing the melatonin receptors were co-transfected with wild-type Gαs or Gαs–αx chimaeras and a cyclic AMP responsive luciferase reporter construct. Receptor–Gαs–αx chimaera coupling in response to melatonin was then measured via stimulation of cyclic AMP and activation of luciferase monitored by a luminescent assay. Cyclic AMP levels were also measured by radioimmunoassay in the Gαs–αx chimaera transfected cell lines to determine temporal and kinetic differences in receptor–Gαs–αx coupling efficiency to the different Gα C-termini between the MT_1 and MT_2 receptor subtypes.

2. Methods

2.1. Materials

All chemical reagents were obtained from Sigma International, UK, unless otherwise stated. Restriction and DNA modifying enzymes and enzyme buffers were obtained from Promega, Southampton, UK. Cell culture media, sera and antibiotics were obtained from Gibco Invitrogen Corporation, Paisley, UK. Random and synthetic oligo primers were purchased from Genosys, Cambridgeshire, England. Antibodies were obtained from Santa Cruz Biotechnology.

2.2. Construction of Gαs–αx chimaeras

First strand cDNA prepared from HEK293, as described previously [24], was used as a template to generate a Gαs subunit cDNA minus the C-terminal 15 bases encoding the last five amino acids. PCR was performed using 100 pmol of primers (5′-gcaagcttgccgccgccatgggctgc-3′, 5′-acgaaggtg-catgcgctgaatgatg-3′), 2.5 U Turbo Pfu polymerase (GibcoBRL, Paisley, Scotland) and 200 μM dNTPs. The PCR reaction cycles consisted of 94 °C for 1 min, 65 °C for 1 min and 74 °C for 3 min for 30 cycles. The PCR product was cleaved at the 5′ end with *Hin*dIII. Oligo pairs encoding the C-terminal five amino acids of the known Gα subunits were annealed by incubating 500 pmol of each oligo at 100 °C for 1 min in 0.15 M NaCl and allowing to cool to room temperature. The annealed oligo pairs generated double-stranded fragments (see Fig. 1) that were ligated to the Gαs PCR product. Ligation of the truncated Gαs PCR product and the annealed oligo pairs was designed to facilitate subcloning of the Gαs chimeric constructs directly into a *Hin*dIII/*Kpn*I cleaved mammalian expression vector pcDNA3.1 (Gibco Invitrogen). The resulting chimaeras were sequenced using DNA prepared by a Wizard DNA purification kit (Promega) and an Applied Biosystems model 377 DNA sequencer.

2.3. Cell culture and transfection

The HEK293 cell lines stably expressing the human MT_1 (HEKh1a) and MT_2 (HEKh1b) receptors and HEK293 culture were described previously [24]. Cells were transiently co-transfected with pGL3(CRE_2-TK) [25] and a Gαs–x chimaera cloned in pcDNA3.1 (Gibco Invitrogen) or an equivalent amount of pcDNA3.1 plasmid. The plasmid, pGL3(CRE_2-TK), consisted of the luciferase reporter, pGL3 (Promega) with a thymidine kinase (TK) basal promoter and two cyclic AMP responsive elements (CRE) cloned upstream of the TK promoter as described previously [25]. Cells were transfected at around 80% confluence on 90 mm plates with 2 μg of each plasmid to be transfected and 3 μl FuGENE 6/μg DNA according to the manufacturers instructions (Boehringer Mannheim).

2.4. Immunodetection of Gαs chimaera expression

Membrane proteins were extracted from cells transfected as described above, 48 h after transfection. Cells were rinsed with 1 × PBS and pelleted at 700 × *g* for 10 min. The cells were stored at −70 °C prior to extraction of membrane proteins as follows. Cell pellets were resuspended in 0.5 ml homogenate buffer (10 mM Tris pH 7.5, 1 mM EGTA) and homogenised using a glass homogeniser. The homogenate was made up to a volume of 1 ml with homogenate buffer

Gαs wt	CAG TAC GAG CTG CTC TAA GGTAC GTC ATG CTC GAC GAG ATT C Q Y E L L
Gαs-αi1 (Gαi1 and Gαi2 identical)	GAT TGC GGC TTA TAT TAA GGTAC CTA ACG CCG AAT ATA ATT C D C G L F
Gαs-αi3	GAA TGC GGC TTA TTT TAA GGTAC CTT ACG CCG AAT AAA ATT C E C G L Y
Gαs-αo1 (Gαo1 and Gαo2 identical)	GGT TGC GGC TTG TAC TAA GGTAC CCA ACG CCG AAC ATG ATT C G C G L Y
Gαs-αz	TAC ATT GGC CTT TGC TAA GGTAC ATG TAA CCG GAA ACG ATT C Y I G L C
Gαs-αq (Gαq and Gα11 identical)	GAG TAC AAC CTC GTT TAA GGTAC CTC ATG TTG GAG CAA ATT C E Y N L V
Gαs-α12	GAT ATC ATG CTT CAA TAA GGTAC CTA TAG TAC GAA GTT ATT C D I M L Q
Gαs-α13	CAA CTC ATG CTT CAA TAA GGTAC GTT GAG TAC GAA CTT ATT C Q L M L Q
Gαs-α14	GAA TTC AAC TTA GTT TAA GGTAC CTT AAG TTG AAT CAA ATT C E F N L V
Gαs-α16	GAG ATC AAC CTG CTG TAA GGTAC CTC TAG TTG GAC GAC ATT C E I N L L

Fig. 1. Construction of the Gαs chimaeras. The constructed Gαs wt representing the S1 form of human Gαs and the Gαs–αx chimaeras with the annealed oligos used to reconstruct the sequence encoding the C-terminal five residues of Gαs wt are shown.

and spun at 500 × *g* (Juoan) for 15 min. The supernatant was then spun at 38,000 rpm (TL100) for 5 min. The pellet was resuspended in 1 ml of homogenate buffer and spun 38,000 rpm for a further 5 min before resuspending in 1 ml of homogenate buffer. An aliquot was removed for quantitation using a Biorad DC protein assay kit.

For each transfected Gαs chimaera, 20–50 μg of membrane proteins were separated on a 10% polyacrylamide gel in the presence of SDS and electrophoretically transferred to poly-(vinylidene difluoride) (PVDF) membrane. Immunodetection of recombinant Gαs and the Gαi3 by a Gαs (C-terminal) and a Gαi3 (C-terminal) antibody, respectively, was visualised by chemiluminescence using an ECL kit (Amersham).

2.5. Ribosylation of Gαs chimaeras

Membrane proteins isolated as described above were assayed for the presence of Gαs short-form chimaeras by ribosylation with cholera toxin in the presence of [^{32}P]NAD as described by Morgan et al. [26]. Briefly, 50 μg membrane protein was incubated for 30 min at 37 °C with 10 mM thymidine, 1 mM ATP, 2 mM GTP, 10 μM NAD, 20 μCi [^{32}P]NAD (specific activity 800 μCi/mmol; NEN) with or without cholera toxin at 120 μg/ml in 150 mM sodium phosphate pH 7.5, 2.5 mM $MgCl_2$, 1 mM EDTA. The reactions were centrifuged at 4 °C to pellet the ribosylated products and electrophoresed by SDS-PAGE (12.5% separating gel; 6% stacking gel). The SDS-PAGE gels were dried and apposed to film (KODAK X-OMAT).

2.6. Assay of luciferase activity

Cells transfected with pGL3(CRE_2-TK) and either a blank pcDNA3.1 plasmid or a Gαs chimaera were trypsinised and seeded into 96-well view plates (Packard) at 40,000 cells per well the day following transfection. The remaining cells were reseeded onto 90 mm plates and

harvested the following day for protein extraction and Western blotting or ribosylation assay to determine Gαs chimeric expression in the transfected cells. The day following seeding into 96-well plates, the cells were stimulated with or without melatonin, or forskolin. The cells were stimulated at 37 °C for 16–18 h. The induced adenylate cyclase activity was then measured using the Luclite luciferase reporter gene assay system (Boehringer Mannheim) according to the manufacturers instructions and a luminometer (Packard).

2.7. Radioimmunoassay of cyclic AMP

Cells transfected as above were trypsinised and seeded into 24-well plates at 200,000 cells per well the day following transfection. Following a further 24 h incubation, the cells were stimulated in cell culture media supplemented with 10^{-8} M melatonin in the presence of 10 mM 3-isobutyl-1-methylxanthine (IBMX) for various time intervals at 37 °C. Reactions were terminated by addition of trichloroacetic acid to a final concentration of 5%. Cyclic AMP levels were determined as reported previously [15].

3. Results

3.1. Expression of Gαs chimaeras in transfected cells

The human Gs-S1 form minus the C-terminal five amino acids (positions 1–375) was obtained by PCR using Pfu polymerase. Annealed oligos encoding the array of C-terminal five amino acids were ligated to the blunt Gs-S1 PCR product to give Gαs and the Gαs–αx chimaeras listed (Fig. 1). Wild-type HEK293 cells express both the S1 and S2 form of Gαs. Overexpression of transiently transfected wild-type Gαs short form was detectable as a 44 kDa protein at levels greater than those of endogenous Gαs as compared by Western blotting of cells transfected with or without the recombinant Gαs using anti Gαs (C-terminal) antibody (Santa Cruz) (Fig. 2A). Signal detection of endogenous Gαs was only detectable after longer radiograph exposure. The endogenous short form of Gαs, S1, was expressed at lower levels than S2 (unpublished observation). The Gαi3 antibody used was able to detect overexpression of recombinant Gαs–αi3 chimaera subunits (44 kDa) in transfected cells as distinct from endogenous Gαi3 subunits (40.5 kDa) when compared with cells transfected with the pGL3(CRE_2-TK) plasmid only (Fig. 2B). Increased levels of ribosylated G proteins were observed in cells transfected with wild-type Gαs or Gαs–αx chimaeras compared to non-transfected cells and cells transfected with the pGL3(CRE_2-TK) plasmid only (Fig. 2C). A 1-D evaluation of Fig. 2C by AIDA Image Analyser software (Raytest Isotopenmeßgerate GmBH, Germany) was performed and confirmed an enhanced signal for ribosylated short form Gs wt and Gs–x chimaeras in transfected cells.

3.2. Activation of luciferase activity by MT_1 and MT_2 receptors via Gαs chimeric coupling

Previous reports have indicated that melatonin receptors inhibit cyclic AMP via PTX-sensitive G proteins of the Gi family. Considering evidence that the C-terminal five amino acids confer coupling specificity on G protein and G protein-coupled receptor interactions, we tested the array of Gαs chimaeras (Fig. 1) to determine the coupling profile of the human MT_1 and MT_2 receptors. The native HEK293 cells and HEK293 cell lines stably expressing the human MT_1 (HEKh1a) and MT_2 (HEKh1b) receptors were transfected with the cyclic AMP responsive luciferase construct pGL3(CRE_2-TK) alone or together with

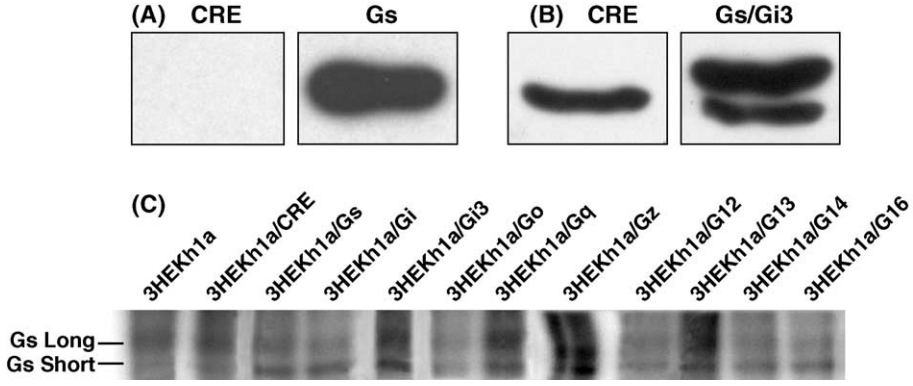

Fig. 2. Expression of recombinant Gαs wt and Gαs chimaeras. The expression of the transfected Gαs wt (A) and Gαs–αi3 (B) chimaeras were ascertained by immunoblotting 20 μg of membrane protein with (A) anti-Gαs (c-terminal) and (B) Gαi3 (c-terminal) antibodies (Santa Cruz). Endogenous Gαi3 is visible as a 40.5 kDa product as opposed to the 44 kDa Gαs–αi3 chimaera. (C) Autoradiograph showing ^{32}P-labelled products ribosylated with cholera toxin (120 μg/ml) in membranes of clonal cell line 3HEKh1a transiently transfected with the Gαs–αx chimaeras. The ribosylated short form Gαs–αx chimaeras are visible above endogenous levels of short form Gαs.

wild-type Gαs or Gαs–αx chimaera constructs. The luciferase activity was then measured as described above in the presence or absence of 10^{-8} M melatonin (Fig. 3). Transfected cells were seeded into 96-well plates to permit four determinations of basal and melatonin-induced luciferase activation. The basal level of luciferase in each transfected cell population was used to calculate activation levels expressed as a ratio (% over basal). Basal activity is a measure of luciferase generation from the TK promoter prior to stimulation via the CRE response elements. This provides a means of determining that sufficient cells have been transfected for the subsequent assay. Calculation of ratios using this approach allow for variations in transfection efficiency and luminescence generated between experiments in the whole series. The ratios obtained were generated from eight luciferase determinations from at least three and up to eight different experiments (24–64 data points) to allow calculation of mean luciferase activation and standard errors. There was no significant activation of luciferase activity in the native HEK293 cell line using wild-type Gαs or Gαs–αx chimaeras (data not shown). There was also no significant activation of luciferase activity in the HEKh1a or HEKh1b cell lines transfected with pGL3(CRE_2-TK) alone or pGL3(CRE_2-TK) and Gαs when compared to the basal levels of luciferase in these transfected cells (Fig. 3A, B). In contrast, luciferase activation was detected on co-transfection with a number of the Gαs–αx chimaeras. HEKh1a cells showed melatonin-stimulated activation of luciferase activity when transfected with Gαs–αGi, Gαs–αGi3 Gαs–αGo, Gαs–αGq, Gαs–αGz, Gαs–αG12 Gαs–αG13 Gαs–αG14 Gαs–αG16 indicating promiscuous coupling of the human MT_1 receptor to the Gαs–αx chimaeras (Fig. 3A). HEKh1b cells showed melatonin activation of luciferase activity to varying degrees when transfected with Gαs–αGi, Gαs–αGi3, Gαs–αGz, Gαs–

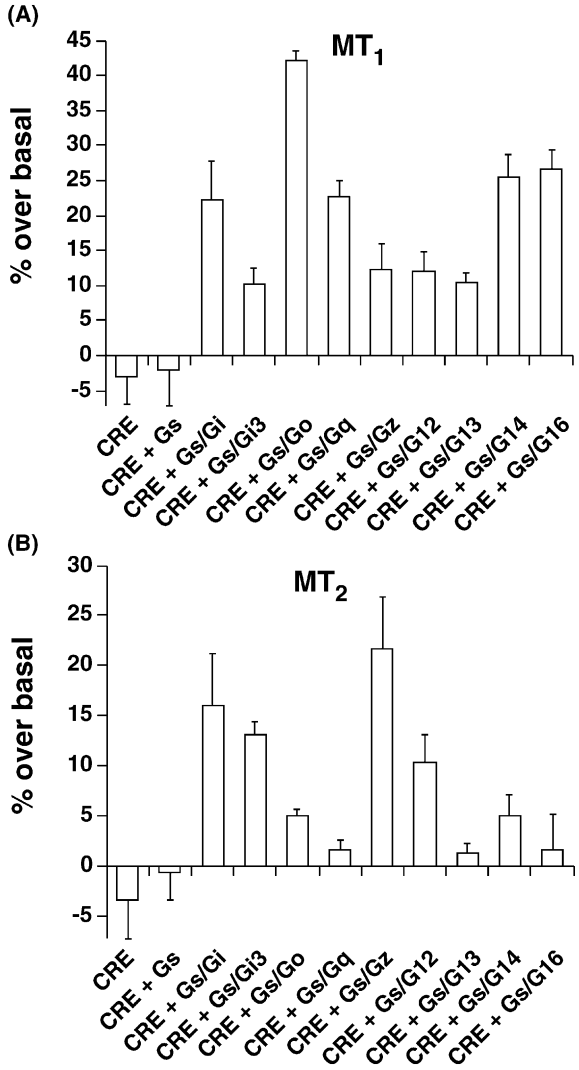

Fig. 3. Coupling profile of the human melatonin receptors MT_1 (A) and MT_2 (B) with the Gαs–αx chimaeras. The activation of luciferase activity in each cell line co-transfected with the pGL3(CRE_2-TK) reporter construct (CRE) and the Gαs–αx chimaeras was measured as the percentage above basal after stimulation with 10^{-8} M melatonin over a period of 16–18 h. Basal levels were obtained from transiently transfected cells that were unstimulated. Values are the means of at least three separate transfections assayed in quadruplicate.

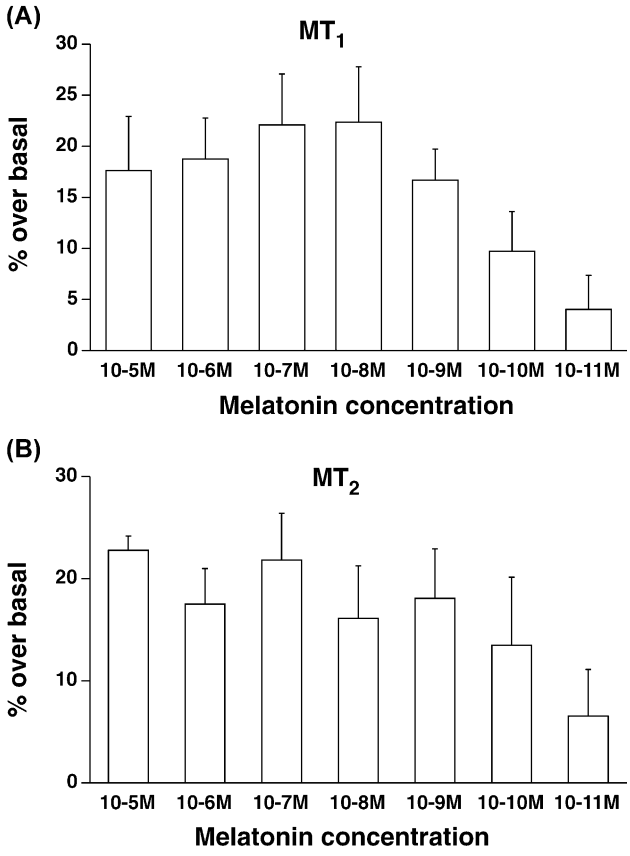

Fig. 4. Dose response of melatonin activation of luciferase response in MT_1 (A) and MT_2 (B) expressing cell lines co-transfected with the pGL3(CRE_2-TK) reporter construct (CRE) and the Gαs–αi chimaera. Values are the means of at least three separate transfections assayed in quadruplicate.

αG12, and to a lesser degree with Gαs–αGo and Gαs–αG14 (Fig. 3B). Activation of Gαs–αGq, Gαs–αG13 or Gαs–αG16 was not achieved to a significant degree (Fig. 3B). Hence, the Gαs–αx chimaeras were successful in converting the usual melatonin cyclic AMP inhibitory signal to a cyclic AMP stimulatory signal as measured by luciferase activity. Potential activation of endogenous cAMP inhibitory Gi proteins is possible in response to the elevation of cAMP via melatonin receptor coupling with Gαs–αx. However, once the luciferase enzyme has been synthesised from the activated reporter construct pGL3(CRE_2-TK) it will remain present and active for many hours. Subsequent degradation of cAMP or its inhibition by other cellular factors does not alter the luciferase already activated via Gαs–αx coupling. Cyclic AMP inhibition via coupling of melatonin to endogenous Gi subunits was not significant in radioimmunoassays of MT_1 and MT_2 expressing cells transfected with the reporter plasmid only (see below). Forskolin was used in early experiments to ensure transfection efficiency was sufficient to give detectable levels of luciferase. Forskolin stimulation was not subsequently used in all of the assays as successful transfection could be ascertained by the luciferase generated from the TK promoter. Forskolin-induced luciferase activity up to 60% over basal was achieved.

Dose response experiments were performed. Dose-dependant stimulation of MT_1 and MT_2 receptors exhibited the expected effect on luciferase activation when coupled to Gαs–αi chimaeras (Fig. 4). Differential coupling efficiency of the melatonin receptors to the chimaeras was observed with differences in activation apparent for different chimaeras and between the two receptors (see Fig. 3).

3.3. Radioimmunoassay of cyclic AMP

The ability of Gαs–αx chimaeras to elevate cyclic AMP via interaction with melatonin receptors and the assay sensitivity was further assessed by performing radioimmunoassay of cyclic AMP levels. Radioimmunoassay confirmed elevation of cyclic AMP levels in response to stimulation of MT_1 receptors in cells transfected with Gαs–αx chimaeras (Fig. 5). Consistent with the luciferase reporter data, increased cyclic AMP levels were not observed in cells transfected with wild-type Gαs. No significant cAMP inhibition via endogenous Gi was detected in cells transfected with the reporter plasmid only. This is consistent with previous reports that melatonin does not significantly affect basal levels of cAMP [27]. Extended incubation periods were necessary to detect the full range of Gαs–αx chimaera coupling interactions predicted by the luciferase assay for MT_1 and MT_2 receptors (unpublished observations).

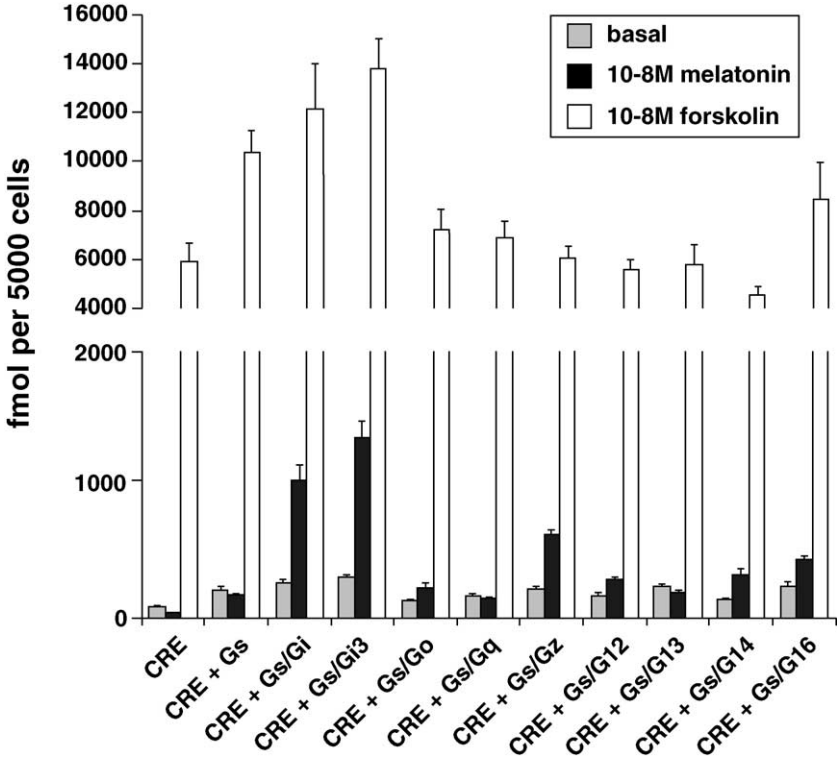

Fig. 5. Measurement of cyclic AMP elevations by radioimmunoassay of HEKh1a transiently transfected with Gαs–x chimaeras. Stimulations with 10^{-8} M melatonin or 10^{-5} M forskolin were performed for 30 min in the presence of 10^{-3} M IBMX. The data shown is a representative experiment performed on samples assayed in duplicate from three experimental replicates.

4. Discussion

This study has illustrated the differential coupling of the human melatonin receptors to the C-terminal five amino acids of the known Gα proteins. Replacement of the C-terminal five amino acids of Gαs allowed paradoxical stimulation of cyclic AMP by melatonin receptors. The broad range of coupling and activation of Gαs−αx chimaeras by the melatonin receptors and the comparative ease of measuring signal activation via the CRE luciferase system provides a useful tool to identify signal activation of Gαi-coupled receptors. Indeed the Gαs−αi chimaera has been used successfully to demonstrate melatonin binding to chimeric melatonin and melatonin-related receptors [28,29]. The work reported here has implications for the study of signalling pathways activated by melatonin receptors and the possibilities for complex G protein interactions dependent on G protein profiles in different cell types. The levels of receptor and Gα protein expression, the time period of ligand activation and sub-localisation of receptors and Gα proteins in the cell membrane may all be contributing factors to determining selection of G protein coupling. The differential coupling of each receptor with the array of possible Gα protein C-terminal five amino acids extends our knowledge of the interaction of G protein-coupled receptors with G proteins and may alter our perception of coupling specificity.

Using the Gαs−αx chimaeras, it was possible to determine a coupling profile for the two human melatonin receptor subtypes to the known Gα C-terminal five amino acids by monitoring cyclic AMP induction via coupling of each receptor subtype to the Gαs−αx chimaeras. The data suggest a strong interaction of the MT_1 and MT_2 receptors with Gi. This is entirely consistent with published data showing that melatonin receptors exhibit cyclic AMP production in a PTX-sensitive manner. The interaction between the MT_1 receptor and Gz is also anticipated, as receptors that couple to Gi often couple Gz. Gz subunits inhibit cyclic AMP in a PTX-insensitive manner and may account for reports of coupling via the PTX-insensitive cyclic AMP inhibitory G proteins [17,18,30]. The observation of differential coupling efficiencies of MT_1 and MT_2 receptor subtypes may have implications for the differential expression of these receptors in vivo. Extensive experiments investigating the interaction of the Gαs−αx chimaeras with MT_1 and MT_2 receptors also exhibit differences that appear to be dependant on the method used to measure signal output. The luciferase assay and radioimmunoassay exhibit differences in temporal and kinetic parameters of the cyclic AMP responses. IBMX is incorporated in the radioimmunoassay to inhibit cyclic AMP phosphodiesterase. If only small elevations in cyclic AMP are generated, it may take several hours to produce detectable accumulations. The luciferase luminescent response is inherently more sensitive and is capable of measuring a wide range in the elevation of cyclic AMP levels. The luciferase luminescent system is also responsive irrespective of whether the response is large and transient (acute) or prolonged with small elevations (chronic). Providing the acute response is sufficiently large, the luciferase produced should be measurable hours later. Alternatively, a chronic effect should provide a steady accumulation of luciferase over a sufficient incubation period. Hence, the CRE luciferase reporter system used in these studies has the capacity to detect a broad range of cellular responses that may be acute or chronic. The CRE luciferase reporter system has the broad detection range necessary for detection of both the large acute responses induced by MT_1 and MT_2 coupling to Gαs−i chimaeras and the chronic responses via coupling of those Gαs−x chimaeras that couple less efficiently. The low levels of cyclic AMP induction by the latter would be difficult to detect by conventional radioimmunoassay of cyclic AMP but are detectable by the CRE-luciferase reporter system that detects luciferase accumulated over the 16 h incubation period. Thus there are important differences in the temporal and kinetic parameters of the cyclic AMP responses and these may influence and account for the different end responses observed. Furthermore, we conclude that the C-terminal five amino acids are not the sole determinants of coupling specificity. Indeed, there have been a number of recent reports revealing further complexities of determinants of G protein receptor coupling [31,32]. However, recognition of the C-terminal five amino acids is an important requisite in the system outlined here.

Acknowledgements

The authors would like to thank Moira Johnston for assistance with DNA sequencing. This work was supported by the Scottish Office Agriculture Environment Fisheries Department and a Strategic Alliance Programme with Servier, France.

References

[1] L. Birnbaumer, J. Abramowitz, K.B. Brown, Receptor–effector coupling by G proteins, Biochim. Biophys. Acta 10331 (1990) 163–224.
[2] P.J. Morgan, L.M. Williams, G. Davidson, H.E. Howell, Melatonin receptors in ovine pars tuberalis: characterisation and autoradiographical localisation, J. Neuroendocrinol. 1 (1989) 1–4.
[3] S.M. Reppert, C. Godson, C.D. Mahle, D.R. Weaver, S.A. Slaugenhaupt, J.F. Gusella, Molecular characterisation of a second melatonin receptor expressed in human retina and brain: the Mel(1b) melatonin receptor, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 8734–8738.
[4] J.E. Drew, L.M. Williams, L.T. Hannah, P. Barrett, D.R. Abramovich, P.J. Morgan, Identification and characterisation of 2-[^{125}I]iodomelatonin binding and mt_1 melatonin receptor expression in the human fetal leptomeninges, Brain Res. 761 (1997) 87–92.
[5] J.E. Drew, L.M. Williams, L.T. Hannah, P. Barrett, D.R. Abramovich, P.J. Morgan, Melatonin receptors in the human fetal kidney: 2-[^{125}I]io-

domelatonin binding sites correlated with expression of mt_1 and MT_2 receptor genes, J. Endocrinol. 156 (1998) 261–267.
[6] P.J. Morgan, L.M. Williams, The pars tuberalis of the pituitary: a gateway for neuroendocrine output, Rev. Reprod. 1 (1996) 153–161.
[7] T. Ebisawa, S. Karne, M.R. Lerner, S.M. Reppert, Expression cloning of a high-affinity melatonin receptor form *Xenopus* dermal melanophores, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 6133–6137.
[8] S.M. Reppert, D.R. Weaver, T. Ebisawa, Cloning and characterisation of a mammalian melatonin receptor that mediates reproductive and circadian responses, Neuron 13 (1994) 1177–1185.
[9] P.J. Morgan, G. Davidson, W. Lawson, H.E. Howell, Melatonin inhibits cyclic AMP in cultured ovine pars tuberalis cells, J. Mol. Endocrinol. 5 (1989) R3–R8.
[10] B.H. White, R.D. Sekura, M.D. Rollag, Pertussis toxin blocks melatonin-induced pigment aggregation in *Xenopus* dermal melanophores, J. Comp. Physiol. 157 (1987) 153–159.
[11] J. Vanecek, L. Vollrath, Melatonin modulates diacylglycerol and arachidonic acid metabolism in the anterior pituitary of immature rats, Neurosci. Lett. 110 (1990) 199–203.
[12] D.R. Weaver, L.L. Carlson, S.M. Reppert, Melatonin receptors and signal transduction in melatonin-sensitive and melatonin insensitive populations of white-footed mice (*Peromyscus leucopus*), Brain Res. 506 (1990) 353–357.
[13] L.L. Carlson, D.R. Weaver, S.M. Reppert, Melatonin signal transduction in hamster brain: inhibition of adenylyl cyclase by pertussis toxin-sensitive G protein, Endocrinology 125 (1991) 2670–2676.
[14] A.J. McArthur, A.E. Hunt, M.U. Gillette, Melatonin action and signal transduction in the rat suprachiasmatic circadian clock; activation of protein kinase C at dusk and dawn, Endocrinology 138 (1997) 627–634.
[15] P.J. Morgan, G. Davidson, W. Lawson, P. Barrett, Both pertussis toxin-sensitive and insensitive G-proteins link melatonin receptor to inhibition of adenylate cyclase in the ovine pars tuberalis, J. Neuroendocrinol. 2 (1990) 773–776.
[16] P.J. Morgan, W. Lawson, G. Davidson, Internal action of forskolin and melatonin on cyclic AMP generation in pars tuberalis cells of ovine pituitary, J. Neuroendocrinol. 3 (1991) 497–501.
[17] L.Y. Yung, S.T. Tsim, Y.H. Wong, Stimulation of cyclic AMP accumulation by the cloned *Xenopus* melatonin receptor through Gi and Gz proteins, FEBS Lett. 372 (1995) 99–102.
[18] L. Brydon, F. Roka, L. Petit, P. de Coppet, M. Tissot, P. Barrett, P.J. Morgan, C. Nanoff, A.D. Strosberg, R. Jockers, Dual signaling of human Mel1a melatonin receptors via G(i2), G(i3), and G(q/11) proteins, Mol. Endocrinol. 13 (1999) 2025–2038.
[19] B.R. Conklin, Z. Farfel, K.D. Lustig, D. Julius, H.R. Bourne, Substitution of three amino acids switches receptor specificity of Gq alpha to that of Gi alpha, Nature 363 (1993) 274–276.
[20] K. Komatsuzaki, Y. Murayama, U. Giambarella, E. Ogata, S. Seino, I. Nishimoto, A novel system that reports the G-proteins linked to a given receptor: a study of type 3 somatostatin receptor, FEBS Lett. 406 (1997) 167–170.
[21] J. Blahos, S. Mary, J. Perroy, C. de Colle, I. Brabet, J. Bockaert, J.P. Pin, Extreme C terminus of G protein alpha-subunits contains a site that discriminates between G(i)-coupled metabotropic glutamate receptors, J. Biol. Chem. 273 (1998) 25765–25769.
[22] S. Osawa, N. Dhanasekaran, C.W. Woon, G.L. Johnson, G-alpha-I-G-alpha-s chimeras define the function of alpha-chain domains in control of G-protein activation and beta–gamma-subunit complex interactions, Cell 63 (1990) 697–706.
[23] C.H. Berlot, H.R. Bourne, Identification of effector-activating residues of Gs-alpha, Cell 68 (1992) 911–922.
[24] S. Conway, S.J. Canning, P. Barrett, B. Guardiola-Lemaitre, P. Delagrange, P.J. Morgan, The roles of valine 208 and histidine 211 in ligand binding and receptor function of the ovine $Mel1_{1a\beta}$ melatonin receptor, Biochem. Biophys. Res. Commun. 239 (1997) 418–423.
[25] S. Conway, S.J. Canning, H.E. Howell, E.S. Mowatt, P. Barrett, J.E. Drew, P. Delagrange, D. Lesieur, P.J. Morgan, Characterisation of human melatonin mt_1 and MT_2 receptors by CRE-luciferase reporter assay, Eur. J. Pharmacol. 390 (2000) 15–24.
[26] P.J. Morgan, L.M. Williams, P. Barrett, W. Lawson, L. Hannah, A. MacLean, Differential regulation of melatonin receptors in sheep, chicken and lizard brains by cholera toxin and pertussis toxins and guanine nucleotides, Neurochem. Int. 28 (1995) 259–269.
[27] P.J. Morgan, W. Lawson, G. Davidson, H.E. Howell, Melatonin inhibits cyclic AMP in cultured ovine pars tuberalis cells, J. Mol. Endocrinol. 5 (1989) R3–R8.
[28] S. Conway, J.E. Drew, E.S. Mowat, P. Barrett, P. Delagrange, P.J. Morgan, Identification of domains and residues participating in ligand binding and receptor activation of the melatonin mt_1 receptor, J. Biol. Chem. 275 (2000) 20602–20609.
[29] S. Conway, E.S. Mowat, J.E. Drew, P. Barrett, P. Delagrange, P.J. Morgan, Serine residues 110 and 114 are required for agonist binding but not antagonist binding to the melatonin MT1 receptor, Biochem. Biophys. Res. Commun. 282 (2001) 1229–1236.
[30] M.K.C. Ho, Y.H. Wong, Functional role of amino terminal serine16 and serine27 of Gαz in receptor and effector coupling, J. Neurochem. 68 (1997) 2514–2522.
[31] J. Blahos, T. Fischer, I. Brabet, D. Stauffer, G. Rovelli, J. Bockaert, J.-P. Pin, A novel site on the Gα-protein that recognizes heptahelical receptors, J. Biol. Chem. 276 (2001) 3262–3269.
[32] J. Perroy, G.J. Gutierrez, V. Coulon, J. Bockaert, J.-P. Pin, L. Fagni, The C terminus of the metabotropic glutamate receptor subtypes 2 and 7 specifies the receptor signalling pathways, J. Biol. Chem. (2001) 45800–45805.