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Loss of activation of G_s but not G_i following expression of an α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein

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Abstract Both the porcine α_{2A}-adrenoceptor and a fusion protein between this receptor and a pertussis toxin-resistant form of $G_{i1}\alpha$ were stably expressed in Rat-1 fibroblasts. The agonist UK14304 mediated a biphasic regulation of adenylyl cyclase activity via the isolated receptor with inhibition of the enzyme activity at low concentrations of the compound which was subsequently reversed at higher concentrations. By contrast, stimulation of the fusion protein with this agonist could only produce inhibition of enzyme activity. This inhibition was produced by activation of endogenous G_i rather than the fused α subunit of G_{i1}, as pertussis toxin treatment obliterated inhibitory regulation of adenylyl cyclase via the fusion construct. Pertussis toxin treatment potentiated stimulation of adenylyl cyclase via the isolated receptor but such treatment was unable to uncover capacity of the fusion protein to produce such an effect.

Key words: Receptor; G protein; Adrenaline; Adenylyl cyclase

1. Introduction

A number of G protein-coupled receptors (GPCRs) have the capacity to interact with multiple guanine nucleotide binding proteins (G proteins) and thus regulate more than one effector pathway [1,2]. Furthermore, certain GPCRs have the capacity to interact with G proteins which have opposite effects on the same effector pathway [3,4].

Although the α_{2A}-adrenoceptor is one prototypic example of a GPCR which interacts with the G_i-family of G proteins to produce inhibition of adenylyl cyclase (AC) activity [5,6], a number of studies have noted the capacity of this GPCR to interact also with G_s. This receptor is then able, particularly when expressed at high levels and in the presence of high concentrations of certain agonists, to stimulate AC activity [7,8]. Such observations suggest that the effectiveness of agonists at this receptor may vary between different cells and tissues.

Receptor knock-out studies in mice indicate that the α_{2A}-adrenoceptor is a key element in the central control of sympathetic outflow, as it mediates the central hypotensive response to α_2-adrenoceptor agonists [9,10]. Using such transgenic models it has been shown that the α_{2A}-adrenoceptor is also the major contributor to the analgesic effects of α_2-adrenoceptor agonists [11].

We have recently generated a fusion protein between the porcine α_{2A}-adrenoceptor and a pertussis toxin-resistant (C351G) mutant of the α subunit of G_{i1}. Following stable expression of this construct in Rat-1 fibroblasts, we observed that a range of α_2-adrenoceptor agonists were able to stimulate high affinity GTPase activity in membranes of these cells. This effect was produced via both the receptor-linked and endogenous $G_i\alpha$ G proteins [12].

Herein we demonstrate that although the free porcine α_{2A}-adrenoceptor can interact with $G_s\alpha$, the fusion construct prevents interaction of the receptor with this G protein resulting in a situation in which UK14304 can only mediate inhibition of AC activity.

2. Materials and methods

2.1. Materials

All tissue culture reagents were from Sigma or Life Technologies (Paisley, Scotland). [^{3}H]Adenine (15–20 Ci/mmol) and [^{3}H]RS-79948-197 (80–90 Ci/mmol) were purchased from Amersham Pharmacia Biotech. All other chemicals were from Sigma or Fison and were of the highest purity available.

2.2. Cell culture

Ragi 77 [12], Tag WT [13], as well as parental Rat-1 fibroblast cells were cultured in DMEM containing 10% (v/v) newborn calf serum, in a 5% CO_2 atmosphere at 37°C. Two days before the AC assays, cells were plated in 12-well plates to obtain full confluency on the day of assay. For binding measurements cells were plated on 10-cm tissue culture dishes.

In some experiments cells were treated with 25 ng/ml of pertussis toxin or 1 μg/ml of cholera toxin 16 h before assay.

2.3. Whole cell binding

Cells were grown to confluency in 10-cm tissue culture dishes. They were harvested in 2 ml of 0.5 mM EDTA in PBS, centrifuged at $1000\times g$ and washed twice with PBS. The final pellet was then gently resuspended in binding buffer (75 mM Tris-HCl, pH 7.4, 1 mM EDTA, 12.5 mM $MgCl_2$). An aliquot of cells was counted and the cells diluted with binding buffer to obtain approximately 200 000 cells/assay.

Cells were incubated with a saturating concentration of [^{3}H]RS79948-197 (2 nM) [14] for 1 h at 30°C in the presence or absence of 1×10^{-4} M idazoxan (non-specific binding). Binding was terminated by filtration through GF/C membranes using a Brandel cell harvester. Free radioactivity was removed by 3×5-ml washes of cold TE buffer (75 mM Tris-HCl, pH 7.4, 1 mM EDTA). Filters were soaked in scintillant fluid and counted in a liquid scintillation counter. The apparent number of receptor/cell was then calculated.

2.4. Whole cell adenylyl cyclase activity

Whole cell AC activity was measured as previously described [15,16]. Briefly, cells loaded with 0.5 mCi of [^{3}H]adenine were washed in assay medium containing IBMX and stimulated for 30 min with agonist. The reaction was stopped with a TCA solution containing ATP and cAMP. cAMP was separated from the other adenosine metabolites by means of double column chromatography on Dowex followed by alumina.

Concentration-response curves to UK14304 and oxymetazoline were performed in the presence of 50 μM forskolin. In assays with

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Abbreviations: GPCR, G protein-coupled receptor; G protein, guanine nucleotide binding protein; AC, adenylyl cyclase

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cholera toxin only the effect of maximal doses of each compound (i.e. 1×10^{-4} M) was assessed.

2.5. Analysis of the data

Data are expressed as % of inhibition or stimulation of forskolin-induced production of cAMP. Concentration-response curves were fitted using Kaleidagraph according to the equation: m1−(((m1−m6)*(m0–m2))/((m0–m2)+(m3–m2))) where m6 is the maximum inhibition (or stimulation), m2 the slope of the curve, m1 the origin of the curve and m3 represents the EC_{50} value.

3. Results

Comparisons were made between parental Rat-1 fibroblasts and clones derived from these cells which express either the porcine α_{2A}-adrenoceptor (clone 'Tag WT' [13]) or a fusion protein between the α_{2A}-adrenoceptor and a pertussis toxin-resistant mutant (C351G) of the α subunit of G_{i1} (clone 'Ragi 77' [12]).

Saturation binding studies using the potent and highly selective α_{2A}-adrenoceptor antagonist [^{3}H]RS-79948-197 [14] showed that it was impossible to detect any expression of the receptor in the parental cell line but that clone Tag WT and clone Ragi 77 expressed respectively some $108\,000\pm10\,000$ and $104\,000\pm13\,000$ copies per cell.

Intact cells were labelled overnight with [^{3}H]adenine (0.5 μCi/ml) and regulation of forskolin-stimulated AC activity by varying concentrations of UK14304 subsequently measured. As anticipated, UK14304 had no effect on forskolin-stimulated AC activity in the parental cell line (Fig. 1a). By contrast, in cells expressing the isolated α_{2A}-adrenoceptor, UK14304 produced a biphasic response. Low concentrations of UK14304 induced a marked, concentration-dependent, inhibition ($IC_{50}=2.9\times10^{-10}$ M) leading to a maximum of 70% reduction of the forskolin-stimulated AC activity at 10^{-8} M UK14304. Addition of higher concentrations of UK14304 resulted in a reversal of this effect, such that no inhibition was observed at concentrations above 10^{-6} M (Fig. 1a).

In cells expressing the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein, the effect of UK14304 was very different. Clear concentration-related inhibition of forskolin-induced AC activity was observed but with lower potency ($IC_{50}=1.2\times10^{-8}$ M). However, even at concentrations up to 10^{-4} M there was no indication of a reversal of the inhibition (Fig. 1a).

In our previous work with this cell line expressing the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein we demonstrated that the endogenous G_i G protein was responsible for all the inhibition of forskolin-stimulated AC activity rather than the receptor-linked C351G-$G_{i1}\alpha$ [12]. Following pertussis toxin-treatment of the cells to cause ADP-ribosylation of the endogenous G_i-population, in clone Tag WT expressing the porcine α_{2A}-adrenoceptor alone, UK14304 was unable to modify AC activity at low concentrations (under 10^{-8} M) but at higher concentrations, a very substantial stimulation of forskolin-amplified AC activity (routinely 5–6-fold) was noted with an EC_{50} of

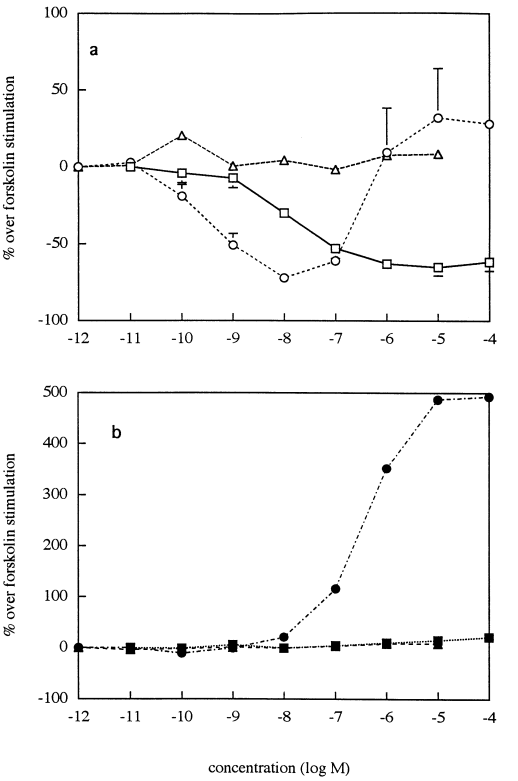

Fig. 1. The effects of UK14304 on forskolin-stimulated adenylyl cyclase activity. Concentration-effect curves to measure the capacity of UK14304 to regulate forskolin (50 μM)-amplified adenylyl cyclase activity were performed on untreated cells (panel a) or following pretreatment of the cells with pertussis toxin (25 ng/ml, 16 h) (panel b), in Rat-1 fibroblasts (triangles), Tag WT cells (circles) and clone Ragi 77 cells (squares).

5×10^{-7} M (Fig. 1b). As anticipated, pertussis toxin treatment did not uncover such an effect of UK14304 in parental Rat-1 fibroblasts. Equally, however, although abolishing the inhibitory regulation of AC activity in cells expressing the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein, pertussis toxin treatment also failed to result in stimulation of AC activity in response to high concentrations of UK14304 (Fig. 1b).

Oxymetazoline is a selective, but partial, agonist of the α_{2A}-adrenoceptor [17]. This compound was again without effect on forskolin-amplified AC activity in membranes of parental Rat-1 fibroblasts (Fig. 2a). Low concentrations of oxymetazoline ($IC_{50}=4.2\times10^{-10}$ M) produced a large inhibition (75%) (Table 1) of such activity in cells expressing the isolated α_{2A}-adrenoceptor but higher concentrations of this agonist were unable to produce reversal of the inhibition in the manner observed for UK14304 (Fig. 2a). In RAGI 77 cells expressing the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein, inhibition of the forskolin-amplified AC activity was observed over the full range of concentrations of oxymetazoline. However, unlike UK14304, the maximal degree of inhibition in response to

Table 1
EC_{50} and maximal effects for UK14304 and oxymetazoline for G_i and/or G_s coupling

Cell line	UK14304	Oxymetazoline	Receptors/cell
TagWT (G_i)	2.9×10^{-10} M (−71%)	4.2×10^{-10} M (−75%)	$108\,000\pm10\,000$
TagWT (G_s)	5×10^{-7} M (+527%)	7.5×10^{-7} M (+30%)	
Ragi 77 (G_i)	1.2×10^{-8} M (−64%)	1.1×10^{-8} M (−25%)	$104\,000\pm13\,000$

Receptor number/cell was obtained in cells which had not been treated with pertussis toxin or cholera toxin.

oxymetazoline was substantially poorer (25%) (Table 1) and a lower potency of the compound ($IC_{50} = 1.1 \times 10^{-8}$ M) was observed. Following pertussis toxin treatment, oxymetazoline-mediated inhibition of forskolin-amplified AC activity was attenuated in cells expressing either the isolated α_{2A}-adrenoceptor or the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein (Fig. 2b). Furthermore, oxymetazoline, at high concentrations, was able to induce only a trivial stimulation of forskolin-amplified AC activity, in cells expressing the isolated α_{2A}-adrenoceptor (Fig. 2b).

Cholera toxin catalyses ADP-ribosylation of $G_s\alpha$ resulting in persistent activation of this protein and its subsequent insensitivity to the effects of receptor agonists. Following treatment of cells with cholera toxin, 1×10^{-4} M UK14304 caused marked inhibition of forskolin-amplified AC activity (Fig. 3) in the cell lines expressing both the isolated α_{2A}-adrenoceptor or the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein. Oxymetazoline also caused inhibition of AC activity under these conditions in both cell types, although as anticipated from the lower efficacy described in untreated conditions with the fusion construct, a lower maximal effect was observed at the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein than at the isolated α_{2A}-adrenoceptor (Fig. 3).

4. Discussion

The results presented herein demonstrate, for the first time, that although the porcine α_{2A}-adrenoceptor has the capability to interact in an agonist-dependent manner with both endogenously co-expressed G_i and G_s, and thus biphasically regulate AC activity, this is selectively modulated following construction of a fusion protein in which the α subunit of a pertussis toxin-resistant mutant of G_{i1} is linked in frame to the C-terminal of the receptor.

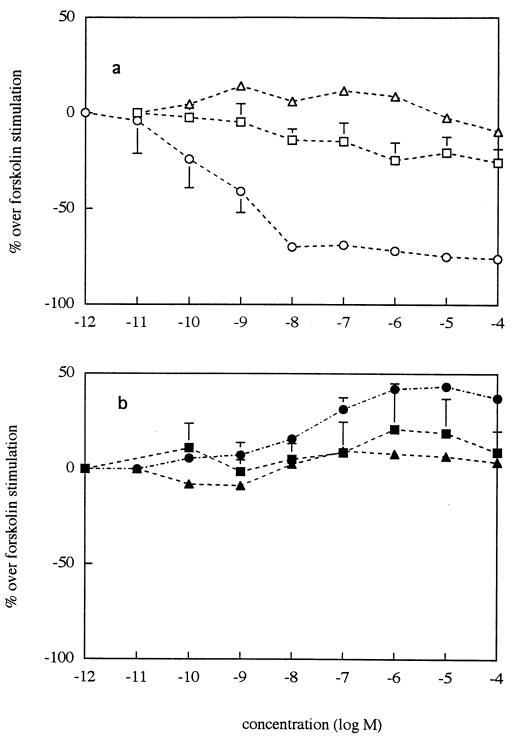

Fig. 2. The effects of oxymetazoline on forskolin-stimulated adenylyl cyclase activity. Concentration-effect curves to measure the capacity of oxymetazoline to regulate forskolin (50 μM)-amplified adenylyl cyclase activity were performed on untreated cells (panel a) or following pretreatment of the cells with pertussis toxin (panel b), in Rat-1 fibroblasts (triangles), Tag WT cells (circles) and clone Ragi 77 cells (squares).

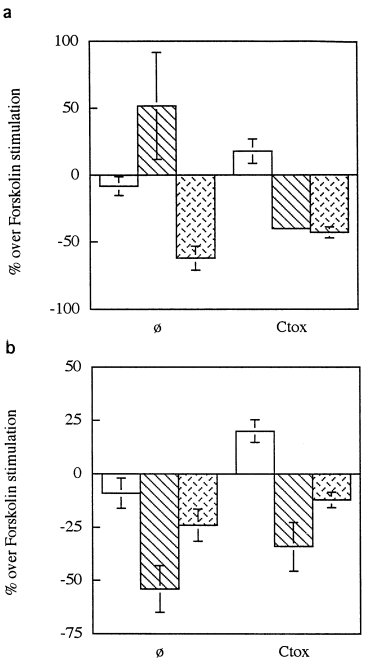

Fig. 3. The effects of cholera toxin on agonist regulation of adenylyl cyclase activity. Cells (Rat-1 fibroblasts (open bars), Tag WT (hatched bars) and Ragi 77 (stippled bars)) were either untreated (left hand side) or treated with cholera toxin (Ctox) (1 μg/ml, 16 h) (right hand side). Subsequently the effects of UK14304 (panel a) and oxymetazoline (panel b) (each at 1×10^{-4} M) on forskolin-stimulated adenylyl cyclase activity were measured.

Following stable expression of this fusion protein in Rat-1 fibroblasts we have previously demonstrated that agonists are able to activate both endogenous and the fusion protein-linked G_i [12]. This was assessed by agonist stimulation of membrane high affinity GTPase activity which was partially pertussis toxin-sensitive and partly resistant to the actions of this ADP-ribosyltransferase. Despite the capacity of the receptor in the fusion protein to cause guanine nucleotide exchange and hydrolysis in both the receptor-linked and free endogenous pool of G_i, the observed agonist-mediated inhibition of AC activity was transduced only via the endogenous G_i as the effect was completely attenuated by pertussis toxin treatment [12].

Stable expression of either the isolated α_{2A}-adrenoceptor or the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein to similar levels has now allowed examination of the relative capacity of the receptor to interact with G_i and G_s and thus regulate AC activity. As previously reported for the human α_{2A}-adrenoceptor [7,18], at low concentrations the agonist UK14304 produced inhibition of forskolin-amplified AC activity in intact cells expressing the porcine α_{2A}-adrenoceptor. As might be anticipated, this effect was attenuated by prior treatment of the cells with pertussis toxin. At concentrations of UK14304 greater than 1×10^{-7} M, the inhibitory regulation was reversed and indeed, following pertussis toxin treatment to prevent productive interaction of the receptor with G_i the stimulation of AC activity produced by activation of $G_s\alpha$ could be easily observed. Interestingly, in cells expressing the α_{2A}-adre-

noceptor-$G_{i1}\alpha$ fusion protein we were unable to produce any evidence of the capacity of UK14304 to activate $G_s\alpha$ and then stimulate AC activity, even following pertussis toxin treatment, which allowed this aspect of the function of UK14304 at the isolated receptor to be observed more easily (see Fig. 1).

It was also noticeable that higher concentrations of UK14304 were required to produce half-maximal inhibition of forskolin-amplified AC activity in the cells expressing the fusion protein. One obvious interpretation of this finding is that the fusion protein is less effective in activating the endogenous pool of G_i than is the isolated receptor and therefore agonist occupancy of a greater number of the receptor sites is required to produce the same degree of inhibition. By comparing agonist-induced stimulation of the GTPase activity of the fusion protein associated G protein and the endogenous G protein population, we have previously calculated that the fusion protein is able to activate some 6 mol G_i/mol of receptor [12]. Although we do not have suitable means to produce an equivalent analysis for the isolated receptor, the results presented herein indicate that this ratio may be substantially higher.

Eason and colleagues have previously indicated that not all agonists at the human α_{2A}-adrenoceptor are able to produce both inhibitory and stimulatory regulation of AC activity [19]. They reported that oxymetazoline is able to produce only the inhibitory phase of the signal. We thus examined the effects of this agonist on the regulation of forskolin-amplified AC activity in both cell lines. In that expressing the isolated receptor, inhibition was observed in a concentration-dependent manner, which reached the same extent as produced by UK14304. However, no matter what the concentration used, no reversal of this effect was observed (Fig. 2). After pertussis toxin treatment, to remove the interaction of the receptor with endogenous G_i, only a small interaction of the receptor with G_s could be induced by oxymetazoline (30% stimulation of AC activity vs. 530% observed with UK14304). Again, results were different in the cells expressing the α_{2A}-adrenoceptor-$G_{i1}\alpha$ fusion protein. As anticipated, no evidence for interaction of this construct with $G_s\alpha$ could be obtained. A concentration-dependent inhibition of forskolin-amplified AC activity could be recorded. However, this also displayed a significantly greater EC_{50} than for the isolated receptor and now the maximal degree of inhibition was much lower than that produced either by UK14304 at the fusion construct or indeed by oxymetazoline in the cells expressing the receptor alone. This is likely to reflect a lower effectiveness of the coupling of the fusion protein to the endogenous G_i population compared to that produced by the isolated receptor.

In many cell systems AC is expressed at relatively low levels when compared to those of the G proteins that regulate this effector (see [20] for review). As such, maximal regulation of AC activity can routinely be produced by activation of only a small fraction of the cellular G protein population. As the levels of expression of both the isolated receptor and the fusion protein are high and equivalent in our cell systems (Table 1), UK14304 was able to activate enough endogenous G_i in both cell types to induce the same maximal degree of inhibition of the forskolin-stimulated AC activity. By contrast, as oxymetazoline is known as a partial agonist at the α_{2A}-adrenoceptor [17], good inhibition of the AC activity in the cells expressing the isolated receptor could be reached. However, because of a possible lower quantitative capacity of the fusion protein to catalytically activate endogenous G_i, then only partial inhibition could be achieved in the cells expressing the fusion protein, even at high concentrations of oxymetazoline where the entire population of receptors should be occupied.

The molecular basis for these observations remains to be elucidated but two possibilities can be considered. The first is that the construction of the fusion protein induced some physical constraint to the receptor such that sites for interaction with $G_s\alpha$ are masked whereas those for interaction with $G_i\alpha$ are perturbed to a lesser degree. Analysis of elements of the second and third intracellular loop of the human α_{2A}-adrenoceptor using chimaeric receptor constructs have indeed indicated that resolvable domains are central for effective interactions with these two classes of G proteins [21]. Furthermore, a mutagenesis study [22] showed that mutation of Asp^{130} in the α_{2A}-adrenoceptor ablated agonist-dependent stimulation of AC activity but not the inhibition. Overall these results, as well as those of Eason et al. [19], indicate that the binding of different agonists within the receptor binding pocket may cause different conformational changes and thus different degrees of activation of G_i and G_s.

Secondly, it has become steadily more clear in recent times that compartmentalisation of signalling elements within specific domains of the plasma membrane is an important contributor to the effectiveness and specificity of signal transduction processes [23]. It is clearly possible that differences in plasma membrane distribution of the isolated α_{2A}-adrenoceptor and the α_{2A}-adrenoceptor-C351G $G_{i1}\alpha$ fusion protein limit the availability and access of these two forms of the receptor to the endogenous G protein pools. Future studies will explore these possibilities.

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References

[1] Evans, P.D., Robb, S., Cheek, T.R., Reale, V., Hannan, F.L., Swales, L.S., Hall, L.M. and Midgley, J.M. (1995) Prog. Brain Res. 106, 259–268.
[2] Kenakin, T. (1995) Trends Pharmacol. Sci. 16, 232–238.
[3] Gurwitz, D., Haring, H., Heldman, E., Fraser, C.M., Manor, D. and Fisher, A. (1994) Eur. J. Pharmacol. 267, 21–31.
[4] Berg, K.A., Loh, E.D., Cropper, J.D., Maayani, S. and Clarke, W.P. (1996) Soc. Neurosci. Abstr. 22, 1777.
[5] Lomasney, J.W., Cottechia, S., Lefkowitz, R.L. and Caron, M.G. (1991) Biochim. Biophys. Acta 1095, 127–139.
[6] Limbird, L.E. and Sweatt, J.D. (1985) in: The Receptor, Vol. II (Conn, P.M., Ed.) pp. 281–305, Academic Press, Orlando, FL.
[7] Eason, M.G., Kurose, H., Holt, B.D., Raymond, J.R. and Liggett, S.B. (1992) J. Biol. Chem. 267, 15795–15801.
[8] Airriess, C.N., Rudling, J.E., Midgley, J.M. and Evans, P.D. (1997) Br. J. Pharmacol. 122, 191–198.
[9] MacMillan, L.B., Hein, L., Smith, M.S., Piascik, M.T. and Limbird, L.E. (1996) Science 273, 801–803.
[10] Link, R.E. et al. (1996) Science 273, 803–805.
[11] MacDonald, E., Kobilka, B.K. and Scheinin, M. (1997) Trends Pharmacol. Sci. 18, 211–219.
[12] Burt, A.R., Sautel, M., Wilson, M.A., Rees, S., Wise, A. and Milligan, G. (1998) J. Biol. Chem. 273, 10367–10375.
[13] Wilson, M., Burt, A.R., Milligan, G. and Anderson, N. (1996) J. Biol. Chem. 271, 8537–8540.
[14] Gillard, N.P., Linton, C.J., Milligan, G., Carr, I.C., Patmore, L. and Brown, C.M. (1996) Br. J. Pharmacol. 117, 298P.
[15] Wong, Y.G. (1994) Methods Enzymol. 238, 81–94.
[16] Mercouris, M., Mullaney, I., Georgoussi, Z. and Milligan, G. (1997) J. Neurochem. 69, 2115–2122.

[17] Jasper, J.R. et al. (1998) Biochem. Pharmacol. 55, 1035–1043.
[18] Fraser, C.M., Arakawa, S., McCombie, W.R. and Venter, J.C. (1989) J. Biol. Chem. 264, 11754–11761.
[19] Eason, M.G., Jacinto, M.T. and Liggett, S.B. (1994) Mol. Pharmacol. 45, 696–702.
[20] Milligan, G. (1996) Cell. Signal. 8, 87–95.
[21] Eason, M.G. and Liggett, S.B. (1996) J. Biol. Chem. 271, 12826–12832.
[22] Wang, C.D., Buck, M.A. and Fraser, C.M. (1991) Mol. Pharmacol. 40, 168–179.
[23] Huang, C., Hepler, J.R., Chen, L.T., Gilman, A.G., Anderson, R.G.W. and Mumby, S.M. (1997) Mol. Biol. Cell 8, 2365–2378.