Alanine-261 in intracellular loop III of the human gonadotropin-releasing hormone receptor is crucial for G-protein coupling and receptor internalization

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Gonadotropin-releasing hormone (GnRH) is a decapeptide that regulates reproductive function via binding to the GnRH receptor, which is a G-protein-coupled receptor (GPCR). For several members of this family, the C-terminal domain of intracellular loop III is important in ligand-mediated coupling to G-proteins; mutations in that region can lead to constitutive activity. A specific alanine residue is involved in certain GPCRs, the equivalent of which is Ala-261 in the GnRH receptor. Mutation of this residue to Leu, Ile, Lys, Glu or Phe in the human GnRH receptor did not result in constitutive activity and instead led to complete uncoupling of the receptor (failure to support GnRH-stimulated inositol phosphate production). When this residue was mutated to Gly, Pro, Ser or Val, inositol phosphate production was still supported. All the mutants retained the ability to bind ligand, and the affinity for ligand,

where measured, was unchanged. These results show that Ala-261 cannot be involved in ligand binding but is critical for coupling of the receptor to its cognate G-protein. Coupling is also dependent on the size of the residue in position 261. When the amino acid side chain has a molecular mass of less than 40 Da efficient coupling is still possible, but when its molecular mass exceeds 50 Da the receptor is uncoupled. Internalization studies on the Ala²⁶¹ → Lys mutant showed a marked decrease in receptor internalization compared with the wild type, indicating that coupling is necessary for effective receptor internalization in the GnRH receptor system. Activation of protein kinase C (with PMA), but not protein kinase A (with forskolin) markedly increased the internalization of the mutant receptor while having a small effect on the wild-type receptor.

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a central regulator of the reproductive hormone cascade. By activating the GnRH receptor (GnRHR) on pituitary gonadotrope cells, the GnRH decapeptide regulates the release of the gonadotropins, luteinizing hormone and follicle-stimulating hormone, which in turn regulate the synthesis of gonadal steroid hormones. The GnRHR is a member of the G-protein-coupled receptor (GPCR) superfamily, which is characterized by seven putative transmembrane helices [1]. Biochemical analysis of the signalling pathway has shown that the GnRHR activates phospholipase C via coupling to the G_{q/11} class of G-proteins [2,3]. The cloning of the GnRHR has provided the opportunity of dissecting the molecular mechanisms of ligand binding and receptor activation.

Some insights into the molecular mechanism of receptor activation have emerged from site-directed mutagenesis of GPCRs [4,5] and from naturally occurring mutations that cause constitutive activation [6–13]. However, identification of the precise molecular mechanism of ligand-induced receptor activation for coupling to the G-protein in GPCRs has so far eluded researchers, and dissection of this fundamental molecular mechanism will be a major advance, with implications for drug development.

One of the consequences of GPCR activation is an increased rate of internalization. The relationship between receptor activation and internalization from the cell surface is not known for the GnRHR, yet the internalization is a component of the important phenomenon of desensitization that is operative in the

therapeutic application of GnRH agonists. It has been demonstrated that G-protein activation is necessary for efficient internalization for some GPCRs that couple to G_q [14–18] as well as to G_s [19,20]. However, in certain GPCRs, loss of second-messenger activation has had no effect on internalization [21,22]. The production of an uncoupled mutant GnRHR should allow further investigation of this important phenomenon.

In this paper we have identified a single amino acid that is crucial for coupling the GnRH receptor to its cognate G-protein. Replacement of this amino acid with bulky amino acids leads to complete uncoupling of the receptor from the intracellular signalling system and decreased receptor internalization, while retaining ligand-binding activity.

METHODS AND MATERIALS

Site-directed mutagenesis

The cDNA of the human GnRHR was cloned into the phagemid pBluescript II SK – (Stratagene) by using the *EcoRI* and *XhoI* restriction enzyme sites. Oligonucleotide-directed mutagenesis was performed by the method of Kunkel et al. [23]. The mutagenesis products were transformed into competent XL-1 Blue *Escherichia coli*. Plasmid DNA was extracted from ampicillin-resistant clones and mutations were confirmed by sequencing the complete gene with a Sequenase 2.0 kit (United States Biochemical Corporation). Mutant cDNA species were subcloned into *EcoRI* and *XhoI* sites of the mammalian expression vector pcDNA1/Amp (Invitrogen).

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GnRH, gonadotropin-releasing hormone; GnRHR, gonadotropin-releasing hormone receptor; GPCR, G-protein-coupled receptor; ICIII, intracellular loop III; IP, inositol phosphate.

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Transfection

Plasmid DNA for transfection was prepared on Nucleobond columns (Macherey-Nagel) or Wizard columns (Promega) in accordance with the manufacturer's instructions. COS-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10% (v/v) fetal calf serum (FCS) (Highveld) in a 37 °C, air/CO₂ (9:1) incubator. Cells were seeded at 2×10^5 cells per well in poly(D-lysine)-coated 12-well plates 24 h before transfection. Cells were transfected by a modified DEAE-dextran method [24]. In brief, cells were washed once with Hepes (20 mM, pH 7.4)-buffered DMEM, then each well was incubated with 0.5 ml of serum-free DMEM containing 0.3 mg/ml DEAE-dextran and 3.3 µg/ml plasmid DNA for 4 h at 37 °C. The medium was replaced with DMEM containing 200 µM chloroquine and 2% (v/v) FCS and incubated for 1 h at 37 °C. This was followed by a 2 min shock with 10% (v/v) DMSO. The cells were washed with Hepes-buffered DMEM and cultured overnight in DMEM with 10% (v/v) FCS.

Inositol phosphate (IP) production

At 24 h after transfection, the cells were washed with Medium 199 (Gibco) containing 2% (v/v) FCS, then each well was incubated overnight in 0.5 ml of Medium 199 containing 2 µCi/ml *myo*-[2-³H]inositol (Amersham) and 2% (v/v) FCS. IP production in the absence or presence of GnRH (pyroGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH₂) was measured as described previously [25].

Radioligand binding

GnRHA (a potent GnRH agonist) ([D-Ala⁶-N-Me-Leu⁷,Pro⁹-NHET]GnRH) was synthesized by conventional solid-phase synthesis. Ant26 (a competitive GnRH antagonist) ([Ac-D-*p*-ClPhe¹,D-*p*-ClPhe²,D-Trp³,D-Lys⁶,D-Ala¹⁰NH₂]GnRH) was a gift from D. Coy. The peptides were iodinated as described previously [25]. At 48 h after transfection, cells were washed with cold buffer I [140 mM NaCl/4 mM KCl/20 mM Hepes/8 mM glucose/0.1% fatty-acid-free BSA/1 mM CaCl₂/1 mM MgCl₂ (pH 7.4)], then incubated with 0.5 ml of buffer I containing 100 000 c.p.m. [¹²⁵I]GnRHA (50 pM) and various concentrations of unlabelled GnRHA for 2–3 h at 4 °C. For antagonist binding, cells were incubated with 100 000 c.p.m. [¹²⁵I]-Ant26 (50 pM) and various concentrations of unlabelled Ant26 for 1 h at 37 °C. After the incubation, cells were washed twice with cold buffer I containing 0.5% BSA. The cells were dissolved in 0.5 ml of 0.1 M NaOH and the amount of bound label was measured. Non-specific binding was estimated in the presence of 1 µM unlabelled Ant26 for both agonist and antagonist binding assays.

Internalization

At 48 h after transfection, cells were washed with unlabelled Hepes-buffered DMEM, then incubated for 3 h at 4 °C in Hepes-buffered DMEM containing 10% (v/v) FCS and 200 000 c.p.m./ml [¹²⁵I]GnRHA. The cells were transferred to 37 °C and incubated for different periods. After the specified times, cells were washed twice with unlabelled PBS. Externally bound ligand was removed by a 10 min acid wash (50 mM acetic acid/150 mM NaCl) and internalized ligand was measured by hydrolysing cells with 0.1 M NaOH [26].

Data reduction

Graphpad PRISM (Graphpad Software) was used to do four-parameter non-linear curve fitting to estimate the peptide con-

centrations required to stimulate half-maximal IP production (EC₅₀), and to half-maximally inhibit the binding of radioligand (IC₅₀). Dissociation constants and receptor numbers in the competition binding studies were calculated by LIGAND [27]. Figures represent the average of at least three individual experiments (unless stated otherwise) in which data points were calculated in triplicate or duplicate.

RESULTS

IP production

Nine amino acids with a spectrum of side-chain properties were substituted for alanine at position 261 in the third intracellular loop of the human GnRHR by site-directed mutagenesis. Of the nine Ala-261 mutants, the A261E, A261F, A261I, A261K and A261L mutants exhibited a complete lack of agonist-stimulated IP production (Table 1), suggesting that these mutants are uncoupled from the second-messenger signalling system. The remaining four mutants all showed agonist-stimulated IP production to varying degrees (Table 1). The absolute values for wild-type basal and agonist-stimulated maximal IP production were 264 ± 12 c.p.m. and 7210 ± 1861 c.p.m. respectively. The A261G and A261S mutants had wild-type agonist-stimulated IP production, whereas the A261P and A261V mutants produced only 3% and 17% of the wild-type agonist-stimulated IP production respectively, indicating that these mutants can still activate the G-protein in a limited manner. Because these mutants were expressed reasonably well (39% and 47% relative to wild-type) (Table 1), they seem to be partly uncoupled. The IP production in the absence of ligand (basal production) for the mutant receptors was in the range 239–294 c.p.m., i.e. similar to that of the wild type, showing that neither the wild-type nor the mutant receptors exhibited any significant constitutive activity.

Table 1 Agonist-stimulated IP production and specific binding of [¹²⁵I]GnRHA for the Ala-261 mutant receptors

IP production was measured in transfected COS-1 cells labelled with [³H]inositol after incubation for 1 h in the absence (basal IP production) or presence of 0.1 µM GnRH (maximal IP production). The agonist-stimulated IP production was determined by subtracting the basal from the maximal IP production. Values are expressed as percentages of the wild-type control value, and are means $\pm$ S.D. for at least three separate experiments, each performed in duplicate. The absolute values for wild-type basal and agonist-stimulated maximal IP production were 264 ± 12 and 7210 ± 1861 c.p.m. respectively. The basal IP production values for the mutant receptors were similar to the wild type, i.e. in the range 239–294 c.p.m. Maximum binding and non-specific binding were measured in untransfected and transfected COS-1 cells by incubation for 3 h at 4 °C with [¹²⁵I]GnRHA in the absence and the presence of competing unlabelled Ant26 respectively. Specific binding was determined by subtracting the non-specific binding from the maximum binding. Results are expressed as percentages of the wild-type control, and are means $\pm$ S.D. for at least three individual experiments performed in triplicate or duplicate. The absolute maximum binding and non-specific binding values for the wild type were 2890 ± 866 and 772 ± 179 c.p.m. respectively. Non-specific binding values for the mutant receptors were similar to the wild type, i.e. in the range 603–940 c.p.m.

Mutant	Agonist-stimulated IP production (% of wild-type)	[¹²⁵ I]GnRHA binding (% of wild-type)
A261G	114.4 $\pm$ 8.9	101.7 $\pm$ 5.0
A261S	107.5 $\pm$ 9.1	105.2 $\pm$ 6.6
A261P	2.8 $\pm$ 1.0	38.7 $\pm$ 4.9
A261V	17.1 $\pm$ 3.6	47.1 $\pm$ 7.7
A261L	0.4 $\pm$ 0.4	24.3 $\pm$ 5.1
A261I	1.4 $\pm$ 0.9	30.7 $\pm$ 5.0
A261E	0.2 $\pm$ 0.4	40.0 $\pm$ 2.8
A261K	1.0 $\pm$ 2.0	112.3 $\pm$ 11.7
A261F	0.1 $\pm$ 0.6	31.7 $\pm$ 2.2

Figure 1 GnRHA competition binding for the wild-type and A261K receptors

Cells transfected with wild-type (●) and A261K (○) receptor DNA were incubated with [¹²⁵I]GnRHA and the indicated concentrations of unlabelled GnRHA for 3 h at 4 °C. IC₅₀ values for the receptors were: wild-type, 4.6 ± 1.1 nM; A261K, 5.0 ± 1.1 nM. Results are means ± S.D. for duplicate readings in the same experiment.

Table 2 Ligand affinities and receptor numbers for the wild-type and mutant Ala-261 receptors

GnRHA whole-cell binding was performed at 4 °C for 2–3 h, whereas Ant26 binding was done at 37 °C for 1 h. Ligand affinity (K_d) is presented in nM; the receptor number (R_0) is presented as a percentage of the wild-type value. The absolute receptor number measured for the wild type at 4 °C with GnRHA was 228 ± 25 pM, and at 37 °C with Ant26 it was 221 ± 53 pM. Results are means ± S.D. for the indicated number of experiments (*n*). Abbreviation: n.d., not determined.

Mutant	GnRHA			<i>n</i>	Ant26			<i>n</i>
	K_d (nM)	R_0 (% of wild-type)			K_d (nM)	R_0 (% of wild-type)		
Wild-type	2.8 ± 0.7	100 ± 21.3		6	12.2 ± 2.8	100 ± 23.6		2
A261G	1.7 ± 1.3	102.6 ± 55.9		3	n.d.	n.d.		
A261K	3.7 ± 0.5	119.9 ± 9.3		3	11.1 ± 2.7	113.1 ± 26.8		2
A261S	1.7 ± 0.9	73.6 ± 30.0		2	n.d.	n.d.		
A261V	2.4 ± 0.9	56.4 ± 20.1		2	n.d.	n.d.		

Radioligand binding

An initial screening of the mutant receptors was performed, where only the maximum binding and the non-specific binding (on untransfected COS-1 cells) were measured (Table 1). The same percentage binding, relative to the wild type, was obtained when radiolabelled agonist (GnRHA) or radiolabelled antagonist (Ant26) was used. All the mutants showed some binding of the radioligand, indicating that they were all expressed. The A261G, A261K and A261S mutants, however, were the only mutants that showed wild-type binding. The binding on the A261E, A261F and A261L mutants was too low for an accurate determination of ligand affinities. Hence further experiments to improve understanding of the uncoupling mechanism were performed on the A261K mutant as a representative of the other uncoupled mutant receptors. Binding on whole cells with labelled GnRHA and competition with GnRHA at 37 °C (Figure 1) or Ant26 at 4 °C on the wild-type and A261K receptors revealed that the two receptors have the same affinity for GnRHA and Ant26 as well as comparable receptor numbers (Table 2).

Figure 2 Receptor internalization of the wild-type and A261K receptors

Transfected cells were first incubated at 4 °C for 3 h and then transferred to 37 °C for the indicated times. External and internal radioactivity was then measured. In the absence of PMA, the wild type (●) internalized 46% of receptors, whereas the A261K mutant (▲) internalized 15%. In the presence of PMA, the wild type (○) internalized 53% of receptors; the A261K mutant (△) internalized 37%. Results are means ± S.D. for two to four experiments performed in duplicate.

For the other Ala-261 mutants, for which good competition binding data could be obtained, ligand affinities for GnRHA and receptor numbers (Table 2) revealed that these mutants had similar affinities to that of the wild-type receptor, but exhibited a decrease in receptor expression.

Receptor internalization

Transfected COS-1 cells were incubated with [¹²⁵I]GnRHA for 3 h at 4 °C to allow an equilibrium to be reached between bound and unbound ligand. It was assumed that no ligand-stimulated internalization occurred at 4 °C. The cells were then transferred to 37 °C to initiate the internalization process. Internalization was stopped at various time points by transferring the cells to a 4 °C bath (ice) and washing with cold PBS. An acid wash was used to remove receptors on the cell surface. The amount of radioactivity measured in the wash was used as an estimate of the amount of GnRHR on the cell surface. The amount of internalized receptors was measured by dissolving the cells in NaOH and determining the amount of radioactivity in the cells.

In spite of its similar total binding of radiolabelled GnRHA, the A261K mutant receptor showed only 33% of the wild-type internalization (Figure 2). In the presence of the phorbol ester PMA the amount of receptor internalized was increased for both wild-type and mutant receptors (Figure 2). However, there was a greater increase in internalization for the A261K mutant, such that mutant receptor internalization was 70% that of the wild-type receptor. Forskolin had no effect on wild-type or A261K internalization (results not shown).

DISCUSSION

These studies have shown that Ala-261 is essential for coupling of the GnRHR to the second messenger system via the G-protein G_{q/11}. Replacement of Ala-261 with Gly and Ser allow normal receptor coupling, whereas Pro and Val substitution results in partial uncoupling. Leu, Ile, Phe, Glu and Lys substitutions result in complete uncoupling, but these mutants retain the ability to bind ligand. Hence a small amino acid is necessary for

normal receptor function, whereas large amino acids cause uncoupling of the receptor. A plot of the molecular mass of the amino acid side chain against coupling efficiency (Table 1, and results not shown) indicates that a molecular mass of less than 40 Da is required for full coupling efficiency; between 40 and 50 Da the receptor is partly uncoupled; and side chains of over 50 Da cause complete uncoupling. The charge of the side chain seems to be unimportant.

In other GPCRs, alanine residues in intracellular loop III have been mutated such that the receptor becomes constitutively active [10,13,28]. In the α_{1B} -adrenoreceptor, mutation of Ala-293 to any other amino acid constitutively activates the G_q/G_{11} -coupled receptor [28]. The thyroid-stimulating hormone receptor constitutively activates G_s when Ala-623 is mutated to Ile or Val [10] and the apparently homologous alanine residue in the related luteinizing hormone receptor confers constitutive activation of G_s when mutated to Val [13]. It seems that Ala-261 of the GnRHR is the equivalent residue of these other alanines, as it is in precisely the same position relative to the conserved proline residue in transmembrane helix IV of the receptors.

From the uncoupling results it seems that a small residue is necessary for maintaining a conformation that presents the G-protein-coupling domains correctly for binding and/or activation of $G_{q/11}$. The replacement of the small residue with a large residue disrupts coupling, either by changing a specific conformation, e.g. an α -helix or β -sheet, or by preventing G-protein binding/activation by steric hindrance. We are unable to distinguish between the inability of the G-protein to bind the receptor and the inability of the G-protein to be activated. Conventionally, this is investigated by binding experiments in the presence of non-hydrolysable GTP analogues. However, our GnRHR membrane receptor-binding assays done at 37 °C in the presence of non-hydrolysable GTP analogues have not shown any decrease in binding for the wild-type GnRHR (results not shown). This has also been observed for the serotonin-2C receptor and might be due to the low intrinsic GTP turnover rate of the G_q family of proteins [29].

We have observed that a close correlation exists between the nature of the amino acid residue that causes uncoupling of the GnRHR and those that cause constitutive activity of the α_{1B} -adrenoreceptor [28]. As the size of the substituted amino acid increases, so does its ability to cause uncoupling in the GnRHR (Table 1) or constitutive activity in the α_{1B} -adrenoreceptor [28]. Although the effects are opposite, this suggests that a common mechanism is involved. In the α_{1B} -adrenoreceptor, large substituted amino acids might force the loop into the active conformation, normally stabilized by agonist binding. In the GnRHR, the same large groups might prevent the loop from assuming the active conformation, possibly because of steric hindrance owing to nearby amino acids in the N-terminal domain of intracellular loop III or in other closely opposed loops. These apparently opposite effects of the same mutation in different receptors offer the possibility of gaining insight into loop activation by studying the behaviour of chimaeric receptors.

The A261K mutant showed a marked decrease in receptor internalization compared with the wild type. The addition of the phorbol ester PMA partly restored receptor internalization, implying that protein kinase C is involved in the GnRHR internalization process. In contrast, forskolin had no effect on

wild-type or A261K internalization, implying that protein kinase A is not involved. The fact that the A261K mutant has decreased internalization and is also uncoupled indicates that coupling is necessary for effective internalization in the GnRHR system, which has not previously been shown for the GnRHR.

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REFERENCES

- Sealfon, S. C., Weinstein, H. and Millar, R. P. (1997) *Endocr. Rev.* **18**, 180–205
- Stojilkovic, S. S., Reinhart, J. and Catt, K. J. (1994) *Endocr. Rev.* **15**, 462–499
- Naor, Z., Shacham, S., Harris, D., Seger, R. and Reiss, N. (1995) *Cell. Mol. Neurobiol.* **15**, 527–544
- Dohlman, H. G., Thorner, J., Caron, M. G. and Lefkowitz, R. J. (1991) *Annu. Rev. Biochem.* **60**, 653–688
- Baldwin, J. M. (1993) *EMBO J.* **12**, 1693–1703
- Clapman, D. E. (1993) *Cell* **75**, 1237–1239
- Parma, J., Duprez, L., Van Sande, J., Cochaux, P., Gervy, C., Mockel, J., Dumont, J. and Vassart, G. (1993) *Nature (London)* **365**, 649–651
- Robbins, L. S., Nadeau, J. H., Johnson, K. R., Kelly, M. A., Roselli-Rehfsuss, L., Baack, E., Mountjoy, K. G. and Cone, R. D. (1993) *Cell* **72**, 827–834
- Shenker, A., Laue, L., Kosugi, S., Merendino, Jr., J. J., Minegishi, T. and Cutler, Jr., G. B. (1993) *Nature (London)* **365**, 652–654
- Paschke, R., Tonacchera, M., Van Sande, J., Parma, J. and Vassart, G. (1994) *J. Clin. Endocrinol. Metab.* **79**, 1785–1789
- Pollak, M. R., Brown, E. M., Estep, H. L., McLaine, P. N., Kifor, O., Park, J., Hebert, S. C., Seidman, C. E. and Seidman, J. G. (1994) *Nature Genet.* **8**, 303–307
- Parma, J., Van Sande, J., Swillens, M. T., Dumont, J. and Vassart, G. (1995) *Mol. Endocrinol.* **9**, 725–733
- Latronico, A. C., Anastasi, J., Arnhold, J. P., Mendonca, B. B., Domenice, S., Albano, M. C., Zachman, K., Wajchenberg, B. L. and Tsigos, C. (1995) *J. Clin. Endocrinol. Metab.* **80**, 2490–2494
- Benya, R. V., Akeson, M., Mrozinski, J., Jensen, R. T. and Battey, J. F. (1994) *Mol. Pharmacol.* **46**, 495–501
- Arora, K. K., Sakai, A. and Catt, K. J. (1995) *J. Biol. Chem.* **270**, 22820–22826
- Laporte, S. A., Servant, G., Richard, D. E., Escher, E., Guillemette, G. and Leduc, R. (1996) *Mol. Pharmacol.* **49**, 89–95
- Lameh, J., Phillip, M., Sharma, Y. K., Moro, O., Ramachandran, J. and Sadee, W. (1992) *J. Biol. Chem.* **267**, 13406–13412
- Nussenzweig, D. R., Heinfinkel, M. and Gershengorn, M. C. (1993) *Mol. Endocrinol.* **7**, 1105–1111
- Dhanwada, K. R., Vijapurkar, U. and Ascoli, M. (1996) *Mol. Endocrinol.* **10**, 544–554
- Cheung, A. H., Sigal, I. S., Dixon, R. A. and Strader, C. D. (1989) *Mol. Pharmacol.* **35**, 132–138
- Cheung, A. H., Dixon, R. A., Hill, W. S., Sigal, I. S. and Strader, C. D. (1990) *Mol. Pharmacol.* **6**, 775–779
- Hausdorff, W. P., Hnatowich, M., O'Dowd, B. F., Caron, M. G. and Lefkowitz, R. J. (1990) *J. Biol. Chem.* **265**, 1388–1393
- Kunkel, T. A., Bebenek, K. and McClary, J. (1987) *Methods Enzymol.* **204**, 125–139
- Keown, W. A., Campbell, C. R. and Kucherlapati, R. S. (1990) *Methods Enzymol.* **185**, 527–537
- Millar, R. P., Davidson, J., Flanagan, C. and Wakefield, I. (1995) in *Methods in Neurosciences*, vol. 25 (Sealfon, S. C., ed.), pp. 145–162, Academic Press, London
- Hazum, E., Meidan, R., Liscovitch, M., Kainan, D., Linder, H. R. and Koch, Y. (1993) *Mol. Cell. Endocrinol.* **30**, 291–301
- Munson, P. J. and Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Kjelsberg, M. A., Cotecchia, S., Ostrowski, J., Caron, M. G. and Lefkowitz, R. J. (1992) *J. Biol. Chem.* **267**, 1430–1433
- Burns, C. M., Chu, H., Rueter, S. M., Hutchinson, L. K., Canton, H., Sanders-Bush, E. and Emeson, R. B. (1997) *Nature (London)* **387**, 303–308