Expression of a rat neurotensin receptor in *Escherichia coli*

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With the goal of obtaining sufficient quantities of seven-helix G-protein-coupled receptors for structural analysis, we have studied the functional expression of a rat neurotensin receptor cDNA in *Escherichia coli* with and without a signal sequence and as a fusion with the gene coding for maltose-binding protein. The addition of an N-terminal signal peptide resulted in increased expression levels. *In vitro* translation at a high level revealed that the codon usage of the rat neurotensin receptor cDNA was not critical for overproduction. Expression of neurotensin receptor cDNA fused to the 3' end of the gene encoding maltose-binding protein resulted in a 40-fold increase in neurotensin-binding

sites. Binding of [³H]neurotensin to intact bacteria or *E. coli* membranes was saturable, with a dissociation constant, K_D , of 0.23 nM (B_{max} = 450 sites/bacterium or 15 pmol/mg of crude membrane protein). The binding properties of all recombinant receptors presented in this study were similar and corresponded to those of the high-affinity binding sites in rat brain. For immunological detection and future purification of neurotensin receptor, a C-terminal pentahistidine/*c-myc* tail was introduced. Western-blot analysis revealed the association of neurotensin receptor with *E. coli* membranes.

INTRODUCTION

Neurotensin and other neuropeptide receptors belong to the large family of seven-helix G-protein-coupled receptors and are present in very small quantities in the cell. The crystallization and subsequent structure determination by either X-ray diffraction or electron microscopy require considerable amounts of neurotensin receptor protein. From the topological group of seven-helix proteins, only bacteriorhodopsin (a light-driven proton pump from *Halobacterium halobium* that does not interact with heterotrimeric G-proteins) and rhodopsin can be obtained in reasonable quantities from natural sources. Therefore it is desirable to overexpress neuropeptide receptor genes as a basis for large-scale purification and for crystallization experiments, and to understand the factors responsible for successful high-level expression.

Neurotensin is a 13-amino acid peptide which probably functions as a neurotransmitter/neuromodulator in the nervous system and as a hormone in some endocrine tissues (Kitabgi et al., 1985). It displays a wide spectrum of biological activities leading to analgesia, hypotension, hyperglycaemia, hypothermia and increased vascular permeability. The diverse activities of neurotensin are evoked by its interaction with specific receptors, resulting in the stimulation of inositol phospholipid metabolism (Goedert et al., 1984; Snider et al., 1986). In addition, the modulation by neurotensin of the levels of the second messengers cyclic AMP (Bozou et al., 1986) and cyclic GMP (Gilbert and Richelson, 1984; Amar et al., 1985) has been observed.

Recently, the cDNA encoding a rat neurotensin receptor (NTR) has been isolated (Tanaka et al., 1990). NTR is an integral membrane protein and belongs to the large family of G-protein-coupled receptors which includes rhodopsin and receptors for hormones, peptides, neurotransmitters and odours (Henderson and Schertler, 1990; Buck and Axel, 1991; Kerlavage, 1991; Hargrave and McDowell, 1992; Kobilka, 1992). Hydropathy analysis of NTR shows the existence of seven stretches of hydrophobic amino acid residues, which are thought to represent seven transmembrane-spanning helices. There are

three potential *N*-glycosylation sites in the N-terminal region. The cDNA does not include a coding region for an N-terminal signal peptide. NTR contains two cysteine residues in outer loops 1 and 2, which are conserved among seven-helix receptors and are thought to form a disulphide bridge (Dixon et al., 1987; Karnik et al., 1988; Hulme et al., 1990). A third conserved cysteine residue following transmembrane domain VII may be palmitoylated (O'Dowd et al., 1989).

The heterologous overexpression in *Escherichia coli* of genes encoding membrane proteins has so far proven difficult (Schertler, 1992). No general rules for the overproduction of membrane proteins in bacteria are available. Here, we present a series of expression plasmids for the production of functional receptors inserted in the *E. coli* cytoplasmic membrane. The replacement of a cysteine residue of NTR and *in vitro* translation experiments were performed to study possible factors limiting expression. Furthermore, we have analysed the direct expression of NTR cDNA and the effect of an N-terminal signal peptide as well as the influence of a fusion to an N-terminal periplasmic protein on the insertion of NTR into the cytoplasmic membrane. A detailed pharmacological characterization of functional recombinant NTR is provided.

MATERIALS AND METHODS

Bacterial strains and plasmids

E. coli strain DH5 α (Gibco-BRL) (Sambrook et al., 1989) was used as the host for recombinant plasmids and grown in 2 $\times$ TY medium (Sambrook et al., 1989) or on TY agar plates containing the appropriate antibiotics (100 μ g/ml ampicillin, 50 μ g/ml kanamycin). Expression of recombinant NTR cDNA was performed in *E. coli* strains DH5 α and CAG627 [*lacZ*(am) *trp*(am) *pho*(am) *supC*^{ts} *mal rpsL lon*] (Spiro and Guest, 1987), with a possible non-characterized periplasmic protease deficiency (Trageser et al., 1990).

The cDNA encoding a rat NTR (Tanaka et al., 1990) was modified by standard cloning techniques and PCR in order to remove the 3'-untranslated region and to introduce a *Hind*III

(Whatman). The data were analysed by non-linear least-squares curve-fitting (Munson and Rodbard, 1980; McPherson, 1985).

Quantification of NTR expression levels

The number of cells growing in suspension was estimated by measuring A_{600} ; 10^9 cells/ml were assumed to correspond to an A_{600} of 1.0. This approach in combination with ligand-binding analysis led to the calculation of the parameter 'receptors/cell', with a possibility of inherent systematic errors. The parameter 'pmol of receptors/mg of crude membrane protein' was determined as follows. Ligand-binding analysis was performed with intact cells. In a parallel experiment, crude membranes were prepared by incubation of *E. coli* cells in ice-cold buffer (25 mM Tris/HCl, pH 7.4, 20 mM EDTA, 50 mM glucose) for 15 min followed by three freeze/thaw cycles. The cells were broken by sonication (Sonicator XL, Heat Systems, large tip, 10 min at level 5 at 25%) after addition of an equal volume of water. The membranes were isolated by ultracentrifugation and washed with buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA). Protein was assayed as described by Schaffner and Weissmann (1973) with BSA as the standard.

In vitro translation

Amersham's prokaryotic DNA-directed translation kit was used as recommended by the manufacturer. The reactions were performed with 3 µg of a mixture containing the repressor plasmid pDMI,1 and the respective pDS12/RBSII expression vector in the presence of 0.3 mM isopropyl β-thiogalactoside (IPTG). The 35 S-labelled proteins were analysed by SDS/10% PAGE (Laemmli, 1970). The gels were soaked in enhancer before fluorography.

Subcellular localization

For analysis of the subcellular localization of recombinant proteins in *E. coli*, cells were harvested, washed and resuspended in ice-cold buffer [50 mM Tris/HCl pH 8, 10 mM EDTA, 20% (w/v) glucose] containing 1 mg of lysozyme/ml. After incubation for 30 min on ice and subsequent centrifugation (3400 g, 10 min) the cells were resuspended in ice-cold buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 40 µg of bacitracin/ml) and broken by sonication (Sonicator XL, Heat Systems, large tip, 5 min at level 2.5 at 25%). Cell debris, unbroken cells and putative inclusion bodies were separated from membranes and soluble proteins by centrifugation (500 g, 15 min). Membranes were isolated from the supernatant by ultracentrifugation (130000 g).

Western-blot analysis

Electrophoretic transfer of proteins (Towbin et al., 1979) to Immobilon membranes (Millipore) was performed with a fast blot apparatus (Hoefer Scientific Instruments) for 1 h at 100 mA in blot buffer [25 mM Tris/HCl, pH 8.3, 150 mM glycine, 0.05% SDS, 5% (v/v) methanol]. Recombinant NTR with c-myc tail synthesized in *E. coli* was detected with the mouse monoclonal antibody 9E10 (Evan et al., 1985) essentially as described (Marks et al., 1991). The membrane was blocked and incubated with 9E10 in 3% (w/v) BSA in PBS. The Western blot was developed by using rabbit anti-mouse IgG conjugated to horseradish peroxidase (Sigma) employing the substrates

diaminobenzidine and H_2O_2 . Alternatively, rabbit anti-(maltose-binding protein) serum (New England Biolabs) and goat anti-rabbit peroxidase-conjugated IgG (Sigma) were used for detection of the MalE-NTR fusion protein.

RESULTS

Expression of rat NTR in *E. coli*

The cDNA encoding NTR (Tanaka et al., 1990) was modified by standard cloning techniques and PCR to obtain a gene cassette flanked by a *Bam*HI-recognition site at the 5' end and a *Hind*III site at the 3' end (Figure 1). The *Bam*HI site coding for the amino acids Gly-Ser was in frame with the codon for His-2 of NTR. To aid immunological detection and to facilitate future purification, a pentahistidine/c-myc tail (encoded by synthetic oligonucleotides) was introduced at the C-terminus of NTR (Figure 1). The c-myc tag (Munro and Pelham, 1986) is recognized by the mouse monoclonal antibody 9E10; the pentahistidine tail is suitable for purification by means of immobilized metal affinity chromatography (Hochuli et al., 1988; Arnold, 1991). The presence of the pentahistidine/c-myc-encoding DNA at the 3' end of the NTR cDNA in plasmids is indicated by the appendage -HMTX.

The NTR gene cassettes were inserted into pDS12/RBSII expression vectors (Bujard et al., 1987) encoding NTR with and without an N-terminal enterotoxin B signal peptide (Figure 2). Maximum expression levels of active ligand-binding NTR were found with *E. coli* DH5α at 30 °C, induced for 1 h with 0.4 mM IPTG. The presence of a signal peptide appeared to facilitate the correct insertion of NTR into the bacterial inner membrane. No active NTR was detected in the absence of IPTG.

Western-blot analysis using the monoclonal antibody 9E10 showed the association of NTR with *E. coli* membranes (DH5α/pDS-S-NTR-HMTX) after induction (Figure 3). A membrane-associated *E. coli* protein with a molecular mass of about 30 kDa cross-reacted non-specifically with 9E10 antibodies. Interestingly, NTR could not be detected by Western-blot

Figure 3 Immunological detection of NTR synthesized in *E. coli*

E. coli DH5α cells containing the plasmid pDS-S-NTR-HMTX were grown at 30 °C and induced for 1 h with 0.4 mM IPTG. The cells were harvested and broken by sonication. Cell debris, unbroken cells and putative inclusion bodies were separated from membranes and soluble proteins by low-speed centrifugation. Membranes were isolated from the soluble proteins by ultracentrifugation. Western-blot analysis (SDS/12.5% PAGE) was performed with the monoclonal antibody 9E10 directed against the C-terminal c-myc tag. Lane 1, prestained molecular-mass markers (Gibco-BRL); lanes 2 and 3, lysed cells, non-induced and induced respectively; lanes 4 and 5, membrane fractions; lanes 6 and 7, soluble fractions; lanes 8 and 9, low-speed centrifugation fractions of subcellular localization analysis from non-induced and induced cells respectively.

Figure 4 Expression of NTR cDNA using an *in vitro* prokaryotic translation system

Coupled *in vitro* transcription and translation reactions were performed as described in the Materials and methods section. The ^{35}S -labelled translation products were separated by SDS/10% PAGE and visualized by fluorography. Reactions 3–9 (lanes 3–9) were performed in the presence of the repressor plasmid pDM1.1 in addition to the respective pDS12/RBSII expression vectors. Lane 1, no plasmid DNA in reaction; lane 2, control plasmid pAT153 (Amersham); lanes 3 and 4, parental expression vectors pDS12/RBSII-S (enterotoxin B signal sequence) and pDS12/RBSII respectively; lanes 5–8, expression plasmids pDS-S-NTR, pDS-NTR, pDS-S-NTR-HMTX and pDS-NTR-HMTX respectively, encoding various modified NTRs (see Figure 2); lane 9, vector pDS-SE31 encoding a fusion protein with the molecular mass of 34 kDa consisting of the enterotoxin B signal peptide and the shortened cytochrome subunit of the photosynthetic reaction centre from *Rhodospseudomonas viridis*.

analysis in DH5 α /pDS-NTR-HMTX after induction, whereas ligand-binding analysis clearly established the presence of active NTR. Use of *E. coli* CAG 627, a protease-deficient strain, with plasmid pDS-NTR-HMTX for expression restored detection of NTR by 9E10 antibodies (results not shown). The C-terminal c-myc tag therefore represents a convenient monitor for the presence of proteolysis of NTR in *E. coli*. Furthermore, the presence of the histidine tail, an essential prerequisite for purification by metal affinity chromatography, is thus checked. Precise quantitative estimation of the NTR expression level by Western-blot analysis was difficult because of the lack of purified NTR. However, taking into account the sensitivity of the Western-blot system used, there seems to be more immunologically detectable NTR than active receptor.

In vitro translation experiments

Expression of rat NTR cDNA under the control of a tightly regulated strong promoter yielded low expression levels. To find whether the codon usage of NTR cDNA might be a limiting factor for high-level expression in *E. coli*, combined *in vitro* transcription/translation experiments were performed using a prokaryotic system (Devries and Zubay, 1967). As a control, the plasmid pDS-SE31 (Grisshammer et al., 1991) was included which is predicted to express a protein with a molecular mass of 34 kDa, consisting of the enterotoxin B leader peptide and the shortened cytochrome subunit of the photosynthetic reaction centre from *Rhodospseudomonas viridis*. The plasmid was chosen because induction of *E. coli* containing pDS-SE31 led to formation of inclusion bodies in large quantities (Grisshammer et al., 1991).

The results of the *in vitro* translation experiment are shown in Figure 4. The intensity of the autoradiography signals corresponding to various modified NTRs (Figure 4, lanes 5–8) was

comparable with that of the cytochrome subunit (Figure 4, lane 9), indicating high-level expression. However, the apparent molecular masses of the respective NTR translation products do not exactly correspond to the expected values. Also, the *in vitro* NTR translation products encoded by the plasmids pDS-S-NTR-HMTX and pDS-NTR-HMTX could not be detected by Western-blot analysis with 9E10 antibodies, although the amount of protein as estimated from the intensity of the autoradiographic signals should have been sufficient for such an analysis (results not shown). These results point towards a possible action of proteases present in the *E. coli* extract used in this analysis. In summary, the *in vitro* translation experiment showed that the codon usage of NTR cDNA does not seem to be a principal problem for overproduction.

Replacement in NTR of Cys-332 with leucine

The rat NTR contains three cysteine residues on the extracellular side of the receptor. Two of them are conserved among seven-helix receptors and are thought to form a disulphide bridge. The third cysteine at position 332 is located at the extracellular C-terminus of helix VI and might interfere with correct disulphide-bridge formation. In the case of turkey β -adrenergic receptor expression in insect cells, exchange of a cysteine codon for a leucine codon in an analogous position in outer loop 1 led to a tenfold increase in production (Parker et al., 1991). Therefore the codon for Cys-332 in the NTR cDNA was replaced by the codon CTG for leucine. Expression of the mutant cDNA inserted into pDS12/RBSII plasmids resulted in functional ligand-binding receptors with expression levels similar to native NTR (results not shown).

Fusion of NTR with maltose-binding protein

It is possible that insertion of NTR into the cytoplasmic membrane is too slow, and that this leads to proteolytic degradation of most of the receptor molecules because of recognition as malfolded protein by *E. coli*. It was observed that the expression level of active NTR without signal peptide is roughly half of the expression level of NTR with signal peptide using the pDS12/RBSII expression vector system (see above). This points towards a possible positive effect of an N-terminal signal peptide on insertion of receptors into the inner membrane. Consequently, higher insertion efficiency might be obtained by using a fusion protein consisting of an N-terminal *E. coli* periplasmic protein such as maltose-binding protein (MalE) followed by NTR. The MalE–NTR fusion protein might be rapidly targeted to the membrane with the MalE moiety promoting a fast translocation.

To test this hypothesis, the NTR-HMTX gene cassette was fused to the 3' end of the gene coding for maltose-binding protein with its own signal sequence under the control of a *tac* promoter (expression plasmid pMalp-NTR-HMTX) (Figure 2). A 40-fold increase in neurotensin-binding sites was achieved using *E. coli* DH5 α or CAG627/pMalp-NTR-HMTX at 20 °C after induction for 18 h in the late exponential phase (450 neurotensin-binding sites/bacterium). The same results were obtained after growth of *E. coli* at 20 °C for about 43 h in the absence of IPTG. Induction of *E. coli* bearing pMalp-NTR-HMTX at 30 °C for 1 h resulted in 60 neurotensin-binding sites per cell, whereas without induction at this temperature 20 sites per bacterium were found.

Western-blot analysis was performed using the monoclonal antibody 9E10 or polyclonal anti-(maltose-binding protein) antibodies. An immunoreactive protein with the molecular mass of 95 kDa, corresponding to the expected mass of the MalE–NTR fusion protein, could be detected with both antibodies in induced

Figure 5 Saturation isotherm of specific binding of $[^3\text{H}]$ neurotensin to intact *E. coli* cells

(a) Strain DH5 α harbouring the plasmid pMalp-NTR-HMTX coding for a MalE-NTR fusion protein was grown at 20 °C and induced for 18 h with 0.3 mM IPTG. $[^3\text{H}]$ Neurotensin-binding experiments were performed with 6×10^7 cells/ml as described in the Materials and methods section. The results are from a typical experiment performed in triplicate. The specific binding activity amounted to 79.1–99.9% of the total binding activity. (b) Scatchard transformation of the data.

cells at 30 °C. In addition, lower-molecular-mass degradation products were observed with anti-(maltose-binding protein) antibodies (results not shown). The MalE-NTR fusion protein was membrane-associated. Interestingly, the MalE-NTR fusion protein could be detected immunologically only with anti-(maltose-binding protein) antibodies, but not with 9E10 antibodies in bacteria grown at 20 °C.

Pharmacological characterization of neurotensin receptors produced in *E. coli*

Ligand-binding analysis was performed with intact *E. coli* cells. The presence of EDTA in the ligand-binding analysis buffer is likely to destabilize the outer membrane and to allow neurotensin to interact with NTR located in the cytoplasmic membrane. No difference in binding properties of neurotensin to NTR was observed when crude membrane preparations were used instead of intact cells. Freezing intact cells in liquid nitrogen did not cause loss of binding activity (results not shown).

The saturation curve for neurotensin binding to intact *E. coli* expressing the MalE-NTR fusion protein is shown in Figure 5(a). Scatchard transformation of the saturation curve yielded a straight line (Figure 5b) with a calculated dissociation constant,

K_D , of $0.23 \text{ nM} \pm 0.07 \text{ nM}$. An average B_{max} of 450 binding sites/cell or 15 pmol/mg of crude membrane protein ($n = 6$) was calculated. Ligand-binding analysis performed with *E. coli*/pDS-NTR resulted in a dissociation constant, K_D , of $0.29 \text{ nM} \pm 0.01 \text{ nM}$ (one experiment in triplicate, results not shown). The neurotensin-binding properties of intact *E. coli* harbouring the various expression plasmids were similar within the experimental error (results not shown) and comparable with those measured in COS cells expressing NTR (Tanaka et al., 1990). The binding properties of NTR produced in *E. coli* correspond to those of the high-affinity binding sites in rat brain (Mazella et al., 1983).

DISCUSSION

Overexpression of membrane proteins

So far, several seven-helix receptors have been synthesized in *E. coli*. For example, the bacterio-opsin gene has been expressed directly or as fusions containing various signal sequences or part of the *lacZ* gene (Dunn et al., 1987; Karnik et al., 1990; Shand et al., 1991). β -Adrenergic receptors and 5-hydroxytryptamine receptor have been pharmacologically characterized after expression in *E. coli* (Breyer et al., 1990; Chapot et al., 1990; Bertin et al., 1992). High-level production of receptors in *E. coli* depends on a variety of factors that are not well understood. The choice of an individual receptor, for example, might considerably influence the results for unknown reasons. Insertion efficiency into the *E. coli* inner membrane seems to be crucial for production of large amounts of active receptors and will be discussed here in more detail.

There is evidence that some *E. coli* membrane proteins assemble in a manner dependent on secretion machinery, whereas others are independent of *sec* gene products (McGovern and Beckwith, 1991). Both mechanisms can even be found within the same protein, as suggested for the *E. coli* leader peptidase (Lee et al., 1992). Leader peptidase and seven-helix receptors both possess extracellular N-termini and are synthesized without an N-terminal signal peptide. The N-terminus and the first transmembrane domain of leader peptidase do not require the SecA or SecY proteins for insertion (Lee et al., 1992). Von Heijne (von Heijne, 1986; von Heijne and Gavel, 1988) has suggested that generally short polar segments with few lysine and arginine residues are quickly translocated across the membrane independently of the secretion machinery in both bacteria and eukaryotic cells. One might speculate that, in analogy to leader peptidase, translocation of the N-terminus of NTR and insertion of the first transmembrane domain is also *sec*-independent and furthermore might influence the overall insertion efficiency of the remaining NTR moiety. Direct expression of NTR in *E. coli* harbouring plasmids pDS-NTR or pDS-NTR-HMTX resulted in only a few active receptors. Addition of an N-terminal signal peptide and therefore targeting the NTR fusion protein to the secretion apparatus increased the number of correctly folded receptors. However, the unusual combination of a signal peptide fused to the relatively short (63 amino acids) NTR N-terminus might not be sufficient for efficient translocation. It has been shown that the molecular chaperone SecB binds to multiple internal sites of the periplasmic maltose-binding protein rather than to the signal peptide alone in order to maintain it in an export-competent state (Randall et al., 1990). Indeed, fusing maltose-binding protein and its N-terminal signal peptide with NTR resulted in a dramatic increase in neurotensin-binding sites in *E. coli*. It will be interesting to see whether this strategy can be applied to other neuropeptide receptors.

Pharmacology of recombinant neurotensin receptors

Rat brain has been shown to contain two types of binding sites with a high and a low affinity for neurotensin (Mazella et al., 1983). The low-affinity site is sensitive to levocabastine (an antihistamine agent) (Kitabgi et al., 1987), whereas the high-affinity site is not. Both in mouse and human, only high-affinity binding sites are present in the brain at birth, whereas the low-affinity binding sites appear at a later stage (Zsürger et al., 1992). Only the high-affinity binding sites in human brain are modulated by GTP and Na⁺ ions, as already observed for other G-protein-coupled receptors. By contrast, the physiological significance of the low-affinity sites which are insensitive to GTP remains unknown.

Transfection of monkey kidney COS cells with rat NTR cDNA (Tanaka et al., 1990) resulted in a single set of high-affinity binding sites for neurotensin. This study demonstrates that rat NTR can be functionally produced in a prokaryotic host with the same properties as NTR expressed in eukaryotic cells.

Interaction of heterotrimeric G-proteins with seven transmembrane-domain receptors leads to an increased affinity of the receptor for the ligand. Conversely, the absence of a G-protein coupling to the receptor results in low-affinity binding of the agonist (Ross, 1989). *E. coli* does not naturally contain seven-helix receptors or heterotrimeric G-proteins. Therefore one would expect only low-affinity binding sites of receptors heterologously expressed in *E. coli*. This has been demonstrated for the human 5-hydroxytryptamine 5-HT_{1A} receptor (Bertin et al., 1992). Functional coupling of the 5-hydroxytryptamine receptor to G-proteins using an *in vitro* reconstitution system resulted in high-affinity agonist binding which could be reversed by addition of a GTP analogue. Similarly, an enhanced potency of the synthetic adrenergic receptor agonist (–)-isoprenaline in competing with prebound antagonist iodocyanopindolol was observed on addition of G-proteins to *E. coli* membranes containing human β_2 - or β_1 -adrenergic receptors (Freissmuth et al., 1991).

NTRs synthesized in *E. coli* elicit the properties of the high-affinity binding sites in rat brain and of NTR produced in COS cells. Solubilization of recombinant NTR produced in *E. coli* and *in vitro* reconstitution with G-proteins will provide further information about the interaction of the ligand with its receptor and might reveal the high- and low-affinity states of the high-affinity NTR.

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REFERENCES

- Amar, S., Mazella, J., Checlier, F., Kitabgi, P. and Vincent, J.-P. (1985) *Biochem. Biophys. Res. Commun.* **129**, 117–125
- Arnold, F. H. (1991) *Bio/Technology* **9**, 151–156
- Bertin, B., Freissmuth, M., Breyer, R. M., Schütz, W., Strosberg, A. D. and Marullo, S. (1992) *J. Biol. Chem.* **267**, 8200–8206
- Bozou, J.-C., Amar, S., Vincent, J.-P. and Kitabgi, P. (1986) *Mol. Pharmacol.* **29**, 489–496
- Breyer, R. M., Strosberg, A. D. and Guillet, J.-G. (1990) *EMBO J.* **9**, 2679–2684
- Buck, L. and Axel, R. (1991) *Cell* **65**, 175–187
- Bujard, H., Gentz, R., Lanzer, M., Stueber, D., Mueller, M., Ibrahim, I., Haeuptle, M.-T. and Dobberstein, B. (1987) *Methods Enzymol.* **155**, 416–433
- Certa, U., Bannwarth, W., Stüber, D., Gentz, R., Lanzer, M., Le Grice, S., Guillot, F., Wendler, I., Hunsmann, G., Bujard, H. and Mous, J. (1986) *EMBO J.* **5**, 3051–3056
- Chapot, M.-P., Eshdat, Y., Marullo, S., Guillet, J.-G., Charbit, A., Strosberg, A. D. and Delavier-Klutchko, C. (1990) *Eur. J. Biochem.* **187**, 137–144
- Devries, J. K. and Zubay, G. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 1010–1012
- Dixon, R. A. F., Sigal, I. S., Candelore, M. R., Register, R. B. and Scattergood, W. (1987) *EMBO J.* **6**, 3269–3275
- Dunn, R. J., Hackett, N. R., McCoy, J. M., Chao, B. H., Kimura, K. and Khorana, H. G. (1987) *J. Biol. Chem.* **262**, 9246–9254
- Duplay, P., Bedouelle, H., Fowler, A., Zabin, I., Saurin, W. and Hofnung, M. (1984) *J. Biol. Chem.* **259**, 10606–10613
- Evan, G. I., Lewis, G. K., Ramsey, G. and Bishop, J. M. (1985) *Mol. Cell. Biol.* **5**, 3610–3616
- Freissmuth, M., Selzer, E., Marullo, S., Schütz, W. and Strosberg, A. D. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 8548–8552
- Gilbert, J. A. and Richelson, E. (1984) *Eur. J. Pharmacol.* **99**, 245–246
- Goedert, M. (1989) *Methods Enzymol.* **168**, 463–481
- Goedert, M., Pinnock, R. D., Downes, C. P., Mantyh, P. W. and Emson, P. C. (1984) *Brain Res.* **323**, 193–197
- Grisshammer, R., Deckl, C. and Michel, H. (1991) *Biochim. Biophys. Acta* **1088**, 183–190
- Hargrave, P. A. and McDowell, J. H. (1992) *FASEB J.* **6**, 2323–2331
- Henderson, R. and Schertler, G. F. X. (1990) *Philos. Trans. R. Soc. London B* **326**, 379–389
- Hochuli, E., Bannwarth, W., Döbeli, H., Gentz, R. and Stüber, D. (1988) *Bio/Technology* **6**, 1321–1325
- Hulme, E. C., Birdsall, N. J. M. and Buckley, N. J. (1990) *Annu. Rev. Pharmacol. Toxicol.* **30**, 633–673
- Karnik, S., Sakman, T. P., Chen, H. B. and Khorana, H. G. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8459–8463
- Karnik, S., Doi, T., Molday, R. and Khorana, H. G. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 8955–8959
- Kerlavage, A. R. (1991) *Curr. Opin. Struct. Biol.* **1**, 394–401
- Kitabgi, P., Checlier, F., Mazella, J. and Vincent, J.-P. (1985) *Rev. Clin. Basic Pharmacol.* **5**, 397–486
- Kitabgi, P., Rostene, W., Dussaillant, M., Schotte, A., Laduron, P. M. and Vincent, J.-P. (1987) *Eur. J. Pharmacol.* **140**, 285–293
- Kobilka, B. (1992) *Annu. Rev. Neurosci.* **15**, 87–114
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lee, J.-I., Kuhn, A. and Dalbey, R. E. (1992) *J. Biol. Chem.* **267**, 938–943
- Marks, J. D., Hoogenboom, H. R., Bonner, T. P., McCafferty, J., Griffiths, A. D. and Winter, G. (1991) *J. Mol. Biol.* **222**, 581–597
- Mazella, J., Poustis, C., Labbe, C., Checlier, F., Kitabgi, P., Granier, C., van Rietschoten, J. and Vincent, J.-P. (1983) *J. Biol. Chem.* **258**, 3476–3481
- McGovern, K. and Beckwith, J. (1991) *J. Biol. Chem.* **266**, 20870–20876
- McPherson, G. A. (1985) *J. Pharmacol. Methods* **14**, 213–228
- Munro, S. and Pelham, H. R. B. (1986) *Cell* **46**, 291–300
- Munson, P. J. and Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- O'Dowd, B. F., Hnatowich, M., Caron, M. G., Lefkowitz, R. J. and Bouvier, M. (1989) *J. Biol. Chem.* **264**, 7564–7569
- Parker, E. M., Kameyama, K., Higashijima, T. and Ross, E. M. (1991) *J. Biol. Chem.* **266**, 519–527
- Randall, L. L., Topping, T. B. and Hardy, S. J. S. (1990) *Science* **248**, 860–863
- Ross, E. M. (1989) *Neuron* **3**, 141–152
- Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Schaffner, W. and Weissmann, C. (1973) *Anal. Biochem.* **56**, 502–514
- Schertler, G. F. X. (1992) *Curr. Opin. Struct. Biol.* **2**, 534–544
- Shand, R. F., Miercke, L. J. W., Mitra, A. K., Fong, S. K., Stroud, R. M. and Betlach, M. C. (1991) *Biochemistry* **30**, 3082–3088
- Snider, R. M., Forray, C., Pfenning, M. and Richelson, E. (1986) *J. Neurochem.* **47**, 1214–1218
- Spiro, S. and Guest, J. R. (1987) *J. Gen. Microbiol.* **133**, 3279–3288
- Tanaka, K., Masu, M. and Nakanishi, S. (1990) *Neuron* **4**, 847–854
- Trageser, M., Spiro, S., Duchêne, A., Kojro, E., Fahrenholz, F., Guest, J. R. and Uden, G. (1990) *Mol. Microbiol.* **4**, 21–27
- Towbin, H., Staehlin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- von Heijne, G. (1986) *EMBO J.* **5**, 3021–3027
- von Heijne, G. and Gavel, Y. (1988) *Eur. J. Biochem.* **174**, 671–678
- Zsürger, N., Chabry, J., Coquerel, A. and Vincent, J.-P. (1992) *Brain Res.* **586**, 303–310