Immunoprecipitation of opioid receptor–G_o-protein complexes using specific GTP-binding-protein antisera

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Solubilization of opioid receptors from rat cortical membranes that retained high-affinity guanine nucleotide-sensitive agonist binding was achieved using 10 mM CHAPS. We report the nature of the interactions of μ and δ opioid receptors with the guanine nucleotide-binding protein G_o by immunoprecipitation of CHAPS extracts with selective G_o-subunit protein antisera. Antiserum IM1 raised against amino acids 22–35 of G_o α selectively co-immunoprecipitated G_o α – μ and G_o α – δ opioid receptor complexes detected in the immunoprecipitates by specific [³H][D-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin and [³H][D-Ser²,Leu⁵,Thr⁶]enkephalin binding respectively. By contrast, antisera

directed against the C-terminal decapeptide (OC2) and the N-terminal hexadecapeptide (ON1) of isoforms of G_o α were unable to immunoprecipitate solubilized opioid receptor–G_o complexes, although both were able to immunoprecipitate solubilized G_o α and have been shown to reduce the affinity of [D-Ala²,D-Leu⁵]enkephalin for opioid receptors in rat cortical membranes [Georgoussi, Carr and Milligan (1993) *Mol. Pharmacol.* **44**, 62–69]. These findings demonstrate that CHAPS-solubilized μ and δ opioid receptors from rat cortical membranes form stable complexes with one or more variants of G_o.

INTRODUCTION

Agonist activation of all three opioid receptor subtypes μ , δ and κ has been reported to result in the inhibition of adenylate cyclase and/or the regulation of ion channels by activation of one or more pertussis-toxin-sensitive guanine nucleotide-binding proteins (G-proteins) acting as signal transducers [1]. Although there has been extensive analysis of opioid receptor function and pharmacology, our understanding of the molecular basis of these properties is limited. All subtypes of opioid receptors have been recently cloned [2–4]. Comparison of amino acid sequences of the three cloned opioid receptors reveals high sequence similarity of the intracellular loops, suggesting that they might interact with similar G-proteins.

Reconstitution studies with the μ opioid receptor solubilized from rat brain have indicated the potential of this receptor to interact with G_i or G_o [5], whereas in NG108–15 hybrid cells the δ opioid receptor appears to activate G_{i2} [6], G_o [7] and possibly G_{i3} [8]. Studies in neuronal cell lines and brain have noted that coupling of μ opioid receptors to adenylate cyclase was maximally blocked by antibodies to G_o α [9]. Using selective G-protein antisera in native brain membranes, we have previously demonstrated that both the C- and N-terminal regions of G_o α play a key role in opioid receptor–G_o-protein interaction [10].

Various laboratories have attempted to solubilize opioid receptors that retain binding characteristics similar to those in intact membranes. In most cases, occupancy of the receptor by a ligand before solubilization or high concentrations of NaCl were required for the receptor to display high-affinity guanine nucleotide-sensitive binding [11–14].

In the present study active opioid receptors from brain cortical membranes were solubilized with 10 mM CHAPS and immunoprecipitated with selective G_o α -protein antisera. This allowed for the first time the observation of stable soluble μ and δ opioid receptor–G_o-protein complexes derived from rat brain mem-

branes. Furthermore, it is shown that the binding of the C- and N-terminal-directed antisera that uncouple opioid receptor–G_o-protein complexes in native membranes [10] cannot immunoprecipitate soluble opioid receptors, whereas an antiserum directed against an internal region of G_o α can.

MATERIALS AND METHODS

Materials

[³H]Diprenorphine (37 Ci/mmol) and [³H][D-Ser²,Leu⁵,Thr⁶]enkephalin (DSLET) (52.6 Ci/mmol) were from DuPont–New England Nuclear. [³H][D-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin (DAMGO) (60 Ci/mmol) was purchased from Amersham. CHAPS was from Pierce. Guanosine 5'-[β -imidol]triphosphate (GppNHp) and all other reagents were of analytical grade from Sigma.

Solubilization of membranes

Rat brain cortical membranes were prepared as described by Georgoussi et al. [10] in the presence of 0.1 mM EGTA and 50 mM Tris/HCl, pH 7.5. Plasma membranes were diluted with buffer containing 50 mM Tris/HCl, pH 7.5, 50 μ g/ml trypsin inhibitor and 0.1 mM phenylmethanesulphonyl fluoride (PMSF) (buffer A) and spun for 15 min at 165000 g. The pellet was solubilized as described by Simonds et al. [11] in buffer A containing 10 mM CHAPS for 1 h at 4 °C with gentle stirring. The solubilized preparation was centrifuged at 100000 g for 1 h at 4 °C. The clear supernatant was removed, diluted 10-fold in buffer B (50 mM Tris/HCl, pH 7.5, 10 mM MgCl₂, 0.1 mM PMSF and 50 μ g/ml trypsin inhibitor) and concentrated to half volume using a CentriCell. The solubilized material was then

Abbreviations used: DAMGO, [D-Ala²,N-Me-Phe⁴,Gly⁵-ol]enkephalin; DSLET, [D-Ser²,Leu⁵,Thr⁶]enkephalin; DADLE, [D-Ala²,D-Leu⁵]enkephalin; GppNHp, guanosine 5'-[β -imidol]triphosphate. PMSF, phenylmethanesulphonyl fluoride.

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used for binding or immunoprecipitation experiments. The concentration of protein in each sample was approx. 250–350 $\mu\text{g}/\text{ml}$, and 1 ml samples were used for binding experiments.

Binding experiments

Binding experiments were performed at 30 °C for 30 min in buffer made up of 10 mM MgCl_2 , 100 mM NaCl and 50 mM Tris/HCl, pH 7.5. In saturation experiments using [^3H]diprenorphine the concentration of ligand was varied between 0.25 and 5 nM. Data were analysed using the LIGAND program [15] modified by McPherson [16]. Non-specific binding was assessed in parallel assays containing either 10 μM naloxone or 10 μM diprenorphine. When [^3H]DAMGO and [^3H]DSLET were used, non-specific binding was assessed in the presence of 10 μM unlabelled DAMGO and DSLET respectively. Binding was terminated by rapid filtration through Whatman GF/B filters, presoaked in 0.3 % polyethylimine at 4 °C, followed by (3×4 ml) washes with ice-cold 50 mM Tris/HCl, pH 7.5. A similar procedure was used to detect immunoprecipitated opioid receptors. In experiments in which uncoupling of opioid receptors from G-proteins was performed the membranes were preincubated for 45 min at 30 °C with 100 μM GppNHp before binding.

Immunoprecipitation of opioid receptor–G-protein complexes

To immunoprecipitate opioid receptor–G-protein complexes, a sample of solubilized receptor (1 ml) was incubated with the appropriate G-protein antiserum (20 μl) under constant rotation at 4 °C. After 6 h, 100 μl of 50 % (w/v) Protein A–agarose beads was added to each sample and the mixture incubated overnight. Finally another portion of antiserum was added to bring the final antiserum dilution to 1:30. These samples were incubated for a further 3–4 h and then centrifuged in an Eppendorf micro-centrifuge for 2 min. The supernatant was removed, and the immune complex was washed by the addition of 1 ml of buffer B and re-centrifuged. The immunoprecipitate was resuspended in 1 ml of buffer B and the opioid receptors were detected by binding assays or the immune complex was resuspended in 30 μl of Laemmli sample buffer and resolved by SDS/PAGE [10 % (w/v) acrylamide].

Immunological experiments

The generation and specificity of the antisera used in this study have been described extensively [10,17,18]. Crude antisera were chromatographed on Protein A–Sephrose (Sigma) as described previously [10]. After elution of the IgG fractions with 100 mM citric acid, pH 4.0, into 2 M Tris/HCl, pH 7.5, they were dialysed against 10 mM Tris/HCl, pH 7.5, containing 0.1 mM EDTA then lyophilized and reconstituted with the same buffer before use. Immunoblotting was performed [6] using horseradish peroxidase-linked donkey anti-rabbit IgG fraction as the secondary antibody. The conditions for gel electrophoresis and the transfer of protein to nitrocellulose membranes were as described previously [6].

Statistical analysis

The results are expressed as means $\pm$ S.E.M.; statistical analysis was performed by Student's *t* test, accepting $P < 0.05$ as significant.

RESULTS

Solubilization of opioid receptor–G-protein complexes from rat cortical membranes with CHAPS

Active high-affinity opioid receptors were solubilized from brain cortical membranes with 10 mM CHAPS in the absence of NaCl as described by Simonds et al. [11]. Saturation analysis of specific [^3H]diprenorphine binding in the solubilized material indicated a K_D of 1.3 ± 0.2 nM, with specific [^3H]diprenorphine binding (B_{max}) of 90 ± 7 fmol/mg of membrane protein (Figure 1). Under

Figure 1 Saturation analysis of [^3H]diprenorphine binding to the solubilized rat cortical membranes

Rat brain soluble membranes (140 μg) were incubated with various concentrations of [^3H]diprenorphine and binding was determined as described in the Materials and methods section. Non-specific binding was assessed in the presence of 10 μM naloxone.

Figure 2 Displacement of [^3H]diprenorphine by DADLE from solubilized opioid receptors in the presence or absence of GppNHp

Rat brain cortical membranes were solubilized as described in the Materials and methods section. CHAPS extracts were incubated in the absence (○) or presence (△) of 10 μM GppNHp for 45 min at 30 °C before binding, which was performed as described in the Materials and methods section. Non-specific binding was assessed in the presence of 10 μM naloxone. Specific binding of [^3H]diprenorphine (2 nM) of 100% was 54 ± 7 fmol/mg of protein for solubilized membranes (control) and 46 ± 5 fmol/mg of protein for the membranes incubated in the presence of 100 μM GppNHp. Data are pooled from two individual experiments.

Figure 3 Anti-G_oα sera immunoprecipitate G_oα from solubilized rat cortical membranes

Rat brain cortical membranes were solubilized and immunoprecipitated at a final antiserum dilution of 1:30 with antisera OC2 (lane 1) or IM1 (lane 3) or preimmune serum (lanes 2 and 4) as described in the Materials and methods section. Both the solubilized membranes (lane 5) and the immunoprecipitates were subjected to SDS/PAGE [10% (w/v) acrylamide] and immunoblotted using antiserum ON1 as the primary antiserum. Immunoglobulin was present in all the immunoblots after immunoprecipitation.

these conditions 35% of the total membrane-associated opioid receptor population could be detected in the solubilized preparation, indicating that either the remaining receptors were not solubilized or, more plausibly, they could not be detected in the soluble fraction by binding experiments [11,13].

The CHAPS extracts displayed high-affinity guanine nucleotide-sensitive agonist binding as assessed by displacement of [³H]diprenorphine by [D-Ala²,D-Leu⁵]enkephalin (DADLE) in the presence or absence of the poorly hydrolysed analogue of GTP, GppNHp. As indicated in Figure 2, GppNHp produced a marked shift to the right in DADLE displacement of the specific binding of [³H]diprenorphine (2 nM) compared with the displacement observed in the absence of exogenous guanine nucleotide. These data indicate that, even in the absence of agonist, it was possible to solubilize a complex of opioid receptors and G-protein(s) that remained stable in the presence of CHAPS.

Immunoprecipitation of solubilized rat cortical membranes with specific anti-G_oα sera

Anti-peptide sera IM1, OC2 and ON1 generated against peptides common to polypeptides corresponding to products from both the G_o1 and G_o2 splice variants of the G_oα gene have been previously characterized [17]. Both antisera IM1 and OC2 were able to immunoprecipitate a 39 kDa polypeptide in the solubilized material which could be identified as G_oα by immunoblotting with antiserum ON1 (Figure 3). Antiserum ON1 was also able to immunoprecipitate a 39 kDa polypeptide which was confirmed to be G_oα by immunoblotting with antiserum OC2 (results not shown but see ref. [10]). These results indicate that each of these antisera could bind G_oα in the CHAPS extracts of rat cortical membranes, and therefore, if the opioid receptors were coupled to G_o, it should be possible to co-immunoprecipitate the receptors using the G_oα antisera.

Antiserum OC2, which is directed against the C-terminal region of G_oα, failed to immunoprecipitate specific [³H]diprenorphine-binding sites from the CHAPS extract when compared with a preimmune serum control (Table 1). A similar lack of opioid receptor immunoprecipitation was observed with

Table 1 Immunoprecipitation of soluble opioid receptors from rat cortical membranes with anti-G_o-protein sera

Soluble opioid receptors from rat cortical membranes were immunoprecipitated with antiserum OC2, ON1 or IM1 or preimmune serum at a final dilution of 1:30 as described in the Materials and methods section. The presence of opioid receptors in the immunoprecipitate was detected by the specific binding of [³H]diprenorphine (2.3 nM) as described in the Materials and methods section. In experiments in which GppNHp was present, the membranes were preincubated with 100 μM GppNHp for 45 min at 30 °C before solubilization. Values are presented as the amount of specific binding (d.p.m.) and are means ± S.E.M. of four different experiments performed in triplicate for the OC2 and ON1 antisera and seven experiments for the IM1 antiserum. **P* < 0.025 compared with preimmune serum (Student's *t* test).

	Specific [³ H]diprenorphine binding (d.p.m.)
Preimmune serum	170 ± 35
OC2 antiserum	60 ± 12
ON1 antiserum	70 ± 16
IM1 antiserum	550 ± 110*
Preimmune serum + GppNHp	150 ± 25
IM1 antiserum + GppNHp	120 ± 30

antiserum ON1 which is directed against the N-terminal hexadecapeptide of isoforms of G_oα (Table 1). By contrast, antiserum IM1, generated against amino acids 22–35 of G_oα, was able to immunoprecipitate effectively G_oα-opioid receptor complexes from solubilized cortical rat brain membranes, measured by specific [³H]diprenorphine binding in the immunoprecipitate. Approx. 64 ± 7% (mean ± S.E.M., *n* = 5) of the solubilized opioid receptors were immunoprecipitated with antiserum IM1. Immunoprecipitation of the opioid receptors with G_oα was specific, as pretreatment of the membranes with GppNHp before solubilization and immunoprecipitation blocked the ability of antiserum IM1 to co-immunoprecipitate G_oα-opioid receptor complexes (Table 1).

Ability of selective anti-G_oα sera to immunoprecipitate μ and δ opioid receptor-G_o-protein complexes

In order to define which opioid receptors interact with G_oα we tested for the presence of both μ and δ opioid receptors in IM1 immunoprecipitates by using the highly selective opioid ligands [³H]DAMGO (μ agonist) and [³H]DSLET (δ agonist). As shown in Table 2, 740 ± 130 d.p.m. of specific [³H]DAMGO (3 nM) binding was present in the IM1 immunoprecipitate compared with 180 ± 67 d.p.m. of preimmune control, indicating that IM1 antiserum can immunoprecipitate μ opioid receptor-G_oα protein complexes. In a typical experiment, 11% of the total solubilized opioid receptors could be detected with [³H]DAMGO in the immunoprecipitate. This binding was selectively abolished by pretreatment of the membranes with GppNHp, as a result of the failure to immunoprecipitate the uncoupled receptor, suggesting that [³H]DAMGO specifically labels G_o-protein-coupled μ opioid receptors in the immunoprecipitate (Table 2). Similar experiments using [³H]DSLET demonstrated that antiserum IM1 could immunoprecipitate 550 ± 100 d.p.m. of specific [³H]DSLET (3.5 nM) binding compared with 100 ± 21 d.p.m. in the preimmune control. This amount represents 16% of the total solubilized opioid receptors, indicating that δ opioid receptor-G_oα complexes could be detected in the IM1 immunoprecipitates of CHAPS-solubilized rat cortical membranes. These results suggest that identification of functional interactions between G_o and μ and δ opioid receptors can be observed in

Table 2 Immunoprecipitation of μ and δ opioid receptors from solubilized rat cortical membranes with the IM1 antiserum

Concentrated solubilized membranes were immunoprecipitated as described in the Materials and methods section with IM1 antiserum at a final dilution of 1:30. Specific [3 H]DAMGO and [3 H]DSLET binding in the immunoprecipitate was assessed as described in the Materials and methods section. The amount of μ and δ receptors in the soluble material was 31 and 42% respectively, as determined by [3 H]DAMGO (3 nM) and [3 H]DSLET (3.5 nM) binding, compared with the amount of receptor defined by [3 H]diprenorphine binding. IM1 antiserum immunoprecipitated 35 and 38% of μ and δ opioid receptors respectively. In experiments in which GppNHp was included, cortical membranes were incubated with 100 μ M GppNHp before solubilization. Values are presented as the amount of specific binding and are means $\pm$ S.E.M. from four independent experiments. * $P < 0.025$, ** $P < 0.01$ compared with preimmune control (Student's t -test).

	Specific [3 H]DAMGO binding (d.p.m.)	Specific [3 H]DSLET binding (d.p.m.)
Preimmune serum	180 $\pm$ 67	100 $\pm$ 21
IM1 antiserum	740 $\pm$ 130**	550 $\pm$ 100*
Preimmune serum + GppNHp	100 $\pm$ 5	
IM1 antiserum + GppNHp	90 $\pm$ 7	

solubilized rat cortical membranes after immunoprecipitation with selective anti-peptide sera.

DISCUSSION

A widely used approach to assessing the specificity of receptor-G-protein interactions has been to use anti-G-protein sera which are able to interfere with these interactions. This antibody approach has been extensively used to observe the interaction of a range of receptors with G-protein(s) [6,18–20]. Using this method we have previously studied the interaction of rat brain cortex μ -opioid receptors with G_o [10].

Biochemical data supporting a physical association of opioid receptors and G-proteins and the nature of these interactions are limited. The work presented here describes an immunoprecipitation-based approach using a selective anti-peptide serum directed against amino acids 22–35 of forms of G_{α} (IM1), which was shown to identify functional opioid receptor-G-protein interactions after solubilization of rat cortical membranes. Solubilization was carried out in the presence of 10 mM CHAPS as described by Simonds et al. [11] for the δ opioid receptors present in NG108-15 hybrid cells, without altering the affinity of the receptor for diprenorphine (Figure 1). Pretreatment of the solubilized preparation with GppNHp reduced the ability of DADLE to displace [3 H]diprenorphine, indicating interaction between the receptor and a G-protein(s) (Figure 2). These observations demonstrate that the solubilization conditions used did not disrupt the association of the receptor with G-proteins and that occupancy of the receptor by a ligand before solubilization is not required to stabilize the receptor and the formation of receptor-G-protein complex.

The presence of tightly coupled opioid receptor-G-protein complexes in the absence of agonist has also been observed in digitonin-solubilized brain extracts [14]. Similar putative receptor-G-protein complexes have been reported after solubilization of the vasopressin [21], the muscarinic acetylcholine [22] and D_2 -dopamine [23] receptors.

The important finding in the present study is that both μ and δ opioid receptors form functional complexes with one or more variants of G_{α} after solubilization of cortical membranes. Antisera directed against the C-terminal decapeptide (OC2) and

the N-terminal hexadecapeptide (ON1) of forms of G_{α} effectively immunoprecipitated a 39 kDa polypeptide from solubilized rat cortical membranes. Antiserum IM1 also efficiently immunoprecipitated G_{α} from the soluble membranes, as assessed by the detection of a 39 kDa polypeptide in such immunoprecipitates by the highly specific G_{α} antiserum ON1 (Figure 3). These results demonstrate the identification and binding of these antisera to G_{α} in solubilized cortex membranes. However, when we checked the ability of each of the antisera to precipitate opioid receptors along with G_{α} , we noted that only the IgG fraction from antiserum IM1 was able to co-immunoprecipitate opioid receptor-G-protein complexes from the solubilized preparation (Table 1). The inability of both antisera OC2 and ON1 to immunoprecipitate opioid receptor-G α complexes suggest that these antisera identify regions of G_{α} that may be important contact points between receptors and G-proteins. Indeed, we have previously demonstrated that both antisera ON1 and OC2 uncouple opioid receptors from G_o [10], on the basis of the ability of each to reduce the ability of the opioid peptide DADLE to compete for specific [3 H]diprenorphine-binding sites in rat cortical membranes. In contrast with antisera OC2 and ON1, antiserum IM1 did not produce a reduction in affinity of DADLE for the opioid receptors, indicating that binding of antiserum IM1 to G_o does not interfere with receptor-G-protein coupling [10]. In support of this explanation, we demonstrate here that antiserum IM1, which was raised against a region of G_{α} believed not to play a key role in receptor-G-protein interaction, does not uncouple G_o from opioid receptors in cortical membranes and therefore was able to co-immunoprecipitate opioid receptors along with G_{α} . Moreover antiserum IM1 effectively immunoprecipitated μ and δ opioid receptors, as assessed by using [3 H]DAMGO and [3 H]DSLET respectively. [3 H]DAMGO binding in the immunoprecipitate was reduced to basal levels compared with preimmune control (Table 2) by pretreatment of the soluble material with GppNHp. The observed data may reflect the failure of precipitated receptor to bind the ligand or more likely failure to precipitate the uncoupled receptor.

It has been reported that the C-terminal region of G_{α} plays a crucial role in receptor-G-protein interaction and activation of the α -subunit after agonist binding to receptors [24], whereas the N-terminal domain of G-protein is responsible for interaction of the α -subunit with $\beta\gamma$ -subunits [25]. The inability of antisera OC2 and ON1 to immunoprecipitate soluble opioid receptor-G α complexes may be attributed to either uncoupling of the complexes by preventing the formation of the heterotrimer or recognition of these antisera by epitopes of G_{α} which are in direct contact with opioid receptors.

Recent work has utilized antisera directed against the C-terminal region of G_{i1} and G_{i2} to immunoprecipitate somatostatin and α_2 -adrenergic receptors with these G-proteins [26,27]. Such studies might have been predicted to be ineffectual, as antisera of this type have been widely used to interfere with receptor signalling pathways [20,29]. Although it has been assumed that such antisera uncouple G-proteins from their receptors, their effect may be to uncouple receptors and G-proteins only functionally rather than physically.

The G-protein content of rat brain resembles that of SH-SY5Y cells which contain both μ and δ opioid receptors which, on activation, lead to inhibition of adenylate cyclase and/or Ca^{2+} channel regulation [9,28–31]. Various studies have revealed profound differences in coupling of μ and δ opioid receptors to pertussis-toxin-sensitive G-proteins [6,7,29–31]. However, as activated μ and δ opioid receptors have identical effects on several known effector systems, it is tempting to speculate that

the two receptors might also interact with identical G-protein(s). In the present study we demonstrate that both μ and δ opioid receptors form stable complexes with one or more variants of G_o. The intracellular events mediated by these interactions remain to be clearly defined but, given the ability of both opioid receptors and the isoforms of G_o to regulate voltage-operated Ca²⁺ channels [30,32], this might represent an obvious target.

REFERENCES

- Childers, S. R. (1991) *Life Sci.* **48**, 1991–2003
- Evans, C. J., Keith, D. E., Morrison, H., Magendzo, K. and Edwards, R. H. (1992) *Science* **258**, 1952–1955
- Kieffer, B. L., Befort, K., Gavériaux-Ruff, C. and Hirth, C. G. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 12048–12052
- Reisine, T. and Bell, G. I. (1993) *Trends Neurosci.* **16**, 506–510
- Ueda, H., Harada, H., Nozaki, M., Katada, T., Ui, M., Satoh, M. and Takagi, H. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7013–7017
- McKenzie, F. R. and Milligan, G. (1990) *Biochem. J.* **267**, 391–398
- Offermanns, S., Schultz, G. and Rosenthal, W. (1991) *J. Biol. Chem.* **266**, 3365–3368
- Roerig, S. C., Loh, H. H. and Law, P. Y. (1992) *Mol. Pharmacol.* **41**, 822–831
- Carter, B. D. and Medzhradsky, F. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4062–4066
- Georgoussi, Z., Carr, C. and Milligan, G. (1993) *Mol. Pharmacol.* **44**, 62–69
- Simonds, W. F., Koski, G., Streaty, R. A., Hjelmeland, L. M. and Klee, W. A. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4623–4627
- Ofri, D., Ritter, A. M., Liu, Y., Giannini, T. L., Hiller, J. M. and Simon, E. J. (1992) *J. Neurochem.* **58**, 628–635
- Cote, T. E., Gosse, M. E. and Weems, H. B. (1993) *J. Neurochem.* **61**, 973–978
- Wong, Y. H., Demoliou-Mason, C. D. and Barnard, E. A. (1989) *J. Neurochem.* **52**, 999–1009
- Munson, P. J. and Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- McPherson, G. A. (1983) *Comput. Prog. Biomed.* **17**, 107–114
- Goldsmith, P. K., Gierschik, P., Milligan, G., Unson, C. G., Vinitzky, R., Malech, H. L. and Spiegel, A. M. (1987) *J. Biol. Chem.* **262**, 14683–14688
- Mullaney, I. and Milligan, G. (1990) *J. Neurochem.* **55**, 1890–1898
- Simonds, W. F., Goldsmith, P. K., Woodward, C. J., Unson, C. G. and Spiegel, A. M. (1989) *FEBS Lett.* **249**, 189–194
- Simonds, W. F., Goldsmith, P. K., Codina, J., Unson, C. G. and Spiegel, A. M. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7809–7813
- Fitzgerald, T. J., Uhing, R. J. and Exton, J. H. (1986) *J. Biol. Chem.* **261**, 16871–16877
- Matesic, D. F., Manning, D. R., Wolfe, B. B. and Luthin, G. R. (1989) *J. Biol. Chem.* **264**, 21638–21645
- Senogles, S. E., Spiegel, A. M., Padrell, E., Iyengar, R. and Caron, M. G. (1990) *J. Biol. Chem.* **265**, 4507–4514
- Masters, S. B., Sullivan, K., Miller, R., Beiderman, B., Lopez, N., Ramachandran, J. and Bourne, H. R. (1988) *Science* **241**, 448–451
- Schmidt, C. J. and Neer, E. J. (1991) *J. Biol. Chem.* **266**, 4538–4544
- Law, S. F., Manning, D. and Reisine, T. (1991) *J. Biol. Chem.* **266**, 17885–17897
- Okuma, Y. and Reisine, T. (1992) *J. Biol. Chem.* **267**, 14826–14831
- Sadee, W., Yu, V. C., Richards, M. L., Preis, P. N., Schwab, M. R., Brodsky, F. M. and Biedler, J. L. (1987) *Cancer Res.* **47**, 5207–5212
- McFadzean, I., Mullaney, I., Brown, D. A. and Milligan, G. (1989) *Neuron* **3**, 177–182
- Seward, E., Hammond, C. and Henderson, G. (1991) *Proc. R. Soc. London Ser. B* **244**, 129–135
- Laugwitz, K.-L., Offermanns, S., Spicher, K. and Schultz, G. (1993) *Neuron* **10**, 233–242
- Surprenant, A., Shen, K. Z., North, R. A. and Tatsumi, H. (1990) *J. Physiol. (London)* **431**, 585–608