

## Identification of both G<sub>i</sub>2 and a novel, immunologically distinct, form of G<sub>o</sub> in rat myometrial membranes

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Immunoblotting of rat myometrial membranes with an antiserum (SG1) which recognizes the  $\alpha$ -subunits of both G<sub>i</sub>1 and G<sub>i</sub>2 indicated the presence of detectable levels of an apparently single form of some 40 kDa. A second antiserum (LE2) specific for G<sub>i</sub>2 also recognized this protein, confirming its identity. Immunoblotting of the myometrial membranes with a series of antipeptide (OC1, IM1, ON1) and polyclonal (RV3) antisera, all of which recognize rat brain G<sub>o</sub>, produced a more complex pattern. Antisera OC1 and ON1 recognized a single polypeptide which essentially comigrated with rat brain G<sub>o</sub>. In contrast, antisera RV3 and IM1 did not recognize the myometrial protein. These data provide evidence for the presence of G<sub>i</sub>2 and of a novel G-protein, related immunologically to G<sub>o</sub>, in rat myometrial membranes.

Guanine nucleotide-binding protein; Pertussis toxin; (Myometrium, Brain)

### 1. INTRODUCTION

The interaction of many hormones and neurotransmitters with specific receptors in the plasma membranes of target cells leads to alterations in levels of particular intracellular second messengers. In every case, communication between the agonist-occupied receptor and the second messenger generation system involves the obligatory participation of members of a family of guanine nucleotide-binding proteins [1,2].

The application of cDNA cloning technology to the field of transmembrane signalling has led to the

identification of a considerable number of these G-proteins [3–6]. Because of the extreme homology between the individual G-proteins, conventional protein purification protocols have been unable to unambiguously identify products corresponding to each of the known cDNA clones. A convenient approach to the detection of the individual G-proteins has thus been to produce antipeptide antisera which based on the predicted primary structures of the different G-proteins should be able to discriminate between the various forms [7–9].

We have previously produced a number of antipeptide antisera which are directed against distinct epitopes within the sequence of G<sub>o</sub> [10]. G<sub>o</sub> is a member of the subfamily of G-proteins which are substrates for pertussis toxin-catalysed ADP-ribosylation [1,2] but despite the high concentrations of this protein in a number of tissues, especially brain and heart [11,12], its function remains a matter of debate [13,14] although it may well function to link receptors to voltage-sensitive Ca<sup>2+</sup> channels. Recently it was noted that two individual mRNAs could be identified on Northern analyses using a G<sub>o</sub> probe [4]. Here we

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*Abbreviations:* G-protein, guanine nucleotide-binding protein; G<sub>o</sub>, a G-protein of unknown function which is particularly abundant in brain; G<sub>i</sub>1, G<sub>i</sub>2, G<sub>i</sub>3, highly homologous G-proteins which may be involved in the control of inhibition of adenylate cyclase (these are named for the chronology of identification of cDNA sequences containing the coding potential for these proteins)

demonstrate the presence of a polypeptide in rat myometrial membranes which has some, but not all, of the immunological characteristics associated with  $G_o$  from rat brain. We suggest that this protein represents a novel G-protein which is immunologically related to  $G_o$ . We further demonstrate that myometrial membranes express high levels of  $G_{i2}$ .

## 2. MATERIALS AND METHODS

The sources of all materials used in this study have previously been recorded [14–16,18].

### 2.1. Antibody production and characterization

The antisera used in this study were as follows: antiserum OC1 was raised in a New Zealand white rabbit against a glutaraldehyde conjugate of Keyhole limpet haemocyanin with a synthetic peptide (ANNLRGCGLY) corresponding to the C-terminal decapeptide of the  $\alpha$ -subunit of  $G_o$  [3,4]. Antisera ON1 and IM1 were produced in a similar fashion except that the synthetic peptides were NLKEDGISAAKDVK (amino acids 22–35 of  $G_{o\alpha}$ ) for antiserum IM1 and GCTLSAEERAALERSK

(amino acids 2–17 of  $G_{o\alpha}$ ) for antiserum ON1 [4]. Antiserum RV3 is a polyclonal antiserum raised against partially purified bovine brain  $G_o$ . This antiserum contains populations of antibodies against both  $G_{o\alpha}$  and the  $\beta$ -subunit. Production and characterization of this antiserum has previously been described [11]. Antiserum SG1 is an anti-peptide antiserum produced against the same conjugate as we have previously used to raise antisera AS6 and AS7 [8,9]. These antisera recognize the  $\alpha$ -subunits of the 'G<sub>i</sub>-like' G-proteins,  $G_{i1}$  and  $G_{i2}$  [8,17]. The production of antiserum LE2 has previously been described [8]. This antiserum displays specificity for  $G_{i2}$  [8].

### 2.2. Membrane preparations

Membranes were prepared from the cerebral cortices of rat brain as previously described for other tissues [14,15]. Myometrial membranes were prepared from uteri obtained from estrogen-dominated rats as in [18]. Immunoblotting studies using the various antisera were performed, unless otherwise detailed, using 1:200 dilutions of the primary antisera as previously described in some detail [8,10,16].

## 3. RESULTS

Rat myometrial membranes have previously been shown to express significant levels of (a) per-

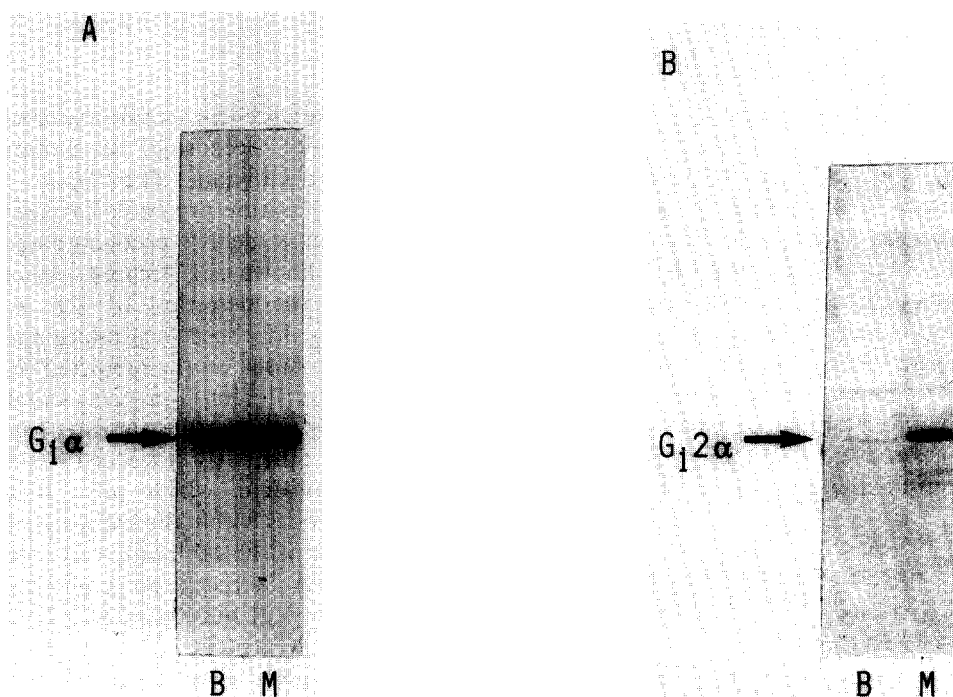


Fig.1. Identification of the major G<sub>i</sub>-like protein of rat myometrial membranes as  $G_{i2}$ . (A) Membranes from either rat brain (B) (100  $\mu$ g) or rat myometrium (M) (100  $\mu$ g) were resolved on an SDS-polyacrylamide gel (10% (w/v) acrylamide). Following electrophoretic transfer to nitrocellulose and blocking with 3% gelatin, the blot was incubated overnight with a 1:200 dilution of antiserum SG1. The immunoblot was developed using *O*-dianisidine following treatment with a horseradish peroxidase-linked donkey anti-rabbit second antibody. (B) A similar experiment to that described in A was performed except that the primary antiserum was a 1:200 dilution of LE2, an antiserum specific for  $G_{i2}$  [8].

tussis toxin-sensitive G-protein(s) of some 40 kDa [18]. However, the molecular identity of this (these) polypeptide(s) has not been established. We first used an antipeptide antiserum (SG1) capable of interacting with the  $\alpha$ -subunits of the  $G_i$ -like proteins,  $G_{i1}$  and  $G_{i2}$  to probe Western blots of both brain and myometrial tissue from rats. Using this antiserum, membranes from rat brain could be shown to express two immunoreactive polypeptides, a more prominent 41 kDa band ( $G_{i1}$ ) and a less prominent polypeptide with slightly greater mobility in the gels ( $G_{i2}$ ) (fig.1A). In contrast, myometrial membranes at this level appeared to contain detectable quantities of only a single form of  $G_i$  (fig.1A). The use of the selective  $G_{i2}$   $\alpha$ -antiserum, LE2, showed, as previously noted, that this polypeptide was present at only low levels in brain [8] but to be present in much higher levels in the myometrial membranes (fig.1B). These ex-

periments thus also confirmed the identity of the rat myometrial  $G_i$  as  $G_{i2}$ .

Experiments on the specificity of antiserum ON1 demonstrated that it recognized a single polypeptide of some 39 kDa in rat brain membranes and that this polypeptide migrated further in the gel than the predominant form of  $G_i$  in brain ( $G_{i1}$ ) as identified with antiserum SG1 (fig.2). Immunoblotting of rat brain membranes with a mixture of antisera SG1 and ON1 further demonstrated that the polypeptides identified by these antisera were mutually exclusive (fig.2) hence confirming that these antisera do not display cross-reactivity. Further experiments with partially purified pertussis toxin-sensitive G-proteins from bovine brain confirmed these observations (results not shown). Similar experiments confirmed the specificity of antiserum OC1 for  $G_o$  (fig.3), whilst mixtures of antisera ON1 and OC1 identified an

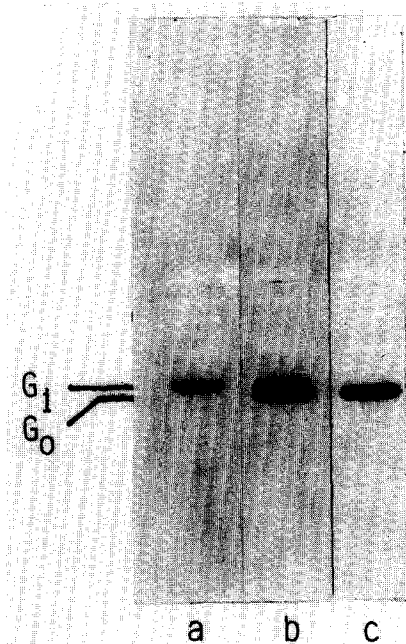


Fig.2. Characterization of the specificity of antiserum ON1. Rat brain membranes (50  $\mu$ g) were resolved as in fig.1 following alkylation of the membranes with *N*-ethyl maleimide as detailed in [21]. The resultant blots were incubated with (a) antiserum SG1 (1:300 dilution), (b) antiserum SG1 (1:300 dilution) + antiserum ON1 (1:1500 dilution), or (c) antiserum ON1 (1:1500 dilution). The immunoblots were then developed as in fig.1A. The two antisera identified mutually exclusive polypeptides.

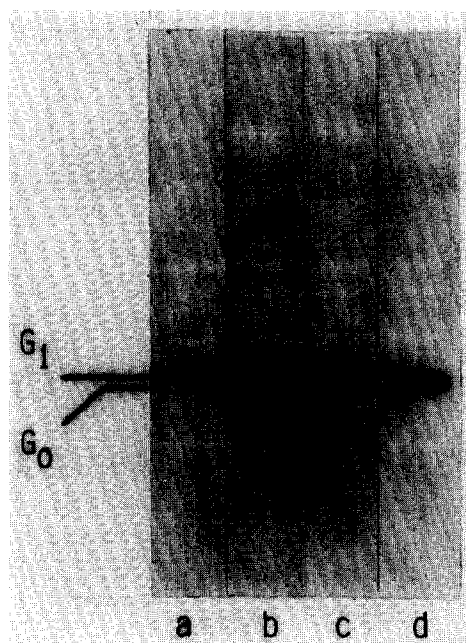


Fig.3. Characterization of the specificity of antiserum OC1. Rat brain membranes (50  $\mu$ g) were resolved following alkylation as described in fig.2 and transferred to nitrocellulose as in fig.1. Following blocking, the samples were treated with (a) antiserum SG1 (1:300 dilution), (b) antiserum SG1 (1:300 dilution) + antiserum OC1 (1:1500 dilution), (c) antiserum OC1 (1:1500 dilution), (d) antiserum OC1 (1:1500 dilution) + antiserum ON1 (1:1500 dilution). The immunoblots were then developed as in fig.1.

apparently single polypeptide in rat brain (fig.3).

Immunoblotting of rat myometrial membranes with both antiserum OC1 and with antiserum ON1 led to the identification of a single polypeptide of some 39 kDa which migrated with similar mobility to  $G_{o\alpha}$  in rat brain (fig.4). In a number of experiments, however, the myometrial protein was noted to have a slightly retarded mobility on SDS-PAGE in comparison to rat brain  $G_{o\alpha}$  (data not shown). In contrast, however, antiserum IM1 did not identify a polypeptide of this size in the myometrial membranes (fig.4). Furthermore, the polyclonal antiserum RV3, which was produced using partially purified, holomeric bovine brain  $G_o$

as antigen [11] equally did not identify the 39 kDa polypeptide in rat myometrial membranes although, as this antiserum also contains a population of antibodies directed against the  $\beta$ -subunit of G-proteins, it did identify a 36 kDa  $\beta$ -subunit in myometrium as well as in the brain membranes (fig.4).

We wished to confirm that the polypeptide in myometrium identified by antisera OC1 and ON1 was not  $G_{i2}$ . We have previously demonstrated that rat glioma C6 BU1 cells express high levels of  $G_{i2}$  and that this is the predominant pertussis toxin-sensitive G-protein present in these cells [19]. We thus immunoblotted membranes of C6 BU1 cells and of rat brain, with each of antisera SG1, OC1 or ON1, under SDS-PAGE conditions in

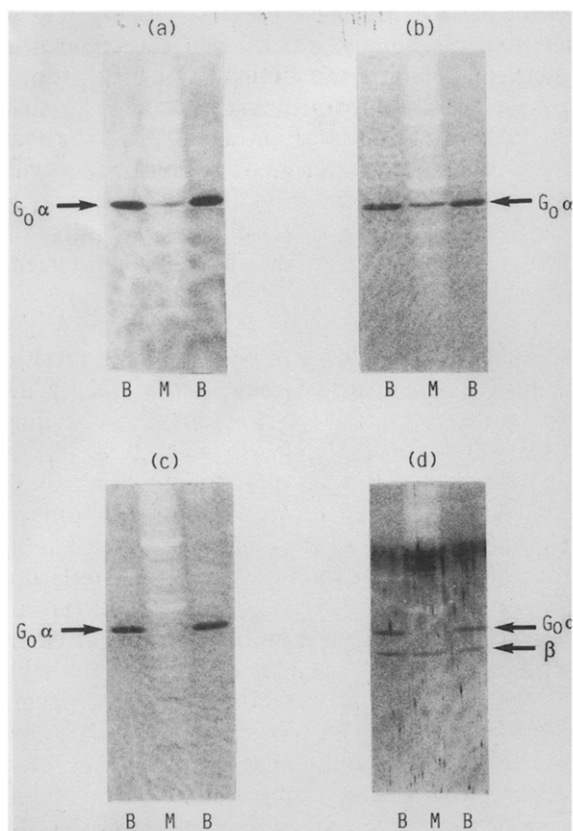


Fig.4. Immunological analysis of a form of  $G_o$  in rat myometrial membranes. Rat brain (B) (50  $\mu$ g) and rat myometrial (M) membranes (200  $\mu$ g) were resolved as in fig.1 and immunoblotted with 1:200 dilutions of a range of antipeptide (a-c) or polyclonal (d) antisera against  $G_o$ . Whilst each of the antisera, (a) ON1, (b) OC1, (c) IM1 and (d) RV3 identified a 39 kDa polypeptide in the rat brain membranes, only ON1 and OC1 identified a polypeptide with similar, if slightly reduced, mobility in rat myometrial membranes.

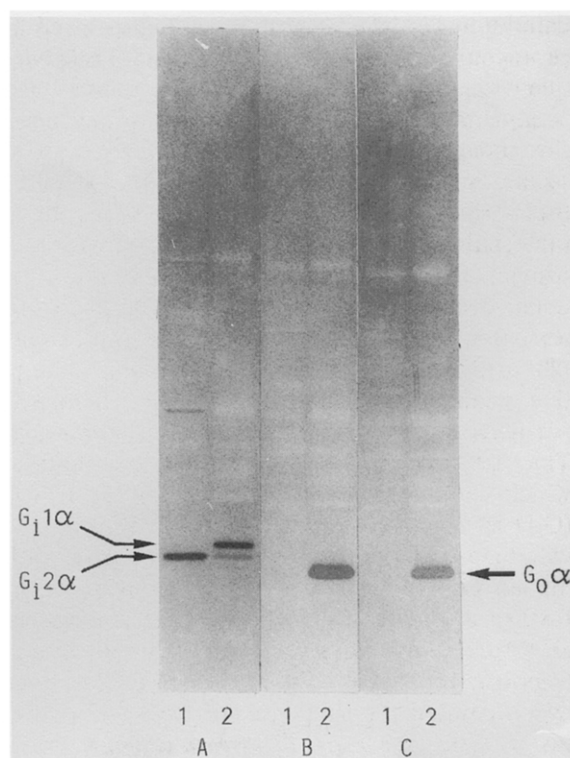


Fig.5. Antisera ON1 and OC1 do not identify  $G_{i2}$ . Membranes of rat glioma C6 BU1 cells (50  $\mu$ g) (1) and rat brain (50  $\mu$ g) (2) were resolved on SDS-PAGE slab gels (12.5% acrylamide, 0.15% bisacrylamide) as described in fig.3 and immunoblotted with (A) antiserum SG1 (1:200 dilution), (B) antiserum ON1 (1:1000 dilution), (C) antiserum OC1 (1:1000 dilution). Whilst antiserum SG1 identified  $G_{i2}$  in C6 membranes and both  $G_{i1}$  and  $G_{i2}$  in brain, both OC1 and ON1 identified only  $G_o$  in brain and did not identify a polypeptide with mobility equivalent to  $G_{i2}$  in the C6 membranes.

which good resolution of  $G_{i1}$ ,  $G_{i2}$  and  $G_o$  can be achieved (12.5% acrylamide, 0.15% bisacrylamide). Antiserum SG1 identified a single polypeptide of 40 kDa in C6 membranes ( $G_{i2}$ ), but two polypeptides in rat brain, a 40 kDa polypeptide which comigrated with  $G_{i2}$  from C6 cells and a more prevalent polypeptide of 41 kDa ( $G_{i1}$ ) (fig.5). Both antisera OC1 and ON1 identified a single band of 39 kDa in brain but failed to identify a 40 kDa polypeptide in the C6 membranes. As such, these antisera do not cross-react with  $G_{i2}$  and thus the polypeptide identified by these antisera in rat myometrium is not  $G_{i2}$ .

#### 4. DISCUSSION

The observation that pertussis toxin was able to catalyse ADP-ribosylation of the inhibitory G-protein of the adenylate cyclase cascade and consequently attenuate receptor-mediated inhibition of adenylate cyclase [20] provided a major advance in the understanding of hormonal regulation of second messenger generation and also provided a tool with which to identify  $G_i$ . However, with the purification of two pertussis toxin-sensitive G-proteins from bovine brain [21,22], and the subsequent demonstrations that these proteins were at least immunologically [22,23], if not functionally [24] distinct, it became apparent that a more selective means of assessing the molecular identity of pertussis toxin-sensitive G-proteins was required. The further demonstration that neither antisera which selectively identified the major form of  $G_i$  ( $G_{i1}$ ) or  $G_o$  from brain were able to cross-react with the major pertussis toxin-sensitive substrate in human neutrophils [25] or in rat glioma cells [26] further added to the complexity of the system. cDNA cloning studies have now identified at least 6 potential G-protein  $\alpha$ -subunit gene products which contain the characteristic signature of pertussis toxin substrates, that is, a cysteine residue located 4 amino acids from the C-terminus [4]. These products are rod and cone specific forms of transducin, 3 forms of  $G_i$  ( $G_{i1}$ ,  $G_{i2}$ ,  $G_{i3}$ ) and  $G_o$  [4]. There is no a priori reason to believe that this should represent the entire family of these proteins and more may yet be identified.

The data reported herein demonstrate the presence of forms of both  $G_i$  and  $G_o$  in the rat myometrium. In contrast to rat brain, where two

distinct polypeptides ( $G_{i1}$ ,  $G_{i2}$ ) can be identified with a 'pan- $G_i$ ' antiserum (SG1), we were able to detect considerable amounts of an apparently single immunoreactive polypeptide in rat myometrium. This polypeptide was further identified with the  $G_{i2}$  specific antiserum LE2 [8]. In this study we further demonstrate that  $G_o$  may not represent a homogeneous population. This conclusion is based on the immunoreactivity of a series of antipeptide antisera which we have generated based on the predicted primary sequence of  $G_o$  as derived from cDNA sequences for rat  $G_o$  reported by both Itoh et al. [3] and Jones and Reed [4]. Two of these antipeptide antisera, directed against either the extreme C-terminus (OC1) or extreme N-terminus (ON1) of the  $\alpha$ -subunit of  $G_o$  identified a single polypeptide of some 39 kDa in rat myometrial membranes which had similar, if slightly reduced mobility, in SDS-polyacrylamide gels in comparison with  $G_o$  of rat brain. However, a third antipeptide  $G_o$  antiserum (IM1), whilst identifying the same polypeptide in rat brain membranes as antisera OC1 and ON1 did not identify the polypeptide present in the rat myometrial membranes.

Intriguingly, a polyclonal antiserum (RV3) [11] raised against a mixture of pertussis toxin-sensitive G-proteins isolated from bovine brain [22], which we have previously characterised to contain populations of antibodies against both the  $\alpha$ -subunit of  $G_o$  and against the  $\beta$ -subunit of G-proteins but not against the  $\alpha$ -subunits of forms of  $G_i$ , did not identify the  $G_o$  in rat myometrium but did identify the  $\beta$ -subunit. This is not a reflection of species diversity in  $G_o$  as this polyclonal antiserum did identify rat brain  $G_o$ . It might be anticipated that a polyclonal antiserum should possess populations of antibodies directed against different regions of a protein and as such should have at least some antibodies directed against areas in common if the forms of  $G_o$  in brain and myometrium were markedly similar. However, this need not be so. We have previously characterised a polyclonal antiserum to rod transducin (CW6) [23] which cross-reacts with the major form of brain  $G_i$  ( $G_{i1}$ ), which on epitope mapping appeared to be directed against an epitope(s) within a very limited region of the protein [23,27]. This polyclonal antiserum shows little or no reactivity against the  $\alpha$ -subunit of  $G_{i2}$  [25,26] despite the fact

that G<sub>i1</sub> and G<sub>i2</sub> are some 88% homologous at the primary sequence level. In this particular case one region (epitope) of the protein appeared to be immunodominant. This may be a reflection that the major areas of sequence diversity in the primary sequences of these two proteins are clustered and may represent areas (domains) involved in the contact of the G-protein with receptors and effector systems. As such, these areas must be exposed at the surface of the protein and hence potentially available to be recognized by the immune system. Whilst antiserum RV3 has not been epitope mapped in the same fashion, similar arguments may well apply. We have also noted that antiserum RV3 shows little or no immunoreactivity towards G<sub>o</sub> in membranes of rat white adipocytes ([16] and unpublished observations), whilst antiserum OC1 identifies a 40 kDa polypeptide in this tissue (unpublished observations). It is thus likely that this novel form of G<sub>o</sub> is not limited in distribution to the myometrium.

In the light of the number of identified cDNA clones which presumptively code for pertussis toxin-sensitive G-proteins [4], it is of further interest to note that two mRNAs which hybridize with a G<sub>o</sub> probe have been identified [4]. Further, it has been possible to purify two separate forms of G<sub>o</sub> from bovine brain [28]. Based on immunological characterization of each of these forms, however, it appears that they may represent no more than post-translationally modified forms of one another [28].

The observed complexity of the family of pertussis toxin-sensitive G-proteins highlights the need for further selective means for their molecular identification. The burgeoning numbers of identified G-proteins also implies that detailed biochemical analysis must be performed before specific functions can be attributed to the individual forms.

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## REFERENCES

- [1] Spiegel, A.M. (1987) *Mol. Cell. Endocrinol.* 49, 1–16.
- [2] Gilman, A.G. (1987) *Annu. Rev. Biochem.* 56, 615–649.
- [3] Itoh, H., Kozawa, T., Nagata, S., Nakamura, S., Katada, T., Ui, M., Iwai, S., Ohtsuka, E., Kawasaki, H., Suzuki, K. and Kaziro, Y. (1986) *Proc. Natl. Acad. Sci. USA* 83, 3776–3780.
- [4] Jones, D.T. and Reed, R.R. (1987) *J. Biol. Chem.* 262, 14241–14249.
- [5] Bray, P., Carter, A., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. and Nirenberg, M. (1987) *Proc. Natl. Acad. Sci. USA* 84, 5115–5119.
- [6] Didsbury, J.R., Ho, Y.-S. and Snyderman, R. (1987) *FEBS Lett.* 211, 160–164.
- [7] Mumby, S.M., Kahn, R.A., Manning, D.R. and Gilman, A.G. (1986) *Proc. Natl. Acad. Sci. USA* 83, 265–269.
- [8] Goldsmith, P., Gierschik, P., Milligan, G., Unson, C., Vinitzky, R., Malech, H. and Spiegel, A. (1987) *J. Biol. Chem.* 262, 14683–14688.
- [9] Gawler, D., Milligan, G., Spiegel, A.M., Unson, C.G. and Houslay, M.D. (1987) *Nature* 327, 229–232.
- [10] McArdle, H., Mullaney, I., Magee, A., Unson, C. and Milligan, G. (1988) *Biochem. Biophys. Res. Commun.* 152, 243–251.
- [11] Gierschik, P., Milligan, G., Pines, M., Goldsmith, P., Codina, J., Klee, W. and Spiegel, A. (1986) *Proc. Natl. Acad. Sci. USA* 83, 2258–2262.
- [12] Huff, R.M., Axton, J.M. and Neer, E.J. (1985) *J. Biol. Chem.* 260, 10864–10871.
- [13] Hescheler, J., Rosenthal, W., Trautwein, W. and Schultz, G. (1987) *Nature* 325, 445–447.
- [14] McKenzie, F.R., Kelly, E.C.H., Unson, C.G., Spiegel, A.M. and Milligan, G. (1988) *Biochem. J.* 249, 653–659.
- [15] Milligan, G. (1987) *Biochem. J.* 245, 501–505.
- [16] Milligan, G., Spiegel, A., Unson, C.G. and Saggerson, E.D. (1987) *Biochem. J.* 247, 223–227.
- [17] Goldsmith, P., Rossiter, K., Carter, A., Simonds, W., Unson, C.G., Vinitzky, R. and Spiegel, A.M. (1988) *J. Biol. Chem.* 263, 6476–6479.
- [18] Tanfin, Z. and Harbon, S. (1987) *Mol. Pharmacol.* 32, 249–257.
- [19] Milligan, G., Mullaney, I., Unson, C.G., Marshall, L., Spiegel, A.M. and McArdle, H. (1988) *Biochem. J.* 254, 391–396.
- [20] Katada, T. and Ui, M. (1981) *J. Biol. Chem.* 256, 8310–8317.
- [21] Sternweis, P.C. and Robishaw, J.D. (1984) *J. Biol. Chem.* 259, 13806–13813.
- [22] Milligan, G. and Klee, W.A. (1985) *J. Biol. Chem.* 260, 2057–2063.
- [23] Pines, M., Gierschik, P., Milligan, G., Klee, W. and Spiegel, A. (1985) *Proc. Natl. Acad. Sci. USA* 82, 4095–4099.
- [24] Florio, V.A. and Sternweis, P.C. (1985) *J. Biol. Chem.* 260, 3477–3483.
- [25] Gierschik, P., Falloon, J., Milligan, G., Pines, M., Gallin, J. and Spiegel, A. (1986) *J. Biol. Chem.* 261, 8058–8062.
- [26] Milligan, G., Gierschik, P., Spiegel, A.M. and Klee, W.A. (1986) *FEBS Lett.* 195, 225–230.
- [27] Milligan, G., Gierschik, P. and Spiegel, A.M. (1987) *Biochem. Soc. Trans.* 15, 42–45.
- [28] Goldsmith, P., Backlund, P.S., jr, Rossiter, K., Carter, A., Milligan, G., Unson, C.G. and Spiegel, A. (1988) *Biochemistry* 27, 7085–7090.