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# Persistent activation of the $\alpha$ subunit of $G_s$ promotes its removal from the plasma membrane

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As assessed both by cholera-toxin-catalysed ADP-ribosylation and by immunoblotting with an anti-peptide antiserum raised against the *C*-terminal decapeptide of forms of $G_s\alpha$ (the $\alpha$ subunit of the stimulatory guanine nucleotide-binding protein), rat glioma C6 BU1 cells express two forms of $G_s\alpha$: a major 44 kDa form and a much less prevalent 42 kDa form. We examined the effects of guanine nucleotides on the interaction of the 44 kDa form with the plasma membrane. Incubation of membranes of C6 BU1 cells with poorly hydrolysed analogues of GTP, but not with analogues of either ATP or GDP, caused the release of this $G_s\alpha$ from the membrane fraction. Release of $G_s\alpha$ was observed within 5 min, and continued throughout the incubation period. After treatment with guanosine 5'-[$\beta\gamma$-imido]triphosphate for 60 min, some 75% of this polypeptide had been released from its site of membrane attachment. These experiments demonstrate that $G_s\alpha$ need not remain associated invariantly with the plasma membrane.

## INTRODUCTION

Adenylate cyclase can be regulated in both positive and negative fashion by hormones and neurotransmitters. In each case, receptor control of cyclic AMP production is mediated via a G-protein, $G_s$ for the stimulation of adenylate cyclase activity and $G_i$ for inhibition [1,2]. Each of these G-proteins is a heterotrimer in which distinct $\alpha$ subunits share a common population of $\beta/\gamma$ subunits. The traditional means of identifying these G-proteins has relied on the ability of cholera toxin to catalyse the transfer of [$^{32}$P]ADP-ribose from [$^{32}$P]-$NAD^+$ to $G_s\alpha$, and of pertussis toxin to catalyse an analogous ADP-ribosylation of $G_i\alpha$ [3,4]. Limitations on these procedures, based either, for cholera toxin, on the requirement for a proteinaceous cofactor (ARF) [5] or, for pertussis toxin, on the identification of a multiplicity of substrates for the toxin [6,7] have led to the generation of both polyclonal and anti-peptide antisera able to identify specifically a number of different G-proteins [8–11]. Here we use an anti-peptide antiserum, directed against the *C*-terminal decapeptide of $G_s\alpha$, to demonstrate that persistent activation of $G_s$ by analogues of GTP can lead to the release of $G_s\alpha$ from its attachment to the plasma membrane, as had originally been proposed by Rodbell [12].

## MATERIALS AND METHODS

### Cell growth

Rat glioma C6 BU1 cells were grown in tissue culture as previously described [13]. Just before confluency they were either split 1:10 into fresh tissue-culture flasks or harvested. Crude membrane preparations were produced from cell pastes [13] and stored at −80 °C until use. The human glioma cell line G328 was grown and utilized by identical protocols.

### Production of antisera

Antiserum CS1 was produced in a New Zealand White rabbit against a synthetic peptide (RMHLRQYELL), which corresponds to the *C*-terminal decapeptide of $G_s\alpha$. This peptide was coupled to keyhole-limpet haemocyanin with glutaraldehyde before immunization, as previously recorded [10]. Specificity of this antiserum for $G_s\alpha$ was confirmed by immunoblotting SDS/PAGE-resolved membranes of C6 BU1 cells which had been treated with thiol-activated cholera toxin and [$^{32}$P]$NAD^+$ as described below. This procedure identified polypeptides of 44 and 42 kDa (see the Results section). Autoradiography of the developed immunoblot also identified 44 and 42 kDa polypeptides which were labelled only in the presence of cholera toxin. Overlay of the autoradiograph and the immunoblot demonstrated that the polypeptides identified by these two procedures migrated identically. Immunoblotting with this antiserum was performed by a previously described protocol [14].

Antiserum SG2 was produced in a similar fashion against the *C*-terminal decapeptide of rod transducin (KENLKDCGLF). This antiserum identifies forms of $G_i$ as well as transducin, as we have previously described for other antisera raised against this conjugate [9,11]. As transducin is limited in distribution to photoreceptor-containing tissues, this antiserum can be used as a probe for forms of $G_i$ in other tissues [9–11].

For molecular-mass estimations on immunoblots, prestained molecular-mass markers (Bethesda Research

Abbreviations used: G-protein, guanine nucleotide-binding protein; $G_s$ and $G_i$, the G-proteins associated with stimulation and inhibition, respectively, of adenylate cyclase; $G_s\alpha$ and $G_i\alpha$, their $\alpha$ subunit; PAGE, polyacrylamide-gel electrophoresis; p[NH]ppG, guanosine 5'-[$\beta\gamma$-imido]triphosphate; GDP[S], guanosine 5'-[$\beta$-thio]diphosphate; GTP[S], guanosine 5'-[$\gamma$-thio]triphosphate.

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Laboratories) were subjected to SDS/PAGE (10% acrylamide) in parallel with test samples. Immunoblotting with these antisera was performed as previously described [14].

### Release assays

These were performed essentially as described by McArdle *et al.* [15]. Membranes of C6 BU1 cells were incubated for various times at 37 °C in a buffer containing 20 mM-Tris/HCl, pH 7.5, 20 mM-$MgCl_2$, 1 mM-dithiothreitol and 100 $\mu$M-EDTA. The assays also contained various nucleotides (100 $\mu$M, unless otherwise noted) (Boehringer–Mannheim). At the end of incubation, soyabean trypsin inhibitor (25 $\mu$g) was added as a soluble protein carrier and samples were separated into supernatant and the membrane-associated fractions by centrifugation at 25 lb/in$^2$ (approx. 150000 *g*) for 2 min in a TL100 (30°) rotor in an Airfuge (Beckman Instruments). Protein in the supernatant fractions was collected by deoxycholate/trichloroacetic acid precipitation [7] before neutralization and addition of sample buffer. The residual membrane pellets were solubilized with sample buffer and used directly for SDS/PAGE.

### Miscellaneous

Cholera-toxin-catalysed ADP-ribosylation was performed in the presence of exogenous GTP as previously described [16]. Protein concentration was assessed by the method of Lowry *et al.* [17], with bovine serum albumin as standard.

## RESULTS

When [$^{32}$P]ADP-ribosylation of membranes of rat glioma C6 BU1 cells was performed in the presence of exogenously added GTP, then two polypeptides were specifically labelled in response to the presence of cholera toxin. These were a major polypeptide of 44 kDa and a much less prevalent component of 42 kDa (Fig. 1*a*). Immunoblotting of the same membranes with antiserum CS1, which was raised against a synthetic peptide corresponding to the *C*-terminal decapeptide of $G_s\alpha$, equally identified both 44 kDa and 42 kDa polypeptides (Fig. 1*b*). The relative intensity of staining of these two polypeptides indicated a similar ratio of the 44 to 42 kDa forms as did the incorporation of [$^{32}$P]ADP-ribose catalysed by cholera toxin. Owing to the relative abundances of the forms, we concentrated only on the 44 kDa form of $G_s\alpha$ ($G_s\alpha$ 44) in subsequent experiments.

Membranes of C6 BU1 cells were incubated with p[NH]ppG or with GDP[S] (each 100 $\mu$M) for 60 min and resolved into membrane-associated and supernatant fractions, which were resolved by SDS/PAGE and then immunoblotted with antiserum CS1. In the presence of GDP[S], all of the $G_s\alpha$ 44 remained in intimate association with the membrane (Fig. 2). However, after

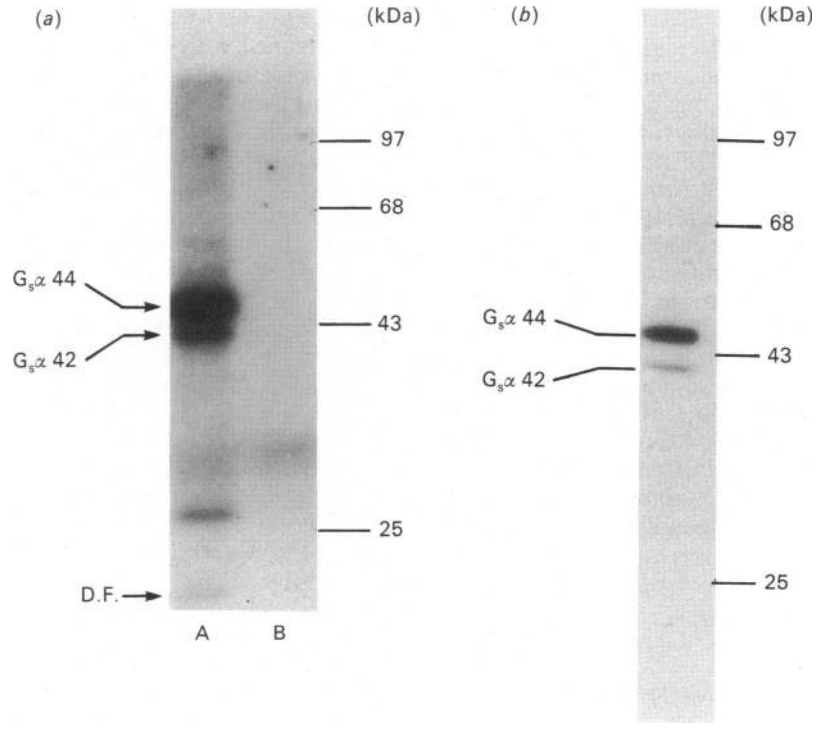


**Fig. 1. Identification of forms of $G_s\alpha$ in membranes of C6 BU1 cells**

(*a*) Cholera-toxin-catalysed ADP-ribosylation of C6 BU1 membranes (20 $\mu$g) was performed in the presence of GTP [16]. Samples were resolved on SDS/PAGE (10% acrylamide) and the dried gels autoradiographed by using Kodak X-O-Mat X-ray film. The third polypeptide containing incorporated radioactivity in the presence of cholera toxin is the A subunit of the toxin itself. Abbreviation D.F., dye front. Lanes: A, in the presence of cholera toxin; B, in the absence of cholera toxin. (*b*) Immunoblotting of membranes of C6 BU1 cells (100 $\mu$g), with a 1:200 dilution of antiserum CS1 as primary antibody. Positions of molecular-mass markers are indicated to the right of the gels.

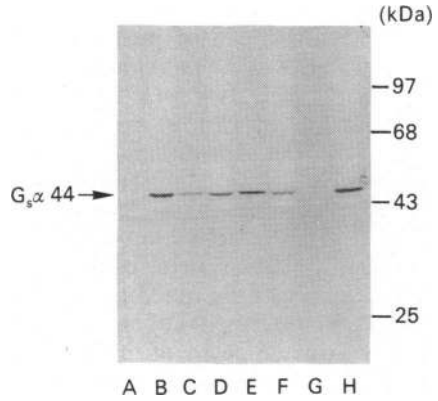


**Fig. 2. Nucleotide specificity of the release of $G_s\alpha$ 44**

Membranes of C6 BU1 cells (100 $\mu$g) were incubated for 60 min at 37 °C with p[NH]ppA (A, B), GTP[S] (C, D), p[NH]ppG (E, F) or GDP[S] (G, H) (all 100 $\mu$M). Samples were separated into membrane (B, D, F, H) and supernatant (A, C, E, G) fractions, resolved by SDS/PAGE and immunoblotted with a 1:200 dilution of antiserum CS1 as primary reagent.

incubation of the membranes with p[NH]ppG, then some 75% of the immunoreactive $G_s\alpha$ 44 was found in the supernatant fraction (Fig. 2). This transfer of $G_s\alpha$ 44 could be noted both by the disappearance of immunoreactivity from the membrane pellet and by its appearance in the supernatant. Incubation of C6 BU1 membranes with a second analogue of GTP, GTP[S], produced qualitatively similar results to those with p[NH]ppG (Fig. 2). However, treatment of the membranes with the adenosine analogue p[NH]ppA produced no release of $G_s\alpha$ 44 from the membrane fraction (Fig. 2). Time courses indicated that the release of $G_s\alpha$ 44 in response to p[NH]ppG was an essentially linear process (Fig. 3). $G_s\alpha$ 44 could be detected in the supernatant fraction at the shortest time point assessed (5 min). The membrane-associated $G_s\alpha$ decreased throughout the time course in parallel with its appearance

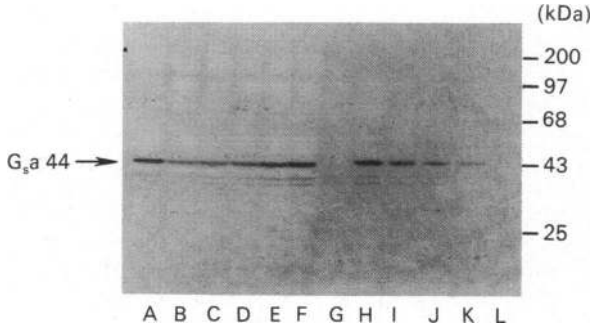


**Fig. 3. Time course of the release of $G_s\alpha$ 44 on incubation with p[NH]ppG**

Membranes of C6 BU1 cells (100 $\mu$g) were incubated with either GDP[S] (100 $\mu$M) for 60 min (A, G) or with p[NH]ppG (100 $\mu$M) for 60 (B, H), 30 (C, I), 15 (D, J), 5 (E, K) or 0 (F, L) min at 37 °C. Samples were separated into membrane-associated (A–F) and supernatant (G–L) fractions, resolved by SDS/PAGE and immunoblotted with antiserum CS1.

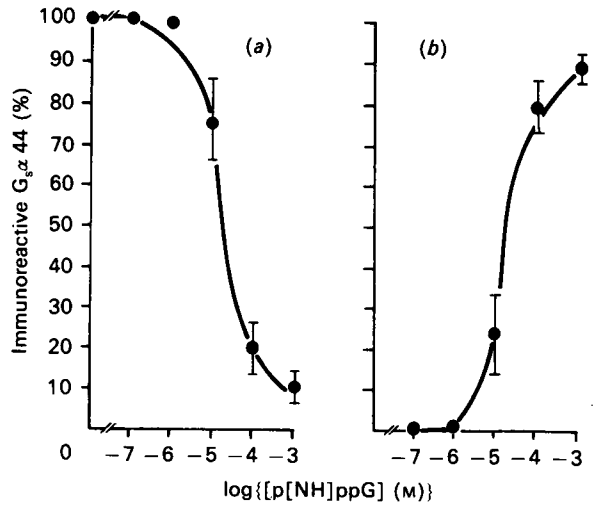


**Fig. 4. Release of $G_s\alpha$ 44 from membranes of C6 BU1 cells by p[NH]ppG: concentration-dependence**

Membranes of C6 BU1 cells (100 $\mu$g) were incubated as described in the Materials and methods section with various concentrations of p[NH]ppG for 60 min at 37 °C. Samples were separated into supernatant and particulate fractions and resolved by SDS/PAGE. After immunoblotting with antiserum CS1 and development, a film positive of the blot was scanned on a Bio-Rad densitometer as described in [26]. (*a*) Immunoreactive $G_s\alpha$ 44 associated with the membrane fraction. (*b*) $G_s\alpha$ 44 present in the supernatant. Results are means ± S.E.M. for three separate experiments.

in the supernatant (Fig. 3). The membrane-associated and soluble forms of $G_s\alpha$ essentially co-migrated through SDS/PAGE, indicating that the release process was not likely to be a reflection of proteolytic cleavage [18].

Release of $G_s\alpha$ from the plasma membrane in response to analogues of GTP was not restricted to C6 BU1 cells. A similar profile of nucleotide sensitivity was noted both for the human glioma cell line G328 and for the rat skeletal myoblast cell line L6 (results not shown). Both of these cell lines expressed each of the 44 and 42 kDa forms of $G_s\alpha$. These two forms were present in similar abundances and relative ratios in both G328 and L6 cells as they were in the C6 BU1 cells (results not shown).

Relatively high concentrations of p[NH]ppG were required to stimulate release of $G_s\alpha$ 44 from the membrane. All of the $G_s\alpha$ 44 remained associated with the membrane after a 60 min incubation with 1 $\mu$M-p[NH]-ppG, but some release was noted with 10 $\mu$M nucleotide. Over this incubation period of maximum of 70–90% of the $G_s\alpha$ could be released from the membrane in the presence of 0.1–1 mM-p[NH]ppG (Fig. 4). Simultaneous determination of the release of both $G_i\alpha$ and $G_s\alpha$ from membranes of C6 BU1 cells, in response to various concentrations of p[NH]ppG, as assessed by mixing antisera CS1 and SG2 (an anti-peptide antiserum which identifies the extreme *C*-terminus of forms of $G_i\alpha$) demonstrated that release of $G_i\alpha$ occurred at 50–100-fold lower concentrations of this nucleotide than did release of $G_s\alpha$ (Fig. 5).

In contrast with the release of $G_s\alpha$ 44 stimulated by the analogues of GTP (some 75% of the immunoreactive polypeptide in 60 min), GTP was much less effective in

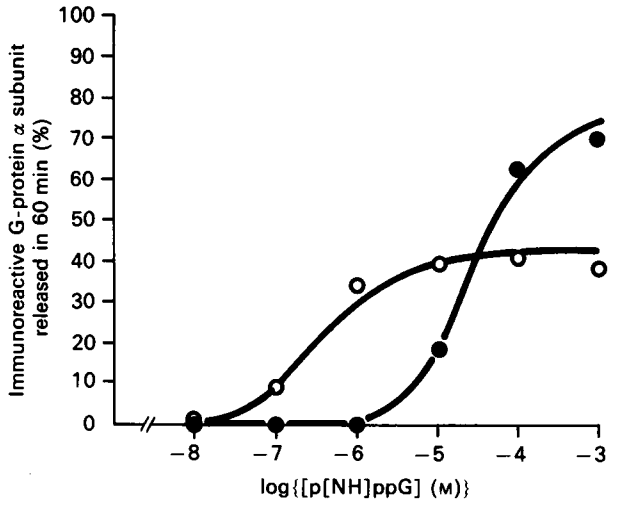


**Fig. 5. Differential ability of p[NH]ppG to promote the release of $G_s\alpha$ 44 and $G_i2\alpha$ from membranes of C6 BU1 cells**

Membranes of C6 BU1 cells (100 $\mu$g) were incubated as described in Fig. 4 for 60 min at 37 °C. Immunoblots of the fractions were performed as in Fig. 4, except that a mixture of antisera CS1 and SG2 (each at 1:200 dilution) was used as primary reagent. A photographic positive was produced from the immunoblot and scanned (described in detail in [26]). The Figure shows the amount of immunoreactive $G_s\alpha$ 44 (●) and $G_i2\alpha$ (○) present in the supernatant fractions as compared with the equivalent polypeptide present in the membrane fraction when the incubation was performed in the absence of p[NH]ppG. Data are derived from a single experiment, but similar results were produced in two other experiments.

this assay (less than 10% release), although the addition of a nucleotide-regeneration system based on phosphocreatine and creatine kinase increased the efficiency of release in response to added GTP (up to 30% release in 60 min) (see also [15]). The regenerating system produced no release of $G_s\alpha$ 44 in the absence of GTP, nor did it increase the amount of release produced by either p[NH]ppG or GTP[S].

## DISCUSSION

G-proteins are located at the cytoplasmic face of the plasma membrane and allow communication between agonist-activated cell surface receptors and effector systems such as those which generate intracellular second messengers [1,2].

In 1985, Rodbell proposed that activation of the $\alpha$ subunit of the stimulatory G-protein of the adenylate cyclase cascade ($G_s$) could lead to the release of this polypeptide from its site of membrane attachment [12]. His hypothesis was based upon the observation that co-incubation of human erythrocyte ghosts (which contain $G_s$) with membranes of the $cyc^-$ varient of the S49 lymphoma cell line (which do not express this polypeptide) led to the functional transfer of $G_s$ activity to the lymphoma membranes. Further approaches to assess the nature of the attachment of the $\alpha$ subunits of G-proteins to the plasma membrane of cells have been limited by the lack of appropriate techniques. This problem has recently been overcome by the generation of antisera directed against synthetic peptides, which, based on analysis of cDNA clones, can be predicted to form part of the primary structure of the individual G-proteins [8–11]. We have recently used such selective antisera to investigate the interaction of various pertusssis-toxin-sensitive G-proteins with the plasma membrane [15,18]. Here we demonstrate that, for $G_s$, the basic requirement for Rodbell's theory of G-proteins as 'programmable messengers' is tenable. That is, activation of $G_s$ causes a release of the $\alpha$ subunit of this protein from the plasma membrane. This process was only noted in the presence of analogues of GTP. Analogues of either ATP or GDP were inactive in this assay. Release of immunoreactive $G_s\alpha$ was not immediate, however. Although detectable amounts of this polypeptide could be monitored within 5 min of treatment with p[NH]ppG, the release process proceeded in an essentially linear fashion with time, some 75% being released within 60 min when 100 $\mu$M-p[NH]ppG was used as the activator. Release was also dependent on the concentration of activator employed and could only be noted at concentrations of p[NH]ppG above 1 $\mu$M, a maximal effect being achieved between 0.1 and 1 mM nucleotide. Although these are relatively high concentrations of the nucleotide, it is known that p[NH]ppG has markedly lower affinity for $G_s$ than for $G_i$, a property which is often utilized to activate selectively the inhibitory arm of the adenylate cyclase cascade [19]. GTP was only very weakly active in the release assay. This presumably reflects the hydrolysis of GTP to GDP, as inclusion of a nucleotide-regenerating system increased the effect of GTP. As the rate-limiting step of G-protein activation and deactivation appears to be the release of bound GDP from the nucleotide-binding site [1], then the addition of GTP will imply that the nucleotide-binding site will be filled with GDP for most of the time, as bound GTP will quickly be hydrolysed by the intrinsic GTPase activity of the G-protein.

Why the release of $G_s\alpha$, after activation with p[NH]ppG, was not immediate is unclear. No evidence has been produced to indicate the covalent attachment of myristic acid to this polypeptide, as has recently been noted for both $G_i\alpha$ and $G_o\alpha$ [20]. However, $G_s\alpha$ appears to be *N*-terminally blocked, as it is resistant to Edman degradation [21]. It may be that other fatty acyl chains are important in the anchorage of $G_s\alpha$ to the plasma membrane. Indeed it is known that the P21 product of the *ras* oncogene contains covalently attached palmitate [22] and that the integrity of this lipid is essential for the interaction of P21 with the plasma membrane [23]. As such, dissociation of $G_s\alpha$ from the $\beta/\gamma$ subunits, which remain in the membrane fraction after treatment with analogues of GTP [24], may not be sufficient in itself to cause release. In each of the cell lines studied in the present work, two separate forms of $G_s\alpha$ were identified, both by the use of cholera-toxin-catalysed ADP-ribosylation and by the use of the anti-peptide antiserum CS1. We have, however, focused exclusively on the more prevalent form, as sensitivity of the assays has limited our ability to detect the 42 kDa form adequately. Individual forms of $G_s\alpha$ can be generated by differential splicing of the pre-mRNA transcribed from a single gene [25]. In all of the individual forms, however, the *C*-terminal decapeptide (which constitutes the synthetic peptide used as antigen to generate antiserum CS1) is conserved. As such this antiserum should identify all of the isotypes equally.

It remains to be ascertained whether release of the $\alpha$ subunits of G-proteins, in response to activation, can occur within a whole cell rather than in the membrane fractions which we have employed in this study. Further, for the theory of G-proteins as 'programmable messengers' to have credence, it would be necessary to demonstrate the release of the $\alpha$ subunits of the G-proteins after activation of relevant hormone receptors on the cell surface. Experiments to assess these points will provide a major challenge.

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## REFERENCES

1. Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
2. Spiegel, A. M. (1987) Mol. Cell. Endocrinol. **49**, 1–16
3. Milligan, G., Simonds, W. F., Streaty, R. A., Tocque, B. & Klee, W. A. (1985) Biochem. Soc. Trans. **13**, 1110–1113
4. Katada, T. & Ui, M. (1982) Proc. Natl. Acad. Sci. U.S.A. **79**, 3129–3133
5. Kahn, R. A., Goddard, C. & Newkirk, M. (1988) J. Biol. Chem. **263**, 8282–8287
6. Sternweis, P. C. & Robishaw, J. D. (1984). J. Biol. Chem. **259**, 13806–13813
7. Milligan, G. & Klee, W. A. (1985) J. Biol. Chem. **260**, 2057–2063
8. Mumby, S. M., Kahn, R. A., Manning, D. R. & Gilman, A. G. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 265–269
9. Backlund, P. S., Jr., Aksamit, R. R., Unson, C. G., Goldsmith, P., Spiegel, A. M. & Milligan, G. (1988) Biochemistry **27**, 2040–2046
10. Goldsmith, P., Gierschik, P., Milligan, G., Unson, C. G., Vinitsky, R., Malech, H. & Spiegel, A. (1987) J. Biol. Chem. **262**, 14683–14688
11. McKenzie, F. R., Kelly, E. C. H., Unson, C. G., Spiegel, A. M. & Milligan, G. (1988) Biochem. J. **249**, 653–659
12. Rodbell, M. (1985) Trends Biochem. Sci. **10**, 461–464
13. Milligan, G. (1987) Biochem. J. **245**, 501–505
14. Gierschik, P., Milligan, G., Pines, M., Goldsmith, P., Codina, J., Klee, W. A. & Spiegel, A. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2258–2262
15. McArdle, H., Mullaney, I., Magee, A., Unson, C. & Milligan, G. (1988) Biochem. Biophys. Res. Commun. **152**, 243–251
16. Milligan, G. (1989) Cell. Signalling **1**, 65–74
17. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
18. Eide, B., Gierschik, P., Milligan, G., Mullaney, I., Unson, C., Goldsmith, P. & Spiegel, A. (1987) Biochem. Biophys. Res. Commun. **148**, 1398–1405
19. Gawler, D., Milligan, G., Spiegel, A. M., Unson, C. G. & Houslay, M. D. (1987) Nature (London) **327**, 229–232
20. Buss, J. E., Mumby, S. M., Casey, P. J., Gilman, A. G. & Sefton, B. M. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 7493–7497
21. Schultz, A. M., Tsai, S.-C., Fung, H.-F., Oroszlan, S., Moss, J. & Vaughan, M. (1987) Biochem. Biophys. Res. Commun. **146**, 1234–1239
22. Magee, A. I., Gutierrez, L., McKay, I. A., Marshall, C. J. & Hall, A. (1987) EMBO J. **6**, 3353–3357
23. Hancock, J. F., Marshall, C. J., McKay, I. A., Gardner, S., Houslay, M. D., Hall, A. & Wakelam, M. J. O. (1988) Oncogene **3**, 187–193
24. Milligan, G., Mullaney, I., Unson, C. G., Marshall, L., Spiegel, A. M. & McArdle, H. (1988) Biochem. J. **254**, 391–396
25. Bray, P., Carter, A., Simons, C., Guo, V., Puckett, C., Kamholz, J., Spiegel, A. & Nirenberg, M. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8893–8897
26. O'Brien, R. M., Houslay, M. D., Milligan, G. & Siddle, K. (1987). FEBS Lett. **212**, 281–288

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