Tissue expression and phylogenetic appearance of the β and γ subunits of GTP binding proteins

Y. Audigier, C. Pantaloni, J. Bigay+, P. Deterre+, J. Bockaert and V. Homburger*

Centre CNRS-INSERM de Pharmacologie-Endocrinologie, Rue de la Cardonille, B.P. 5055, 34033 Montpellier and *+Laboratoire de Biologie Moléculaire et Cellulaire, (ER 199 CNRS) DRF-CEN Grenoble, B.P. 85X, 83041 Grenoble, France*

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Antibodies raised against the T-$\beta\gamma$ dimer of bovine retinal transducin specifically bind to the β and γ subunits of transducin in calf retina. Tissues from different vertebrates, but not from invertebrates, contained a band comigrating with the β subunit of transducin (T-β) which was immunostained. This protein most likely corresponds to the β subunit of GTP binding proteins of hormonal systems (G-β). In non-retinal vertebrate membranes, the antibodies did not recognize the γ subunits of G proteins whereas a band comigrating with bovine T-γ was detected in frog or rat retina. Although T-β was precipitated by the T-$\beta\gamma$ antiserum, we failed to immunoprecipitate the G-β from calf brain.

Guanyl nucleotide binding protein *Adenylate cyclase* *Phototransduction* *Hormonal transduction*

1. INTRODUCTION

Phototransduction [1–3] as well as many hormonal (or neurotransmitter) transduction processes, especially those mediated by cyclic AMP [4–6] and probably also those mediated by phosphatidylinositol breakdown [7], implicated the crucial role of GTP binding proteins.

In retinal rods, the GTP binding protein is transducin (T) [1–3,8], whereas plasma membranes from most cells contain several GTP binding proteins (Gs, Gi and Go); Gs and Gi, respectively, are implicated in hormonal activation and inhibition of adenylate cyclase [4–6], whereas the role of Go is unclear [9,10]. All GTP binding proteins have the same structure composed of 3 distinct subunits, α, β and γ [11–13]. Although all α subunits possess a binding site for guanine nucleotides [8,14,15], they can be differentiated by their molecular mass [16], their amino acid composition [16] and their sensitivity to cholera [17,18] or *Bordetella pertussis* toxins [9,19,20]. In contrast, the β subunits are indistinguishable in regard to the similar biochemical parameters (molecular mass, amino acid composition) and even their electrophoretic pattern of proteolysis [16]. As far as γ subunits are concerned, those in retinal transducin (T-γ) display no evident homology (at the level of the mRNA nucleotide sequence) with γ subunits in other tissues (G-γ) like brain, when studied in the same species [21]. Recently, immunological approaches using antibodies raised against the holoprotein transducin, have confirmed the important differences between α subunits as well as γ subunits from bovine retina and brain and the strong cross-reactivity of the β subunits under denaturating conditions [22,23].

To further delineate the putative identity of the β subunits from plasma membranes (G-β) and retina (T-β), we raised antibodies against the purified T-$\beta\gamma$ dimer of transducin to reveal epitopes which would be masked by the α subunit in the trimeric complex.

* To whom correspondence should be addressed

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2. MATERIALS AND METHODS

2.1. *Purification of T-βγ subunits*

Purification of T-$\beta\gamma$ subunits was performed as described [11,24].

2.2. *Immunization with purified T-βγ*

Three New Zealand white rabbits were injected intradermally at multiple sites with 100 μg of purified T-$\beta\gamma$ subunits per animal in complete Freund's adjuvant, followed by an injection of 50 μg in incomplete Freund's adjuvant, 2 weeks later.

2.3. *Membrane preparations*

Frog and rat erythrocyte membranes were prepared by hypotonic lysis as described [25]. Brains from goldfish, pigeon, rat, calf and cerebral ganglia or retinas from locusts (*Locusta migratoria*) were homogenized at 0°C with a Teflon pestle in a buffer A containing 5 mM EDTA and 50 mM Tris-HCl, pH 7.8, centrifuged at 40000 × *g* for 20 min at 4°C and resuspended in the same buffer. Frog and rat livers were homogenized in buffer A containing 250 mM sucrose at 0°C and centrifuged at 200 × *g* for 5 min at 4°C to remove unbroken cells and then 10000 × *g* for 15 min at 4°C. The viscero-abdominal ganglia of snails (*Helix aspersa*) were isolated and particulate fractions were prepared as described [26]. Retinas from rat, goldfish, calf, frog and pigeon were dissected and ROS were prepared by gently shaking retinas at 0°C in isotonic Ringer's solution. The suspension was filtered through a nylon screen (150 μm pore size) centrifuged at 4°C at 200 × *g* for 5 min and the pellet washed twice in Ringer's solution. ROS were then lysed in 5 mM Hepes, pH 7, 1 mM $MgCl_2$, 1 mM DTT buffer for 10 min at 0°C, homogenized with a Teflon pestle and centrifuged at 10000 × *g* for 15 min at 4°C.

2.4. *Immunoblots*

Membranes of tissues, purified transducin or T-$\beta\gamma$ subunits were analyzed by SDS Laemmli gels (10 or 12% acrylamide) [27]. Transfer onto nitrocellulose paper was performed for 7 h at a constant voltage (60 V) in a transblot apparatus [28]. After transfer, the sheet of nitrocellulose was processed as described [22] with minor modifications. Dilution of the antiserum was 1/50 and incubation was for 15 h at 4°C.

2.5. *Silver staining*

This was performed as described [29].

3. RESULTS

To check the immunospecificity of the antisera raised against the purified T-$\beta\gamma$, we tested the immunoreactivity of purified T-$\beta\gamma$, T-$\alpha\beta\gamma$, or whole ROS homogenates. The comparative analysis of Coomassie blue staining (fig.1A) and immunostaining (fig.1B) clearly shows that the antibodies specifically interact with both T-β and T-γ subunits of transducin but not with the α-subunit

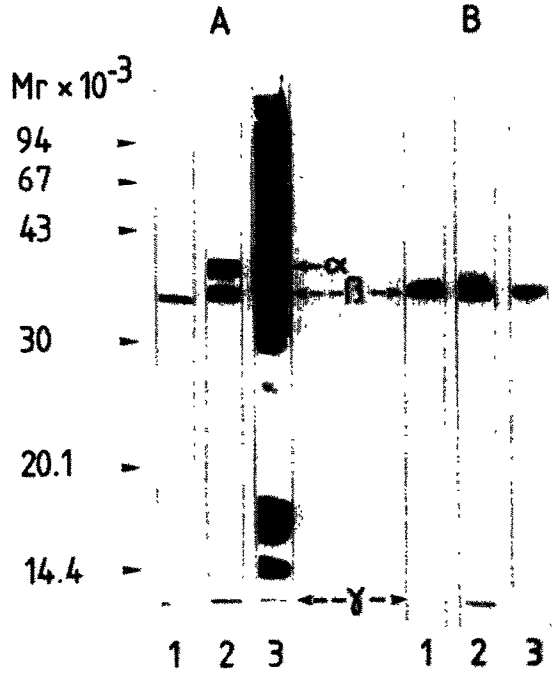

Fig.1. Specificity of T-$\beta\gamma$ antibodies. SDS-polyacrylamide gels (12% gel) of purified T-$\beta\gamma$, T-$\alpha\beta\gamma$ and ROS membranes were stained with Coomassie blue or blotted onto nitrocellulose paper and immunostained as described in section 2. (A) Lanes of the Coomassie stained gels of 8 μg purified T-$\beta\gamma$ (1), 10 μg purified T-$\alpha\beta\gamma$ (2) and 100 μg of ROS membranes (3). (B) Lanes of the immunostaining of 0.8 μg purified T-$\beta\gamma$ (1), 1.2 μg purified T-$\alpha\beta\gamma$ (2) and 100 μg of ROS membranes (3).

of this GTP binding protein (T-α) nor with other retinal proteins (fig.1B).

It is interesting to note that we did not find T-β immunoreactivity in membranes from ram sperm (not shown). Therefore, these membranes are probably not only devoid of Gs [30] but also of other GTP binding proteins.

The antibodies raised against bovine T-$\beta\gamma$ recognized the G-β subunits of crude plasma membranes prepared from different tissues of rat (blood, liver and brain) (fig.2A). A similar pattern of immunostaining for G-β subunits was observed when the same tissues from an amphibian (frog) were analyzed (fig.2A).

In brain from different vertebrate classes (fish, amphibian, bird) the antiserum revealed a single band comigrating with T-β (fig.2B), whereas the nervous tissue from 2 invertebrates (locust and snail) failed to contain any immunoreactive material (fig.2B). Note that T-$\beta\gamma$ from bovine retina also failed to inhibit basal, GTP or forskolin stimulated adenylate cyclase of snail nervous ganglia (not shown).

In non-retinal vertebrate membranes, no band

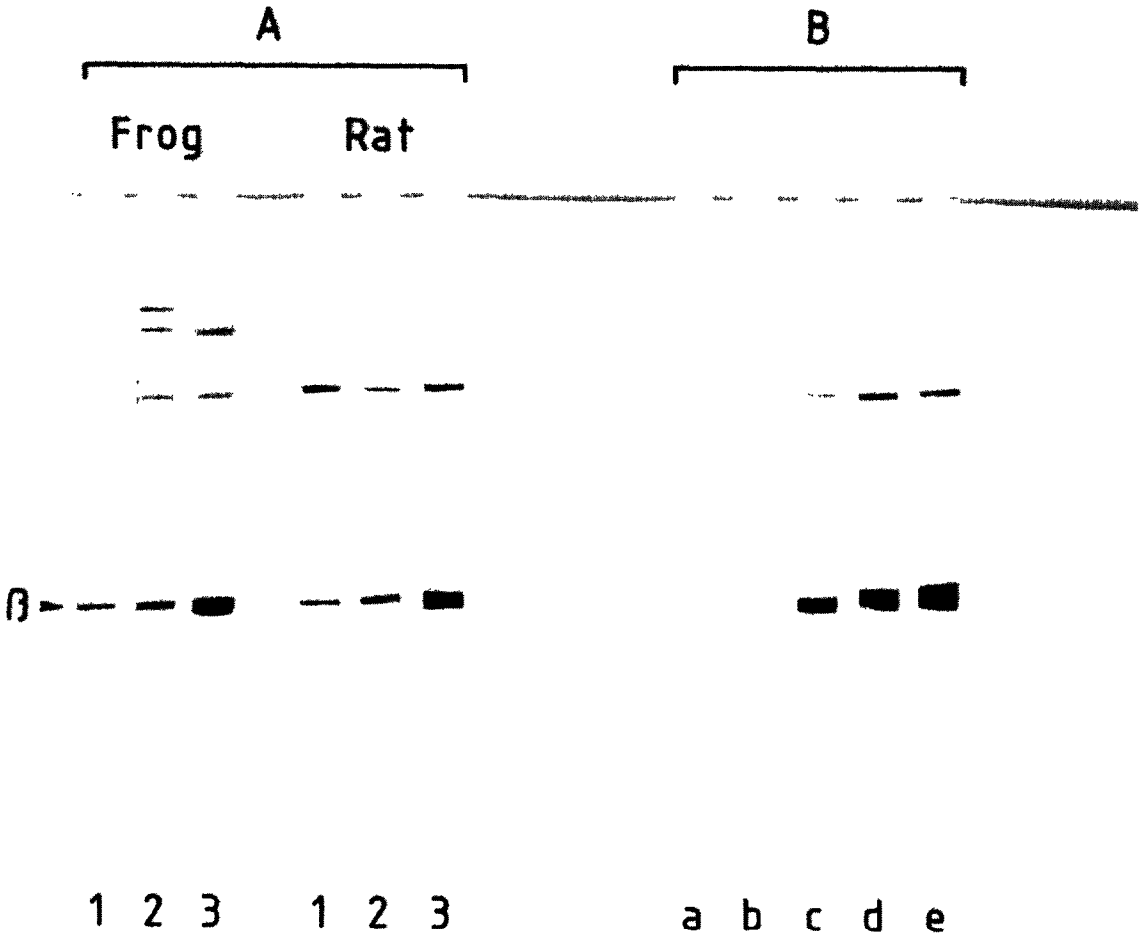

Fig.2. Cross reactivity of T-$\beta\gamma$ antiserum with β subunits of 'GTP binding proteins' from various tissues of different species. Approx. 100 μg protein per lane was loaded onto an SDS-polyacrylamide gel (10%) which was then blotted onto a nitrocellulose sheet. (A) Immunoblots of membranes of erythrocytes (1), livers (2), and brains (3) from frog and rat. (B) Immunoblots of membranes from nerve tissue of locusts (a: cerebral ganglia), snails (b: suboesophageal nervous ganglionic mass), goldfish (c: whole brain), pigeon (d: whole brain) and calf (e: cerebral cortex).

could be immunostained in the region of the dye front, where G-γ or T-γ usually run (fig.2). We therefore decided to search for a possible homology between retinal T-γ subunits from an invertebrate (locust) and different classes of vertebrates (fish, amphibian, bird and mammals). As already found with β subunits, the locust did not contain any cross-reacting material comigrating with bovine T-γ subunits (fig.3). On the other hand, an immunostained band comigrating with bovine T-γ was found in the retina from another mammal (rat) and an amphibian (frog) (fig.3). However, such a band could not be detected in the retina of pigeon and goldfish (fig.3) or trout (not shown).

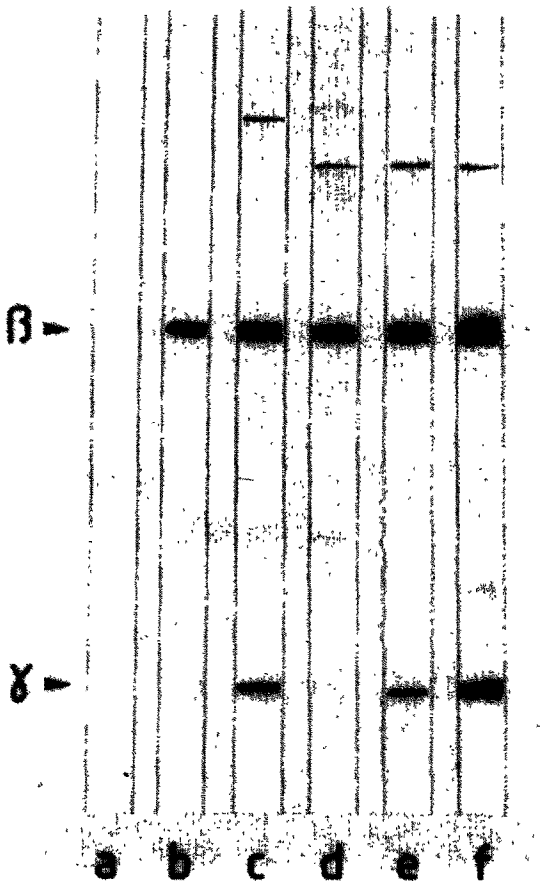

Fig.3. Cross reactivity of the rabbit antiserum with β and γ subunits of transducin from retina of different species. Immunoblots of retina of locust (a) and ROS of goldfish (b), frog (c), pigeon (d), rat (e) and calf (f) (12% polyacrylamide-SDS gel; 100 μg of protein).

Since all these experiments were performed under denaturating conditions, we further tested the similarities between the T-$\beta\gamma$ subunits from retina and the G-$\beta\gamma$ subunits from cerebral tissues by their ability to be immunoprecipitated in their native form in the absence or presence of various dissociating agents. The silver staining of the immunoprecipitates from retina clearly reveals the specificity and efficiency of the antiserum to immunoprecipitate the T-β subunit either in the presence or absence of cholate (fig.4). On the other hand, whatever the conditions tested (several extracting detergents, $MgCl_2$ (50 mM) plus GTP-γ-S (0.1 mM)), we failed to immunoprecipitate the G-β subunits of the GTP binding proteins from calf brain.

4. DISCUSSION

The structural homology between plasma membranes, G proteins, and retinal transducin (T) is likely to be closely related to their functional similarity, suggesting that transduction processes have been basically conserved throughout evolution despite the reception of different signals by distinct receptors and their coupling to specific effectors present inside each type of responsive cells. As regards to the 3 elements of the transducing proteins from retina and plasma membranes, the most striking homology resides in the β subunit [16]. Indeed, antibodies against the bovine T-β subunit cross-react with all β subunits of GTP binding proteins from retina or plasma membranes of other tissues as long as they belong to vertebrates (fig.2). No cross reactivity was seen with a similar protein in plasma membranes from insects or molluscs (fig.2). Stimulations of invertebrate adenylate cyclase by neurotransmitters are a GTP-dependent process and do not appear qualitatively different from those of vertebrates [31,32]. Therefore, the GTP binding proteins of plasma membranes from invertebrates either contained a structurally unrelated but functionally equivalent β subunit or are composed of a completely different oligomeric structure. This question deserves further analysis. It has been demonstrated that T-$\beta\gamma$, as G-$\beta\gamma$, inhibits mammalian adenylate cyclases likely by blocking the stimulatory effect of the α subunit of Gs [33,34]. The structural differences between the subunits of G proteins from verte-

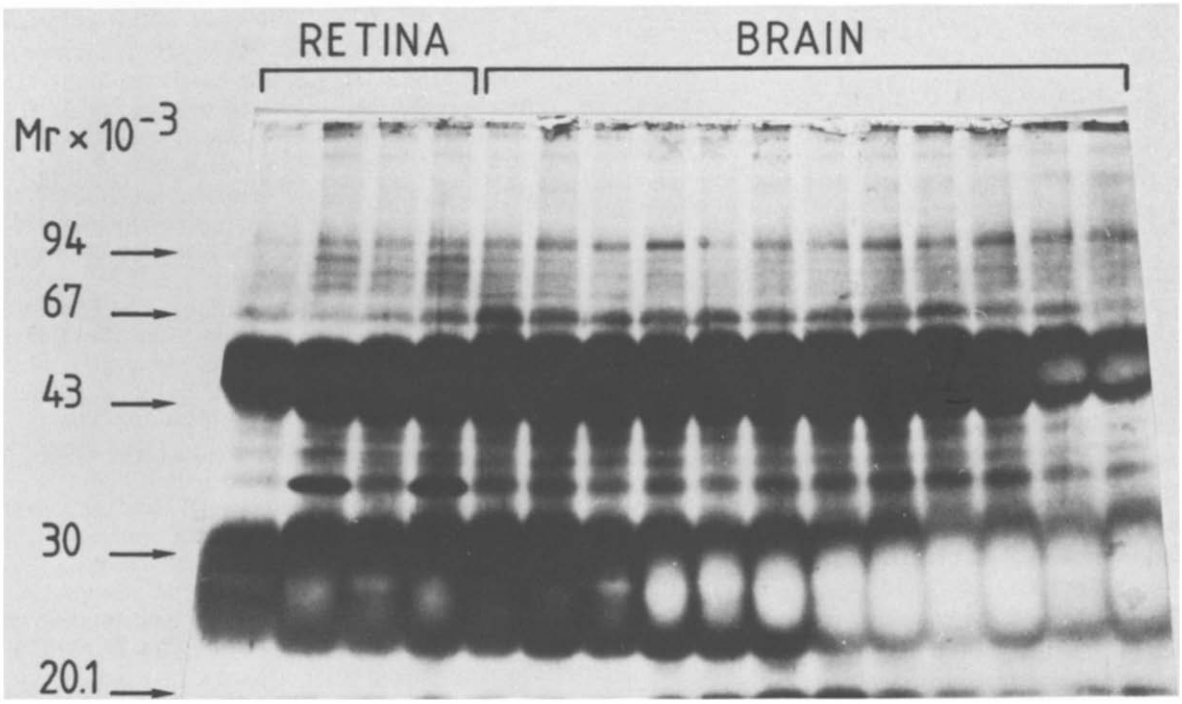

Fig.4. Comparative immunoprecipitation of T-$\beta\gamma$ and G-$\beta\gamma$ from retina and brain extracts. A pre-precipitation reaction was performed by incubating tissue extracts (100 μl) with 10 μl preimmune serum for 60 min at 37°C, and then by adding 70 μl of immunoprecipitin (Bethesda Research Laboratories). After centrifugation at 10000 × g for 5 min, supernatants were incubated with 10 μl of pre- or post-immune sera for 2 h at 37°C. After addition of immunoprecipitin (100 μl) for 10 min at 4°C, the tubes were centrifuged at 6000 × g for 1 min; the pellet was resuspended in 200 μl of 10 mM Hepes, pH 8, 150 mM NaCl, glycerol (10% v/v) containing Nonidet P-40 (0.1% v/v) and layed upon a 0.5 ml cushion of 1 M sucrose. After centrifugation at 10000 × g for 1 min, the pellet was washed 4-times with the same buffer (500 μl) containing successively 0.1% Nonidet P-40, 0.05% SDS, 0.5% Triton X-100, or altogether. The final pellet was incubated with 100 μl elution buffer (625 mM Tris-HCl, pH 6.8, 2.5% SDS, 10% glycerol) for 2 h at 37°C, centrifuged at 10000 × g for 5 min. 30 μl of the supernatant was boiled at 100°C for 3 min in the presence of half-diluted Laemmli sample buffer. The different immunoprecipitates from retina and brain extracts were loaded onto 10% polyacrylamide gels. SDS-polyacrylamide gel electrophoresis and silver staining were performed as described. For each experimental condition, immunoprecipitations were performed with pre-immune serum (odd lanes) and post-immune serum (even lanes). Lanes 1 and 2: retinal homogenate; lanes 3 and 4: retinal homogenates in 1% cholate; lanes 5 and 6: 1% cholate extract of brain membranes; lanes 7 and 8: 1% cholate extract of brain membranes after 60 min incubation with 0.1 mM GTP-γ-S and 50 mM $MgCl_2$; lanes 9 and 10: 1% cholate extract of brain membranes treated with 0.1 mM GTP-γ-S and 50 mM $MgCl_2$ before extraction; lanes 11 and 12: 1% Nonidet P-40 extract of brain membranes; lanes 13 and 14: 1% Triton X-100 extract of brain membranes; lanes 15 and 16: 1% Triton X-100 extract of brain membranes after 60 min incubation with 0.1 mM GTP-γ-S and 50 mM $MgCl_2$.

brates and invertebrates is further strengthened by the observation that bovine T-$\beta\gamma$ did not inhibit adenylate cyclase from snail nervous ganglia.

It has been shown that in bovine species, the T-γ subunit is not homologous to the G-γ subunit at the amino acid [23] as well as the nucleotide sequence level [21]. We can confirm and extend this observation to different tissues of different vertebrates (fig.2). Although no immuno-cross reactivity could be detected between T-γ from retina of mammals and a hypothetical T-γ of pigeon or fish (goldfish, trout) retina, it is interesting to note a homology between T-γ from frog and mammals (fig.3).

Therefore, the T-β subunits of different vertebrates have been relatively conserved whereas

earlier evolutionary divergences had occurred between T- and G-γ. As no antibodies have been raised against individual T- or G-γ subunits, the presence of a conserved domain (masked when the T-$\beta\gamma$ complex has been injected) cannot be excluded, which would be implicated in their strong interaction with the common 'ubiquitous' β subunit.

There are clear differences between the detergent extractability [11,12] and the interaction with the membrane [33,34] of T- and G-$\beta\gamma$ complexes, although they appear to inhibit the membrane bound adenylate cyclase with similar characteristics [33,34]. Two main hypotheses could be proposed to explain these differences. The first one is that these differences reside in the primary structure of the γ subunit as revealed by their lack of immunological cross reactivity. In this context, it could be pointed out that the water-extractable T-$\beta\gamma$ complex contains a highly hydrophilic γ subunit [35]. If this first hypothesis is correct, one should expect a hydrophobic G-γ subunit. The second hypothesis is that although very similar, the β subunit would not be structurally identical, as revealed by their differential immunoprecipitation (fig.4). Such a difference could be due to a post-translational modification of β subunits which could modulate their final conformation and/or their membrane insertion. Such a hypothesis is not improbable since the α and β subunits of GTP binding proteins are blocked at their NH_2 terminus [36], a region which is known to be a potential site for fatty acid addition, like *N*-myristylation [37,38] which enhances the affinity for membranes and/or reinforce intramolecular hydrophobic interactions.

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REFERENCES

[1] Chabre, M., Pfister, C., Deterre, P. and Kühn, H. (1984) in: Hormone and Cell Regulation (Dumont, J.E. ed.) vol.8, pp.87–98, Elsevier, Amsterdam, New York.
[2] Kühn, H. (1984) in: Progress in Retinal Research (Osborne, N. and Chader, G.J. eds) vol.3, pp.123–156, Pergamon, New York.
[3] Stryer, L., Hurley, J.B. and Fung, B.K.K. (1981) Curr. Top. Membr. Transp. 15, 93–108.
[4] Rodbell, M. (1980) Nature 284, 17–22.
[5] Hildebrandt, J. and Birnbaumer, L. (1982) in: Hormones and Cell Regulation (Dumont, J.E. et al. eds) vol.6, pp.111–130, Elsevier, Amsterdam, New York.
[6] Gilman, A.G. (1984) J. Clin. Invest. 73, 1–4.
[7] Litosch, I., Wallis, C. and Fain, J.N. (1985) J. Biol. Chem. 260, 5464–5471.
[8] Fung, B.K.K., Hurley, J.B. and Stryer, L. (1981) Proc. Natl. Acad. Sci. USA 78, 152–156.
[9] Sternweis, P.C. and Robishaw, J.D. (1984) J. Biol. Chem. 259, 13806–13813.
[10] Neer, E.J., Lok, J.M. and Wolf, L.G. (1984) J. Biol. Chem. 259, 14222–14229.
[11] Kühn, H. (1980) Nature 283, 587–589.
[12] Northup, J.K., Sternweis, P.C., Smigel, M.D., Schlafer, L.S., Ross, E.M. and Gilman, A.G. (1980) Proc. Natl. Acad. Sci. USA 77, 6516–6520.
[13] Hildebrandt, J.D., Codina, J., Risinger, R. and Birnbaumer, L. (1984) J. Biol. Chem. 259, 2039–2042.
[14] Northup, J.K., Smigel, M.D. and Gilman, A.G. (1982) J. Biol. Chem. 257, 11416–11423.
[15] Bokoch, G.M., Katada, T., Northup, J.K., Hewlett, E.L. and Gilman, A.G. (1983) J. Biol. Chem. 258, 2072–2075.
[16] Manning, D.R. and Gilman, A.G. (1983) J. Biol. Chem. 258, 7059–7063.
[17] Abood, M., Hurley, J., Pappone, M.C., Bourne, H.R. and Stryer, L. (1982) J. Biol. Chem. 257, 10540–10543.
[18] Cassel, D. and Pfeuffer, T. (1978) Proc. Natl. Acad. Sci. USA 75, 2669–2673.
[19] Van Dop, C., Yamanaka, G., Steinberg, F., Sekura, R.D., Manclark, C.R., Stryer, L. and Bourne, H.R. (1984) J. Biol. Chem. 259, 23–26.
[20] Katada, T. and Ui, M. (1982) J. Biol. Chem. 257, 7210–7216.
[21] Van Dop, C., Medinski, D., Sullivan, K., Wu, A.M., Fung, B.K.K. and Bourne, H.R. (1984) Biochem. Biophys. Res. Commun. 124, 250–255.
[22] Gierschik, P., Simons, C., Woodard, C., Somers, R. and Spiegel, A. (1984) FEBS Lett. 172, 321–325.
[23] Gierschik, P., Codina, J., Simons, C., Birnbaumer, L. and Spiegel, A. (1985) Proc. Natl. Acad. Sci. USA 82, 727–731.
[24] Deterre, P., Bigay, J., Pfister, C. and Chabre, M. (1984) FEBS Lett. 178, 228–232.
[25] Dickinson, K.E.J., Richardson, A. and Nahorski, S.R. (1981) Mol. Pharmacol. 19, 194–204.

[26] Deterre, P., Gozlan, H. and Bockaert, J. (1983) J. Biol. Chem. 258, 1467–1473.
[27] Laemmli, U.K. (1970) Nature 227, 650–685.
[28] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[29] Merril, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Science 211, 1437–1441.
[30] Stengel, D. and Hanoune, J. (1981) J. Biol. Chem. 256, 5394–5398.
[31] Drummond, A.H., Bucher, F. and Levitan, I.B. (1980) J. Biol. Chem. 255, 6679–6686.
[32] Deterre, P., Gozlan, H. and Bockaert, J. (1983) J. Biol. Chem. 258, 1467–1473.
[33] Northup, J.K., Sternweis, P.C. and Gilman, A.G. (1983) J. Biol. Chem. 258, 11361–11368.
[34] Bockaert, J., Deterre, P., Pfister, C., Guillon, G. and Chabre, M. (1985) EMBO J. 4, 1413–1417.
[35] Hurley, J.B., Fong, H.K.W., Teplow, D.B., Dreyer, W.J. and Simon, M.I. (1984) Proc. Natl. Acad. Sci. USA 81, 6948–6952.
[36] Hurley, J.B., Simon, M.I., Teplow, D.B., Robishaw, J.D. and Gilman, A.G. (1984) Science 226, 860–862.
[37] Carr, S.A., Biemann, K., Shoji, S., Parmelee, D.C. and Titani, K. (1982) Proc. Natl. Acad. Sci. USA 79, 6128–6131.
[38] Schultz, A.M., Henderson, L.E., Oroszlan, S., Garger, E.A. and Hanafusa, H. (1985) Science 227, 427–429.