Distinct biochemical properties of the native members of the G_{12} G-protein subfamily

Characterization of $G\alpha_{12}$ purified from rat brain

Rainer HARHAMMER*, Bernd NÜRNBERG, Christian HARTENECK, Daniela LEOPOLDT, Torsten EXNER and Günter SCHULTZ

Institut für Pharmakologie, Freie Universität Berlin, Thielallee 67-73, D-14195 Berlin, Germany

G_{12} and G_{13} are insufficiently characterized pertussis toxin-insensitive G-proteins. Here, we describe the isolation of $G\alpha_{12}$ from rat brain membranes. $G\alpha_{12}$ was purified to apparent homogeneity by three steps of conventional chromatography, followed by two cycles of subunit-exchange chromatography on immobilized G subunits. Purified $G\alpha_{12}$ bound guanosine 5'-[γ -thio]triphosphate slowly and substoichiometrically. For isolation of functionally active $G\alpha_{12}$, it was mandatory to use sucrose monolaurate as a detergent. Comparative studies of both rat-brain-derived members of the G_{12} subfamily revealed differences in the affinity of $G\alpha_{12}$ and $G\alpha_{13}$ for $G\beta\gamma$. $G\alpha_{12}$ required a higher Mg^{2+} concentration for AlF_4^- -induced dissociation from immobilized $G\beta\gamma$ than did $G\alpha_{13}$. In addition, the G_{12} subfamily

members differed in their sedimentation velocities, as determined by sucrose-density-gradient centrifugation. Analysis of sedimentation coefficients revealed a higher tendency of G_{12} to form supramolecular structures in comparison to G_{13} and other G-proteins. These G_{12} structures were stabilized by sucrose monolaurate, which in turn may explain the necessity for this detergent for purification of functionally active $G\alpha_{12}$. Despite these distinct biochemical characteristics of G_{12} and G_{13} , both purified G-proteins coupled to a recombinant thromboxane A_2 (TXA₂) receptor reconstituted into phospholipid vesicles. These data indicate, (1) significant differences in the biochemical properties of native members of the G_{12} subfamily, and (2) their specific coupling to TXA₂ receptors.

INTRODUCTION

G-proteins are molecular switches mediating signals from a plethora of heptahelical transmembrane receptors to an increasing number of cellular effectors, including adenylyl cyclases, phospholipases, kinases and ion channels [1,2]. They are composed of a guanine nucleotide-binding $G\alpha$ subunit and a $G\beta\gamma$ dimer. All G-proteins follow the same principle activation/inactivation cycle for reversible and specific signal transmission. Activation of the receptor induces an exchange of GDP for GTP, allowing functional coupling of the dissociated GTP-bound $G\alpha$ subunit and/or the free $G\beta\gamma$ dimer to their effector targets. Hydrolysis of GTP and subunit reassociation inactivates the G-protein subunits. To date, cDNAs for 23 distinct $G\alpha$ subunits (the products of 17 genes), at least 5 $G\beta$ and 11 $G\gamma$ subunits, are known. G-proteins are classified in four subfamilies (G_s , G_i , G_q and G_{12}), based on amino acid similarity of their $G\alpha$ subunits [3]. Whereas the functions of most proteins belonging to the G_s , G_i and G_q subfamilies are known, we still remain ignorant of the physiological roles of the members of the G_{12} subfamily, i.e. G_{12} and G_{13} . Interestingly, this subfamily exhibits a considerable degree of heterogeneity since their $G\alpha$ subunits share less than 50% amino acid identity with other G-protein subfamilies. Even the amino acid identity between $G\alpha_{12}$ and $G\alpha_{13}$ amounts to only 67%, which is less than that observed among members of the other G-protein subfamilies [4]. Both proteins are ubiquitously expressed at very low abundance [5]. Our laboratory provided evidence that stimulation of human platelet thrombin and

thromboxane A_2 (TXA₂) receptors, as well as human TSH receptors, activates G_{12} and G_{13} ; this effect was slower than activation of the other G-protein subfamilies by the same or other receptors [6,7]. Other groups have reported data on overexpressed recombinant wild type or mutationally activated $G\alpha_{12}$ and $G\alpha_{13}$, suggesting an involvement in the regulation of Na^+/H^+ -exchangers [8,9], and a potent cell-transforming activity for both G-proteins which appears to be mediated by the activation of the small GTPase Rho and/or Jun N-terminal kinases [10–13].

Previously, we purified $G\alpha_{13}$ from bovine and rat brain membranes and recognized a particularly low affinity of GDP-bound $G\alpha_{13}$ for $G\beta\gamma$ dimers [14]. As reported for several other $G\alpha$ subtypes, we found that the N-terminus of $G\alpha_{13}$ is important for subunit interaction [15]. Interestingly, this region of $G\alpha_{13}$ differs markedly from other $G\alpha$ isoforms, including $G\alpha_{12}$. Furthermore, we realized that the protocol applied for purification of $G\alpha_{13}$ did not allow isolation of functionally active G_{12} , which points to different biochemical properties of both proteins. In contrast, purification of the overexpressed recombinant members of the G_{12} subfamily from baculovirus-infected Sf9 cells described previously exhibited no indication of the different biochemical features of G_{12} and G_{13} [16,17]. This discrepancy prompted us to establish conditions for the purification of G_{12} from brain membranes and to compare the native members of the G_{12} subfamily. Here we present for the first time evidence of significant differences between G_{12} and other G-proteins, including G_{13} , concerning their affinity of $G\alpha$ for $G\beta\gamma$ and their

Abbreviations used: AMF, mixture of $AlCl_3$, $MgCl_2$ and NaF; DTT, dithiothreitol; ECL, enhanced chemiluminescence; GTP[S], guanosine 5'-[γ -thio]-triphosphate; Lubrol PX, polyoxyethylene(9) lauryl ether; High Five cells (*Trichoplusia ni* egg cells); SQ 29,548, [(1S)1 α ,2 β (5Z)3 α ,4 β]-7-[3-[2-(phenylamino)carbonyl-hydrazino-methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid; TXA₂, thromboxane A_2 ; TSH, thyrotropin or thyroid stimulating hormone; U 46,619, 15S-hydroxy-11 α ,9 α (epoxymethano)prosta-5Z,13E-dienoic acid.

* To whom correspondence should be addressed.

hydrodynamic properties. Purified G_{12} and G_{13} are both specifically activated by a recombinant expressed human TXA_2 receptor.

MATERIALS AND METHODS

Materials

[35 S]Guanosine 5'-[γ -thio]-triphosphate ([35 S]GTP[S]) (1150–1400 Ci/mmol), [α - 32 P]GTP (3000 Ci/mmol) and [3 H]([1S]1 α ,2 β (5Z)3 α ,4 β)-7-[3-[2-(phenylamino)carbonyl-hydrazino-methyl]-7-oxabicyclo[2.2.1]-hept-2-yl]-5-heptenoic acid; ([3 H]SQ 29,548) (50.4 Ci/mmol) were obtained from Du Pont NEN (Bad Homburg, Germany). 15S-hydroxy11 α ,9 α (epoxymethano)prosta-5Z,13E-dienoic acid (U 46,619) was purchased from Sigma (Deisenhofen, Germany), whereas unlabelled SQ 29,548 was from Research Biochemicals International (Natick, MA, U.S.A.). Sucrose monolaurate was obtained from Fluka (Neu-Ulm, Germany). The sources of all other reagents used were as described previously [14,15].

Purification of $G\alpha_{12}$

Typically, frozen rat brain membranes (4.6 g of protein and 960 nmol of GTP[S]-binding activity) were thawed and suspended in buffer A (20 mM Tris/HCl, pH 8.0/1 mM EDTA/20 mM 2-mercaptoethanol/10 μ M GDP/1.7 μ g/ml PMSF/10 μ g/ml Na α -p-tosyl-L-lysine chloromethyl ketone/10 μ g/ml soybean trypsin inhibitor) plus sucrose monolaurate (0.5%, w/v). All steps of purification were carried out at 4 °C unless stated otherwise. The mixture was incubated for 1 h and subsequently centrifuged at 200 000 g for 1 h. The clear supernatant (1.0 g of protein; 530 nmol of GTP[S]-binding activity) was removed, mixed with ethylene glycol (final concentration 30%, v/v) and applied to a 600 ml column of DEAE-Sepharose Fast Flow (Pharmacia, Freiburg, Germany), which had been equilibrated with 2.4 litres of buffer A, omitting proteinase inhibitors but including 0.12% sucrose monolaurate. Elution was accomplished employing a linear gradient of NaCl in buffer A containing 0.12% sucrose monolaurate and 30% ethylene glycol (2.4 litres; 0–800 mM NaCl). The eluate was fractionated (12 ml each fraction) and analysed for G_{12} by immunoblotting. Pooled fractions containing the major peak of $G\alpha_{12}$ -immunoreactivity were concentrated to 40 ml by pressure filtration through an Amicon PM 10 filter (Danvers, MA, U.S.A.) and loaded onto a 900 ml column of Ultrogel AcA 34 (Serva, Heidelberg, Germany). Proteins were eluted in buffer A containing 0.12% sucrose monolaurate, 30% ethylene glycol and 100 mM NaCl. Peak fractions (6 ml each) of G_{12} were concentrated and diluted with 15 vol. of buffer A containing 0.12% sucrose monolaurate. The solution was applied to an equilibrated FPLC Mono Q column (1 ml; Pharmacia). The column was washed with buffer A containing 0.12% sucrose monolaurate, and proteins were eluted with two consecutive linear gradients of NaCl (0–400 mM, 70 ml; 400–00 mM, 4 ml). Fractions enriched with G_{12} were pooled and stored at –80 °C. Final purification of G_{12} required subunit-exchange chromatography using $G\beta\gamma$ -agarose which was synthesized as described [14]. G_{12} -rich fractions from FPLC were mixed with appropriate amounts of $G\beta\gamma$ -agarose in the presence of 30 μ M $AlCl_3$ /6 mM $MgCl_2$ /10 mM NaF (AMF). This mixture was incubated for 90 min at room temperature with stirring. Following addition of EDTA to a final concentration of 20 mM and further incubation for 60 min, the suspension was washed with at least eight vol. of buffer B [20 mM Na-Hepes (pH 8.0), 1 mM EDTA, 0.12% sucrose monolaurate, 3 mM dithiothreitol (DTT), 100 mM NaCl and 5 μ M GDP]. $G\alpha_{12}$ was eluted with

buffer B containing AMF with a high concentration of Mg^{2+} (30 μ M $AlCl_3$ /50 mM $MgCl_2$ /10 mM NaF). Eluates containing $G\alpha_{12}$ were subjected to a second cycle of subunit-exchange chromatography. Final eluates were stored at –80 °C.

Gel electrophoresis, immunoblotting and antibodies

For detection of G-proteins, SDS/PAGE, as described by Laemmli [18], was employed with separating gels containing 10% (w/v) acrylamide. Samples obtained from $G\beta\gamma$ -affinity chromatography were prepared as described by Schagger and von Jagow [19]. Immunoblotting and visualization of filter-bound proteins with peroxidase-coupled anti-rabbit IgG (Sigma, Deisenhofen, Germany) and the enhanced chemiluminescence (ECL) Western blotting system (Amersham, Braunschweig, Germany) have been described previously [20]. Antibodies AS 6 (anti- α_{common}), AS 8 (anti- α_{common}), AS 11 (anti- $\alpha_{1/2}$), AS 164 (anti- α_2), AS 232 (anti- α_{12}), AS 266 (anti- α_1), AS 311 (anti- α_{12}), AS 343 (anti- α_{13}) and AS 348 (anti- α_s) are characterized elsewhere [5,14,20]. AS 369 (anti- $\alpha_{q/11}$) was raised against a peptide (LQLNLKEYNLV) corresponding to amino acids 349–359 of $G\alpha_q$, showing similar properties to the previously published antiserum AS 368 [5].

Cloning of recombinant baculoviruses and expression of human recombinant TXA_2 receptor in High Five cells

Monolayer *Trichoplusia ni* egg cells (High Five/BTI-TN-5B1-1 from Invitrogen, Leek, The Netherlands) were propagated in TNM-FH medium (Sigma, Deisenhofen) supplemented with 10% (v/v) fetal calf serum. Recombinant baculoviruses containing the cDNA of a human TXA_2 receptor isolated from placental tissue and murine $G\alpha_{12}$ were generated as previously described [21,22]. For expression of recombinant TXA_2 receptors, cells were infected with three plaque-forming units per cell for viruses and harvested 48–52 h post-infection.

Preparation of cell membranes and receptor-binding assay

High Five cells were scraped from culture flasks and harvested by centrifugation at 2500 g for 15 min. Pellets were resuspended in a buffer containing 20 mM Tris/HCl, pH 8.0, 1 mM EDTA, 20 mM 2-mercaptoethanol and 10 μ M PMSF. Cells were subjected to nitrogen cavitation at 28×10^5 Pa for 40 min. The homogenate was centrifuged at 700 g for 2 min to remove nuclei and intact cells. Membranes were obtained by spinning at 80 000 g for 30 min and stored at –80 °C. Expression of TXA_2 receptors was confirmed by receptor binding of the TXA_2 antagonist SQ 29,548. Aliquots of membranes (50 μ g) were incubated with [3 H]SQ 29,548 (0.3–100 nM) in a buffer containing 50 mM Tris/HCl, pH 7.4, 100 mM NaCl, 20 mM $MgCl_2$ and 1% (w/v) BSA in a final volume of 100 μ l at 25 °C.

After 30 min, the samples were quickly filtered through Whatman GF/B filters and subsequently washed with 2×4 ml of ice-cold 50 mM Tris/HCl, pH 7.4/100 mM NaCl/20 mM $MgCl_2$. All filtration procedures were completed within 10 s. The filters were dried, and bound radioactivity was measured by liquid scintillation counting. Non-specific binding, determined by the addition of 10 μ M unlabelled SQ 29,548, was less than 10% of total binding.

Reconstitution of TXA_2 receptors with G-proteins

TXA_2 receptors and G-proteins were reconstituted into phospholipid vesicles as described previously [23] with modifications.

Briefly, $G\alpha$ subunits were mixed with bovine brain $G\beta\gamma$ for heterotrimer formation, and subsequently co-incubated with azolectin and CHAPS-extracted recombinant TXA_2 receptors. Thereafter, mixtures were subjected to gel-filtration chromatography on a Sephadex G-50 column (Pharmacia) and eluted in 20 mM Na-Hepes, pH 7.4/0.1 mM EDTA/1 mM DTT/100 mM NaCl. Reconstituted proteins appearing in the void volume were concentrated and immediately used in subsequent experiments.

Photolabelling and immunoprecipitation of reconstituted proteins

Photolabelling of reconstituted G-proteins by [α - 32 P]GTP azidoanilide was performed according to published procedures [6]. For maximal activation of G-proteins, samples containing G_{12} or G_{13} were preincubated with U 46,619 or buffer, followed by incubation with [α - 32 P]GTP azidoanilide (5×10^6 c.p.m. per tube) at 30 °C for 30 min, whereas G_{10} -containing liposomes were exposed for 3 min. After addition of glutathione to a final concentration of 2 mM, proteins were irradiated for 15 s with a 254 nm UV lamp. Subsequent immunoprecipitation was performed as detailed elsewhere [24]. Precipitated proteins were subjected to SDS/PAGE and gels were subsequently dried and exposed to X-ray films.

Miscellaneous methods

$G\alpha_{13}$, $G\alpha_{q/11}$, $G\alpha_{i1}$, $G\alpha_o$ and $G\beta\gamma$ were purified from rat or bovine brain as described [14,25]. The GTP[S]-binding activity of proteins eluted from a DEAE-Sepharose Fast Flow column was monitored as described by Sternweis and Pang [26]. Samples were incubated with 500 nM GTP[S] (100 000 c.p.m. of [35 S]GTP[S] per tube) at 32 °C for 30 min. For the determination of GTP[S]-binding to purified $G\alpha_{12}$ and $G\alpha_o$, the reaction mixtures containing 50 mM Na/Hepes (pH 8.0), 1 mM EDTA, 1 mM DTT, 0.05 % sucrose monolaurate, 0.5 mM $MgSO_4$ and 10 μ M GTP[S] (1×10^6 c.p.m. of [35 S]GTP[S] per sample) were incubated at 32 °C for the times indicated. Non-specific binding was estimated by the addition of 2 or 20 mM GTP.

Sucrose-density-gradient centrifugation was performed as detailed previously [16]. Briefly, G-proteins were supplemented with standard proteins and layered over linear sucrose gradients (5–20 %, w/v) in either 0.12 % sucrose monolaurate or 0.1 % polyoxyethylene(9) lauryl ether (Lubrol PX) or 0.9 % cholate. After centrifugation (175 000 g, 15–16 h, 4 °C), the fractions obtained were analysed by SDS/PAGE and immunoblotting. Protein quantification was analysed by the method of Lowry et al. [27] with modifications [28].

RESULTS

Purification of $G\alpha_{12}$

The use of specific antibodies against murine $G\alpha_{13}$ peptide sequences allowed us to isolate rat brain $G\alpha_{13}$ [14]. At the same time, we had to bear in mind that G_{12} aggregated even in the crude detergent extract of the membranes. Similar results were obtained when G_{12} was solubilized with Lubrol PX or CHAPS instead of cholate. Whereas several other approaches, including supplementation with AMF or GTP[S], were unsuccessful in stabilizing this protein, eventually applying sucrose monolaurate allowed extraction of functionally active G_{12} . For further purification, extracts were loaded onto a DEAE-Sepharose anion-exchange column. The profile of the resolved proteins is illustrated in Figure 1(A). $G\alpha_{12}$ -immunoreactive polypeptides eluted in a broad peak centred at 280 mM NaCl. $G\alpha_{13}$ eluted slightly

Figure 1 Chromatography of membrane proteins extracted from rat brain on DEAE-Sepharose Fast Flow and Ultragel AcA 34

(A) DEAE-Sepharose Fast Flow chromatography. Sucrose monolaurate-extracted proteins (1.0 g) were applied to a 600 ml DEAE-Sepharose Fast Flow column and eluted by an NaCl gradient as described in the Materials and methods section. Protein elution profile (○) and GTP[S]-binding activity (●) are shown. GTP[S]-binding was performed as follows: aliquots of the fractions (3 μ l) were incubated at 32 °C for 30 min with 500 nM GTP[S] and [35 S]GTP[S] (100 000 c.p.m./tube) in a total volume of 60 μ l. Non-specific binding was determined by the addition of 2 mM GTP. The broken line indicates the gradient from 0 to 800 mM NaCl. The indicated fractions (numbers 68–81) containing the peak of $G\alpha_{12}$ immunoreactivity were pooled and subjected to gel filtration. (B) Ultragel AcA 34 chromatography of G_{12} . Pooled fractions of DEAE-Sepharose were subjected to gel-filtration chromatography as described in the Materials and methods section. Portions of fractions (120 μ l) were acetone-precipitated, separated by SDS/PAGE and analysed by immunoblotting using AS 232. A molecular-mass marker (kDa) is indicated on the left.

differently, with peak concentrations found at 300 mM NaCl. Most other G-proteins, including G_i , G_o and $G_{q/11}$, peaked at higher NaCl concentrations, as determined by immunological detection with antibodies AS 8, AS 11, AS 343 and AS 369. The profile of GTP[S]-binding activity confirmed this immunological finding (see Figure 1A). Notably, $G\alpha_{12}$ coeluted with $G\beta\gamma$ dimers. Because of contamination by other G-proteins, we pooled only the peak fractions of $G\alpha_{12}$ -immunoreactivity and subjected them to gel-filtration chromatography. Screening the eluate with AS 232 revealed two different portions of G_{12} -immunoreactivity (see Figure 1B). The first one obviously represented aggregated protein appearing in the void volume, so that only the second portion contained functionally active G_{12} . The latter proteins containing the bulk of G_{12} were pooled and subjected to FPLC on a Mono Q anion-exchange column. G_{12} eluted in a peak centred at 180 mM NaCl, whereas most of the other G-proteins required higher NaCl concentrations. Accordingly, G_{12} -rich fractions eluting at NaCl-concentrations between 120 and 180 mM were virtually free of $G\alpha_i$ -, $G\alpha_o$ -, $G\alpha_{q/11}$ -, $G\alpha_z$ - and $G\alpha_s$ -immunoreactive polypeptides but contained small quantities of $G\alpha_{13}$ -immunoreactive polypeptides which were recognized by

Figure 2 Affinity chromatography on immobilized $G\beta\gamma$ complexes

Aliquots of $G\alpha_{12}$ -immunoreactive material, obtained by FPLC on a Mono Q column, were incubated with 500 μ l of $G\beta\gamma$ -agarose. Procedures of incubation, washes and elution are described in the Materials and methods section. **(A)** Elution profile of $G\alpha_{12}$ from the affinity column. Aliquots (250 μ l) of wash fractions containing GDP (500 μ l each) and eluate fractions containing AMF (500 μ l each) were supplemented with 10 μ g of BSA, precipitated with acetone, resuspended in 50 μ l of sample buffer [17] and heated to 40 °C for 30 min. Samples were separated by SDS/PAGE (10% acrylamide), immunoblotted and probed with anti- α_{12} antiserum AS 232 (dilution 1:150). The ECL system was used for detection of filter-bound antibodies. **(B)** Silver stains of the purified proteins. Silver-stained SDS/PAGE pattern of proteins eluted with buffer containing AMF (30 μ M $AlCl_3$ /10 mM NaF/50 mM $MgCl_2$) from a single cycle of subunit-exchange chromatography (left lane) and of $G\alpha_{12}$ obtained after two cycles of $G\beta\gamma$ -agarose chromatography (right lane). Molecular masses (kDa) of marker proteins are indicated.

the respective antibodies. At this stage G_{12} was still associated with $G\beta\gamma$ complexes, as confirmed by $G\beta$ -immunoreactivity and sucrose-density-gradient centrifugation of the respective fractions.

To achieve an efficient separation of the two G_{12} subfamily members, we tried additional chromatographic steps. In contrast to the purification procedures for many other G-proteins, neither hydrophobic interaction nor hydroxyapatite chromatography was satisfactory. Ultimately, final purification of $G\alpha_{12}$ was possible by affinity chromatography on immobilized $G\beta\gamma$. Hence, G_{12} obtained from the Mono Q column was first activated and dissociated by AMF and then, while incubating with $G\beta\gamma$ -agarose, inactivated by the addition of EDTA, thereby allowing subunit reassociation. After washing procedures, bound proteins were eluted with AlF_4^- in the presence of 50 mM Mg^{2+} , resulting in the release of a 42 kDa protein which was only immunoreactive to anti- α_{12} -antibodies (Figure 2A). Since the applied G_{12} -rich Mono Q pool still contained small amounts of $G\alpha_{13}$, we carefully looked for $G\alpha_{13}$ contaminants. Surprisingly, immunoblots using AS 343 detected $G\alpha_{13}$ only in flow-through and washes of the $G\beta\gamma$ chromatography, but not in the eluates. This observation appeared not to be an artefact due to detection limits, since the same results were obtained when crude sucrose monolaurate extracts containing sufficient amounts of $G\alpha_{13}$ -immunoreactivity were subjected to $G\beta\gamma$ -agarose chromatography. Hence, it is most likely that $G\alpha_{13}$ did not bind as tightly as $G\alpha_{12}$ to immobilized $G\beta\gamma$ in the presence of sucrose monolaurate. In contrast, when affinity chromatography on $G\beta\gamma$ -agarose was performed in the presence of Lubrol PX instead of sucrose monolaurate, $G\alpha_{13}$ but not $G\alpha_{12}$ bound to the affinity matrix. Silver-stained SDS/PAGE revealed that $G\alpha_{12}$ eluted from the $G\beta\gamma$ -agarose was contaminated with small quantities of 53 and 28 kDa proteins. Removal of these impurities was achieved by a second chromatography on $G\beta\gamma$ -agarose (see Figure 2B). The present chromatographic procedure allowed the purification of $G\alpha_{12}$, providing 10–14 μ g of purified $G\alpha_{12}$ from 4.5–5.0 g of membrane proteins, as roughly estimated from silver-stained gels.

Table 1 Time dependence of [^{35}S]GTP[S] binding to purified $G\alpha_{12}$ and $G\alpha_o$

Binding was carried out at 32 °C in 50 mM Na-Hepes (pH 8.0) 1 mM EDTA, 1 mM DTT, 0.05% sucrose monolaurate, 0.5 mM $MgSO_4$ and 10 μ M GTP[S] (1×10^6 c.p.m. of [^{35}S]GTP[S]). Assays contained 5 pmol of $G\alpha_o$ and 4 pmol of $G\alpha_{12}$. At the indicated times, samples were filtered, and filter-bound radioactivity was counted. Non-specific binding was estimated by the addition of 20 mM GTP. Means $\pm$ S.D. (number of experiments in parentheses) are shown.

Incubation time (min)	GTP[S] bound (pmol)	
	$G\alpha_o$	$G\alpha_{12}$
0	0.2 $\pm$ 0.05 (3)	0.1 $\pm$ 0.11 (3)
30	5.0 $\pm$ 0.38 (3)	0.4 $\pm$ 0.06 (2)
135	5.0 $\pm$ 0.15 (2)	2.0 $\pm$ 0.09 (3)

Figure 3 AMF-induced dissociation of $G\alpha_{12}$ and $G\alpha_{q/11}$ from $G\beta\gamma$, depending on the Mg^{2+} concentration

Sucrose monolaurate extracts from rat brain membranes (900 μ g of protein) were incubated with $G\beta\gamma$ -agarose (500 μ l), as described in the Materials and methods section. Elution of $G\alpha_{12}$ (upper panel) or $G\alpha_{q/11}$ (lower panel) was performed with AlF_4^- and different concentrations of Mg^{2+} , i.e. 5, 10 or 50 mM as indicated. Aliquots of eluates (250 μ l) were subjected to SDS/PAGE and analysed by immunoblotting using antibodies AS 232 (anti- α_{12}) and AS 369 (anti- $\alpha_{q/11}$). Both antisera were diluted 1:150. The positions of a 43 kDa marker protein are indicated. Consecutive eluted fractions for each set of conditions are shown.

Although the selective interaction of $G\alpha_{12}$ with immobilized $G\beta\gamma$ subunits provided convincing evidence for a functionally active protein, we were interested in examining the binding of guanine nucleotides to isolated rat brain $G\alpha_{12}$, which is a hallmark of G-protein function. The rate of [^{35}S]GTP[S] binding (10 μ M GTP[S]) was low and substoichiometric, and a saturation was not obtained even after 135 min of incubation (Table 1). For comparison, we determined GTP[S] binding to purified $G\alpha_o$ and found maximal values after 30 min. The slow kinetics of GTP[S] binding to the purified native $G\alpha_{12}$ agree with the binding kinetics to the isolated recombinant murine protein and the kinetics of [α - ^{32}P]GTP azidoanilide-binding to native $G\alpha_{12}$ in membranes [7,17].

Mg^{2+} -dependence of AMF-induced activation of G_{12}

Purification of the native members of the G_{12} subfamily indicated differences in the affinity of the $G\alpha$ subunit and $G\beta\gamma$. Therefore, we studied AMF-induced release of $G\alpha_{12}$ bound to immobilized $G\beta\gamma$, in comparison to other $G\alpha$ isoforms, in more detail. Figure 3 depicts a typical experiment showing the release of $G\alpha_{12}$, compared with $G\alpha_{q/11}$, from $G\beta\gamma$ -agarose, depending on the Mg^{2+} concentration. As expected, low mM concentrations of Mg^{2+} , i.e. 5 and 10 mM, induced dissociation of $G\alpha_{q/11}$ from $G\beta\gamma$ in the presence of AlF_4^- (see Figure 3, lower panel). In contrast, an Mg^{2+} concentration of 50 mM was required to release $G\alpha_{12}$ from $G\beta\gamma$ complexes, suggesting a tight association of subunits (see Figure 3, upper panel). This behaviour of $G\alpha_{12}$ is apparently different from Lubrol PX-solubilized $G\alpha_{13}$ bound to immobilized

Table 2 Sedimentation coefficients of G₀, G₁₂ and G₁₃ in the presence of different detergents

Proteins were centrifuged through 5 ml sucrose density gradients (5–20%, w/v). Gradients were fractionated, and the fractions were subjected to SDS/PAGE and analysed by immunoblotting with antibodies AS 6 (anti- α_0), AS 232 (anti- α_{12}) and AS 343 (anti- α_{13}). Sedimentation values ($S_{20,w}$) of G α subunits were calculated by comparison with $S_{20,w}$ values of the marker proteins BSA (4.2 S) and cytochrome *c* (1.8 S). The $S_{20,w}$ values shown represent means $\pm$ S.D. (number of experiments given in parentheses). n.d., not determined.

	G ₀	G ₁₂	G ₁₃
Sucrose monolaurate	6.4 $\pm$ 0.19 (4)	6.6 $\pm$ 0.30 (5)	5.9 $\pm$ 0.14 (3)
Sucrose monolaurate + GTP[S] (100 μ M)	5.2 $\pm$ 0.32 (3)	6.1 $\pm$ 0.16 (3)	n.d.
Lubrol PX	4.3 $\pm$ 0.10 (4)	5.4 $\pm$ 0.27 (4)	4.2 $\pm$ 0.10 (3)
Cholate	4.0 $\pm$ 0.18 (4)	n.d.	3.3 $\pm$ 0.03 (3)

G $\beta\gamma$ complexes, which was easily released by AlF₄[−] in the presence of 10 mM Mg²⁺ [14].

Characterization of G₁₂ in sucrose density gradients

As mentioned above, solubilization of functionally active G₁₂ required sucrose monolaurate, a detergent that inhibits, to a lesser extent than others, interactions between associated proteins [29]. This points to the possibility that the formation of supra-molecular structures, such as associations of G₁₂ with other proteins, or multimers of G₁₂, may be important. In order to examine this assumption, we analysed the sedimentation velocity of G₁₂ in comparison to other G-proteins, e.g. G₀ and G₁₃, using sucrose-density-gradient centrifugation. After application of sucrose monolaurate extracts to gradients, G₁₂ displayed a sedimentation value of 6.6 S (Table 2). Under the same conditions, G₀ and G₁₃ showed slightly smaller coefficients of 6.4 S and 5.9 S respectively. Interestingly, addition of 100 μ M GTP[S] in the presence of sucrose monolaurate lowered the sedimentation coefficient for G₀ to 5.2 S, whereas the sedimentation value for G₁₂ was only slightly reduced to 6.1 S. Nevertheless, all these values were considerably larger than the coefficients of 4.0–4.5 S reported for G-protein heterotrimers of approx. 85 kDa [30]. Since sucrose monolaurate micelles are very small, these results support the idea that sucrose monolaurate may promote or stabilize supra-molecular structures of G-proteins in a similar manner as described for digitonin or octyl glycoside [31,32]. In contrast, Lubrol PX failed to stabilize structures of G-proteins that were larger than heterotrimers [32]. Accordingly, the application of freshly prepared rat brain Lubrol PX extracts to Lubrol PX-containing gradients produced a different pattern of sedimentation velocities of the G-proteins tested. G₀ and G₁₃ exhibited values of 4.2–4.3 S, which would be expected for G-protein heterotrimers of approx. 85 kDa. G₁₂ differed significantly from calculated sedimentation coefficients of 5.4 $\pm$ 0.27 S (n = 4). Therefore, it appears that Lubrol PX-solubilized G α_{12} shows a tendency to form complex molecular structures. This feature is in clear contrast to G₁₃. Solubilized, functionally active G₁₃ neither requires sucrose monolaurate nor displays a tight subunit association. Conversely, G α_{13} dissociates very easily from its G $\beta\gamma$ complexes, thereby remaining functionally active [14]. Accordingly, cholate facilitated subunit dissociation of G₁₃, since application of cholate-extracted proteins to gradients resulted in sedimentation velocities of 3.3 S and 4.0 S for G₁₃ and G₀ respectively (see Table 2).

Figure 4 Immunoblot analysis and photolabelling of membranes of High Five cells expressing the TXA₂ receptor

(A) Cell membranes (150 μ g of protein/lane) were acetone-precipitated, separated by SDS/PAGE and analysed by immunoblotting using anti- $\alpha_{q/11}$ (AS 369) (lanes 1 and 2), anti- α_{12} (AS 232) (lanes 3 and 4) and anti- α_{13} (AS 343) (lane 5) antisera. Antisera were preincubated with the peptides (10 μ g/ml) used for their generation (lanes 2 and 4). The ECL system was used for detection of filter-bound antibodies. The position of a 43 kDa marker protein is indicated. (B) High Five cell membranes were solubilized with CHAPS and reconstituted into phospholipid vesicles, as described in the Materials and methods section. Reconstituted proteins (lanes 3–6) and platelet membranes (lanes 1 and 2) were photolabelled with [α -³²P]GTP azidoanilide in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 30 μ M U 46,619. Immunoprecipitation was then performed with AS 232 (lanes 1–4) or AS 343 (lanes 5 and 6). Precipitated proteins were subjected to SDS/PAGE. An autoradiogram of an SDS gel is shown.

Characterization of the recombinant expressed TXA₂ receptors

In order to study the interaction of G₁₂-subfamily members with receptors, we expressed a human TXA₂ receptor [21] in the baculoviruses/High Five cell expression system. Isolated cell membranes exhibited a saturable binding of the TXA₂ receptor-selective radioligand [³H]SQ 29,548. Analysis of the [³H]SQ 29,548 equilibrium binding, based on the mass-action law, yielded a single class of binding sites with a K_D of 14 $\pm$ 2 nM and a B_{max} of 8.1 $\pm$ 0.3 pmol/mg of protein (mean $\pm$ S.D.; n = 4) using non-linear regression curve fits and transformation of binding data by Scatchard analysis. These results indicate a high level of receptor overexpression in High Five cell membranes as compared with data communicated from studies with human platelets (K_D approx. 8 nM; B_{max} approx. 1.5 pmol/mg), or stably transfected TXA₂ receptors in CHO cells (K_D approx. 10 nM; B_{max} 20–40 fmol/mg) [33,34].

Membranes of High Five cells expressing the human TXA₂ receptor were devoid of detectable G α_{13} , but contained endogenous G α_{12} - and G α_q -like proteins, as proven by immunoblot analysis (Figure 4A, lanes 1, 3 and 5). Interestingly, insect G α_{12} migrated with an apparent molecular mass of 45 kDa, which is higher than the apparent molecular mass of murine G α_{12} . Preincubation of the antisera with the peptides (10 μ g/ml) used for their generation abolished detection of insect G α_{12} - and G α_q -like proteins (see Figure 4A, lanes 2 and 4). In order to determine whether endogenous G-proteins of High Five cells interact with the expressed human TXA₂ receptor, cell membranes were solubilized with CHAPS, reconstituted into liposomes and subsequently incubated with [α -³²P]GTP azidoanilide and immunoprecipitated with specific G₁₂ and G₁₃ antibodies. They showed no incorporation of the GTP analogue into proteins, even after receptor stimulation with the specific TXA₂ receptor agonist U 46,619, whereas in the same experiment in platelet membranes a receptor-stimulated incorporation of [α -³²P]GTP azidoanilide into G α_{12} was visible (see Figure 4B). Since the insect G-proteins failed to couple to the expressed human receptors, no further

Figure 5 Effect of U 46,619 on photolabelling of G α -subunits reconstituted with TXA₂ receptors

G-proteins were reconstituted with TXA₂ receptor into phospholipid vesicles, as described in the Materials and methods section. Proteins were photolabelled with [α -³²P]GTP azidoanilide in the presence and absence of 30 μ M U 46,619 and immunoprecipitated with anti- α_{12} (AS 232), anti- α_{13} (AS 343), anti- α_o (AS 6), anti- α_i (AS 266) and anti- $\alpha_{q/11}$ (AS 369) antisera. Precipitated proteins were subjected to SDS/PAGE and visualized by exposure to X-ray films. Autoradiograms of SDS gels without (–) and with (+) agonist stimulation are shown.

purification of the extracted TXA₂ receptor was performed for reconstitution experiments with purified G-proteins.

Reconstitution of TXA₂ receptors with G-proteins

For studies on the interaction of G₁₂ subfamily members with a recombinant TXA₂ receptor, we reconstituted G-proteins with the receptor into phospholipid vesicles produced by gel filtration. Protein-containing vesicles eluted in the void volume were free of detergent. This is important, since sucrose monolaurate was found to diminish markedly the functional coupling between G₁₂ and the TXA₂ receptor, even at concentrations of 0.02 %. As a functional parameter, we measured [α -³²P]GTP azidoanilide incorporation into the respective G-protein, following activation of the receptor by the agonist U 46,619. For proof of specificity, G-proteins were subsequently immunoprecipitated with specific antibodies.

Figure 5 shows basal and receptor-stimulated incorporation of [α -³²P]GTP azidoanilide into the reconstituted G α subunits of G₁₂, G₁₃, G_{q/11}, G_{i1} and G_o proteins under optimized conditions. Whereas G₁₂ and G₁₃ incorporated small amounts of radioactivity in the absence of the receptor agonist even after 30 min of incubation, in the presence of U 46,619 the increase in [α -³²P]GTP azidoanilide incorporation into either protein was similar, indicating functional coupling of the receptor to these G-proteins (see Figure 5). As expected, G_{q/11} was activated after incubation with ligand-occupied receptor for 10 min. In contrast, G_{i1} and G_o proteins, which exhibited a reasonable basal guanine nucleotide turnover, showed no increase in the incorporation of [α -³²P]GTP azidoanilide in response to the receptor agonist (see Figure 5). These results indicate a specific functional interaction between both members of the G₁₂ subfamily and the reconstituted TXA₂ receptor.

DISCUSSION

Our knowledge of the biochemical properties and physiological functions of the G₁₂ subfamily of proteins is very limited. In order to gain more information, we have purified these proteins from rat brain membranes. Initially, isolation of functional, active, rat brain G₁₃ was possible, whereas G₁₂ was found to be

irreversibly denatured [14]. The different behaviours of G₁₂ and G₁₃ suggested distinct biochemical properties of the native proteins, which were not observed during purification of over-expressed recombinant G₁₂-proteins [16,17]. We therefore sought appropriate conditions for isolating native functionally active G₁₂. Previously, we observed species differences in the apparent molecular mass of G₁₃ [15]. In order to assure a species-independent comparison of purified members of the G₁₂ subfamily, rat brain membranes were chosen as the starting material for isolation of G₁₂. The native G α_{12} was purified by a combination of conventional column chromatography and affinity chromatography on immobilized G $\beta\gamma$ subunits in the presence of sucrose monolaurate. It turned out that the use of sucrose monolaurate was mandatory for isolation of functionally active G₁₂. This is quite different from purification protocols of G₁₃ using detergents commonly used for G-protein isolation, i.e. cholate and Lubrol PX. Speculating about the reasons, one has to consider that sucrose monolaurate is a detergent that preserves protein–protein interactions. Hence, it is not surprising that, with sucrose monolaurate, we observed sedimentation coefficients for G₁₂, G₁₃ and G_o that point to the formation of supramolecular structures by these proteins (see Table 2). Activation of G₁₂ and G_o by GTP[S] decreased their sedimentation coefficients to values which were still larger than those reported for G-protein heterotrimers, suggesting that in the presence of sucrose monolaurate even GTP[S]-activated G-proteins form supramolecular structures. Obviously, these structures can be associations between G-proteins and other proteins or, as suggested by Nakamura and Rodbell [31] and Jahangeer and Rodbell [32], multimeric structures of G-proteins. The latter possibility is supported by structural data showing that the N-terminus of crystallized, GDP-bound G α_{11} forms extensive quaternary contacts with neighbouring G α subunits [35]. Using Lubrol PX, which has been shown to facilitate disaggregation of supramolecular structures of G-proteins [32], the sedimentation behaviour of three tested G-proteins differed markedly. G₁₃ and G_o showed coefficients typical for G-protein heterotrimers, whereas G₁₂ displayed values indicating the formation of supramolecular complexes. However, Lubrol PX appeared to be insufficient to stabilize G₁₂ in its conformation, since the protein denatured with time. Cholate, known to reduce the affinity of G α subunits for G $\beta\gamma$ complexes [36], revealed the differences between the two G₁₂ subfamily members even more impressively. It produced a rapid aggregation of G₁₂ and allowed dissociation of G₁₃ into functional, active G α and G $\beta\gamma$ subunits ([14]; and see Table 2). Hence, we suppose that heterotrimeric G₁₂ may have a higher tendency to form supramolecular structures and exhibits a stronger subunit association than other G-proteins including G₁₃.

Therefore, we tested the subunit interaction between G α_{12} and immobilized G $\beta\gamma$ complexes in more detail. G α_{12} required a higher concentration of Mg²⁺ than G α_{13} or G $\alpha_{q/11}$ for AlF₄[–]-induced release from G $\beta\gamma$. Considering the observation that the Mg²⁺ concentration required for AlF₄[–]-induced release correlates with the affinity of a G α subunit for G $\beta\gamma$ [37], it is feasible to conclude that G α_{12} displays an enhanced affinity for G $\beta\gamma$ in comparison to other isoforms, including G α_{13} . This difference in affinity is most likely due to differences in the N-terminal region, a domain reported to be important not only for interaction of G α_{13} subunits with G $\beta\gamma$ dimers [15], but also for several other G α isoforms [38–41].

Both members of the G₁₂ subfamily have an extremely low basal exchange rate of guanine nucleotides in common, as shown for the native proteins in this report and for the recombinant proteins described in [16,17]. This points to the possibility that critical differences in guanine nucleotide-binding domains exist

between members of the G₁₂ subfamily and other G-protein subfamilies. The three-dimensional structures of the active conformations of G α_i and G α_{11} and of the heterotrimeric G_i and G_t were determined recently [42–45]. It appeared that Cys-321 and Thr-323 in G α_i (Cys-325 and Thr-327 in G α_{11}) are involved in binding of the guanine ring of GTP[S]. Both amino acids are conserved in most G-proteins, but not in members of the G₁₂ subfamily. In the case of G α_{12} and G α_{13} , Cys-321 of G α_i is replaced by Thr, and Thr-323 of G α_i is replaced by Ile.

In order to show the ability of purified members of the G₁₂ subfamily to couple functionally to receptors, we reconstituted them with recombinant expressed TXA₂ receptors. In contrast to G_o, a G-protein that was not expected to couple functionally to this receptor [6], both G₁₂ and G₁₃ were activated via the recombinant receptor. These results support previous findings under *in situ* conditions demonstrating the functional coupling of more than one G-protein isoform, e.g. G_q, G₁₂ and G₁₃ to TXA₂ receptors in human platelets [6]. Considering that G α_{12} activated the Na⁺/H⁺-exchanger via a protein kinase C pathway, whereas G α_{13} stimulated this effector in a protein kinase C-independent manner [9], one may speculate that effectors rather than receptors are able to discriminate between members of the G₁₂ subfamily.

We are grateful to Dr. M. I. Simon (Pasadena, CA, U.S.A.) for G α_{12} cDNA, and to Drs. P. V. Halushka and C. J. Allan (Charleston, SC, U.S.A.) for TXA₂ receptor cDNA. We thank Dr. K.-L. Laugwitz for synthesis of [α -³²P]GTP azidoanilide, and Drs. K. Spicher and K.-D. Hinsch for providing antisera. Thanks to the Institut für Toxikologie und Embryonalpharmakologie, Freie Universität Berlin, for providing rat brains. R. H. was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft (DFG). D. L. was supported by a predoctoral fellowship of the State of Berlin (NaFöG). This work was supported by grants from the DFG and by the Fonds der Chemischen Industrie.

REFERENCES

- Neer, E. J. (1995) *Cell* **80**, 249–257
- Nürnberg, B., Gudermann, T. and Schultz, G. (1995) *J. Mol. Med.* **73**, 123–132
- Simon, M. I., Strathmann, M. P., and Gautam, N. (1991) *Science* **252**, 802–808
- Strathmann, M. P. and Simon, M. I. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5582–5586
- Spicher, K., Kalkbrenner, F., Zobel, A., Harhammer, R., Nürnberg, B., Söling, A. and Schultz, G. (1994) *Biochem. Biophys. Res. Commun.* **198**, 906–914
- Offermanns, S., Laugwitz, K.-L., Spicher, K. and Schultz, G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 504–508
- Laugwitz, K.-L., Allgeier, A., Offermanns, S., Spicher, K., van Sande, J., Dumont, J. E. and Schultz, G. (1995) *Exp. Clin. Endocrinol.* **2**, A19
- Voyno-Yasenetskaya, T., Conklin, B. R., Gilbert, R. L., Hooley, R., Bourne, H. R. and Barber, D. L. (1994) *J. Biol. Chem.* **269**, 4721–4724
- Dhanasekaran, N., Vara Prasad, M. V. V. S., Wadsworth, S. J., Dermott, J. M. and Rossum, G. (1994) *J. Biol. Chem.* **269**, 11802–11806
- Xu, N., Bradley, L., Ambudkar, I. and Gutkind, J. S. (1993) *Proc Natl Acad Sci U.S.A.* **90**, 6741–6745
- Jiang, H., Wu, D. and Simon M. I. (1993) *FEBS Lett.* **330**, 319–322
- Vara Prasad, M. V. V. S., Dermott, J. M., Heasley, L. E., Johnson, G. L. and Dhanasekaran, N. (1995) *J. Biol. Chem.* **270**, 18655–18659
- Buhl, A. M., Johnson, N. L., Dhanasekaran, N. and Johnson, G. L. (1995) *J. Biol. Chem.* **270**, 24631–24634
- Harhammer, R., Nürnberg, B., Spicher, K. and Schultz, G. (1994) *Biochem. J.* **303**, 135–140
- Harhammer, R., Nürnberg, B., Spicher, K. and Schultz, G. (1994) *Biochem. Biophys. Res. Commun.* **204**, 835–840
- Singer, W. D., Miller, R. T. and Sternweis, P. C. (1994) *J. Biol. Chem.* **269**, 19796–19802
- Kozasa, T. and Gilman, A. G. (1995) *J. Biol. Chem.* **270**, 1734–1741
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Schägger, H. and von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- Spicher, K., Nürnberg, B., Jäger, B., Rosenthal, W. and Schultz, G. (1992) *FEBS Lett.* **307**, 215–218
- Harteneck, C., Obukhov, A. G., Zobel, A., Kalkbrenner, F. and Schultz, G. (1995) *FEBS Lett.* **358**, 297–300
- Veit, M., Nürnberg, B., Spicher, K., Harteneck, C., Ponimaskin, E., Schultz, G. and Schmidt, M. F. G. (1994) *FEBS Lett.* **339**, 160–164
- Körner, C., Nürnberg, B., Uhde, M. and Bräulke, T. (1995) *J. Biol. Chem.* **270**, 287–295
- Pfeifer, A., Nürnberg, B., Kamm, S., Uhde, M., Schultz, G., Ruth, P. and Hofmann, F. (1995) *J. Biol. Chem.* **270**, 9052–9059
- Nürnberg, B., Spicher, K., Harhammer, R., Bosserhoff, A., Frank, R., Hilz, H. and Schultz, G. (1994) *Biochem. J.* **300**, 387–394
- Sternweis, P. C. and Pang, I.-H. (1990) in *Receptor–Effector Coupling: A Practical Approach* (Hulme, E. J., ed.), pp. 1–30, Oxford University Press, Oxford
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Im, M.-J., Holzhoefer, A., Boettlinger, H., Pfeuffer, T. and Helmreich, E. J. M. (1988) *FEBS Lett.* **227**, 225–229
- Iyengar, R., Rich, K. A., Herberg, J. T., Premont, R. T. and Codina, J. (1988) *J. Biol. Chem.* **263**, 15348–15353
- Nakamura, S.-I. and Rodbell, M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6413–6417
- Jahangeer, S. and Rodbell, M. (1993) *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8782–8786
- Hedberg, A., Hall, S. E., Ogletree, M. L., Harris, D. N. and Liu, E. C. K. (1988) *J. Pharm. Exp. Ther.* **245**, 786–792
- Raychowdhury, M. K., Yukawa, M., Collins, L. J., McGrail, S. H., Kent, K. C. and Ware, J. A. (1994) *J. Biol. Chem.* **269**, 19256–19261
- Mixon, M. B., Lee, E., Coleman, D. E., Berghuis, A. M., Gilman, A. G. and Sprang, S. R. (1995) *Science* **270**, 954–960
- Hepler, J. R., Kozasa, T., Smrcka, A. V., Simon, M. I., Rhee, S. G., Sternweis, P. C. and Gilman, A. G. (1993) *J. Biol. Chem.* **268**, 14367–14375
- Pang, I.-H. and Sternweis, P. C. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7814–7818
- Navon, S. E. and Fung, B. K. K. (1987) *J. Biol. Chem.* **262**, 15746–15751
- Neer, E. J., Pulsifer, L. and Wolf, L. G. (1988) *J. Biol. Chem.* **263**, 8996–9000
- Journot, L., Pantaloni, C., Bockaert, J. and Audiger, Y. (1991) *J. Biol. Chem.* **266**, 9009–9015
- Graf, R., Mattera, R., Codina, J., Estes, M. K. and Birnbaumer, L. (1992) *J. Biol. Chem.* **267**, 24307–24314
- Noel, J. P., Hamm, H. E. and Sigler, P. B. (1993) *Nature (London)* **366**, 654–663
- Coleman, D. E., Berghuis, A. M., Lee, E., Linder, M. E., Gilman, A. G. and Sprang, S. R. (1994) *Science* **265**, 1405–1412
- Wall, M. A., Coleman, D. E., Lee, E., Iniguez-Lluhi, J. A., Posner, B. A., Gilman, A. G. and Sprang, S. R. (1995) *Cell* **83**, 1047–1058
- Lambright, D. G., Sondek, J., Böhm, A., Skiba, N. K., Hamm, H. E. and Sigler, P. B. (1996) *Nature (London)* **379**, 311–319