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Reconstitution of β-adrenergic receptor with components of adenylate cyclase

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β_1-Adrenergic receptor proteins were extracted from turkey erythrocyte membranes with lauroyl sucrose and digitonin and purified by affinity chromatography on a column of alprenolol agarose Affi-gel 10 or 15. The 5000-fold purified receptor is able to couple functionally with the stimulatory GTP-binding protein (G_s) from either turkey or duck erythrocytes. Functional coupling was achieved by three different approaches. (i) Purified β-receptor polypeptides were coupled in phospholipid (asolectin) vesicles with G_s from a crude cholate or lauroyl sucrose extract of turkey erythrocyte membranes. The detergent was removed and vesicles were formed with SM-2 beads. (ii) Purified β-receptor was reconstituted with pure, homogeneous G_s in asolectin vesicles. (iii) Purified β-receptors were either coupled in asolectin vesicles with a mixture of pure, homogeneous Gpp(NH)p-activated G_s and a lauroyl sucrose extract of turkey erythrocyte membranes, or with pure, homogeneous Gpp(NH)p-activated G_s alone. The decay of activity was measured on addition of GTP and hormone. In (ii) and (iii), the detergent was removed and vesicles were formed by gel filtration on Sephadex G-50 columns. In each of the three different experimental conditions, the β-receptor was activated with l-isoproterenol and activation was blocked with d,l-propranolol. Activated G_s were measured separately by means of their capacity to activate a crude Lubrol PX-solubilized adenylate cyclase preparation from rabbit myocardial membrane. The kinetics of G_s activation by purified β-receptors occupied by l-isoproterenol was first order and activation was linearly dependent on receptor concentration. This indicates that in the reconstituted system the β_1-adrenergic receptor acts catalytically, as it does in native membranes. Gpp(NH)p-preactivated duck erythrocyte G_s can be deactivated by GTP to the basal activity state only in the presence of the β (γ) subunits of G_s. This observation indicates that α-β (γ) subunit interactions are necessary for guanylnucleotide exchange.

Key words: adenylate cyclase/β-adrenergic receptor/GTP-binding proteins/reconstitution

Introduction

The development of methods for purification of β_1-adrenergic receptors (Shorr *et al.*, 1982; Homcy *et al.*, 1983; Feder *et al.*, 1984) and of the GTP-binding regulatory component G_s (Northup *et al.*, 1980; Hanski *et al.*, 1981) together with the development of reconstitution techniques (Pedersen and Ross, 1982; Citri and Schramm, 1980, 1982; Gal *et al.*, 1983; Feder *et al.*, 1984) has opened the way for studies of the stoichiometry and kinetics of interaction with isolated, purified components of the signal transduction chain from β-receptor to adenylate cyclase. The first successful reconstitution assay with purified components was developed by Pedersen and Ross (1982) and Brandt *et al.* (1983). They followed hormone β-adrenergic receptor-dependent induction of GTPase activity in G_s, binding of [^{35}S]GTP(γS) to G_s, and activation of G_s. We have measured β-receptor-dependent activation and deactivation of G_s and have assayed G_s activity with a crude, soluble adenylate cyclase preparation from rabbit myocardial membranes (Pfeuffer *et al.*, 1983). β-Receptor G_s coupling was carried out in the absence of adenylate cyclase in most instances. This approach is valid, since the mode of coupling between β-receptor and G_s in native membranes is the same, regardless of whether the catalytic component is active or inactivated (Gal *et al.*, 1983).

The purpose of the present experiments was to test whether reconstituted systems made up of isolated purified β-receptor and G_s-proteins in phospholipid vesicles retain the properties of β-receptor-linked adenylate cyclase in the native state (see Levitzki and Helmreich, 1979). These properties are: (i) catalytic action of hormone-activated β-receptor which can activate many G- and C-units; (ii) decay of activated adenylate cyclase to an inactive state on hydrolysis of GTP to GDP and P_i by G_s (Cassel and Selinger, 1976). We present results obtained with β-receptors purified by affinity chromatography: (i) on the kinetics of activation of G_s preparations at various stages of purification including homogeneous G_s; and (ii) on reversal of Gpp(NH)p-activated G_s in the presence of GTP and β-adrenergic agonist.

Results

With one exception, all experiments were carried out with purified β-adrenergic receptor (see Figure 1). 60-70% of the β_1-adrenergic receptors present in turkey erythrocyte membranes were made soluble with 0.3% lauroyl sucrose. When extraction was carried out with 1.0% digitonin, only 30-40% of the receptors were solubilized. The receptor proteins were adsorbed on the affinity gel and removed from the gel by 0.1% digitonin and 0.5 mM alprenolol (see Feder *et al.*, 1984). This step provides an ~5000-fold purified receptor preparation with 40-50% yield, starting from the lauroyl sucrose extract of the membranes. Therefore, this receptor preparation is at least 10% pure. Figure 1 documents also the purity of the G_s preparation from turkey erythrocyte membranes. The molar ratio of G_s-α to G_s-β was ~1:1. The incorporations of purified receptor in the presence of various detergents (such as lauroyl sucrose, cholate, desoxycholate, n-octylglucoside alone or in combination) into soybean phospholipid vesicles in the presence of SM-2 beads, or using G-50 gel filtration, were compared. Incorporation of functional β-adrenergic receptor proteins was measured by binding of [^{125}I]iodocyanopindolol ([^{125}I]ICYP). The extent of incorpor-

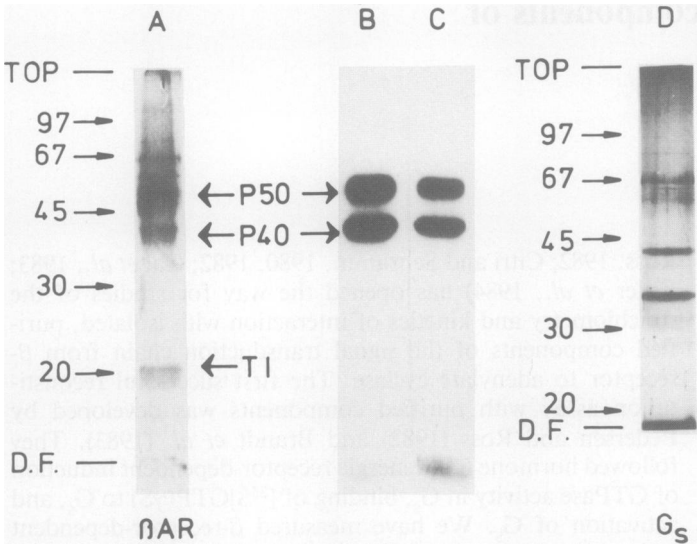

Fig. 1. Purity of β-receptor and G_s. Receptor and G_s were purified from turkey erythrocyte membranes as described in Materials and methods. Photoaffinity labeling with [^{125}I]ICYP azide-2, SDS-PAGE and silver staining are described in Materials and methods. Mol. wt. standards are listed in Materials and methods. **(A)** Silver staining of affinity chromatographically purified β-receptor. The two upper bands in **A** and **D** at 65 kd are artifacts of the silver stain, the band in **A** at 22 kd is soybean trypsin inhibitor. **(B)** Photoaffinity-labeled purified β-receptor in 0.02% digitonin. **(C)** Photoaffinity-labeled crude β-receptor in a 1% digitonin extract of turkey erythrocyte membranes. There are two forms with mol. wts. 50 and 40 kd, respectively, of the β_1-adrenergic receptor in turkey erythrocyte membranes which are apparently indistinguishable with regard to ligand binding (cf. Shorr *et al.*, 1982; Hekman *et al.*, 1984). **(D)** Silver staining of G_s. The band at 42 kd is G_s-α and the band at 35 kd is the β subunit. The arrows to the left of lanes **A** and **D** mark mol. wt. standards in kd.

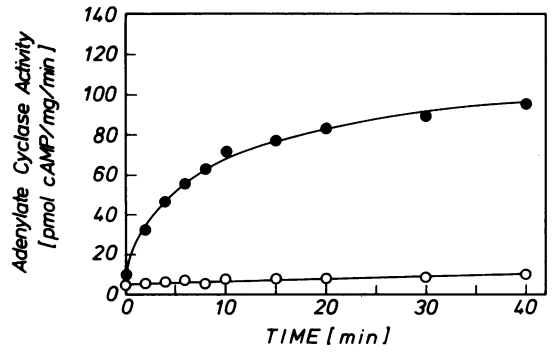

Fig. 2. Functional reconstitution of the adenylate cyclase complex. β-Receptor, G_s and adenylate cyclase from turkey erythrocyte membranes were extracted with 0.3% lauroyl sucrose in buffer B and inserted into asolectin vesicles using the SM-2 beads method. The amount of incorporated β-receptor was 1.6 pmol binding sites/ml. Activation was carried out with (●) 100 μM Gpp(NH)p and 100 μM l-isoproterenol; (○) is the control with 100 μM Gpp(NH)p and 100 μM d,l-propranolol.

ation ranged from 35 to 65%, depending on the detergent and the method used for vesiculation.

Lauroyl sucrose deserves a comment because it has apparently not yet been used for β-receptor solubilization. Figure 2 shows that one can solubilize from turkey erythrocyte membranes all three components (R, G and C) of the adenylate cyclase complex. The lauroyl sucrose extract from turkey erythrocyte membranes contains 2.0–2.5 pmol β-receptor binding sites/mg protein (60–70% yield) and 0.6–0.7 nmol/mg/min cyclase specific activity determined by activation

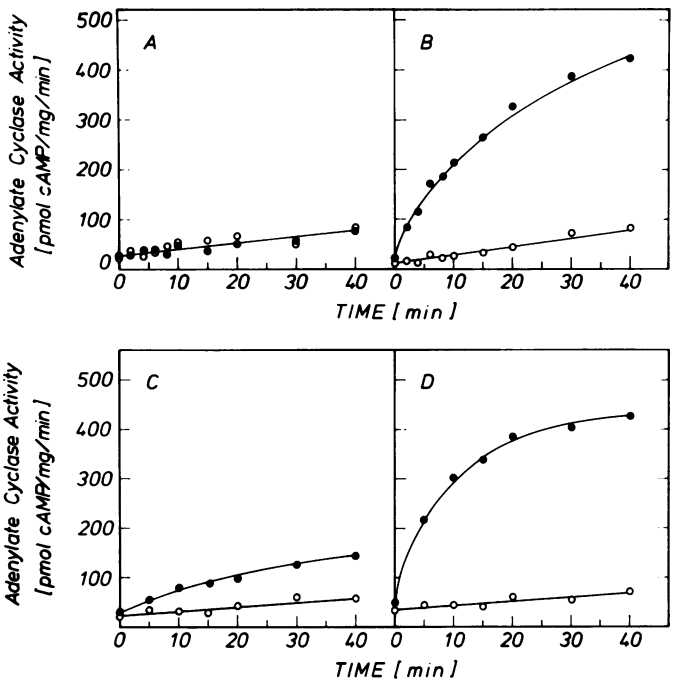

Fig. 3. Reconstitution of purified β-receptor with crude G_s. **(A)** and **(B)** are experiments with cholate-solubilized crude G_s. **(C)** and **(D)** are comparable experiments with a lauroyl sucrose extract from turkey erythrocyte membranes. Affinity chromatographically purified β-receptor was added in **(B)** and **(D)**. Final concentration of receptor binding sites was in **(A)** 0.15 pmol/ml, in **(B)** 1.70 pmol/ml, in **(C)** 1.05 pmol/ml and in **(D)** 1.95 pmol/ml. Reconstitution was carried out in asolectin vesicles by the SM-2 beads method. Activation was carried out with (●) 100 μM Gpp(NH)p and 100 μM l-isoproterenol; (○) is the control with 100 μM Gpp(NH)p and 100 μM d,l-propranolol.

with Gpp(NH)p. The extracted components could be functionally coupled on incorporation into asolectin vesicles (final concentration of asolectin lipids: 0.8 mg/ml of the reconstitution mixture). After vesicles were formed and detergent removed by SM-2 beads, all three components became functionally coupled giving the hormonally stimulated adenylate cyclase activity shown in Figure 2. It should be noted that the lauroyl sucrose-extracted crude components, after incorporation into asolectin vesicles, recombine with high efficiency. Thus, the half-time of adenylate cyclase activation by isoproterenol in the reconstituted system was ~6 min which compares favorably with a $t_{1/2}$ ~1–1.5 min for the native turkey erythrocyte membrane under comparable conditions. Moreover, the hormone receptor-independent activation in the presence of propranolol and Gpp(NH)p was small. Hence, the use of lauroyl sucrose allows the simultaneous extraction of the β-receptor, G_s and C components. Moreover, this detergent solubilizes efficiently β-adrenergic receptor with retention of specific binding activity. The fact that lauroyl sucrose-solubilized C can be incorporated into asolectin vesicles and effectively coupled to R and G_s, after removal of detergent, makes this assay attractive for future reconstitution experiments with pure, homogeneous components.

Extraction of turkey erythrocyte membranes with a mixture of 1.2% cholate and 0.4 M NaCl provides a source of crude G_s. This crude G_s preparation was reconstituted with purified β-receptor in soybean lipid vesicles and activated with Gpp(NH)p and isoproterenol. Vesicles were formed on

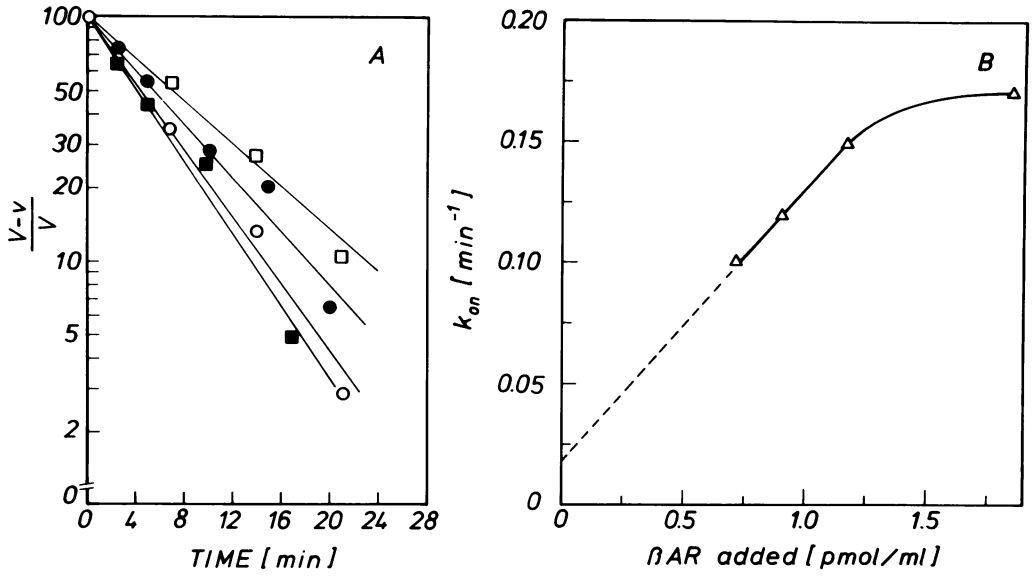

Fig. 4. Activation of crude G_s as a function of added receptor. **(A)** Kinetics of activation: purified β-receptor was added to a 0.3% lauroyl sucrose extract of turkey erythrocyte membranes as in Figure 3D. The total concentrations of receptor binding sites were (■) 2.75 pmol/ml; (○) 2.06 pmol/ml; (●) 1.80 pmol/ml; and (□) 1.64 pmol/ml. **(B)** The first order rate constant of activation, k_{on}, is plotted against the concentration of added receptor.

treatment of the mixture with SM-2 beads (see Figure 3A and B). Crude G_s may also be extracted from turkey erythrocyte membranes with lauroyl sucrose (Figure 3C and D). Lauroyl sucrose-extracted G_s can likewise be effectively coupled to purified β-receptor using either soybean lipids or a mixture of soybean and turkey erythrocyte membrane lipids. Reconstitution in lipid vesicles was again carried out with SM-2 beads. In these experiments, activation was a first order reaction and was dependent on the concentration of added purified β-receptor (Figure 4). The half-time of activation ranged from 4 to 7 min, whereas the half-time for adenylate cyclase activation in intact erythrocyte membranes, under comparable conditions, is ~1–1.5 min.

These successful reconstitution experiments with affinity chromatographically purified β-receptor and crude soluble G-proteins from turkey erythrocyte membranes set the stage for reconstitution experiments with pure, homogeneous G_s (see Figure 1). Activation and kinetics of the coupling reaction with purified components are shown in Figure 5. Coupling was carried out in a mixture containing cholate, deoxycholate, asolectin and bovine serum albumin (BSA) essentially as described by Brandt *et al.* (1983). It is of interest that comparable results (not shown) were also obtained when cholate/deoxycholate was replaced by 0.3% lauroyl sucrose. One experimental detail should be noted: with pure G_s it was necessary to pass the reconstitution mixture through a Sephadex G-50 column rather than to use SM-2 beads. Coupling kinetics with purified G_s was, as in the case of crude G_s, of the first order, with k_{on} of ~0.06/min and $t_{1/2}$ of activation of ~11 min.

Figure 6A and B describes reversal of activation using Gpp(NH)p-activated G_s. We first did experiments with purified G_s, pre-activated with Gpp(NH)p and purified β-receptor proteins in a reconstitution mixture with soybean lipids fortified with a 0.3% lauroyl sucrose extract of turkey erythrocyte membranes. For removal of detergent and vesicle formation, the SM-2 bead method was used. Although 35–40% deactivation was routinely observed, 10–15% of the reversal of activity was independent of added purified β-receptor and therefore due to β-receptor carried over with the lauroyl

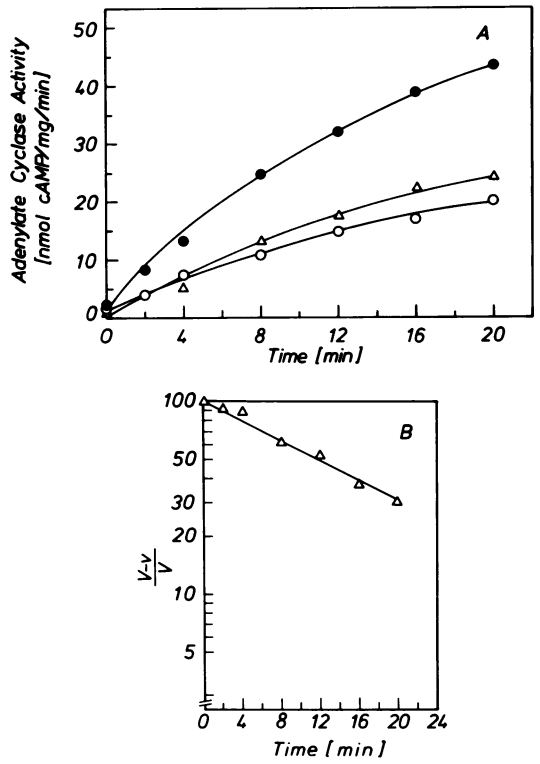

Fig. 5. Functional reconstitution with purified β-receptor and pure G_s. **(A)** (●) Activation by Gpp(NH)p and l-isoproterenol; (○) is the control with Gpp(NH)p and d,l-propranolol; (△) is net specific activation due to l-isoproterenol. **(B)** Kinetics of isoproterenol-induced activation. Purified β-receptor was in 0.02% digitonin; G_s was in 1 mM Lubrol PX. Reconstitution was carried out with the G-50 gel filtration method. Activation was at 37°C with 10 μM Gpp(NH)p, 100 μM l-isoproterenol and 0.1 mM Mg^{2+}. The control received 10 μM Gpp(NH)p, 100 μM d,l-propranolol and 0.1 mM Mg^{2+}. The molar ratio of β-receptor to G_s was 1:4.

sucrose extract of the membranes. In native turkey erythrocyte membranes Gpp(NH)p-activated G_s is deactivated on addition of GTP. Deactivation requires a functional β-receptor and an agonist, i.e., l-isoproterenol. This applies to

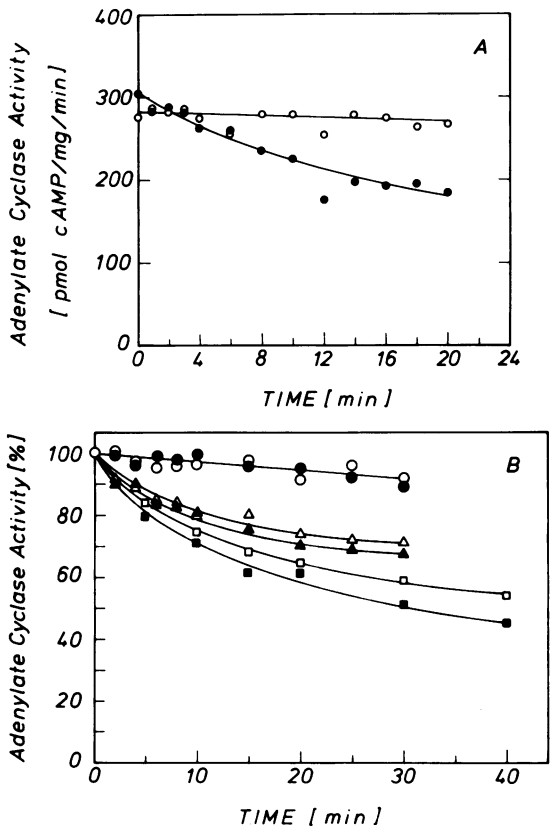

Fig. 6. Reversal of activation. **(A)** In the presence of a lauroyl sucrose extract. Purified β-receptor, 7.2 pmol binding sites in 0.02% digitonin, was mixed with 17.5 pmol of pure Gpp(NH)p-activated duck erythrocyte G_s in 1 mM Lubrol PX and with soybean lipids and a 0.3% lauroyl sucrose extract of turkey erythrocyte membranes. The reconstitution mixture was treated with SM-2 beads. Reversal of activity was followed at 37°C on addition of (●) 100 μM l-isoproterenol and 100 μM GTP, (○) is the control with 100 μM d,l-propranolol and 100 μM GTP. After the reaction was stopped, the remaining G_s activity was determined. **(B)** In the absence of a lauroyl sucrose extract. The Sephadex G-50 gel filtration method was used. (●, ▲, ■) Reversal in the presence of 100 μM l-isoproterenol and 10 μM GTP; (○, △, □) are controls in the presence of 100 μM d,l-propranolol and 10 μM GTP. Reversal of activity at 37°C is given in percent of original activity. 100% corresponds to an activity of 150 nmol cAMP/min/mg. In (▲, △), the pure β subunit was added at a molar rate of β to G_s-α of 4:1 and in (■, □) at a ratio of 26:1. Residual G_s activity was assayed with rabbit myocardial adenylate cyclase. The endogenous adenylate cyclase activity (0.04 nmol/mg/min) was deducted in each case.

native turkey erythrocyte membranes (Sevilla and Levitzki, 1977; Arad *et al.*, 1981) and to a crude Lubrol PX-treated membrane extract (Keenan *et al.*, 1982). The data in Figure 6B indicate that no reversal of activity occurred when a pure homogeneous Gpp(NH)p-activated G_s preparation was reconstituted with purified β-receptors in cholate-deoxycholate in the presence of soybean lipids, but without addition of a lauroyl sucrose extract. In the experiment in Figure 6B, Sephadex G-50 gel filtration was used to remove detergent and induce vesiculation, instead of SM-2 beads. Figure 6B shows also that effective (40–60%) reversal under these conditions required addition of a 4- to 26-fold excess of the 35-kd β-subunit over the GTP binding 42-kd α-subunit of G_s. However, reversal in the presence of the β-subunit was only marginally (5–10%) dependent on isoproterenol-activated purified β-receptor. A possible explanation is given in the Discussion.

Discussion

The first attempts to reconstitute the initial sequence of the signal transmission chain from β-adrenergic receptor to adenylate cyclase using isolated, purified β-receptor and G_s preparations proved successful (Brandt *et al.*, 1983), and other laboratories (Kelleher *et al.*, 1983; Feder *et al.*, 1984; Caron *et al.*, 1984) have followed. This report presents our attempts to construct a viable reconstitution system which allows coupling of purified β-receptor and pure G_s in lipid vesicles. The main differences between our procedures and those of other laboratories are detailed below.

(i) Purification of the β-receptor by affinity chromatography. We have developed a simple chemical procedure for derivatisation of β-adrenergic ligands which involves modification of primary amine derivatives of β-blockers. This versatile method was applied to the synthesis of fluorescent alprenololamine derivatives (Henis *et al.*, 1982), photoaffinity labels (Burgermeister *et al.*, 1982, 1983) and to preparation of effective affinity gels (Feder *et al.*, 1984). With these affinity gels we have purified 4000- to 5000-fold β-adrenergic receptor polypeptides from turkey erythrocyte membranes starting from a detergent extract (Figure 1) (Feder *et al.*, 1984). This shows that the β-adrenergic receptor purified by our affinity chromatographic procedure and activated with l-isoproterenol, a potent β-adrenergic agonist, is capable of effectively coupling with G_s after insertion into asolectin vesicles.

(ii) The use of lauroyl sucrose. We have synthesized several acylesters of sucrose and raffinose, differing in chain length and extent of derivatisation. The intention was to replace digitonin by a more appropriate detergent. Digitonin has an unfavorably low critical micellar concentration and interferes with coupling. Lauroyl sucrose proved to be more effective for solubilizing the β-adrenergic receptor from turkey erythrocyte membranes. Moreover, lauroyl sucrose does not interfere with the coupling reaction. This detergent does not affect binding of crude soluble receptor preparations to the affinity support, but for specific removal of the receptor bound to the gel with alprenolol, digitonin (0.1%) is still required. The experiment in Figure 2 shows that one can solubilize with lauroyl sucrose all components (R, G and C) of the signal transduction chain present in turkey erythrocyte membranes and reconstitute the transduction pathway after removal of the detergent by inserting the crude components into lipid vesicles. The fact that lauroyl sucrose neither interferes with coupling of crude and purified R and G_s components in asolectin vesicles, nor with the catalytic moiety of adenylate cyclase (Figure 2) makes this detergent valuable for the reconstitution of pure components.

Purified β-receptors in lauroyl sucrose (or in mixtures of lauroyl sucrose and n-octylglucoside or cholate) can be incorporated with good yields into phospholipid vesicles following removal of the detergent by absorption on SM-2 beads or by the Sephadex G-50 method. The G-50 method was, however, far superior to the SM-2 bead method when purified β-receptor was reconstituted with pure, homogeneous G_s (Figure 5). We have not yet studied the lipid requirement for effective coupling between β-receptor and G_s, although admixture of turkey erythrocyte membrane lipids to soybean lipids, or replacement of soybean lipids by dimyristoylphosphatidylcholine (DMPC) had no effect on the binding capacity of the incorporated β-receptors. A study of the vesicles by electron microscopy did show variations in size and structure depen-

dent on lipids, detergents and on whether hydrophobic beads or gel filtration were used to remove detergent. For example, the vesicles were mostly unilamellar with an average diameter of d = 16–50 nm if the G-50 method was used. On the other hand, a mixture of unilamellar vesicles with d = 54–82 nm and multilamellar vesicles with d = 200–300 nm were observed with SM-2 beads, but a systematic relationship of vesicle size and receptor incorporation and functional coupling was not apparent.

The functional characteristics of β-receptor G_s interactions in the reconstituted system allow us to conclude that the kinetics of β-receptor G_s coupling are not qualitatively different from that in the native system. (a) This follows because the activation kinetics of G_s by the hormone receptor complex is first order. (b) The first order rate constant of activation, k_{on}, is linearly dependent on receptor concentration (Figure 4). This kinetic behavior is in accordance with the 'collision coupling mechanism' described in native membranes by Tolkovsky and Levitzki (1978) and Arad *et al.* (1981). This gives assurance that the reconstituted system has retained the characteristic functional properties of the transduction system in native membranes. The rate constant of activation using purified β-receptor and pure G_s is 2–3 times smaller than with crude G_s. The reasons for the less than optimal coupling rate in the case of pure G_s are not known. We do not know for instance whether replacement of the SM-2 bead method, which is less effective with pure, homogeneous G_s as coupling partner, by the Sephadex G-50 gel filtration method is responsible for the coupling impairment, or whether the topological requirements for the coupling reaction are more stringent in the case of pure components. This and the fact that reversal of activity of Gpp(NH)p-activated pure G_s on addition of GTP is more responsive to β-adrenergic receptor interactions in the presence of a lauroyl sucrose extract of turkey erythrocyte membranes than in its absence (compare Figure 6A and B) indicates that further work is needed to optimise the reconstituted system. Although reversal of activation occurred without addition of a lauroyl sucrose membrane extract with an up to 26-fold excess of pure β subunits (Figure 6B), the dependence of the reversal reaction on agonist-activated β-receptor was still rather poor. Addition of agonist accelerated the rate of reversal by only ~10%. In reversal experiments, rates of association of β and pre-activated G_s-α-Gpp(NH)p might be faster than rate of coupling of activated β-adrenergic receptor and activated G_s-α. But other possibilities have not yet been excluded.

A more general comparison of activation and deactivation experiments with pure components alone, or with the addition of a lauroyl sucrose extract containing proteins and lipids present in the native membrane, reveals some differences (compare Figures 2, 3 and 5). (a) the hormonally independent activation of G_s is more sensitive towards Mg^{2+} and Gpp(NH)p in a reconstituted system containing only purified components than in the same system supplemented with the crude membrane extract. (b) Deactivation of G_s-Gpp(NH)p and its decay to inactive G_s-GDP was more responsive to β-receptor action when membrane extract was added, resembling more the native state, than reversal of activity carried out with pure components alone (compare Figure 6A and B). Thus, at present we cannot exclude that factor(s) present in the lauroyl sucrose extract of membranes, which are missing when only pure components are coupled, are required for efficient hormone receptor-coupled deactivation. Whether this factor(s) is an additional coupling factor, the inhibitory G or special lipids is a matter of speculation. There is, however, little doubt that the further study of deactivation kinetics will open new and attractive possibilities to learn more about the specific requirements for effective coupling of G-proteins and β-receptor. The final goal is to develop an assay which allows the study of the interaction in lipid vesicles of all components of the signal transmission chain purified to homogeneity, including a pure adenylate cyclase preparation.

Materials and methods

Chemicals

GTP, guanyl-5'-yl-imidodiphosphate [Gpp(NH)p], creatine kinase and creatine phosphate were purchased from Boehringer, Mannheim, FRG. BSA, ATP, 3',5'-cAMP, Lubrol PX, sodium deoxycholate, trypsin inhibitor, soybean phospholipids (asolectin), l-alprenolol, d,l-propranolol, bacitracin, n-ocytlglucoside and DMPC were obtained from Sigma, Munich, FRG. l-Isoproterenol, benzamidine and lauroyl chloride were from Ega-Chemie, Steinheim, FRG. Hydroxyapatite (DNA-Grade Bio-Gel HTP), SM-2 beads and Affi-gels 10 and 15 were from Bio-Rad, Munich, FRG. Silica gel 60 was a product of Merck, Darmstadt, FRG. Anion exchanger DE 52 was obtained from Whatman, UK, and phenylmethylsulfonyl fluoride (PMSF) was from Fluka, Buchs, Switzerland. Pre-mixed mol. wt. standards for gel electrophoresis from Pharmacia, Uppsala, Sweden, were phosphorylase (97 400), albumin (67 000), ovalbumin (43 000), carbonic anhydrase (30 000), soybean trypsin inhibitor (20 100) and α-lactalbumin (14 400). [^{125}I]ICYP (2000 Ci/mmol) was from Amersham Buchler, Braunschweig, FRG. [^{3}H]Dihydroalprenolol ([^{3}H]DHA; 35.6 Ci/mmol), [α-^{32}P]adenosine-5'-triphosphate ([α^{32}P]ATP; 600 Ci/mmol) and [^{3}H]fluorodinitrobenzene ([^{3}H]FDNB; 15 Ci/mmol) were obtained from New England Nuclear Corp., Boston, USA. [^{125}I]-ICYP azide-2 (2000 Ci/mmol) was synthesized according to Burgermeister *et al.* (1982). Sodium cholate, digitonin, SDS and dithiothreitol (DTT) were from Serva, Heidelberg, FRG. Digitonin was further purified by heating a 2% aqueous solution at 95–100°C for 5 min. The solution was then filtered through membrane filters (Sartorius, Type SM-41) and lyophylized. All other chemicals were of the highest commercially available grade. Buffers: Buffer A; 50 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA. Buffer B; 10 mM Tris-HCl, pH 7.4, 90 mM NaCl, 10 μg/ml soybean trypsin inhibitor, 200 μM benzamidine, 100 μg bacitracin/ml, 30 μM PMSF.

Lauroyl sucrose

The method of Osipow *et al.* (1956) modified as follows was used. Sucrose (0.3 mol) was dissolved in N,N-dimethyl formamide (600 ml) and pyridine (20 ml). The solution was cooled in an ice-bath. Lauroyl chloride (0.086 mol) was added dropwise with stirring keeping the temperature below 4°C. After 4 h at 4°C with stirring, the unreacted lauroyl chloride was decomposed by adding saturated sodium hydrogen carbonate (20 ml) and the organic solvent wes removed by distillation *in vacuo* at 70°C. The viscous residue was treated with acetone (600 ml) and the non-dissolved sucrose filtered off. Acetone was removed by distillation and the viscous material (~40 g) dissolved in 120 ml chloroform. A chloroform solution containing 5 g of lauroyl sucrose was applied to a silica gel 60 column (2 x 50 cm, 70–230 mesh, Merck). The silica gel column was eluted in the following order. Firstly, (a) with 200 ml chloroform; (b) with 200 ml chloroform/acetone (7:3 v/v); (c) with 200 ml chloroform/acetone (4:6 v/v); (d) with 200 ml acetone, and finally (e) with 200 ml acetone/methanol (9:1 v/v). Fractions were collected and dried by evaporation and the white powder was dissolved in water. A 1% solution was applied to the anion exchanger DE 52 column (1 x 5 cm) and the collected fraction was lyophylized. The total amount of lauroyl sucrose was 18–22 g. The substance was identified by t.l.c. chromatography on silica gel 60 plates (Merck). 2-Propanol/NH_3/H_2O (7:1:2 v/v) was used as solvent. The R_f value of the synthesized product was 0.47 and identical to a reference sample of monolauroyl sucrose, kindly supplied by Dr Hermann Kühn, Jülich, FRG.

Affinity gels

Affinity gels for purification of β-receptors were prepared starting with a primary amine derivative of alprenolol (Henis *et al.*, 1982). Affinity gels 10 and 15 were loaded with ~15 μmol of activated ester groups/g gel. The activated gels (18 g) were reacted with 8–10 μmol alprenololamine/g gel in 100 ml dioxan at room temperature for 3 h. Excess-activated ester groups were destroyed by stirring the gel with 0.8 ml ethanolamine for 2 h at 10°C. The gel was washed exhaustively with 25 mM Tris HCl, 90 mM NaCl, 2 mM EDTA buffer, pH 7.4.

Adenylate cyclase activity measurements

G_s activity was measured with a crude Lubrol PX-solubilized adenylate cyclase preparation from rabbit myocardial membranes. Rabbit myocardial

membranes (10 mg/ml) were solubilized with Lubrol PX according to Pfeuffer and Metzger (1982). The specific activity of the solubilized adenylate cyclase following reconstitution with Gpp(NH)p-activated G_s was 1–1.5 nmol/mg/min. The solubilized myocardial membrane adenylate cyclase preparation used by us proved to be an effective and convenient substitute for the cyc$^-$ S49 lymphoma cell membrane preparations used by others (Hanski *et al.*, 1981). The myocardial adenylate cyclase preparation does contain G_s and most likely also β-receptor, but the functions of the endogenous components are not expressed in the presence of excess GTP and propranolol. Under these conditions, the soluble crude myocardial adenylate cyclase only becomes activated when exogenous G_s activated by Gpp(NH)p is added. The assay mixture, final volume 150 μl, contained 40–60 μl of the G_s vesicle preparation, 30 μl of rabbit heart myocardial Lubrol PX extract, 0.2 mM [α-^{32}P]ATP (0.5–1.0 μCi/tube), 4 mM $MgCl_2$, 2 mM theophylline, 6 mM creatine phosphate and 0.2 mg/ml creatine kinase in buffer A. Where indicated, 100 μM GTP was added. Incubations were for 20 min at 30°C and [^{32}P]cAMP was separated according to Salomon *et al.* (1974). Under all conditions where G_s activities were measured with the crude Lubrol PX-solubilized adenylate cyclase preparation (see Figures 3–6), the basal adenylate cyclase activity was in the range of 0.02–0.04 nmol/mg/min.

Radioligand β-receptor binding assay

Radioligand binding to β-receptor incorporated in vesicles was measured with [^{125}I]ICYP: 10–20 μl of a β-receptor containing vesicle preparation were incubated with 220 pM [^{125}I]ICYP in 20 mM N-2-hydroxyethylpiperazine-N′-2-ethane sulfonic acid (Hepes) buffer, pH 8.0, 2 mM $MgCl_2$ and 1 mM EDTA in a final volume of 500 μl for 45 min at 30°C. Non-specific binding was assessed in the presence of 1 μM d,l-propranolol. Bound ligand was separated from free ligand by filtration on Whatman GF/F filters. Quantitation of detergent-dispersed β-receptor was carried out with 50 nM [^{3}H]DHA as described by Vauquelin *et al.* (1979).

Other assays

Protein concentrations were measured with the [^{3}H]fluorodinitrobenzene method of Schultz *et al.* (1978). Phospholipid concentrations were assayed colorimetrically according to Stewart (1980). Photoaffinity labeling of β-receptor polypeptides was carried out with 200 pM [^{125}I]ICYP azide-2 according to Burgermeister *et al.* (1982) and Hekman *et al.* (1984). Asolectin vesicles were inspected by electron microscopy after staining with phosphotungstate. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (1970) using a 12.5 or 11% acrylamide separating gel. Proteins were stained with the silver method of Oakley *et al.* (1980). For autoradiography, gels were dried and exposed to Kodak OG-1 films at −80°C for 1–2 days.

Preparation of membranes

Plasma membranes from turkey and duck erythrocytes were purified as described (Puchwein *et al.*, 1974) and stored in liquid nitrogen. Myocardial membranes from New Zealand rabbits were prepared according to Pfeuffer and Metzger (1982).

Purification of β-receptor

Turkey erythrocyte membranes, 2.5 mg protein/ml, containing about 1.3 ± 0.2 pmol [^{3}H]DHA binding sites/mg protein were solubilized with 0.3% lauroyl sucrose in buffer B for 20 min at 4°C. The solubilized material, 100 ml, 2–2.5 pmol binding sites/ml, was stirred with 5 g affinity gel 10 or 15 for 3 h at room temperature. The loaded gel was washed five times with 50 ml each of 25 mM Tris-HCl, pH 7.4, 250 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 2 μM PMSF, 0.05 μg/ml soybean trypsin inhibitor and 0.2% digitonin at 4°C. About 75–85% of receptor added to the gel remained bound after washing. Receptor was eluted with 20 ml of a solution containing 500 μM l-alprenolol in 25 mM Tris-HCl, pH 7.5, 250 mM NaCl, 2 mM EDTA, 1 mM $MgCl_2$, 2 μM PMSF, 0.05 μg/ml soybean trypsin inhibitor and 0.1% digitonin. Elution was carried out at room temperature for 4 h at a continuous flow rate of 5 ml/h. The eluate was concentrated to ~3–4 ml with Immersible-CX^R ultrafilters and excess ligand was removed by G-50 chromatography (1.2 x 35 cm column) with 10 mM Tris-HCl, pH 7.4, 40 mM NaCl, 0.1 mg/ml BSA and 0.03% digitonin. This procedure gave reproducibly 10.5–12.5 nmol specific β-adrenergic binding sites per mg protein with ~40–50% yield.

Purification of G_s

G_s was purified from turkey erythrocyte membranes according to Hanski *et al.* (1981) with the exception that ATP was replaced by 20 μM $AlCl_3$. The β subunit was separated as described by Northup *et al.* (1983) by an additional hydroxyapatite chromatography step. Gpp(NH)p-pre-activated G_s was prepared from duck erythrocyte membranes according to Pfeuffer *et al.* (1983). Maximal activity of G_s purified from turkey erythrocyte membranes was attained on incubation with 50 mM $MgCl_2$ and 100 μM Gpp(NH)p for 25 min at 30°C.

Experimental protocols

All reconstitution experiments were carried out with characterised separate components with varying purity. The purity of the β-receptor and the G_s preparations is documented in Figure 1.

Reconstitution with crude components. Purified turkey erythrocyte membranes (2.5 mg/ml, 1.3 ± 0.2 pmol/mg [^{3}H]DHA binding sites) were extracted with 0.3% lauroyl sucrose in buffer B for 40 min at 4°C. The extract was centrifuged at 39 000 *g* for 30 min at 4°C. The supernatant solution contained 0.8–1.0 mg protein/ml and 2–2.5 pmol/ml [^{3}H]DHA binding sites and had an adenylate cyclase activity of 0.6–0.7 nmol/mg. G_s in the extract produced when maximally activated 1.6–1.8 nmol cAMP/mg/min. For reconstitution 1.44 ml of the supernatant solution were mixed with 60 μl of a soybean phospholipid mixture (20 mg/ml) in 10 mM Tris-HCl, pH 7.8, 0.1 mM EDTA. The phospholipid mixture was sonicated for 30 min at 30°C in a bath sonicator (model Bransonic 220). The final concentration of lipids was 0.8 mg/ml. SM-2 beads (1.8 g) were added and the reconstitution mixture was incubated for 1 h at 4°C with gentle shaking. The beads were removed by centrifugation at 2000 *g* for 3 min at 4°C. Activation was achieved on addition of the activating solution in a ratio of 1:4 (v/v). The activation solution contained 500 μM Gpp(NH)p, 10 mM $MgCl_2$ and 500 μM l-isoproterenol and/or 500 μM d,l-propranolol in buffer A. Before adding the activating solution, the two solutions were pre-incubated separately at 37°C, rapidly mixed and incubated at 37°C for the times stated in Figure 2. At various time points, 60 μl aliquots were withdrawn and activation was stopped by rapidly mixing with 20 μl of d,l-propranolol solution giving a final concentration of d,l-propranolol of 100 μM in the assay.

Reconstitution of purified β-adrenergic receptors with crude G_s. 500 μl of the lauroyl sucrose extract of turkey erythrocyte membranes described above were mixed with 280 μl purified β-receptor, 15 pmol binding sites/ml in 10 mM Tris-HCl, pH 7.4, 40 mM NaCl, 0.1 mg/ml BSA, 0.02% digitonin and 40 μl of an asolectin solution (20 mg/ml) in 10 mM Tris-HCl, pH 7.8, and 0.1 mM EDTA. The final lipid concentration in the reconstitution mixture was 800 μg/ml asolectin and ~200 μg/ml turkey erythrocyte membrane lipids. The reconstitution mixture was brought to 1 ml with the same Tris buffer, 1.2 g SM-2 beads were added and the mixture treated as described above. Incorporation of β-receptors into vesicles was measured by binding of [^{125}I]ICYP. Activation was effected by mixing the lipid vesicle suspension (70% of the volume) with 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA solution containing 500 μM Gpp(NH)p (20% of the volume) and 1 mM l-isoproterenol or 1 mM d,l-propranolol in H_2O (10% of the volume). The reaction was carried out as described above. Instead of lauroyl sucrose, cholate was also used to solubilize G_s from turkey erythrocyte membranes. Membranes (10 mg protein/ml) were incubated for 1 h at 4°C with 1.2% cholate, 0.4 M NaCl in 10 mM Tris-HCl, pH 7.8, containing 10 μg/ml soybean trypsin inhibitor, 200 μM benzamidine, 100 μg/ml bacitracin and 30 μM PMSF. The mixture was centrifuged at 39 000 *g* for 30 min at 4°C. The cholate extract served as source of crude G_s for coupling to purified β-receptor. For reconstitution, 600 μl cholate extract containing 1.4 mg protein/ml were mixed with 300 μl of purified β-receptor (12 pmol binding sites/ml) and 38 μl of a sonicated soybean lipid mixture (20 mg/ml). The reconstitution mixture was treated with SM-2 beads, activated and assayed as described.

Reconstitution of purified β-adrenergic receptor with pure G_s. The preparation from turkey erythrocyte membranes contained 16 μg pure, homogeneous G_s/ml in 25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 100 mM NaCl with 0.1 mg/ml BSA and 1 mM Lubrol PX. For reconstitution, 380 μl of the purified β-receptor preparation (12 pmol binding sites/ml), 200 μl of G_s (16 μg/ml), 100 μl cholate (1.7 mg/ml), 100 μl deoxycholate (15.4 mg/ml) and 30 μl asolectin solution (20 mg/ml) giving a final lipid concentration of 0.6 mg/ml were mixed at 4°C. The mixture was filled up to 1 ml with 10 mM Tris-HCl, pH 8.0 and 45 mM NaCl. In some experiments, cholate-deoxycholate was replaced by 0.3% lauroyl sucrose. The reconstitution mixture was passed at 4°C with a flow rate of 0.5 ml per min through a 10 ml Sephadex G-50 column (fine grade, 0.8 x 20 cm). The column was eluted with 10 mM Tris-HCl and 45 mM NaCl buffer, pH 8.0, containing 0.05 μg/ml soybean trypsin inhibitor but no BSA. Activation was achieved at 37°C by mixing the vesicle suspension with 0.2 volumes of a solution containing 0.5 mM $MgCl_2$, 50 μM Gpp(NH)p in 10 mM Tris-HCl, 45 mM NaCl, pH 8.0, and 0.1 volumes of a solution containing 1 mM l-isoproterenol or 1 mM d,l-propranolol in H_2O. Other details are described above.

Reconstitution of purified β-adrenergic receptor with pure Gpp(NH)p-activated G_s. Reconstitution in the presence of a lauroyl sucrose extract of turkey erythrocyte membranes: 600 μl of a solution containing purified β-receptor (12 pmol binding sites/ml), 0.1 mg/ml BSA and 0.02% digitonin were mixed with 1 ml of a 0.3% lauroyl sucrose extract of turkey erythrocyte membranes and 68 μl of a soybean lipid (asolectin) solution containing 20 mg

lipids/ml in 10 mM Tris-HCl, pH 7.8, 0.1 mM EDTA. The final lipid concentration was 800 μg asolectin/ml and ~200 μg/ml turkey erythrocyte membrane lipids. To this mixture were added 70 μl of a solution containing 20 μg/ml of pure, homogeneous Gpp(NH)p-activated G_s prepared from duck erythrocyte membranes in 10 mM 3-N-morpholino-propane sulfonic acid (Mops), pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$ and 1 mM Lubrol PX. SM-2 beads (2 g) were added and reconstitution was carried out as described.

Reconstitution without lauroyl sucrose extract: 380 μl of purified β-receptor (12 pmol binding sites/ml) and 200 μl of pure, homogeneous Gpp(NH)p-activated G_s (16 μg/ml) in 25 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, 1 mM Lubrol PX and aliquots of 100 μl each of a 0.17% cholate and of 1.54% deoxycholate solutions and 30 μl of an asolectin lipid mixture (20 mg/ml) giving a final lipid concentration of 0.6 mg/ml were mixed and filled up to 1.0 ml with 10 mM Tris-HCl, 45 mM NaCl, pH 8.0. In some experiments, 6 and 40 μg of pure β subunit were added to the reconstitution mixture. The reconstitution mixture was passed over a Sephadex G-50 column at 4°C and the column was eluted as described and the eluate assayed for reversal of activity. Reversal assays were carried out at 37°C as reported by Kennan *et al.* (1982) and started with addition of 10 μM GTP and 100 μM l-isoproterenol or 100 μM d,l-propranolol.

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References

Arad,H., Rimon,G. and Levitzki,A. (1981) *J. Biol. Chem.*, **256**, 1593-1597.
Brandt,D.R., Asano,T., Pedersen,S.E. and Ross,E.M. (1983) *Biochemistry (Wash.)*, **22**, 4357-4362.
Burgermeister,W., Hekman,M. and Helmreich,E.J.M. (1982) *J. Biol. Chem.*, **257**, 5306-5311.
Burgermeister,W., Nassal,M., Wieland,T. and Helmreich,E.J.M. (1983) *Biochim. Biophys. Acta*, **729**, 219-228.
Caron,M.G., Cerione,R.A., Codina,J., Benovic,J.L., Birnbaumer,L. and Lefkowitz,R.J. (1984) *Fed. Proc.*, **43**, 1579 (abstract).
Cassel,D. and Selinger,Z. (1976) *Biochim. Biophys. Acta*, **452**, 538-551.
Citri,Y. and Schramm,M. (1980) *Nature*, **287**, 297-300.
Citri,Y. and Schramm,M. (1982) *J. Biol. Chem.*, **257**, 13257-13262.
Feder,D., Arad,H., Gal,A., Hekman,M., Helmreich,E.J.M. and Levitzki,A. (1984) *Adv. Cycl. Nucl. Prot. Phosphoryl. Res.*, **17**, 61-71.
Gal,A., Braun,S., Feder,D. and Levitzki,A. (1983) *Eur. J. Biochem.*, **134**, 391-396.
Hanski,E., Sternweis,P.C., Northup,J.K., Dromerick,A.W. and Gilman, A.G. (1981) *J. Biol. Chem.*, **256**, 12911-12919.
Hekman,M., Schiltz,E., Henis,Y.I., Elson,E.L. and Helmreich,E.J.M. (1984) *Adv. Cycl. Nucl. Prot. Phosphoryl. Res.*, **17**, 47-60.
Henis,Y.I., Hekman,M., Elson,E.L. and Helmreich,E.J.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2907-2911.
Homcy,C.J., Rockson,S.G., Countaway,J. and Egan,D.A. (1983) *Biochemistry (Wash.)*, **22**, 660-668.
Keenan,A.K., Gal,A. and Levitzki,A. (1982) *Biochem. Biophys. Res. Commun.*, **105**, 615-623.
Kelleher,D.J., Rashidbaigi,A., Ruoho,A.E. and Johnson,G.L. (1983) *J. Biol. Chem.*, **258**, 12881-12885.
Laemmli,U.K. (1970) *Nature*, **227**, 680-685.
Levitzki,A. and Helmreich,E.J.M. (1979) *FEBS Lett.*, **101**, 213-219.
Northup,J.K., Sternweis,P.C., Smigel,M.D., Schleifer,L.S., Ross,E.M. and Gilman,A.G. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 6516-6520.
Northup,J.K., Sternweis,P.C. and Gilman,A.G. (1983) *J. Biol. Chem.*, **258**, 11361-11368.
Oakley,B.R., Kirsch,D.R. and Morris,N.R. (1980) *Anal. Biochem.*, **105**, 361-363.
Osipow,K., Dee Snell,F., York,W.C. and Finchler,A. (1956) *Ind. Eng. Chem.*, **48**, 1459-1462.
Pedersen,S.E. and Ross,E.M. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 7228-7232.
Pfeuffer,T. and Metzger,H. (1982) *FEBS Lett.*, **146**, 369-375.
Pfeuffer,T., Gaugler,B. and Metzger,H. (1983) *FEBS Lett.*, **164**, 154-160.
Puchwein,G., Pfeuffer,T. and Helmreich,E.J.M. (1974) *J. Biol. Chem.*, **249**, 3232-3240.
Salomon,Y., Londos,C. and Rodbell,M. (1974) *Anal. Biochem.*, **58**, 541-548.
Schultz,R.M., Bleil,J.D. and Wasserman,P.M. (1978) *Anal. Biochem.*, **91**, 354-356.
Sevilla,N. and Levitzki,A. (1977) *FEBS Lett.*, **76**, 129-134.
Shorr,R.G.L., Strohsacker,M.W., Lavin,T.N., Lefkowitz,R.J. and Caron, M.G. (1982) *J. Biol. Chem.*, **257**, 12341-12350.
Stewart,J.C.M. (1980) *Anal. Biochem.*, **104**, 10-14.
Tolkovsky,A.M. and Levitzki,A. (1978) *Biochemistry (Wash.)*, **17**, 3795-3810.
Vauquelin,G., Geynet,P., Hanoune,J. and Strosberg,A.D. (1979) *Eur. J. Biochem.*, **98**, 543-556.

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