Human β -adrenergic receptors

Simultaneous purification of β_1 - and β_2 -adrenergic-receptor peptides

Suleiman W. BAHOUTH* and Craig C. MALBON

Department of Pharmacological Sciences, School of Medicine, Health Sciences Center, State University of New York at Stony Brook, Stony Brook, NY 11794-8651, U.S.A.

β -Adrenergic receptors from basal membranes of human placenta were purified from digitonin extracts by sequential rounds of affinity chromatography, hydrophobic chromatography, ion-exchange chromatography and steric-exclusion h.p.l.c. Basal membranes display both β_1 - and β_2 -adrenergic receptors, in the ratio 65:35. Affinity chromatography, hydrophobic chromatography on heptylamine–Sephacel and ion-exchange chromatography on DEAE–Sephacel removed most of the contaminating proteins, and final purification of the receptor to apparent homogeneity was achieved by steric-exclusion h.p.l.c. The purified receptors showed M_r 67 000 on SDS/polyacrylamide-gel electrophoresis under reducing conditions. Specific binding of radioligand to the purified β -adrenergic receptors displayed stereoselectivity, and the agonist competition profiles demonstrated the presence of both β_1 - and β_2 -receptors. By using the subtype-selective ligands CGP-20712A (β_1 -selective) and ICI-118,551 (β_2 -selective), the purified M_r -67 000 species was shown to be composed of equivalent amounts of β_1 - and β_2 -adrenergic receptors. Affinity chromatography on Sepharose–alprenolol and sequential elution with 1 μ M-CGP-20712A followed by 100 μ M-(–)-alprenolol permitted β_1 -adrenergic receptors to be resolved from the mixture of β_1 -/ β_2 -adrenergic receptors. The pharmacologically distinct human β_1 - and β_2 -adrenergic receptors are shown to be structurally very similar peptides.

INTRODUCTION

The β -adrenergic receptor is an integral membrane protein to which catecholamines bind and trigger the activation of adenylate cyclase (for reviews, see Ross & Gilman, 1980; Stiles *et al.*, 1984b). β -Adrenergic receptors are subdivided into two distinct subclasses, termed β_1 - and β_2 -, based on pharmacological criteria (Lands *et al.*, 1967). Unlike the differences observed between amphibian β_2 - and avian β_1 -adrenergic receptors (Stiles *et al.*, 1984a), for mammalian β_1 - and β_2 -receptors many structural features appear in common (Cubero & Malbon, 1984; Benovic *et al.*, 1984; Graziano *et al.*, 1985; George & Malbon, 1985). Both receptor subtypes appear to be glycoproteins (Stiles *et al.*, 1984a; Benovic *et al.*, 1984; George *et al.*, 1986) and contain intramolecular disulphide bridges (Moxham & Malbon, 1985; Moxham *et al.*, 1986).

Little information derived from larger-scale purifications is available about the nature of human β -adrenergic receptors. The relatively low abundance of these receptors in most human tissues and the lack of suitable post-mortem sources for use as a starting material for the isolation of human β -adrenergic receptors have been major obstacles to large-scale isolation. The human placenta, in contrast, is an unusually good source for β -adrenergic receptors: it is a rich source of both β_1 - and β_2 -adrenergic receptors (Moore & Whitsett, 1981). Except for the rat fat-cell, the placenta represents the only other suitable source for the isolation of mammalian β_1 -adrenergic receptors. Moreover, the speci-

fic activities of crude and purified basal membranes of human placenta are 1–2 and 3–5 pmol of receptor/mg of protein respectively (Kelley *et al.*, 1983). The high specific activity of the membranes, the presence of both β_1 - and β_2 -adrenergic-receptor subtypes and the availability of fresh tissue make the placenta an excellent source for isolation and characterization of human β -adrenergic receptors.

In this report we describe the large-scale isolation of human β -adrenergic receptors. A novel method is described for resolving receptor subtypes. The purified pharmacologically distinct receptor subtypes both display M_r 67 000 on SDS/polyacrylamide-gel electrophoresis. Further evidence supporting the structural similarity between β_1 - and β_2 -adrenergic receptors that have been isolated simultaneously from this human source is provided.

EXPERIMENTAL

Preparation of human placenta basal cell membranes

Crude basal membranes were prepared from fresh placenta by the procedure of Kelley *et al.* (1983). Leupeptin (2 μ g/ml), soya-bean trypsin inhibitor (5 μ g/ml), benzamidine (15 μ g/ml) and 1 mM-PMSF were included in all buffers.

Solubilization procedure

Basal membranes of fresh human placenta were adjusted to a final concentration of 6.25 mg of protein/

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; TEN buffer, 10 mM-Tris (pH 7.4)/2 mM-EDTA/100 mM-NaCl/0.05% digitonin/1 mM-PMSF; C₇-Sephacel, heptylamine–Sephacel; [³H]DHA, tritiated (–)-dihydroalprenolol; [¹²⁵I]ICYP, (–)-[¹²⁵I]iodocyanopindolol.

* To whom all correspondence should be directed.

ml in 60 ml of 300 mM-NaCl/6.25 mM-EDTA/12.5 mM-Tris (pH 7.4, 4 °C), containing leupeptin (12.5 µg/ml), aprotinin (12.5 µg/ml) and PMSF (1.25 mM). Then 15 ml of 5% (w/v) digitonin was added dropwise and the mixture stirred for 1 h in an ice bath. The mixture was centrifuged at 100000 g_{av} for 60 min at 4 °C, and the supernatant was decanted and held in an ice bath.

Affinity-chromatography procedure

Sephacel-alprenolol was synthesized by the procedure of Caron *et al.* (1979). The digitonin extract (75 ml) was loaded at a flow rate of 40 ml/h on to a 75 ml (1.6 cm × 40 cm) Sepharose-alprenolol column equilibrated in TEN buffer at room temperature. The column was placed at 4 °C and washed overnight with 300 ml of TEN buffer at 25 ml/h. After washing, the column was returned to 22 °C, eluted with a 180 ml linear gradient of 0–100 µM-(–)-alprenolol in TEN buffer at 37 ml/h. Portions (0.5 ml) of each fraction (8 ml) were dialysed at 4 °C in TEN buffer without digitonin and assayed for β -adrenergic-receptor activity by radioligand binding.

Heptylamine-Sepharose chromatography

C₇-Sepharose was synthesized by the method of Shaltiel (1974), except that Sepharose 4BCL was employed as the insoluble support. Fractions eluted from the Sepharose-alprenolol matrix were applied twice consecutively to a 5 ml column (1 cm × 6 cm) of C₇-Sepharose that had been equilibrated at 4 °C with TEN buffer. The column was then washed successively with 20 ml of TEN buffer, followed by 20 ml of 500 mM-NaCl/2 mM-EDTA/10 mM-Tris (pH 7.4)/0.05% digitonin/1 mM-PMSF. β -Adrenergic receptors were eluted with a 50 ml linear gradient starting with 250 mM-NaCl/2 mM-EDTA/10 mM-Tris (pH 7.4)/1 mM-PMSF/0.1% digitonin and ending with 50 mM-NaCl/2 mM-EDTA/10 mM-Tris (pH 7.4)/1 mM-PMSF/1% digitonin.

DEAE-Sepacel chromatography

Fractions containing receptor were pooled, diluted with 10 mM-Tris (pH 8.0)/1 mM-EDTA/0.05% digitonin/1 mM-PMSF to a final NaCl concentration of 10 mM, and applied twice to a 1 ml DEAE-Sepacel column (1.1 cm × 1.1 cm). The column was washed with the dilution buffer, and receptors were eluted from the column with 1–1.5 ml of 0.2 M-NaCl/0.1 M-Na₂HPO₄/NaH₂PO₄ (pH 7.0)/1 mM-EDTA/0.1% digitonin/0.1 mM-PMSF.

Steric-exclusion h.p.l.c.

A sample (0.5 ml) of DEAE-Sepacel eluate was subjected to steric-exclusion h.p.l.c. as described by Cubero & Malbon (1984). The mobile phase was composed of 0.2 M-NaCl, 0.1 M-Na₂HPO₄/NaH₂PO₄ (pH 7.0), 1 mM-EDTA, 0.1 mM-PMSF and 0.1% digitonin.

Affinity chromatography of h.p.l.c.-purified receptors

H.p.l.c.-purified β -adrenergic receptors (1 ml) were diluted 4-fold with 10 mM-Tris (pH 7.4)/1 mM-EDTA/0.05% digitonin/1 mM-PMSF and applied to a 1 ml (0.6 cm × 1.4 cm) Sepharose-alprenolol column. The column was washed at 4 °C with 20 ml of TEN buffer at 2 ml/h, then returned to room temperature and eluted sequentially with 2.5 ml of 1 µM-CGP-20712A in TEN

buffer, followed by 2.5 ml of 100 µM-(–)-alprenolol in TEN buffer. The eluates were dialysed in TEN buffer without digitonin to permit measurement of β -receptors by [¹²⁵I]ICYP binding.

Radioligand-binding assays

Basal membranes of human placenta (75 µg) suspended in 50 mM-Tris (pH 7.4)/10 mM-MgCl₂ were incubated with 1.5 nM-[³H]DHA in the absence or presence of competing ligands in a total volume of 0.1 ml for 20 min at 22 °C. [¹²⁵I]ICYP binding was performed by incubating 5 µg of membranes with 0.1 nM-[¹²⁵I]ICYP in a total volume of 0.1 ml for 1 h at 22 °C. The incubation was terminated by adding 6 ml of ice-cold 50 mM-Tris (pH 7.4)/10 mM-MgCl₂ and filtering the mixture on to GF/C filters, followed by washing with 2 × 6 ml of buffer. Specific binding was defined throughout this work as the difference between radioligand bound in the absence or the presence of 100 µM-(–)-isoprenaline.

The maximal concentration of β -adrenergic receptors in soluble preparations was determined by radioligand binding with 50 nM-[³H]DHA in the absence or presence of 100 µM-(–)-isoprenaline incubated in a total volume of 0.1 ml for 1 h at 22 °C. The amount of β_1 -adrenergic receptors was calculated from the amount of [³H]DHA competed specifically by 1 µM of the β_1 -adrenergic antagonist CGP-20712A. The competition for [¹²⁵I]-ICYP binding to soluble preparations by β -adrenergic-selective ligands was performed by incubating 0.1 nM-[¹²⁵I]ICYP and the indicated concentrations of the ligands in a total volume of 0.1 ml for 120 min at 22 °C. [³H]DHA and [¹²⁵I]ICYP binding to soluble preparations was assayed by the polyethylene-glycol-precipitation technique of Vauquelin *et al.* (1979).

Analysis of the binding data

All receptor-binding parameters were analysed by computer by using a non-linear least-squares curve-fitting procedure and the modified LIGAND program (Bio-soft-Elsevier, Cambridge, U.K.). Data are presented as means ± S.E.M. from the pooled data of at least three experiments analysed simultaneously.

SDS/polyacrylamide-gel electrophoresis

Fractions were treated with or without agents that chemically reduce disulphide bonds of protein and then subjected to electrophoresis on homogeneous 10% polyacrylamide gels in the presence of 0.1% SDS (Laemmli, 1970). Proteins were made visible by silver staining with the Bio-Rad kit. Glycoprotein staining was performed by the periodic acid-silver stain method (Dubray & Bezard, 1982).

Radioiodination of β -adrenergic receptors

The proteins of fractions from the various steps of purification were radioiodinated with Na¹²⁵I by the chloramine-T method (Greenwood *et al.*, 1963), then desalted on Sephadex G-50 (Graziano *et al.*, 1985) or precipitated in ice-cold 20% (w/v) trichloroacetic acid.

Miscellaneous methods

Protein content of membranes and digitonin extracts was measured by the method of Lowry *et al.* (1951). In highly purified preparations, protein was determined by the Amido Schwarz assay of Schaffner & Weissmann (1973), with bovine serum albumin as a standard.

Table 1. Affinities of different subtype-selective β -adrenergic ligands for β -adrenergic receptors in basal membranes of human placenta

The dissociation constants of the high-affinity (k_{dH}) and low-affinity (k_{dL}) radioligand-binding sites for the drugs, calculated from computer analysis as described in the Experimental section, are presented. Each value is the mean $\pm$ S.E.M. for at least three separate experiments performed in triplicate.

Ligand	Selectivity	Radioligand	k_{dH} (M)	k_{dL} (M)	β_1 (%)
Atenolol	β_1	ICYP	$1.7(\pm 0.4) \times 10^{-7}$	$8.2(\pm 0.6) \times 10^{-6}$	66
Practolol	β_1	ICYP	$1.9(\pm 0.5) \times 10^{-7}$	$6.7(\pm 0.7) \times 10^{-6}$	65
ICI-118,551	β_2	ICYP	$4.2(\pm 0.5) \times 10^{-9}$	$1.1(\pm 0.3) \times 10^{-7}$	70
ICI-118,551	β_2	DHA	$5.0(\pm 0.7) \times 10^{-9}$	$1.3(\pm 0.5) \times 10^{-7}$	64
CGP-20712A	β_1	DHA	$2.2(\pm 0.4) \times 10^{-9}$	$3.2(\pm 0.5) \times 10^{-6}$	65
Metoprolol	β_1	DHA	$5.0(\pm 3.0) \times 10^{-8}$	$2.0(\pm 0.5) \times 10^{-6}$	68
Zinterol	β_2	DHA	$1.0(\pm 0.5) \times 10^{-7}$	$1.2(\pm 0.4) \times 10^{-6}$	64

Materials

All radiochemicals were purchased from New England Nuclear (Boston, MA, U.S.A.). (–)-Alprenolol and CGP-20712A were generously given by Dr. Enar Carlsson of A. B. Hassle (Molndal, Sweden) and Dr. L. Maitre of Ciba-Geigy A.G. (Basel, Switzerland) respectively. All other reagents were purchased from Sigma.

Presentation of results

The data provided are reported from more than 20 large-scale purifications from human placenta. The results presented are either mean determinations from these preparations or single observations that are representative of multiple experiments.

RESULTS

Characterization of β -adrenergic receptors in basal membranes of human placenta

Basal membranes of human placenta displayed a specific activity of β -adrenergic receptors of 1.2 pmol/mg of protein. The subtype distribution of β -adrenergic receptors in these membranes was measured by competition of bound [3 H]DHA and [125 I]ICYP with subtype-selective antagonists (Table 1). All the antagonists, except CGP-20712A, gave shallow competition curves. These curves were analysed by the LIGAND computer program to determine the subtype distribution of β -adrenergic receptors. These analyses indicated that the composition of receptors in basal membranes of human placenta is approx. 65:35 ($\pm 5\%$) β_1 -/ β_2 -adrenergic-receptor subtypes. The inhibition of [3 H]DHA binding to the membranes by CGP-20712A ligand was biphasic, displaying a plateau that extended from 0.1 to 1 μ M (Fig. 1). CGP-20712A is a β -adrenergic-receptor antagonist that is 10000-fold more selective for β_1 - than for β_2 -adrenergic receptors, in a number of tissues (Dooley & Bittiger, 1984; Bahouth *et al.*, 1986). Because CGP-20712A possesses such a high selectivity for β_1 -adrenergic receptors, it displays a plateau in these experiments that accurately differentiates the β_1 - from the β_2 -adrenergic receptor populations (Dooley & Bittiger, 1984). From the position of the plateau at 1 μ M-CGP-20712A, it was inferred that β -adrenergic receptors were composed of β_1 - (65%) and β_2 - (35%) adrenergic receptors. A similar result was obtained by analysing the competition data by

Fig. 1. β -Adrenergic receptor subtype and composition in human placental membranes

Basal membranes (20 μ g of protein) were incubated with increasing concentrations of CGP-20712A in the presence of 1.5 nM-[3 H]DHA for 30 min at 22 $^{\circ}$ C. The percent of the specifically bound radioligand is plotted as a function of the concentration of CGP-20712A. Inset: equilibrium data were analysed by the method of Hofstee (1952): V represents the inhibition (%) of [3 H]DHA binding. The concentration of CGP-20712A is expressed as micromolar. The curvilinear Hofstee plot was analysed by a non-linear curve-fitting program (Munson & Rodbard, 1980) to measure the B_{max} and IC_{50} of each component. The proportions of β_1 - and β_2 -receptors were $65 \pm 3\%$ and $35 \pm 5\%$ respectively. The IC_{50} of CGP-20712A for β_1 -receptors was 4 nM and that for β_2 -receptors 7 μ M. Data are expressed as the mean values of triplicate determinations from a representative experiment that was replicated at least three times.

the method of Hofstee (1952) (Fig. 1, inset). The subtype composition of these receptors was defined throughout the purification by using this strategy and the CGP-20712A ligand.

Table 2. Large-scale isolation of human β -adrenergic receptors

The β -adrenergic receptors in digitonin extracts of basal membranes of human placenta were purified sequentially by affinity chromatography, C_7 -Sephacrose chromatography and DEAE-Sephacel chromatography, followed by h.p.l.c. on steric-exclusion columns; details are outlined in the Experimental section and in the text. The quantity and subtype distribution of β -adrenergic receptors at each step of the isolation were determined. Data are expressed as means $\pm$ s.e.m. from the indicated numbers of large-scale preparations.

Fraction	Protein (μ g)	β -Adrenergic receptors			
		Total (pmol)	Specific activity (nmol/mg)	β_1 -receptors (%)	Purification (fold)
Human placenta basal membranes	233 000 (21)	280 $\pm$ 15	0.001	65 $\pm$ 3 (7)	0
Digitonin extract	91 600 (21)	110 $\pm$ 9	0.001 $\pm$ 0.0002	65 $\pm$ 5 (7)	0
Affinity chromatography	230 (13)	17 $\pm$ 1	0.08 $\pm$ 0.03	62 $\pm$ 4	80
C_7 -Sephacrose chromatography	22 $\pm$ 4 (7)	11 $\pm$ 2	0.5 $\pm$ 0.03	60 $\pm$ 2 (4)	500
DEAE-Sephacel chromatography	9 $\pm$ 2 (5)	9 $\pm$ 1	1.08 $\pm$ 0.1	50 $\pm$ 5 (3)	990
Steric-exclusion h.p.l.c.	1.2 $\pm$ 0.2 (4)	3.7 $\pm$ 0.5	3.08 $\pm$ 0.2	50 $\pm$ 5 (4)	3080

Fig. 2. Affinity chromatography of human β -adrenergic receptors

β -Adrenergic receptors (0.1 nmol) were applied (40 ml/h) to a 75 ml Sepharose 4B-alprenolol column. The column was washed at 4 °C with 5 column volumes. Matrix-adsorbed receptors were eluted with a gradient of (–)-alprenolol (0–100 μ M). Samples of each fraction (8 ml) were dialysed at 4 °C to lower the concentration of alprenolol. Competition of [3 H]DHA binding by 1 μ M-CGP-20712A was used to define receptor-subtype distribution: $\square$, β_1 ; $\blacksquare$, β_2 . (b) Protein content in the fractions eluted by alprenolol.

Fig. 3. Hydrophobic chromatography of affinity-purified human β -adrenergic receptors

(a) β -Adrenergic receptors eluted by alprenolol from the Sepharose 4B-alprenolol matrix (16 pmol) were pooled (80 ml) and applied twice over a 5 ml heptylamine-Sepharose column at 4 °C. The column was washed over 2 h, then eluted with a gradient (50 ml) at 10 ml/h as described in the Experimental section. Ten fractions (each 5 ml) were collected, and a 0.5 ml portion from each was diluted with 100 mM-NaCl/2 mM-EDTA/10 mM-Tris (pH 7.4)/1 mM-PMSF to a final digitonin concentration of 0.1% before quantification of the amount and subtype distribution of receptors: $\square$, β_1 ; $\blacksquare$, β_2 . (b) Total protein content of each fraction. Data are expressed as the means of triplicate determinations from a representative experiment.

Large-scale isolation of human β -adrenergic receptors

Digitonin (1 %, w/v) effectively solubilized 40 % of the β -adrenergic receptors of placental basal membranes (Table 2). Neither the specific activity of the preparation nor the ratio of β_1 -/ β_2 -receptors of the extract was altered with respect to particulate membranes. Receptors solubilized in digitonin were first subjected to affinity chromatography on a Sepharose-alprenolol matrix (Fig. 2). Bound receptors were eluted from the matrix with a linear gradient of (–)-alprenolol (0–100 μ M); the peak of

receptor binding was detected at 35–40 μ M-(–)-alprenolol (Fig. 2). The peak fractions from the affinity matrix were assayed to quantify the amount of β -adrenergic receptor content and subtype composition. Approx. 65 % β_1 - and 35 % β_2 -adrenergic receptors was the distribution at this step.

Fig. 4. Large-scale isolation of human β -adrenergic receptors: analysis by SDS/polyacrylamide-gel electrophoresis

Lanes 1 and 2, a sample (0.05 ml) of Sepharose-alprenolol (lane 1) and C_7 -Sepharose (lane 2) eluates, displaying β -adrenergic-receptor binding activities of 0.1 and 0.5 nmol of receptor/mg of protein respectively, were radioiodinated and subjected to SDS/polyacrylamide-gel electrophoresis. The autoradiograms are 2-day exposures with X-O-Mat film and a Cronex-plus intensifying screen. Lanes 3 and 4, a 0.2 ml sample (200 ng of protein) of peak I of h.p.l.c.-purified receptors was freeze-dried and subjected to SDS/polyacrylamide-gel electrophoresis in the presence of 10% (v/v) 2-mercaptoethanol (lane 3) or in its absence (lane 4). The proteins were made visible by silver staining of the gel. Lane 5, a 0.1 ml sample (100 ng of protein) of peak I of the h.p.l.c.-purified receptors was freeze-dried and subjected to SDS/polyacrylamide-gel electrophoresis in the presence of 2-mercaptoethanol, and the proteins were made visible by the periodic acid-silver staining of the gel.

The affinity-purified receptors were next subjected to hydrophobic chromatography on C_7 -Sepharose. More than 50% of the protein and less than 15% of the β -adrenergic receptors applied to the C_7 matrix were observed in the sum of the fractions from the flow-through and from the column washes. The matrix-bound receptors were eluted with a gradient of increasing concentration of digitonin and decreasing ionic strength (Fig. 3). Receptors (60% β_1 -, 40% β_2 -) were eluted as a sharp single peak at 0.5% digitonin (Table 2). A 7-fold increase in specific activity was obtained by this step.

Radioiodination and autoradiography of the protein in the fractions from the hydrophobic chromatography revealed that the peaks of receptor activity contained two major peptides, a predominant M_r -67000 peptide and a second, minor, peptide of M_r 45000 (Fig. 4, lane 2). Hydrophobic chromatography also provided a means by which the receptors could be concentrated and freed of alprenolol. Rapid quantification of the receptor-binding activity and a decreased lag time for ion-exchange chromatography were additional benefits from this step.

The receptors from the hydrophobic-chromatography step were collected on DEAE-Sephacel and then eluted with a high-ionic-strength buffer. The receptors eluted from the ion-exchange matrix now displayed a 50:50

Fig. 5. Steric-exclusion h.p.l.c. profile of human β -adrenergic receptors

(a) A 0.5 ml sample of the DEAE-Sephacel eluate containing 2 pmol of receptor-binding activity was chromatographed on two TSK-3000 (7.5 mm $\times$ 30 cm) and one TSK-2000 (7.5 mm $\times$ 30 cm) steric-exclusion columns, tandem-linked. Portions of each fraction eluted from the h.p.l.c. were assayed for β -adrenergic-receptor binding activity: $\square$, β_1 ; $\blacksquare$, β_2 . β_1 -Adrenergic receptors were measured in the fractions eluted in peak I only (fractions 22–24). (b) M_r marker proteins in a total volume of 0.25 ml were subjected to h.p.l.c. under conditions identical with those used to purify β -adrenergic receptors; their elution was monitored by measuring their A_{280} . Key: GDH, glutamate dehydrogenase (M_r 290000); LDH, lactate dehydrogenase (M_r 140000); EN, enolase (M_r 67000).

ratio of β_1 - to β_2 -adrenergic receptor subtype. The specific activity was ≥ 1 nmol/mg of protein (Table 2). Autoradiography of the radioiodinated protein separated by SDS/polyacrylamide-gel electrophoresis revealed again two peptides, of M_r 67000 (major) and M_r 45000 (minor) (results not shown).

Receptors isolated by affinity, hydrophobic, and ion-exchange chromatography were subjected to steric-exclusion h.p.l.c. (Fig. 5). Purified receptors displayed M_r 180000 (peak I) and 90000 (peak II). The β -adrenergic-receptor binding activity eluted in peak I (85–90%) was further analysed. The receptor-binding activity in peak II accounted for 10–15% of β -adrenergic receptors of some preparations, but in others no binding activity in peak II was detected. The specific binding activity of the receptors in peak I from the h.p.l.c. was

Fig. 6. Characterization of the h.p.l.c.-purified β -adrenergic receptors by subtype-selective ligands

(a) H.p.l.c.-purified β -adrenergic receptors were incubated with increasing concentrations of the β_1 -selective antagonist CGP-20712A in the presence of 10 nM-[3 H]DHA for 60 min at 22 °C. The percentage of the radioligand specifically bound is plotted as a function of the concentration of CGP-20712A. (b) H.p.l.c.-purified β -adrenergic receptors were incubated with increasing concentrations of the β_2 -selective antagonist ICI-118,551 in the presence of 0.2 nM-[125 I]ICYP for 2 h at 22 °C. The percentage of radioligand specifically bound is plotted as a function of the concentration of ICI-118,551. (c) Equilibrium binding was analysed by the method of Hofstee (1952). On the ordinate and abscissa, V represents the percentage inhibition of [125 I]ICYP binding. The free concentration of ICI-

Table 3. Affinities of membrane-bound, solubilized and purified human β -adrenergic receptors for β -adrenergic ligands

The inhibition of [3 H]DHA binding by the ligands was performed with 2 nM-[3 H]DHA (in membranes and digitonin extracts) and 10 nM (in purified preparations of β -adrenergic receptors). The inhibition of [3 H]DHA binding to basal membranes of human placenta by agonists was performed in 50 mM-Tris (pH 7.4)/10 mM-MgCl₂ in the presence of 0.1 mM-GTP. The concentration of each ligand that inhibited the specific [3 H]DHA binding by 50% (IC_{50}) was determined and used to calculate the apparent k_d of this ligand by the equation of Cheng & Prusoff (1973). Data are expressed as the means of triplicate determinations from representative experiments: ND, not determined.

Ligand	Apparent k_d of ligands for β -adrenergic receptors (μ M)		
	Membrane-bound	Digitonin extract	H.p.l.c.-purified
(-)-Isoprenaline	0.4	0.02	0.06
(+)-Isoprenaline	34	1.2	3.0
(-)-Noradrenaline	30	3.0	5.0
(-)-Adrenaline	20	1.2	2.0
(-)-Propranolol	0.004	0.004	ND
(+)-Propranolol	0.9	0.2	ND

≥ 3 nmol/mg of protein (Table 2), and they were homogeneous by SDS/polyacrylamide-gel analysis (Fig. 4, lane 3).

The binding of [3 H]DHA by the purified receptors was stereoselective; the affinity of (-)-isoprenaline was 50-fold higher than that of (+)-isoprenaline (Table 3). The affinities of the agonist ligands (-)-adrenaline and (-)-noradrenaline were in close agreement to those obtained in digitonin extracts. The affinity of (-)-adrenaline was about 2.5-fold higher than that of (-)-noradrenaline, suggesting the existence of a mixed receptor population in the purified preparations (Lands *et al.*, 1967).

The inhibition curves of [3 H]DHA binding to the h.p.l.c.-purified receptors by the CGP-20712A ligand was biphasic and displayed a well-defined plateau (Fig. 6a). These data suggest that approximately equivalent amounts of β_1 - and β_2 -subtypes are present in the purified receptor preparation. The K_d of CGP-20712A for β_1 -adrenergic receptors was 1.3 nM, and for β_2 -adrenergic receptors approx. 50 μ M. The competition for [3 H]DHA binding to β_1 -adrenergic receptors by CGP-20712A was complete at CGP-20712A concentrations below 1 μ M. CGP-20712A did not compete with [3 H]DHA binding to β_2 -adrenergic receptors except at CGP-20712A concentrations higher than 10 μ M (Fig. 6a).

The distribution of β_1 - and β_2 -adrenergic receptors in this fraction was also assessed by competition of [125 I]-ICYP binding to these receptors by the β_2 -selective antagonist ligand ICI-118,551 (Fig. 6b). The competition

118,551 is expressed as micromolar. Data are expressed as the mean values of triplicate determinations from a representative experiment that was replicated three times.

Fig. 7. Characterization of the h.p.l.c.-purified receptors subjected to affinity chromatography and sequentially eluted with CGP-20712A and alprenolol

H.p.l.c.-purified β -adrenergic receptors (1 pmol in 1 ml) were subjected to affinity chromatography and then sequentially eluted first with 1 μ M-CGP-20712A (0.09 pmol) and secondly with 100 μ M-(–)-alprenolol (0.25 pmol), as described in the Experimental section. (a) Characterization of CGP-20712A-eluted receptors. Samples (0.08 ml) of the CGP-20712A eluate were incubated with 0.2 nM-[125 I]ICYP and increasing concentrations of metoprolol for 2 h at 22 °C. Equilibrium binding was analysed by the method of Hofstee (1952). On the ordinate and abscissa, V represents the percentage inhibition of [125 I]ICYP binding. The concentration of free metoprolol is expressed as micromolar. Inset: the percentage of radioligand specifically bound plotted as a function of the concentration of metoprolol. (b) Characterization of alprenolol-eluted β -adrenergic receptors. Samples (0.08 ml) of the alprenolol eluate were incubated with 0.2 nM-[125 I]ICYP and increasing concentrations of ICI-118,551 for 2 h at 22 °C. Equilibrium binding was analysed by the method of Hofstee (1952). On the ordinate and abscissa, V represents the percentage inhibition of [125 I]ICYP binding. The concentration of free ICI-118,551 is expressed as nanomolar. Inset: the percentage of radioligand specifically bound plotted as a function of the concentration of ICI-118,551.

curves for [125 I]ICYP binding by ICI-118,551 were shallow, with a Hill coefficient of 0.72. The competition data were analysed by the method of Hofstee (1952) to determine the receptor populations and their affinities for ICI-118,551. The binding of ICI-118,551 displayed an affinity of 6 nM for about 45 % of the receptor population and an affinity of 0.16 μ M for the remainder (Fig. 6c). Thus the h.p.l.c.-purified receptors are composed of β_1 - and β_2 -adrenergic receptors in the ratio 55:45 respectively.

β -Adrenergic receptors purified through the h.p.l.c. step (peak I) were further analysed by SDS/polyacrylamide-gel electrophoresis. A single peptide band of M_r 67000 was visible by silver staining (Fig. 4, lane 3). In contrast, in the absence of chemical reduction of disulphide bridges, the purified receptors displayed an increase in their electrophoretic mobility, corresponding to M_r 54000 (Fig. 4, lane 4). Specific staining with periodic acid-silver (Fig. 4, lane 5) revealed the receptor peptides to be glycoprotein in nature.

Resolving β_1 -adrenergic receptors from the purified receptors preparation

The purified preparations composed of both receptor subtypes migrated as a single compact species when subjected to analytical SDS/polyacrylamide-gel electro-

phoresis. Thus the pharmacologically distinct receptor peptides must have structural similarity. The selectivity of the interaction of CGP-20712A to β_1 -adrenergic receptors was exploited to separate the β_1 -subtype from the mixture of subtypes observed in the purified receptor preparations. For this purpose, the h.p.l.c.-purified receptor (peak I) was subjected to a second pass of affinity chromatography on Sepharose-alprenolol. The bound receptor was then eluted sequentially by 1 μ M-CGP-20712A followed by 100 μ M-(–)-alprenolol. The concentration of CGP-20712A selected competed solely for β_1 -adrenergic receptors (see Fig. 6a). The pharmacology of the β -adrenergic receptors eluted by CGP-20712A was examined by equilibrium-binding techniques using [125 I]ICYP. Metoprolol is a β -adrenergic antagonist selective for receptors of β_1 -subtype (Minneman *et al.*, 1979). Competition of [125 I]ICYP binding by metoprolol was performed in order to quantify the amount of receptors present in the CGP-20712A-eluted material that are truly β_1 -subtype. Analysis of the equilibrium-binding data by the method of Hofstee (1952) yielded a straight line (Fig. 7a). These data suggest that metoprolol was binding to a homogeneous population of binding sites. Under the conditions employed, the concentration of metoprolol that inhibited [125 I]ICYP binding by 50 % was 0.4 μ M, indicating a K_d for metoprolol of 80 nM

Fig. 8. SDS/polyacrylamide-gel analysis of purified human β -adrenergic receptors isolated by selective elution from affinity matrix

Samples of h.p.l.c.-purified receptors were subjected to affinity chromatography on Sepharose-alprenolol and eluted sequentially first with 1 μ M-CGP-20712A and then with 100 μ M-(-)-alprenolol. Samples of each eluate (0.5 ml) were concentrated by using a Centricon-30 micro-concentrator (Amicon). The concentrate was radioiodinated and desalted on Sephadex G-50. Samples (0.2 ml) were freeze-dried, solubilized and subjected to SDS/polyacrylamide-gel electrophoresis in the absence (lanes 1 and 2) or the presence (lanes 3 and 4) of disulphide-bridge-reducing reagents ('BME'). Lanes 1 and 3 are 3-day autoradiograms of receptor eluted by 1 μ M-CGP-20712A. Lanes 2 and 4 are 1-day autoradiograms of receptor eluted by 100 μ M-(-)-alprenolol.

(Fig. 7a, inset). These results are in excellent agreement with the reported affinity of metoprolol to β_1 -adrenergic receptors in other tissues (Minneman *et al.*, 1979).

The β -adrenergic agonists (-)-adrenaline and (-)-noradrenaline were equipotent competitors of [125 I]ICYP binding (results not shown). This rank order of potencies of β -adrenergic agonists indicated that the receptor eluted by CGP-20712A was β_1 in nature (Lands *et al.*, 1967). Similar analysis to that outlined in Fig. 6(b) was performed on the receptors eluted by alprenolol from the Sepharose-alprenolol matrix. The competition of [125 I]-ICYP binding to the alprenolol-eluted receptors by ICI-118,551 was shallow, with a Hill coefficient of 0.8. Hofstee analysis of the competition data (Fig. 7b) indicated that ICI-118,551 was binding with $K_d = 3$ nM to a population of β_2 -adrenergic receptors that accounted for 75% of the total receptor population, and to the remaining β_1 -adrenergic receptors (25% of total) with $K_d = 0.1$ μ M. Unlike the situation observed for the receptor eluted with CGP-20712A, these results demonstrate that the receptors eluted with alprenolol after CGP-20712A were a mixed population of β -adrenergic subtypes.

The fractions eluted from the affinity matrix by CGP-20712A as compared with alprenolol were radioiodinated and analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 8). The radioiodinated peptides eluted by either CGP-20712A or alprenolol alone migrated with M_r 67000 under reducing conditions and with M_r 54000 under non-reducing conditions.

DISCUSSION

Progress in elucidating the nature of the human β -adrenergic receptors has been slow, owing to the extremely low abundance of these membrane proteins and the difficulties encountered in obtaining fresh human tissues. In order to expand our knowledge on the structure of human β -adrenergic receptors, we undertook large-scale purification of this receptor from basal membranes of human placenta. The purification of the β -adrenergic receptor from a tissue that contains both β -adrenergic receptor subtypes has not been attempted, because it has been difficult to stabilize both subtypes and to quantify accurately each subtype throughout the purification by using the standard protocols. The specificity of the interaction between the β_1 -adrenergic receptor and CGP-20712A served as the basis for estimation of each β -adrenergic receptor subtype. By using sequential rounds of affinity, hydrophobic and ion-exchange chromatography, as well as h.p.l.c. on steric-exclusion columns, we have purified human β -adrenergic receptors. Hydrophobic chromatography with C_7 -Sepharose, useful in the purification of other membrane proteins such as G_s (Northup *et al.*, 1980), was included in the protocol. Hydrophobic chromatography not only increased the specific activity of the receptor but also concentrated the receptor in a single fraction devoid of alprenolol. After C_7 -Sepharose chromatography, the receptor preparation contained two major peptides, of M_r 67000 and 45000. The M_r -67000 human β -adrenergic receptor species could be purified to apparent homogeneity by steric-exclusion h.p.l.c. Results from partial proteolytic maps of the M_r -45000 species suggest that it is a major proteolytic fragment of the M_r -67000 species (results not shown).

The M_r of purified human β -adrenergic receptors on SDS/polyacrylamide gels is similar to that of purified β_1 -adrenergic receptor from rat fat-cells (M_r 67000) (Cubero & Malbon, 1984) and β_2 -adrenergic receptors of guinea-pig lung (M_r 67000) (Drake & Perkins, 1984), hamster lung (M_r 64000) (Benovic *et al.*, 1984), rat liver (M_r 67000) (Graziano *et al.*, 1985) and mouse lymphoma S49 cells (M_r 67000) (George & Malbon, 1985). Autoradiograms of β -adrenergic receptors identified by photoaffinity labelling of basal membranes prepared from human placenta by [125 I]iodoazidobenzylpindolol also revealed a predominant M_r -65000-67000 peptide, in addition to a lesser quantity of M_r -55000 and a novel M_r -76000 species (Bahouth *et al.*, 1986). Photoaffinity labelling of membranes of mouse lymphoma S49 cells by [125 I]iodoazidobenzylpindolol likewise identified two predominant peptides, of M_r 65000 and 55000 (Rashidbaigi *et al.*, 1983). The M_r -76000 peptide of human placenta has only been recently identified by photoaffinity labelling (Bahouth *et al.*, 1986), and its relationship to other β -adrenergic receptor peptides is currently unknown. In addition to several lower- M_r peptides, a predominant M_r -62000 peptide was identified by photoaffinity label-

ling by using *p*-azido-*m*-[¹²⁵I]iodobenzylcarazolol in membranes prepared from human left-ventricular tissues (Stiles *et al.*, 1983). Our data suggest that the predominant form of human β -adrenergic receptors is a M_r -67000 peptide.

In the absence of treatment with disulphide-bridge-reducing reagents, the human β -adrenergic receptor displayed a greater relative mobility on SDS/polyacrylamide gels. This suggests that the human β -adrenergic receptor, like rat fat-cell β_1 -adrenergic receptor (Moxham & Malbon, 1985) and mouse S49 lymphoma β_2 -adrenergic receptor (George & Malbon, 1985), contains intramolecular disulphide bridges which when chemically reduced permit the receptor to 'unfold', increasing its apparent M_r .

The h.p.l.c.-purified receptors appear homogeneous after electrophoresis on SDS/polyacrylamide gels. The specific activity, however, is less than expected on theoretical calculations. Analysis of chemical amounts of the receptor by silver staining, periodic acid staining and autoradiography revealed no obvious contaminating peptides. We speculate that active and inactive receptors are co-purified through most procedures, except affinity chromatography. The susceptibility of mammalian β -adrenergic receptors to proteolysis and/or inactivation is well documented (Stiles *et al.*, 1984b).

The h.p.l.c.-purified β -adrenergic receptors migrating as a single peptide with M_r 67000 displayed both β_1 - and β_2 -adrenergic binding activities. In an attempt to demonstrate that the two subtype-binding activities resided in physically distinct peptides, it is necessary to separate the receptor subtypes and study the structural characteristics of each peptide. Until now, it has not been possible to purify one subtype of β -adrenergic receptors from tissues or preparations containing relatively similar proportions of both subtypes. This is because the subtype-selective ligands in current use do not possess a sufficiently high selectivity to interact with a single subtype. CGP-20712A is an antagonist ligand selective for β_1 -adrenergic receptors (Dooley & Bittiger, 1984). The selectivity ratio of this ligand is such that a plateau is observed in the competition of CGP-20712A for [³H]DHA binding to mixed populations of β_1 - and β_2 -adrenergic receptors (Figs. 1 and 6a). On the basis of computer modelling studies, the magnitude of the plateau increases as the selectivity ratio of the competing ligand increases (Molinoff *et al.*, 1981). The plateau observed in Fig. 6 extends about two log units, and it separates the range of CGP-20712A concentrations that interact predominantly with β_1 -adrenergic receptors from those that interact also with β_2 -adrenergic receptors (Dooley & Bittiger, 1984). The novel method used to purify β_1 -adrenergic receptors in the present study makes use of the CGP-20712A ligand during the elution step from affinity chromatography. Both receptor subtypes are bound to immobilized alprenolol. A 1 μ M concentration of CGP-20712A was shown to elute specifically only the β_1 -adrenergic receptors bound to Sepharose-alprenolol. This concentration of CGP-20712A competed for radioligand bound to β_1 -adrenergic receptors only (Fig. 6a). The early results of Caron *et al.* (1979) demonstrated that the concentration of agonists and antagonists required for eluting the receptor bound to Sepharose-alprenolol was in the same range as that competing for [³H]DHA bound to soluble β -adrenergic receptors. The receptor eluted by CGP-20712A from the affinity matrix was shown to

indeed be β_1 -, as determined by the established procedures for the assessment of these subtypes. The method outlined here for the purification of β_1 -adrenergic receptors from tissues containing both β_1 - and β_2 -adrenergic receptors is the first such report.

The M_r of purified radioiodinated β_1 -adrenergic receptors on SDS/polyacrylamide gels is similar to the M_r of β -adrenergic receptors eluted by alprenolol or purified by the six-step method outlined above. The proposed structural similarity between the human β_1 - and β_2 -adrenergic receptors is supported by several lines of evidence. Two-dimensional peptide maps generated from purified radioiodinated rat liver β_2 - and rat fat-cell β_1 -adrenergic receptors by *Staphylococcus aureus* V8 protease, α -chymotrypsin and elastase were essentially identical (Graziano *et al.*, 1985). The pI for both rat fat-cell β_1 - and liver β_2 -adrenergic receptors was the same (Moxham *et al.*, 1986). Antibodies prepared against rat fat-cell β_1 - or guinea-pig lung β_2 -adrenergic receptor cross-react with purified β_1 - and β_2 -adrenergic receptors (Moxham *et al.*, 1986). Photoaffinity labelling of basal membranes of human placenta with [¹²⁵I]iodo-azidobenzylpindolol resulted in photoaffinity labelling of a predominant M_r -67000 species representing both β -adrenergic receptor subtypes (Bahouth *et al.*, 1986). The pattern of photoaffinity labelling of the M_r -67000 species was not altered when the photoaffinity labelling was restricted to the β_2 -adrenergic receptor subtype by 1 μ M-CGP-20712A (Bahouth *et al.*, 1986). Taken together, these results represent further proof that a high degree of structural homology exists between the β_1 - and β_2 -adrenergic receptors and that their pharmacological specificity resides in subtle differences in their structure.

The gene encoding human β_2 -adrenergic receptors has been cloned from human placenta (Kobilka *et al.*, 1987). On the basis of the deduced amino acid sequence, the human β_2 -adrenergic receptor is structurally homologous to hamster lung β_2 -adrenergic receptor and to a lesser extent to the photoreceptor rhodopsin (Kobilka *et al.*, 1987; Dixon *et al.*, 1986). This apparent similarity of hamster lung β_2 -adrenergic receptor and human placenta β -adrenergic receptor is also suggested from the M_r value established for both receptor peptides (the present paper, and Benovic *et al.*, 1984). Furthermore, anti-rhodopsin antibodies recognize a M_r -65000 species in human placenta membranes that co-migrates with authentic purified β_1 - and β_2 -adrenergic receptors of mammalian sources (Weiss *et al.*, 1987). The expression of the genes encoding the mammalian β_2 -adrenergic receptor by transfection of cells in culture likewise reveal a gene product with M_r 67000 on SDS/polyacrylamide gels under reducing conditions (Dixon *et al.*, 1987). The identification of the gene encoding mammalian β_1 -adrenergic receptors has not been reported. On the basis of the results provided here and in the references cited above, the β_1 -adrenergic receptor appears to be structurally similar to the β_2 -adrenergic receptor. Further investigation using antibodies directed against peptides whose sequences are deduced from human β_2 -adrenergic receptor to probe for recognition of purified human β_1 -adrenergic receptor will enable further clarification of this matter.

This work was supported by U.S. Public Health Service Grants DK30111 and DK25410 from the National Institutes of Health. C.C.M. is recipient of Career Development Award

(K04 AM00786) from the N.I.H. We express our thanks to Dr. David Baker and Dr. Cynthia Kaplan (Department of Pathology, University Hospital, S.U.N.Y. at Stony Brook) for their assistance in procuring the placentas.

REFERENCES

- Bahouth, S. W., Kelley, L. K., Smith, C. H., Arbabian, M., Ruoho, A. E. & Malbon, C. C. (1986) *Biochem. Biophys. Res. Commun.* **141**, 411–417
- Benovic, J. L., Shorr, R. G. L., Caron, M. G. & Lefkowitz, R. J. (1984) *Biochemistry* **23**, 4510–4518
- Caron, M. G., Srinivasan, Y., Pitha, J., Kocielek, K. & Lefkowitz, R. J. (1979) *J. Biol. Chem.* **254**, 2923–2927
- Cheng, Y.-C. & Prusoff, W. H. (1973) *Biochem. Pharmacol.* **22**, 3099–3108
- Cubero, A. & Malbon, C. C. (1984) *J. Biol. Chem.* **259**, 1344–1359
- Dixon, R. A. F., Kobilka, B. K., Strader, D. J., Benovic, J. L., Dohlman, H. K., Frielle, T., Bolanowski, M. A., Bennett, C. D., Rands, E., Diehl, R. E., Mumford, R. A., Slater, E. E., Sigal, I. S., Caron, M. G., Lefkowitz, R. J. & Strader, C. D. (1986) *Nature (London)* **321**, 75–79
- Dixon, R. A. F., Sigal, I. S., Rands, E., Register, R. B., Candelore, M. R., Blake, A. D. & Strader, C. D. (1987) *Nature (London)* **326**, 73–76
- Dooley, D. J. & Bittiger, H. (1984) *Int. Union Pharmacol. Int. Congr.* 9th, abstr. 1009P
- Drake, F. H. & Perkins, J. P. (1984) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **43**, 874
- Dubray, G. & Bezaud, G. (1982) *Anal. Biochem.* **119**, 325–329
- George, S. T. & Malbon, C. C. (1985) *Prep. Biochem.* **15**, 349–366
- George, S. T., Ruoho, A. E. & Malbon, C. C. (1986) *J. Biol. Chem.* **261**, 16559–16570
- Graziano, M. P., Moxham, C. P. & Malbon, C. C. (1985) *J. Biol. Chem.* **260**, 7665–7674
- Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
- Hofstee, B. H. J. (1952) *Science* **116**, 329–331
- Kelley, L. K., Smith, C. H. & King, B. F. (1983) *Biochim. Biophys. Acta* **734**, 91–98
- Kobilka, B. K., Dixon, R. A. F., Frielle, T., Dohlman, H. G., Bolanowski, M. A., Sigal, I. S., Yang-Feng, T., Francke, U., Caron, M. G. & Lefkowitz, R. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 46–50
- Laemmli, U.K. (1970) *Nature (London)* **227**, 680–685
- Lands, A. M., Arnold, A., McAuliff, J. P., Ludueno, F. P. & Brown, T. G. (1967) *Nature (London)* **214**, 597–598
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Minneman, K. P., Hegstrand, L. R. & Molinoff, P. B. (1979) *Mol. Pharmacol.* **16**, 34–46
- Molinoff, P. B., Wolfe, B. B. & Weiland, G. A. (1981) *Life Sci.* **29**, 427–443
- Moore, J. J. & Whitsett, J. A. (1981) *Placenta, Suppl.* **3**, 103–114
- Moxham, C. P. & Malbon, C. C. (1985) *Biochemistry* **24**, 6072–6077
- Moxham, C. P., George, S. T., Graziano, M. P., Brandwein, H. & Malbon, C. C. (1986) *J. Biol. Chem.* **261**, 14562–14570
- Munson, P. J. & Rodbard, D. (1980) *Anal. Biochem.* **107**, 220–239
- Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M. & Gilman, A. G. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 6516–6520
- Rashidbaigi, A., Ruoho, A. E., Green, D. A. & Clark, R. B. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 2849–2853
- Ross, E. M. & Gilman, A. G. (1980) *Annu. Rev. Biochem.* **49**, 533–564
- Schaffner, W. & Weissmann, C. (1973) *Anal. Biochem.* **56**, 502–514
- Shaltiel, S. (1974) *Methods Enzymol.* **34**, 126–140
- Stiles, G. L., Strasser, R. H., Lavin, T. N., Jones, L. R., Caron, M. G. & Lefkowitz, R. J. (1983) *J. Biol. Chem.* **258**, 8443–8449
- Stiles, G. L., Benovic, J. L., Caron, M. G. & Lefkowitz, R. J. (1984a) *J. Biol. Chem.* **259**, 8655–8663
- Stiles, G. L., Caron, M. G. & Lefkowitz, R. J. (1984b) *Physiol. Rev.* **64**, 661–743
- Vauquelin, G., Geynet, P., Hanoune, J. & Strosberg, A. D. (1979) *Eur. J. Biochem.* **98**, 543–556
- Weiss, E. R., Hadcock, J. R., Johnson, G. L. & Malbon, C. C. (1987) *J. Biol. Chem.* **262**, 4319–4323

Received 5 January 1987/28 July 1987; accepted 17 August 1987