Biochemical Pharmacology, Vol. 32, No. 22, pp. 3375–3380, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

BINDING OF PROPRANOLOL AND IODOCYANOPINDOLOL TO ISOLATED CELLS, HOMOGENATES AND PLASMA MEMBRANES OF RAT LIVER, LUNG, KIDNEY AND HEART

R. ZINI, I. GAULT, S. LEDEWYN, PH. D'ATHIS and J. P. TILLEMENT
Département de Pharmacologie, Faculté de Médecine de Paris XII, 8 Rue du Général Sarrail, F-94010 Créteil Cedex, France

(*Received* 25 *November* 1982; *accepted* 17 *June* 1983)

Abstract—^{3}H-(±) propranolol and ^{125}I-(±) cyanopindolol have been used to characterize β adrenoceptors of liver, lung, kidney and heart of rat. Two main binding parameters, K_D and B_{max} were measured using either cells, homogenates or plasma membranes of each organ (except heart). Results show that the most accurate determination of K_D and B_{max} involves:
(1) a previous extraction of plasma membranes
(2) the use of a ligand of a high affinity for β adrenoceptors
(3) a high specific radioactivity of this ligand.
^{125}I-(±) cyanopindolol seems to be a better ligand than ^{3}H(±) propranolol for such determinations.

During the last five years, β adrenergic receptors have been extensively studied using specific radioligands including ^{3}H-(±) dihydroalprenolol [1], ^{125}I-(±) hydroxybenzylpindolol [2], ^{3}H-(±) carazolol [3] and ^{125}I-(±) cyanopindolol [4]. However, few works are systematically determined and compared binding parameters in tissue homogenates, cells and purified plasma membranes. Therefore, the purpose of this study was to determine binding parameters of the β adrenoceptors with these different materials in an attempt to select the most accurate one. Specific β adrenoceptors ligands were used but non-selective for a subtype β_1 or β_2 [5] as we wanted to measure total amounts of β adrenoceptors and as it is well known that relative proportions of each subtype vary widely between the different organs.

Considering a possible application of this study to human organs, ^{3}H-(±) propranolol was formerly chosen. Indeed this β blocking agent is often used in therapeutics; it is also highly concentrated both in lungs and liver where a high number of β adrenoceptors was thus to be expected [6].

At the reverse, despite the fact it is not used in therapeutics, ^{125}I-(±) cyanopindolol was used considering its high affinity for β adrenoceptors and the fact that a sample of high specific radioactivity was available: this reagent was used to check kidneys and heart which are known to have less β adrenoceptors.

The following binding parameters, B_{max}, the maximal binding capacity and K_D, the corresponding dissociation constant were systematically measured and compared.

1. EXPERIMENTAL PROCEDURES

1.1. *Materials*

(*a*) *Ligands*. ^{3}H-(±) propranolol hydrochloride (23 Ci/mmole) and ^{125}I-(±) cyanopindolol (>2000 Ci/mmole) were supplied by the Radiochemical Centre (Amersham, France). Their radiochemical purities were checked and found higher than 95% as determined by thin-layer chromatography on silica gel in *n*-butanol–water–acetic acid (12:5:3) and ethyl acetate–isopropanol–concentrated ammonia–water (45:35:5:10) respectively.

(*b*) *Buffers*. A saline buffer containing NaCl (115 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (0.8 mM), glucose (5 mM) and HEPES (25 mM) was adjusted at pH 7.4 with 1 N NaOH.

A washing buffer containing NaCl (140 mM), KCl (5.4 mM), $CaCl_2$ (1.8 mM), $MgCl_2$ (0.8 mM) and TRIS (25 mM) was adjusted at pH 7.4 with 1 N HCl.

A HEPES buffer containing NaCl (140 mM), KCl, (6.75 mM) and HEPES (10 mM) was adjusted at pH 7.4 with 1 N NaOH.

1.2. *Methods*

(*A*) *Isolation of rat living cells*

(*a*) *Isolation of hepatocytes*. Isolated hepatocytes were prepared in agreement with Seglen [7]. The cells were washed three times in the saline buffer incubation gassed with a mixture of O_2 (95%) and CO_2 (5%) and readjusted at pH 7.4 with 1N HCl. The cell suspension was stored at 4° in Falcon and gently stirred to avoid cell clustering. Viability of cells was assessed as follows: 85–95% of hepatocytes excluded Trypan Blue (0.4%, w/v); intracellular potassium concentrations determined by flame photometry was 120–150 mEq after isolation. The maximal period of storage was 5 hr. Concentration of cell protein was measured by the biuret method [8].

(*b*) *Isolation of lung cells*. Isolated lung cells were prepared in agreement with Phillips *et al.* [9] with some slight changes. Lung cells were stirred in saline buffer containing collagenase Type I (0.1%) and trypsin (0.1%) at 37° during 30 min. After this first

incubation, the solution was filtered and remaining pieces of lung were reincubated in saline buffer with collagenase Type I (0.5%) and trypsin (0.5%) at 37° during 2 hr. The solution obtained was filtered and the supernatant was centrifuged at 300 *g* at 4° for 5 min. The pellet was resuspended in suspension in 2 ml collagenase and trypsin solution; cells then were gently stirred to avoid clustering. This procedure was repeated twice. Concentration of cells was measured by the biuret method [8].

(*c*) *Isolation of kidney cells*. The procedure of isolation of cortex kidney cells was identical to the lung cell method [9]. However, incubation times were set at 20 min for the first incubation and three periods of 20 min for the second incubation. Concentration of kidney cells was measured also by the biuret method [8].

(*d*) *Isolation of beating heart cells*. Isolated ventricular heart cells were prepared in agreement with Powell *et al.* [10]. However, the beating ventricular heart cells had a short-lived viability and the efficiency derived was too weak. Consequently, binding assays have not been carried out.

(*B*) *Rat homogenates preparation*

Male Wistar rats (100–150 g body weight) were anesthetized with 5 mg/100 g weight pentobarbital and infused with HEPES buffer at 37° for 3 min. Heart, lung and kidney were homogenized at 4° with an Ultraturrax–Ikawerk (5 × 5 sec) in the saline buffer at 10 volumes (w/v). Liver was homogenized at 4° with a Potter–Heidolph homogenizer with a rotating teflon pestle (3 × 10 sec) in the saline buffer at 10 volumes (w/v). For each tissue, concentration of proteins was measured using the biuret method [8].

(*C*) *Rat plasma membranes preparation*

Homogenates of each organ were prepared as described above; however a TSE medium (20 mM Tris-HCl, 0.25 M sucrose and 1 mM EDTA) was used and homogenates were centrifuged in agreement with Paris *et al.* [11]. This procedure allows a final purification of 10-fold and a 6% yield. For each plasma membrane tissue, concentration of proteins was measured by the biuret and Lowry method [12].

(*D*) *Binding assays*

Binding studies to organ cells were performed as soon as possible, usually within 2–4 hr, whereas binding studies to homogenates or plasma membranes were performed either within 2–4 hr or after deep-freeze storage at −35°.

Experiments were routinely carried out in polypropylene tubes. Proteins were diluted between 0.1 and 1 mg/ml in saline solution then preincubated at 25°, with radiolabelled ligand either alone, or with a competing drug at various concentrations, during 6 and 90 min for propranolol and iodocyanopindolol respectively in a final volume of 1 ml. Bound and free ligands were separated by fast ultra-filtration through Whatman GF/F glass fiber filters. Each filter was rapidly washed at 4° with an additional volume of twice 10 ml of the washing buffer. The radioactivity of the filter disc was counted in 10 ml of Picofluor 30 in scintillation spectrometer Packard Tri-Carb 460 CD. Specific binding of the ligands was defined as the amount of the labelled ligand bound in the absence of competing ligand, minus the amount bound in the presence of 10^{-6} and 10^{-4} M-(±) propranolol for ^{125}I-(±) cyanopindolol and ^{3}H-(±) propranolol respectively. Specific binding was a linear function of the protein concentration. For homogenates, cells and membranes, the results are the mean ± S.D. of quadruplicate determinations with five animals.

(*E*) *Swelling method*

When checking ligand binding to living cells, a particular process was used to avoid a possible tissue penetration of the ligand. A swelling method was used [13] which consists, after a previous incubation between cells and the ligand in the isotonic buffer as usual, to remove the buffer and to replace it by an hypotonic solution which prompts the intracellular ligand to flow out.

(*F*) *Data analysis*

Data were processed according to the classical mass action law: free (F) and bound (B_t) labelled drug concentrations measured in the direct binding experiment were supposed to be related to each other by

$$B_t = \frac{KnRF}{1 + KF} + (K'n')RF, \tag{1}$$

where R is the receptor concentration, n and K stand respectively for the number of binding sites and the affinity constant of the drug for specific sites of receptor, and n' and K' stand respectively for the number of binding sites and the affinity constant of drug for non specific sites.

Thus, in the non specific binding experiments, where F values are quite high, equation (1) can be expressed more simply as

$$B_{ns} \simeq (K'n')RF, \tag{2}$$

where B_{ns} represents the new concentration of bound labelled drug.

The two parameters n, K and the product $n'K'$ were assessed by simultaneous coalescence of direct and non-specific binding data to their respective models. The non-linear regression technique based upon a uniformly weighed sum of squares was performed using a Gauss–Newton iterative minimization method [14]. Such an estimating technique is particularly relevant in reducing testing parallelism of direct and non-specific data to testing the suitability of data with the models.

Finally, for practical convenience, exogenous parameters were estimated as follows:

$$B_{max} = nR; \tag{3}$$

dissociation constant at equilibrium

$$K_D = \frac{1}{K} \tag{4}$$

and a specific binding curve was drawn, using

$$B_s = \frac{KnRF}{1 + KF}. \tag{5}$$

Table 1. Association and dissociation rate constants of propranolol and iodocyanopindolol to their respective tissues

Tissue	k_{+1} $M^{-1} \cdot min^{-1}$	k_{-1} min^{-1}	$K_D(k - 1/k + 1)$ nM
Liver	$(2.24 \pm 0.34) \times 10^8$	0.866 ± 0.147	3.86 ± 1.24
Lung	$(1.14 \pm 0.24) \times 10^8$	0.179 ± 0.028	1.57 ± 0.57
Kidney	$(1.50 \pm 0.14) \times 10^9$	0.048 ± 0.006	0.032 ± 0.007
Heart	$(1.37 \pm 0.22) \times 10^9$	0.085 ± 0.005	0.062 ± 0.014

Liver and lung cells (0.2 mg/ml), homogenates (0.5 mg/ml) or membranes (0.1 mg/ml) and ^{3}H-propranolol (10 nM) were incubated in presence and absence of 10^{-4} M (±) propranolol at 25°.

Kidney and heart cells (0.05 mg/ml), homogenates (0.2 mg/ml) or membranes (0.1 mg/ml) and ^{125}I-cyanopindolol (50 pM) were incubated in presence and absence of 10^{-6} M (±) propranolol at 25°.

Non specific binding which did not change significantly with time was less than 10% and was removed from total binding. Results are expressed as the mean (±SD) of two determinations with each preparation, homogenates, cells and membranes of the considered organ.

Table 2. Binding parameters of propranolol to rat liver and lung

	Liver		Lung	
	K_D (nM)	B_{max} (f mole/mg Prot)	K_D nM	B_{max} (f mole/mg Prot)
Cells	4.18 ± 0.46*	19,860 ± 1020*	23 ± 4*	3746 ± 446*
Homogenates	1.33 ± 0.12*	4140 ± 120*	77 ± 9*	479 ± 35*
Membranes	6.54 ± 0.39	24,702 ± 653	1.04 ± 0.15	1295 ± 51

* $P < 0.001$ (Student's test).

The equilibrium dissociation constants (K_D) and the maximum number of binding sites (B_{max}) were determined by means of the non-linear least squares method using Gauss–Newton algorithm. Each value is the mean ± SD of fifteen concentrations estimated from equilibrium studies performed in quadruplicate with five rats.

Table 3. Binding parameters of iodocyanopindolol to rat kidney and heart

	Kidney		Heart	
	K_D (pM)	B_{max} (f mole/mg Prot)	K_D (pM)	B_{max} (f mole/mg Prot)
Cells	88 ± 26*	81 ± 13‡	—	—
Homogenates	35 ± 11	33 ± 7‡	18 ± 5†	36 ± 4‡
Membranes	39 ± 5	174 ± 4	37 ± 5	226 ± 5

* $P < 0.02$
† $P < 0.01$
‡ $P < 0.001$
(Student's test).

Experimental conditions are identical to Table 2.

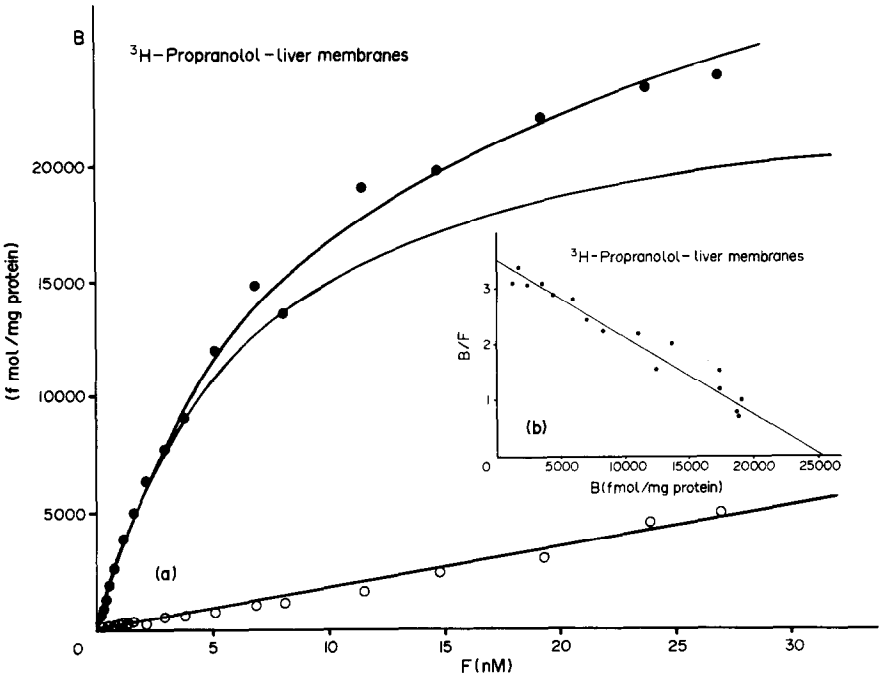

Fig. 1.(a) Binding of propranolol to rat liver plasma membranes. Binding studies were performed at 25° for 10 min, using ^{3}H-propranolol from 0.4 to 40 nM (protein = 0.02 mg/ml). The curve drawn without a symbol represents the specific propranolol binding by gaining the difference between total (●) and non-specific (○) binding in presence of 10^{-4}M (±) propranolol. Each symbol represents the mean of five rats in quadruplicate. The standard deviation of each measure is included in symbols. (b) Scatchard plot of propranolol binding to liver plasma membranes. Propranolol specific binding shows one class of binding sites.

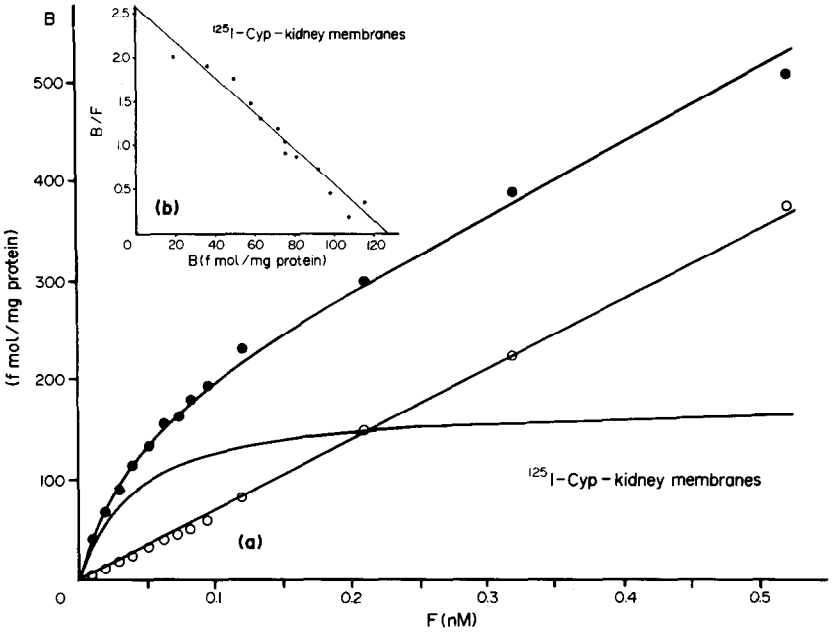

Fig. 2(a) Binding of iodocyanopindolol to rat kidney plasma membranes. Binding studies were performed at 25° for 75 min using ^{125}I-cyanopindolol from 10 to 600 pM (protein = 0.1 mg/ml). The curve drawn without symbol represents the specific iodocyanopindolol binding by gaining the difference between total (●) and non specific (○) binding in presence of 10^{-7} M (±) propranolol. Each point represents the mean of five rats in quadruplicate. The standard deviation of each measure is included in symbols. (b) Scatchard plot of iodocyanopindolol binding to rat kidney plasma membranes. Iodocyanopindolol specific binding shows one class of binding sites.

2. RESULTS

2.1. *Kinetic experiments*

Association and dissociation rate constants, respectively k_{+1} and k_{-1}, are shown on Table 1. Whatever the biological material used, i.e., cells, homogenates or membranes, values obtained are very close to a determined tissue. k_{+1} highest values and k_{-1} lowest values are obtained with iodocyanopindolol which exhibits a higher affinity ($1/K_D$) than propranolol for β adrenoceptors (Table 1). Corresponding K_D are close to the ones measured with equilibrium experiments (Tables 2 and 3).

2.2. *Equilibrium experiments*

The swelling method used was effective for liver cells. The whole propranolol binding to hepatocytes—$K_D = 7.20$ nM and $B_{max} = 96{,}540$ fmole/mg protein—represents both specific and non-specific bindings and passive diffusion inside the cells. The latter was suppressed by the swelling method, leading to a three-fold decrease of initial B_{max}. On the contrary, this method was unsuccessful with other organs.

Two series of typical binding plots are shown in Figs. 1 and 2. For all tissues, whatever the biological material, specific binding plots show only one class of binding sites described by the corresponding Scatchard representation. The binding parameters are listed in Tables 2 and 3. B_{max} and K_D values vary according to either tissues or their preparations. For every tissue, B_{max} increases significantly from homogenates to membranes, then from homogenates to cells. B_{max} values are higher with liver and kidney membranes than the corresponding ones measured with cells, but B_{max} measured with lung cells is higher than those obtained with the corresponding membranes. K_D significant variations are also observed between the different tissues and preparations. The Table 4 emphasizes the relative importance of non-specific binding, as compared to total binding of the two ligands. Therefore it shows that the use of tissue membranes instead of homogenates lowers significantly the non-specific binding of liver, kidney and lung.

3. DISCUSSION

Results are discussed according to either the selected experimental procedure, or the K_D and B_{max} values obtained when compared to other authors' results.

3.1. *Experimental procedures*

So as to be fairly accurate, the calculation of binding parameters requires a precise determination of B_{max}. This is obtained either when the non-specific binding is low, or when B_{max} and/or the relevant signal are high enough. So, the first cause of an inaccurate determination is an important non-specific binding. Our experiments show (Table 4) that the lowest values for this parameter are observed with tissue membranes. Thus, this biological material seems the most accurate for such determinations. Meanwhile, highest significant B_{max} and K_D variations from the different preparations of the same tissue are also observed in liver and lung; this cannot be related to their respective non-specific bindings but to the use of propranolol. The lower affinity of this ligand compared to those of iodocyanopindolol leads to a lower bound ligand concentration and consequently to a lower signal. Moreover, the affinity of ligand seems a decisive factor of accuracy, as shown by more precise K_D and B_{max} values obtained with kidney and heart membranes and iodocyanopindolol in spite of relatively high non-specific binding. The third factor of an accurate determination is obviously a high specific radioactivity which also leads to a higher signal, thus a more precise evaluation of B_{max}, at equilibrium.

Eventually, the use of iodocyanopindolol seems a better ligand than propranolol for K_D and B_{max} measurements of beta adrenoceptors.

Trypsin was used in order to isolate lung and kidney cells. As was previously shown [15] it lowers B_{max} values; it would be surprising that such a treatment led to a B_{max} increase. However, the results clearly show that cells exhibit a higher B_{max} when compared to those of homogenates with the same protein concentration. Moreover, the same result was observed with hepatocytes, when compared to

Table 4. Variations of non specific binding of the two ligands according to the biological materials and the tissues used

	Non specific binding (Percentage of total binding)		
	Cells	Homogenates	Membranes
Liver	40.0 ± 8.0*	45.0 ± 10.0*	12.0 ± 3.0
Lung	26.0 ± 12.0	31.0 ± 5.0*	23.0 ± 7.0
Kidney	72.0 ± 10.0*	64.0 ± 11.0*	43.0 ± 15.0
Heart	—	52.0 ± 15.0	47.0 ± 15.0

* $P < 0.001$ (Student's test).

Propranolol was used to assess β adrenoceptors in liver and lung and iodocyanopindolol for kidney and heart. Percentages of non-specific binding are compared to those of membranes. The results are the mean ± SD of four concentrations measured at plateau in quadruplicate with five rats.

the corresponding homogenates. Since this cell preparation did not require this enzyme, it has probably an insignificant effect, if any, on receptor number determination.

3.2. K_D and B_{max} values

K_D values observed for propranolol and rat liver and lung membranes are in the same range than those reported for the same or different species, for instance with chicken cerebral membranes $K_D = 2 \times 10^{-8}$ M [16], with guinea pig heart ventricle membranes, $K_D = 6.9 \times 10^{-9}$ M [17] and with rat kidney membranes, $K_D = 4 \times 10^{-9}$ M [18]. These similar results point out the likelihood of a common basic structure of the β adrenoceptors.

K_D of iodocyanopindolol is identical for both kidney and heart membranes. It is also between one hundred and one thousand times weaker than the ones of propranolol. This difference has been already shown with guinea pig left ventricle and lung [4].

On the contrary, B_{max} values observed with propranolol are much higher than those usually reported [19–22] especially for liver membranes [21–22]. Such a discrepancy lies in the fact that some rat tissue membranes, especially hepatocytes and adipocytes have many other propranolol binding sites, together with beta adrenoceptors, which are also saturable but non-stereospecific binding sites [23–25]. Since non-specific binding was checked using the same ligand—(±) propranolol—it was impossible to dissociate these sites from the true β stereospecific adrenoceptors. This hypothesis was recently confirmed while using iodocyanopindolol as the ligand of the same biological material, i.e. rat liver membranes.

The corresponding B_{max} value was 90 fmole/mg protein, a result which tallies with other authors [21, 22]. So it is clear that B_{max} determination of liver membranes β adrenoceptors cannot be appraised accurately using (±) ^{3}H-propranolol as ligand.

Quite differently, B_{max} values for lung are close to those of Engel *et al.* [4], i.e. 920 ± 80 fmole/mg protein.

Similarly with heart membranes, our result is close to those of Hoyer *et al.* [26], i.e. 123.9 fmole/mg protein.

Nevertheless, all these results are higher than those obtained by other authors [19, 20, 27, 28]. A tentative explanation is a higher purity of membrane samples; the extraction involves three successive centrifugations in order to discard not only soluble proteins but also the main insoluble impurities like cell fragments, nucleus and mitochondria. Receptor preparation often involves only the elimination of soluble proteins; as a matter of fact our preparations of receptor membranes are more purified. Thus, when compared to the same protein concentration, our B_{max} values are higher than usual.

In conclusion, it appears that the most accurate determination of β adrenoceptors of these tissues involve primarily a previous membrane extraction, than the use of a ligand that offers both a high specific radioactivity and affinity. Hence the more effective iodocyanopindolol property than propranolol.

Acknowledgements—The authors thank Dr. G. Engel (Sandoz Ltd, Basle, Switzerland) for his gift of iodocyanopindolol and for valuable information on the "swelling method" with regard to cellular passive diffusion and ICI company for his generous gift of (±) propranolol hydrochloride. They are indebted to Professor Michel Lazdunski and Dr. Jean-François Renaud for their help and their very stimulating comments all over this work.

This research was supported by a grant from DGRST No. BFM/P.96.

REFERENCES

1. R. J. Lefkowitz, C. Mukherjee, M. Coverstone and M. G. Caron, *Biochem. biophys. res. Commun.* **60**, 703 (1974).
2. G. D. Aurback, S. A. Fedak, C. J. Woodward, J. S. Palmer, D. Hauser and F. Troxler, *Science* **186**, 1223 (1974).
3. R. B. Innis, F. M. A. Correa and S. Snyder, *Life Sci.* **24**, 2255 (1979).
4. G. Engel, D. Hoyer, R. Berthold and H. Wagner, *Naunyn-Schmied. Archs Pharmacol.* **317**, 277 (1981).
5. K. P. Minneman and P. B. Molinoff, *Biochem. Pharmacol.* **29**, 1317 (1980).
6. G. H. Evans, G. R. Wilkinson and D. G. Shand, *J. Pharmacol. Exp. Ther.* **186**, 447 (1973).
7. P. O. Seglen, *Expl. cell. Res.* **82**, 391 (1973).
8. A. G. Gornall, C. J. Bardawill and M. M. David, *J. Biol. Chem.* **177**, 751 (1949).
9. H. J. Phillips, *In vitro.* **8**, 101 (1972).
10. T. Powell, M. F. Sturridge, J. K. Suvarna, D. A. Terrar and V. W. Twist, *Med. J.* **283**, 1013 (1981).
11. S. Paris and M. Fosset, *J. Mol. cell. Cardiol.* **9**, 161 (1977).
12. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Rendall, *J. Biol. chem.* **193**, 265 (1951).
13. E. Paietta, D. Hoyer, G. Engel and J. D. Schwarzmeier, *Mol. cell. Endocrinol.* **25**, 267 (1982).
14. R. Zini, Ph. d'Athis, J. Barre and J. P. Tillement, *Biochem. Pharmacol.* **28**, 2661 (1979).
15. R. Pochet and H. Schmitt, *Nature* **277**, 58 (1979).
16. S. R. Nahorski, *Nature* **259**, 488 (1976).
17. A. Rankin and J. K. Broadley, *Biochem. Pharmacol.* **31**, 1325 (1982).
18. D. E. Brodde, *Biochem. Pharmacol.* **31**, 1743 (1982).
19. E. L. Rugg, D. B. Barnett and S. R. Nahorski, *Mol. Pharmacol.* **14**, 996 (1978).
20. K. P. Minneman, L. R. Hegstrand and P. B. Molinoff, *Mol. Pharmacol.* **16**, 21 (1979).
21. B. B. Wolfe, T. K. Harden and P. B. Molinoff, *Proc. Nat. Acad. Sci., U.S.A.* **173**, 1343 (1976).
22. G. Guellaen, M. Yates-Aggerbeck, G. Vauquelin, D. Strosberg and J. Hanoune, *J. Biol. Chem.* **253**, 1114 (1978).
23. E. M. Dax, J. S. Partilla and R. I. Gregerman, *J. Receptor Res.* **2**, 267 (1981).
24. E. M. Dax and J. S. Partilla, *Mol. Pharmacol.* **22**, 5 (1982).
25. E. M. Dax, J. S. Partilla and R. I. Gregerman, *J. Lipid Res.*, **23**, 1001 (1982).
26. D. Hoyer, G. Engel and R. Berthold, *Naunyn. Schmied. Archs. Pharmacol.* **318**, 319 (1982).
27. J. Tse, R. W. Wrenn and J. F. Kuo, *Endocrinology* **107**, 6 (1980).
28. A. O. Davies, A. de Lean and R. J. Lefkowitz, *Endocrinology* **108**, 720 (1981).