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CHARACTERIZATION OF PERIPHERAL TYPE BENZODIAZEPINE BINDING SITES IN HUMAN AND RAT PLATELETS BY USING [^{3}H]PK 11195. STUDIES IN HYPERTENSIVE PATIENTS

JESUS BENAVIDES, DOMINIQUE QUARTERONET, PIERRE-FRANÇOIS PLOUIN*, FRANÇOISE IMBAULT, TAN PHAN, ANDRE UZAN, CHRISTIAN RENAULT, MARIE-CHRISTINE DUBROEUCQ, CLAUDE GUEREMY and GERARD LE FUR

PHARMUKA Laboratories, 35 Quai du Moulin de Cage, 92231 Gennevilliers, France and *Service d'Hypertension. Hôpital Saint Joseph. 75674 Paris, France

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Abstract—Peripheral type benzodiazepine binding sites have been studied in human and rat platelets and platelet membranes by using PK 11195 (1-(2-chlorophenyl)-*N*-methyl-*N*-(1-methyl propyl)-3-isoquinolinecarboxamide) as a ligand. [^{3}H]PK 11195 binding to the intact cells and membranes is saturable, with high affinity and presents the pharmacological specificity corresponding to the peripheral binding sites (PK 11195 > RO5-4864 > diazepam > clonazepam). [^{3}H]PK 11195 affinity is not affected by cell lysis, but there is a loss of binding capacity, contrarily to RO5-4864 whose affinity is greatly diminished. For this reason [^{3}H]RO5-4864 binding can only be demonstrated in intact cells. Furthermore opposite to RO5-4864, PK 11195 affinity is not decreased by increasing temperatures. No difference was found between binding parameters (K_D and B_{max}) for [^{3}H]PK 11195 between normotensive and hypertensive subjects. The very high binding capacity of human and rat platelets (B_{max} > pmole/10^8 cells) makes them a good biological model for studying the physiological significance of "peripheral type" benzodiazepine binding sites.

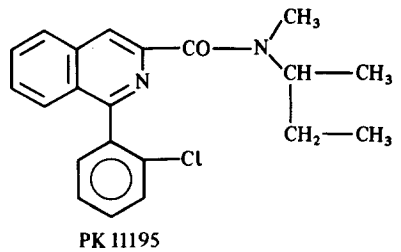
Binding sites for benzodiazepine with a different specificity that the GABA coupled benzodiazepine brain receptors have been recently described [1–3]. These sites were denominated "peripheral type" because they were firstly described in peripheral organs such as kidney, heart and adrenals [1–4]. Nevertheless, by using a specific ligand for the peripheral binding sites, the [^{3}H]RO5-4864, sites with the "peripheral" pharmacological specificity have been described in the brain [3, 5, 6].

A main difference between the brain and peripheral type binding sites is that the potency of displacing agents is 10^{-9} M (clonazepam), 4×10^{-9} M (diazepam) and $>10^{-6}$ M (RO5-4864) for the former and $>10^{-5}$ M (clonazepam), 4×10^{-8} M (diazepam) and 10^{-8} M (RO5-4864) for the later.

An advance in the knowledge of the peripheral binding sites came from the discovering of a compound with an isoquinoline structure, PK 11195 (Fig. 1), which presented a high affinity and specificity for the peripheral type benzodiazepine binding sites [4]. Thermodynamic studies of the binding indicated that RO5-4864 binding induced a conformational change while PK 11195 binding was not accompanied by such a modification [7]. Tritium labelled PK 11195 has been utilized to characterize the "peripheral" type binding sites for benzodiazepines in several organs [6–8].

Peripheral binding sites for benzodiazepines have also been described in several cell types such as macrophages [9] and platelets. Binding sites have been characterized in intact platelets with [^{3}H]diazepam [10] and [^{3}H]RO5-4864 [4] as ligands. Interestingly, binding sites for benzodiazepines are increased in platelets from spontaneous hypertensive rats [11]. Because there was a loss in binding capacity in the platelet membrane fractions it was suggested that binding sites could be localized in intracellular compartment or be destroyed during cell disruption.

For these reasons it seemed interesting to characterize the binding of [^{3}H]PK 11195 to platelets as compared with [^{3}H]RO5-4864. [^{3}H]PK 11195 is a specific and high affinity ligand for the peripheral benzodiazepine binding sites in platelets. Moreover opposite to [^{3}H]RO5-4864 no affinity loss was induced by cell disruption. On the other hand, as previously described in other tissues [^{3}H]PK 11195 but not [^{3}H]RO5-4864 keep unchanged its high affinity at 37°, allowing the characterization of the binding sites at physiological temperatures.



PK 11195

Fig. 1. Chemical structure of PK 11195.

MATERIALS AND METHODS

Preparation of intact platelets and platelets crude membrane fraction. Blood was withdrawn from the abdominal cave vein of ether-anesthetized rats weighing 350 g or from healthy human volunteers and mixed with the anticoagulant as described by Rossi [12]. Platelets were prepared according to the same author and resuspended in Tris-buffered saline (140 mM NaCl, 5 mM KCl, 5 mM glucose, 0.38 mM sodium citrate and 25 mM Tris–HCl, pH = 7.4) at a final concentration of 4×10^8 platelets/ml.

Platelet membrane fraction was prepared from the platelet pellet by lysis in 5 mM Tris–HCl pH = 7.4 and 20 sec ultrasonic disruption in a Branson Sonifier-Cell disruptor B.15 set at 7. Homogenates were then centrifuged at 46,000 *g* for 10 min and pellets resuspended in 50 mM Tris–HCl at a protein concentration of about 10 μg/ml. In some cases platelets were disrupted by glycerol osmotic lysis as described by Barber and Jamieson [13]. Protein was measured by a standard method [14].

Binding assays. Platelets (5×10^7 platelets/ml) or membranes 10 μg prot/ml were incubated in Tris-buffered saline at 25° for 15 min or at 4° for 60 min. Reaction was terminated by the addition of 3 ml of phosphate-buffered saline and vacuum filtration through GF/C filters. Filters were then washed 3 times with 3 ml phosphate-buffered saline. Non-specific binding was defined by the binding non-displaceable by 10 μM RO5-4864, [^{3}H]RO5-4864 binding was measured as described by Le Fur *et al.* [4].

The absence of degradation of tritiated ligands has been determined as previously described [8].

Material. [^{3}H]PK 11195 was prepared by catalytic tritiation of 1-(2-chlorophenyl-*N*-methyl-*N*-(1-methyl 2-propenyl)-3-isoquinolinecarboxamide with a specific activity of 40 Ci/mmole. [^{3}H]RO5-4864 (73 Ci/mmole) was purchased from NEN. RO5-4864 was kindly provided by Hoffmann-La Roche Inc. Nutley, NJ. All the other compounds were from standard sources.

Patients. Fifteen patients, 3 women and 12 men (mean age 47 ± 3 years old) hospitalized at the Hôpital Saint Joseph were diagnosed by conventional methods as having essential hypertension [15]. The patients were free of renal disease, symptomatic heart disease or diabete mellitus. The blood pressure in all patients was consistently above 160/90 torr (177 ± 11/105 ± 11 at admission). All patients and subjects were free of medication for at least 14 days prior to the study. Control normotensive subjects (8 women 6 men) were 34 ± 6 years old.

RESULTS

Specific [^{3}H]PK 11195 binding (i.e. displaceable by PK 11211) to rat and human platelets increases linearly up to 10^8 platelets/ml or 20 μg/ml of platelet membrane protein. Non-specific binding was less than 10% of total binding. At 4° specific binding of 1 nM [^{3}H]PK 11195 to rat platelet membranes reached equilibrium in less than 30 min (Fig. 2). Binding was completely reversible after the addition of 10 μM RO5-4864. Rate constants calculated from association-dissociation kinetics were $k_1 = 0.0063\ \text{nM}^{-1}\text{min}^{-1}$ and $k_{-1} = 0.0823\ \text{min}^{-1}$.

Rate constants have also been calculated for the [^{3}H]PK 11195 binding to human membranes (results not shown) and were at 4° $k_1 = 0.0038\ \text{nM}^{-1}\text{min}^{-1}$ and $k_{-1} = 0.0509\ \text{min}^{-1}$.

Equilibrium binding experiments at 25° indicated that [^{3}H]PK 11195 binds to platelets membranes in a saturable manner (Figs. 3a and 3b).

Scatchard analysis of the saturation isotherms gave similar dissociation constant (K_D) for human (8.6 ± 1.3 nM) (mean of 14 independent determinations) and rat (2.45 ± 0.4 nM) (mean of 4 independent determinations). However B_{max} was 5 times higher in rat platelets membranes as compared to human platelets membranes (Table 1).

K_D values for intact cells are very similar to that found in the membranes (Table 1). However, B_{max}

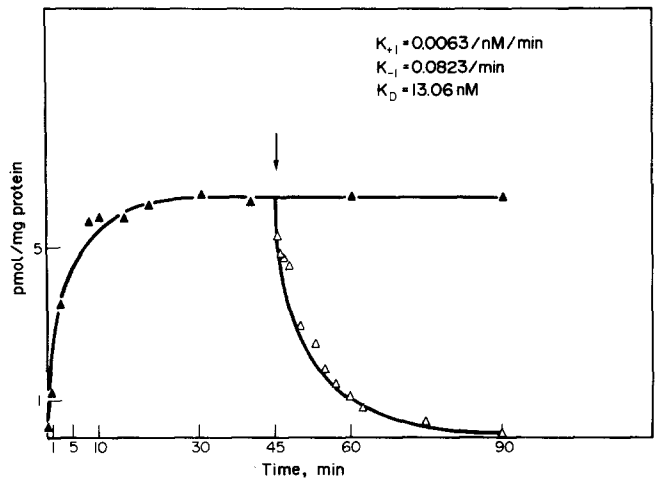


Fig. 2. Reversibility of [^{3}H]PK 11195 binding to rat platelet membranes. Platelet membranes were incubated at 4° in the presence of 1 nM [^{3}H]PK 11195 and filtered and washed at the indicated times. Ten micromolar RO5-4864 was added at the time indicated by the arrow to initiate the dissociation. Values are the mean of 3 experiments.

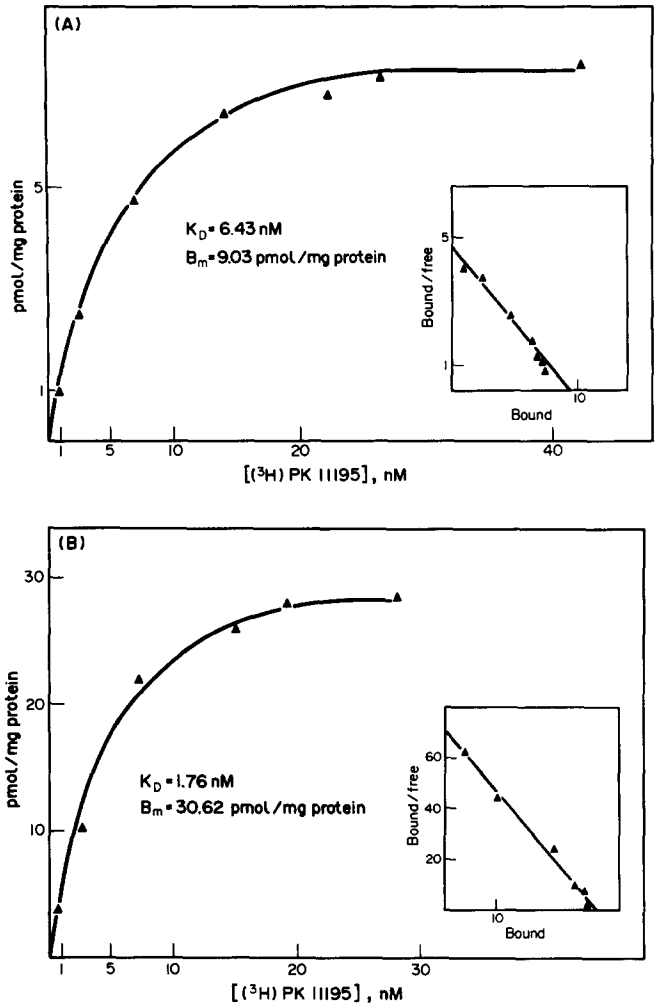


Fig. 3. Saturability of the [^{3}H]PK 11195 binding to human (panel A) and rat (panel B) platelet membranes. Membranes (10 μg prot/ml) were incubated in the presence of several concentrations of [^{3}H]PK 11195 (0.5–15 nM) for 20 min at 25°. Experiments were repeated 4 times and data shown are from one representative experiment. Non specific binding (in the presence of 10 μM RO5-4864) was always less than 5% of total binding. Inset—Scatchard analysis of the specific binding of [^{3}H]PK 11195 calculated from the saturation isotherms. Data are from a representative experiment which was repeated at least 4 times with similar results.

values were higher in intact cells than in membranes. The loss of binding sites due to cell disruption is about 75% in human and 40% in rat platelets.

[^{3}H]PK 11195 binding to human and platelets membranes has also been tested at 4°. Binding capacity and [^{3}H]PK 11195 affinity were the same as at 25° (results not shown).

Specificity of [^{3}H]PK 11195 *binding.* [^{3}H]PK 11195 binding to rat and human intact platelets or platelet membranes is not affected by 10^{-4} M of GABA,

Table 1. [^{3}H]PK 11195 binding to human and rat platelets—comparison between intact cells and membrane fractions

	Human		Rat	
	Intact platelets	Membranes	Intact platelets	Membranes
K_D (nM)	3.74 ± 0.42	8.56 ± 1.29	1.65 ± 0.59	2.45 ± 0.4
B_{max} (pmole/10^8 cells)	1.18 ± 0.06		1.17 ± 0.13	
(pmole/mg prot)	31.1 ± 1.6	7.7 ± 0.4	58.5 ± 6.5	34.9 ± 4.3

[^{3}H]PK 11195 binding was performed at 25°. Platelets concentration was 5 × 10^7 platelets/ml or 10 μg/ml (membranes). K_D and B_{max} values are the mean ± S.E. of at least 4 experiments. Protein content of 10^8 platelets was 38 μg (human) and 20 μg (rat).

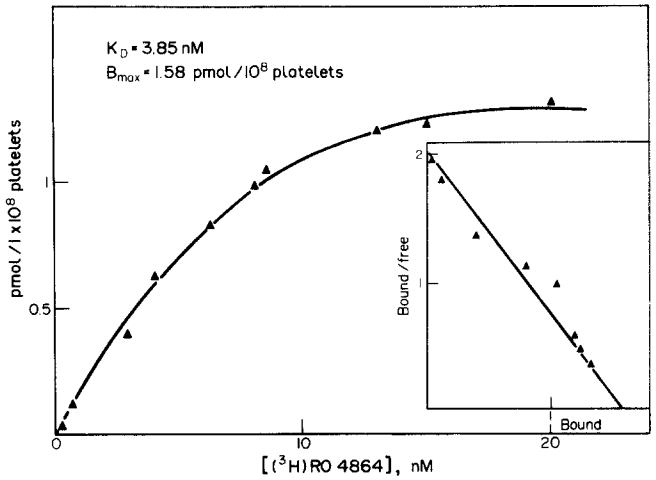


Fig. 4. Saturability of [^{3}H]RO5-4864 binding to intact rat platelets. Rat platelets (5×10^7 cells/ml) were incubated in the presence of several concentrations of [^{3}H]RO5-4864 for 60 min at 4°. Data shown are from a representative experiment which was repeated 4 times with similar results. Non-specific binding (in the presence of 10 μM RO5-4864) was always less than 5% of total binding. Inset—Scatchard analysis of the saturation isotherms.

histamine, serotonine, noradrenaline, dopamine, glycine and glutamate or 10^{-5} M of imipramine, clonidine, cimetidine, morphine, promethazine, atropine, propranolol, yohimbine, haloperidol, chlorpromazine and chloroquine. Metergoline was inactive at 2×10^{-6} M and lysvasopressin at 3 U/ml.

The potency order of displacing agent suggests that [^{3}H]PK 11195 labels the "peripheral type" benzodiazepine binding sites in platelets (Table 2). PK 11195 was always the most potent displacer, followed by RO5-4864, diazepam and dipyridamole, clonazepam being inactive. However, while K_i for PK 11195 was the same in membranes and intact platelets, displacing potency of RO5-4864 undergone a seven times decrease after rat platelet lysis. No such a difference was found between human platelets and membranes. Diazepam also presents a lower affinity in the crude membrane fraction than in the cells. Inhibition by RO5-4864, dipyridamole and diazepam was competitive as demonstrated by the increase of the K_D value without modification in B_{max} when binding was carried out in the presence of these drugs (results not shown).

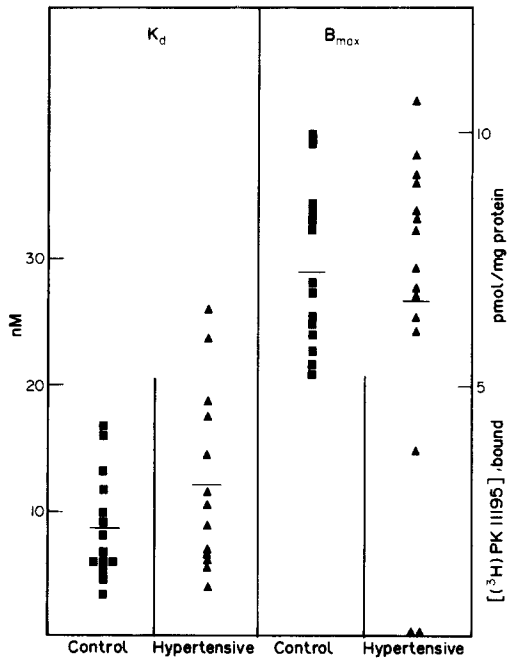


Fig. 5. Platelets benzodiazepine binding sites in normotensive and hypertensive patients. Binding sites in human platelets membranes were measured using [^{3}H]PK 11195 as ligand. Binding parameters were determined from Scatchard plots of the binding data and [^{3}H]PK 11195 concentrations were as in Fig. 3.

"Peripheral type" benzodiazepine binding sites can also be labeled with [^{3}H]RO5-4864 in intact rat platelets (Fig. 4). Binding capacity for this ligand is identical to that of [^{3}H]PK 11195 (1.58 ± 0.11 pmole/10^{-8} platelets) and K_D value agrees with the K_i obtained in the displacement experiments (3.85 ± 0.55 nM). The percentage of RO5-4864 specific binding to rat platelets and human platelets and platelet fraction was very low (≈30%). Because this fact, perhaps due to a lower affinity, no clear saturation could be demonstrated (results not shown). However, 10^{-5} M RO5-4864 completely displaced [^{3}H]PK 11195 specific binding. Thus, our inability to demonstrate saturable [^{3}H]RO5-4864 binding was not due to a loss of binding sites but to a methodological problem.

Rat platelets disruption by glycerol osmotic lysis [14] leads to similar affinity lowering for RO4-4864 (results not shown).

[^{3}H]PK 11195 *binding to platelets from hypertensive patients.* [^{3}H]PK 11195 binding to human platelets membranes have been compared in normotensive and hypertensive subjects. Individual values of the binding parameters were presented in Fig. 5. Mean ± S.E. of K_D values were

Table 2. Inhibition of [^{3}H]PK 11195 binding to human and rat platelets—comparison between intact cells and membrane fractions

Drugs	Human K_i (nM)		Rat K_i (nM)	
	Intact platelets	Membranes	Intact platelets	Membranes
PK 11195	4.2 ± 0.52	5.9 ± 0.78	1.6 ± 0.2	1.4 ± 0.2
RO5-4864	22 ± 3.2	41 ± 2.9	5.0 ± 0.7	39 ± 5.1
Diazepam	194 ± 11	259 ± 31	405 ± 61	2974 ± 233
Dipyridamole	888 ± 103	1851 ± 155	218 ± 17	1555 ± 188
Clonazepam	>10 000	>10 000	>10 000	>10 000

[^{3}H]PK 11195 (1 nM) binding was performed at 25°. K_i were calculated from IC_{50} values obtained from displacement studies with 5 drug concentrations. Previously the competitivity of the inhibition was demonstrated and thus $K_i = (IC_{50} - K_D/S + K_D)$, where K_D and S were respectively the affinity and the concentration of [^{3}H]PK 11195.

8.62 ± 1.29 nM (normotensive) and 12.14 ± 2.01 nM (hypertensive) and for B_{max} values 7.7 ± 0.4 pmole/mg prot (normotensive) and 6.7 ± 0.9 (hypertensive). Thus K_D and B_{max} values were not significantly different between the two groups. Interestingly in the hypertensive group there were two cases with no detectable [^{3}H]PK 11195 binding, even if no correlation could be established with their clinical state. However, the control group was younger than the hypertensive group.

DISCUSSION

The results here reported demonstrate the existence of "peripheral type" benzodiazepine binding sites in rat and human platelets. These sites can be labeled with [^{3}H]PK 11195 both in intact and disrupted cells. [^{3}H]RO5-4864 presents a good affinity in intact rat platelets, but in platelets membranes affinity is 8 times lower as compared to intact cells. The weak affinity of [^{3}H]RO5-4864 in human platelets makes difficult to utilize this ligand to label the "peripheral type" benzodiazepine binding sites in these cells.

Rat platelets lysis causes an affinity loss for RO5-4864 but not for PK 11195. This is an interesting feature which might suggest a different kind of interaction of both ligands with the binding sites.

Wang *et al.* [10], using [^{3}H]diazepam as ligand found that rat platelet lysis affected only the binding capacity but not the K_D value. In our conditions lysis reduces the B_{max} but not the K_D value for [^{3}H]PK 11195. However an affinity loss for RO5-4864 and diazepam was also evident.

In human platelets the effect of cell lysis on RO5-4864 affinity is smaller than in the rat, but a possible explanation is that the affinity of intact cells is already smaller as compared to rat intact platelets. Of interest it is that [^{3}H]PK 11195 binding can always be completely displaced by 10^{-5} M RO5-4864.

It is evident that the lower affinity for [^{3}H]RO5-4864 of rat platelets membranes can not be interpreted as if measured binding in intact platelets was the uptake of this drug into a intracellular organelle such as lysosomes since affinity for [^{3}H]PK 11195 is not modified by cell lysis. Morever chloroquine, an inhibitor of lysosomal uptake processes does not affect [^{3}H]PK 11195 or [^{3}H]RO5-4864 binding to intact cells. Homogenization also decreases the binding capacity for [^{3}H]PK 11195 [8] and other ligands [16].

Thus in agreement with Maloteaux *et al* [17] it can be concluded that binding of [^{3}H]PK 11195 and [^{3}H] RO5-4864 to intact platelets is a membrane process and does not reflect the trapping of these drugs by intracellular organelles.

The reciprocal displacement of RO5-4864 and PK 11195 (Table 2, [4]) as well as a similar B_{max} for both ligands in rat platelets suggest that both compounds label the same binding sites. However the differential effect of cell lysis could be interpreted as if this procedure induces a conformational changes of the binding sites.

Because in a previous report it was shown that spontaneous hypertensive rats presented a higher number of benzodiazepine binding sites in platelets than age matched normotensive (Wistar Kyoto) rats it seemed interesting to test if the same holds true in human. This was not the case because it can not be detected significant differences in platelets benzodiazepine binding sites between normotensive and hypertensive patients.

A possible explanation for this fact could be that spontaneous hypertensive rats represent a homogeneous group with a single origin for hypertension while in human this decrease can have multiple origins. At this respect it is noteworthy that in the hypertensive group there were two cases without any detectable binding even if this lack could not be related to the clinical state.

While no clear biochemical or pharmacological effects can be ascribed to the "peripheral type" binding sites for benzodiazepine the conjoint utilization of PK 11195 and RO5-4864 could help to determine the physiological relevance of these sites.

REFERENCES

1. T. Taniguchi, J. K. T. Wang and S. Spector, *Biochem. Pharmac.* **31**, 589 (1982).
2. L. P. Davies and V. Huston, *Eur. J. Pharmac.* **73**, 209 (1981).
3. H. Schoemaker, R. G. Boles, W. D. Horst and H. I. Yamamura, *J. Pharmac. exp. Ther.* **225**, 61 (1983).
4. G. Le Fur, M. L. Perrier, N. Vaucher, F. Imbault, A. Flamier, J. Benavides, A. Uzan, C. Renault, M. C. Dubroeucq and C. Gueremy, *Life Sci.* **32**, 1839 (1983).

5. P. J. Marangos, J. Patel, J. P. Boulenger and R. Clark-Rosenberg, *Molec. Pharmac.* **22**, 26 (1982).
6. J. Benavides, D. Quarteronet, F. Imbault, C. Malgouris, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy and G. Le Fur, *J. Neurochem.* **41**, 1744 (1983).
7. G. Le Fur, N. Vaucher, M. L. Perrier, A. Flamier, J. Benavides, C. Renault, M. C. Dubroeucq, C. Gueremy and A. Uzan, *Life. Sci.* **33**, 449 (1983).
8. J. Benavides, C. Malgouris, F. Imbault, F. Begassat, A. Uzan, C. Renault, M. C. Dubroeucq, C. Gueremy and G. Le Fur, *Archs. int. Pharmacodyn.* **266**, 38 (1983).
9. T. Taniguchi, J. K. T. Wang and S. Spector, *Life Sci.* **27**, 171 (1980).
10. J. K. T. Wang, T. Taniguchi and S. Spector, *Life Sci.* **27**, 1881 (1980).
11. T. Taniguchi, J. K. T. Wang and S. Spector, *Eur. J. Pharmac.* **70**, 587 (1981).
12. E. C. Rossi, *J. Lab. clin. Med.* **78**, 483 (1971).
13. A. J. Barber and G. A. Jamieson, *J. biol. Chem.* **245**, 6357 (1970).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. J. Menard, C. Devries, A. Tugaye and P. F. Plouin, in *Hypertension* (eds. P. Sleight and E. Freis), p. 117. Butterworths, London (1982).
16. M. J. Kuhar, in *Handbook of Psychopharmacology* (eds. L. L. Iversen, S. D. Iversen and S. H. Snyder), Vol. 15, p. 229. Plenum Press, New York (1982).
17. J. M. Maloteaux, A. Gossuin, C. Waterkey and P. Laduron, *Biochem. Pharmac.* **32**, 2543 (1983).