Biochemical Pharmacology, Vol. 35, No. 3, pp. 515–519, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

THE CHARACTERISTICS OF LOW AND HIGH AFFINITY [^{3}H]-PRAZOSIN BINDING TO MEMBRANES FROM RAT RENAL CORTEX

GRANT A. MCPHERSON* and ROGER J. SUMMERS†

Department of Pharmacology, University of Melbourne, and *School of Pharmacology, Victorian College of Pharmacy, Royal Parade, Parkville, Victoria 3052, Australia

(*Received* 26 *March* 1985; *accepted* 23 *August* 1985)

Abstract—The characteristics of [^{3}H]-prazosin binding in renal cortical membranes of the rat have been assessed under a variety of buffer conditions. At 37°, in Krebs' phosphate and Tris buffer, [^{3}H]-prazosin bound to two sites, a small population of high affinity sites with properties of α_1-adrenoceptors and a much larger population of low affinity sites with different characteristics. High affinity [^{3}H]-prazosin binding was insensitive to Na^+, K^+, Ca^{2+} and Mg^{2+} ions, but low affinity [^{3}H]-prazosin binding was markedly increased in Krebs' phosphate or sodium phosphate buffer and further enhanced in membranes pretreated with EGTA. Binding was decreased in the presence of Ca^{2+}, the decrease in binding mainly being due to a decrease in the number of low affinity sites labelled by the ligand. Low affinity [^{3}H]-prazosin binding was increased at 37° and relatively insensitive to α-adrenoceptor antagonists which were weak competitors while catecholamines failed to compete for low affinity binding. Scatchard plots of [^{3}H]-prazosin binding performed using (−)-noradrenaline (1 mM) to define non-specific binding defined binding only to α_1-adrenoceptors. This provides a means of differentiating high and low affinity [^{3}H]-prazosin binding.

There is now general acceptance of the concept that α-adrenoceptors exist as two subtypes designated α_1 and α_2 and that this nomenclature does not define their anatomical location, but rather their pharmacological specificity in response to drugs [1]. This concept has been strengthened by the development of drugs with selective actions at the receptor subtypes. The α_1-adrenoceptor antagonist prazosin has been shown in pharmacological [2] and radioligand studies [3–5] to display high selectivity for α_1-adrenoceptors. The available of high specific activity [^{3}H]-prazosin has led to its use as a molecular probe to label α_1-adrenoceptors in brain [4, 6, 7], lung [8] and kidney [9–11]. In a recent study of α_1-adrenoceptors in kidney studied by both receptor binding and biochemical techniques, the pharmacological properties and distribution of receptors indicated that high affinity [^{3}H]-prazosin binding is to α_1-adrenoceptors controlling gluconeogenesis [12]. It was also evident from these studies that when binding was examined under physiological conditions (37° and Krebs phosphate buffer), two sites could be differentiated—a small population of high affinity sites with characteristics of α_1-adrenoceptors and a much larger population of low affinity sites. In the study presented here the characteristics and in particular the ion sensitivity of these two binding sites have been further examined.

MATERIALS AND METHODS

[^{3}H]-Prazosin binding to membranes prepared from rat renal cortex was assessed in Krebs phosphate buffer (NaCl, 119; KCl, 4.8; $MgSO_4$, 1.2; NaH_2PO_4, 10; $CaCl_2$, 1.27 mM; pH 7.4). Membranes were prepared as previously described [12].

Aliquots (0.25 ml) of membrane suspension (containing 5 mg tissue wet weight of tissue) were incubated with 1.75 ml of Krebs phosphate buffer containing increasing amounts of [^{3}H]-prazosin (final concentration 0.02–5 nM). All determinations were performed in duplicate. Incubations were carried out for 30 min at 37°. For comparison, identical experiments were performed using Tris buffer (50 mM, pH 7.6) with incubations being carried out at both 25° and 37°. Phentolamine (10 μM) was used to define non-specific binding at each concentration of [^{3}H]-prazosin.

Characterization of [^{3}H]*-prazosin binding to low affinity binding sites.* LIGAND [13] analysis of saturation data indicated that [^{3}H]-prazosin was binding to two sites, one high affinity and another of relatively low affinity. In preliminary competition studies it was found that phentolamine (10 μM) was not adequately defining non-specific binding at higher concentrations of [^{3}H]-prazosin. In addition the full characterization of the low affinity site necessitated using much higher concentrations of prazosin than could practically be achieved using labelled ligand. Because of this, separate saturation experiments were performed to characterize the low affinity binding site. One ml aliquots of the final membrane suspension (20 mg of tissue wet weight) were incubated with an equal volume of Krebs' phosphate buffer with [^{3}H]-prazosin (final concentration approx. 2 nM) and increasing amounts of unlabelled prazosin (final concentrations 1–250 nM). Non-specific binding was defined using phentolamine (1 mM).

† To whom correspondence should be addressed.

Competition for low affinity [^{3}H]*-prazosin binding.* Competition studies characterizing high affinity [^{3}H]-prazosin binding have previously been described [12]. Competition studies characterizing low affinity [^{3}H]-prazosin binding were performed in Krebs' phosphate buffer at 37°. [^{3}H]-Prazosin (1.5 nM) was added to 13.5 nM unlabelled prazosin to give a final concentration of 15 nM of [^{3}H]-prazosin (with reduced specific activity) to predominantly label the low affinity site.

One ml aliquots of membrane suspension were incubated with an equal volume of Krebs' phosphate buffer containing 15 nM [^{3}H]-prazosin in the presence of increasing amounts of unlabelled drug. Non-specific binding was defined using phentolamine (1 mM).

The effect of ions on high and low affinity [^{3}H]*-prazosin binding.* The influence of ions other than Ca^{2+} on both high and low affinity [^{3}H]-prazosin binding was assessed using sodium phosphate buffer (10 mM, pH 7.4) containing EGTA (0.1 mM). The effect of Ca^{2+} was studied in membranes prepared in calcium-free Krebs' phosphate buffer with EGTA (0.1 mM).

Na^+ (10–200 mM), K^+ (0.3–10 mM), Mg^{2+} (0.3–3 mM) and Ca^{2+} (0.1–1.27 mM) were added to duplicate tubes containing 1 ml of the final membrane suspension and 7 ml of phosphate EGTA (or Krebs' phosphate EGTA for Ca^{2+}) buffer containing [^{3}H]-prazosin, 0.1 nM or 15 nM.

In saturation studies investigating the calcium dependence of the low affinity [^{3}H]-prazosin binding site, renal cortical membranes were prepared in the following buffers: (a) Sodium phosphate buffer (10 mM) pH 7.4; (b) Sodium phosphate buffer (10 mM) with EGTA (0.1 mM); (c) Krebs' phosphate, Ca^{2+} free, with EGTA; (d) Krebs' phosphate with EGTA and Ca^{2+} 1.27 mM; and saturation studies performed as described above.

Calculation of results. After incubation, bound and free ligands were separated by filtration through Whatman GF/B filters and washed with 3 × 5 ml aliquots of the appropriate ice cold buffer. Filters were then transferred to scintillation vials and the radioactivity eluted and counted at approximately 55% efficiency by standard liquid scintillation spectrometry. Where appropriate, results were processed by the computer programme "EBDA" [14] which provides preliminary estimates of equilibrium binding parameters by Scatchard, Hill and Hoffstee analysis and then produces a file for use by the non-linear curve fitting programme "LIGAND" [13] which was used to obtain final parameter estimates.

Drugs and chemicals. (−)-α-Methylnoradrenaline, (+)-noradrenaline bitartrate (Sterling-Winthrop), L-phenylephrine hydrochloride (Koch-Light), oxymetazoline hydrochloride (Allen and Hanburys), prazosin hydrochloride (Pfizer), BE2254 (2(β-(4-hydroxyphenyl)ethyl-aminoethyl)-tetralone) (Beiersdorf AG, Hamburg, FRG), indoramin (Wyeth), phentolamine hydrochloride (Ciba, Basel, Switzerland), [^{3}H]-prazosin (20.2 Ci/mmol—Radiochemical Centre, Amersham). All other chemicals were of analytical grade.

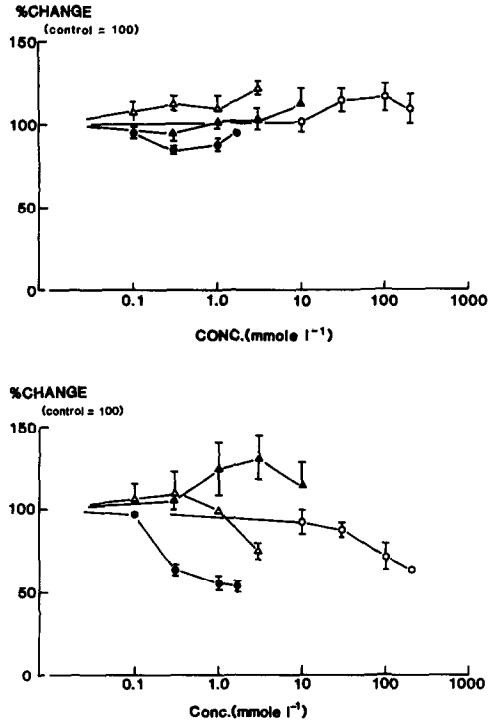

Fig. 1. The effect of mono and divalent cations on specific [^{3}H]-prazosin binding to high affinity (labelled with 0.1 nM ligand) (top) and low affinity sites (labelled with 15 nM ligand) (bottom). Non-specific binding was determined using phentolamine 10 μM. The percentage change in specific binding is shown on the ordinate and the concentration of cation on the abscissa (log scale). Cations tested were calcium (●), sodium (○), magnesium (△) and potassium (▲). Each point is the mean of 3 separate experiments conducted in duplicate ± S.E.M.

RESULTS

Saturation studies

When membranes are prepared and incubated in Krebs' phosphate buffer, [^{3}H]-prazosin binds to both high and low affinity sites in membranes from rat kidney cortex [12] but only the high affinity site has characteristics of α_1-adrenoceptors. The low affinity site can be characterized using a preparation of [^{3}H]-prazosin of low specific activity which allowed much higher ligand concentrations to be reached. The contribution of the high affinity component to binding at low ligand concentration was compensated for by fixing predetermined values for K_D (0.062 nM) and B_{max} (6.2 pmol/g) in "LIGAND". Under these conditions, numerous low affinity sites were observed with a K_D of 173 ± 21 nM and a B_{max} of 382 ± 54 pmol/g (N = 3). Experiments performed with membranes prepared in Tris and incubated at 37° also indicated low affinity sites with a K_D of 102 ± 17 nM but of lower density (B_{max}, 88 ± 35 pmol/g). Numerous low affinity sites are therefore seen in Krebs phosphate at 37°, fewer in Tris at 37° and these sites are virtually absent in Tris at 25° [12].

The effect of cations on high and low affinity [^{3}H]-*prazosin binding*

High and low affinity prazosin binding sites were preferentially labelled with either 0.1 nM or 15 nM of [^{3}H]-prazosin, and the effects of mono and divalent cations tested.

The effect of each concentration of cation is expressed as a percentage of the amount of [^{3}H]-prazosin specifically bound in the absence of ions. The high affinity site was relatively insensitive to cations when compared to the control. Ca^{2+} had a tendency to decrease while Mg^{2+} increased binding when compared to control (Fig. 1). The low affinity site, however, was sensitive to Ca^{2+} which significantly ($N = 3$, $P < 0.05$) suppressed binding by approximately 50%, at near physiological concentrations (0.3–1.7 mM). K^+ facilitated binding, although this result was not significantly different from that of control. Both Mg^{2+} and Na^+ had little effect at lower concentrations but significantly depressed [^{3}H]-prazosin binding at higher concentrations ($N = 3$, $P < 0.05$) (Fig. 1).

The effect of calcium ions on the saturation kinetics of [^{3}H]-*prazosin binding*

In order to obtain a clearer picture of the effect of Ca^{2+}, experiments were conducted with membranes prepared and incubated in sodium phosphate buffer (10 mM, pH 7.4). In the absence of EGTA, [^{3}H]-prazosin binding differed from that observed in Krebs' phosphate. The K_D for the low affinity site was 51 ± 17 nM, some three times higher than that seen in Krebs' phosphate ($P < 0.05$; $N = 3$) whereas the B_{max} was reduced by 50% to 194 ± 38 pmol/g wet weight ($P < 0.01$; $N = 3$) (Table 1). In phosphate buffer with EGTA (0.1 mM) the K_D was unchanged (45 ± 8 nM) whereas the B_{max} was increased to 317 ± 71 pmol/g wet weight of tissue ($P < 0.05$; $N = 3$). Removal of Ca^{2+} therefore significantly increased the maximum number of binding sites without altering K_D.

In Krebs phosphate without Ca^{2+} but with EGTA, the mean K_D was 145 ± 28 nM and the B_{max} was 745 ± 130 pmol/g wet weight tissue. With calcium re-added, the K_D was 114 ± 16 nM, (NS) but the B_{max} was reduced to 392 ± 52 pmol/g wet weight tissue ($P < 0.01$; $N = 4$) (Table 1). Thus in both Krebs' phosphate and phosphate buffer, the removal of calcium significantly increases the maximum number of binding sites but has little effect on the apparent affinity of binding.

The was, however, a marked difference between [^{3}H]-prazosin binding in Krebs' phosphate with EGTA and phosphate with EGTA. The maximum number of binding sites in phosphate is approximately half that seen in Krebs' (317 pmol/g wet weight cf. 745 pmol/g wet weight; $P < 0.05$; $N = 3$) and the affinity of the receptor for [^{3}H]-prazosin had tripled (45 nM cf. 145 nM; $P < 0.05$; $N = 3$). It is possible therefore that the presence of other ions in Krebs' phosphate influences [^{3}H]-prazosin binding under these conditions.

Competition for [^{3}H]-*prazosin binding to low affinity sites*

It has been shown previously that agonists and antagonists having a high affinity for α_1-adrenoceptors compete for high affinity [^{3}H]-prazosin binding to rat renal cortex membranes [12]. In the study reported here low affinity sites were preferentially labelled (approx. 84%) by 15 nM [^{3}H]-prazosin and all of the compounds tested were much less effective competitors.

As shown in Fig. 2, prazosin, BE2254, oxymetazoline and phentolamine competed for [^{3}H]-prazosin binding in a concentration dependent manner. The IC_{50} values (nM) for the compounds tested against [^{3}H]-prazosin binding determined by probit analysis were, prazosin 32, BE2254 300, oxymetazoline 3400, indoramin 13,500 and phentolamine 15,000. Competition curves were shallow and relatively high concentrations of antagonists were needed to inhibit [^{3}H]-prazosin binding.

None of the full agonists previously tested in this system [12], (−)-noradrenaline, (−)-adrenaline, (−)-phenylephrine or (−)-α-methylnoradrenaline (up to 1 mM) had any significant effect on low affinity [^{3}H]-prazosin binding. This was confirmed for (−)-noradrenaline in saturation studies. When (−)-nor-

Table 1. Effects of incubation and buffer conditions on the characteristics of low affinity [^{3}H]-prazosin binding in membranes prepared from rat renal cortex

Buffer	N	K_D (nM)	B_{max} (pmol/g wet weight tissue)
Tris control	3	102 ± 17	88 ± 33
Phosphate	3	51 ± 17*	194 ± 38*
Phosphate + EGTA	3	45 ± 8*	317 ± 71*
KP control	3	173 ± 21	382 ± 54
KP + EGTA	3	145 ± 28	745 ± 130*
KP + EGTA + Ca^{2+} 1.27 mM	3	114 ± 16*	392 ± 52

The apparent dissociation constant (K_D) and maximum density of binding sites (B_{max}) are given as the mean ± S.E.M. Asterisks (*) indicate significant differences compared to control. KP = Krebs' phosphate buffer.

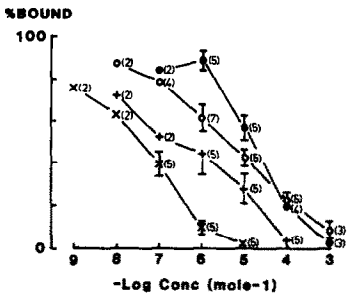

Fig. 2. Competition curves for α-adrenoceptor antagonists against [^{3}H]-prazosin binding to low affinity sites labelled with 15 nM ligand in rat renal cortical membranes. Incubations were performed at 37° in Krebs' phosphate buffer. Binding is expressed as a percentage of that in the absence of competitors and plotted on the ordinate and −log drug concentration on the abscissa. Drugs shown are prazosin (×), BE2254 (+), oxymetazoline (○), and phentolamine (●). Each point is the mean of several determinations (n in parentheses) conducted in duplicate ± S.E.M.

adrenaline (1 mM) was used to define non-specific binding, linear Scatchard plots were obtained and the data were best described by a single site model. The binding parameters, K_D were respectively, 0.169 ± 0.06 nM and 0.171 ± 0.003 nM (NS) and B_{max} 8.75 ± 0.9 pmol/g and 9.13 ± 3 pmol/g (NS). The ability of agonists and antagonists known to act on other receptor systems was also tested on low affinity [^{3}H]-prazosin binding. The β-adrenoceptor antagonists propranolol, H35/25, and metoprolol, the calcium antagonist D600, the nicotinic and muscarinic cholinoceptor antagonists d(+)-tubocurarine, atropine, and pempidine, the histamine H_2 receptor antagonist cimetidine, the MAOI inhibitor pargyline, and the morphine antagonist nalorphine all produced greater than 50% inhibition of specific (defined by phentolamine 1 mM) low affinity binding at 1 mM. Only ouabain, theophylline and the catecholamines produced less than this effect at similar concentrations.

DISCUSSION

In Krebs' phosphate buffer, the binding isotherm obtained for [^{3}H]-prazosin binding to rat renal cortical membranes could not be described by a single site model. Non-linear curve fitting by LIGAND indicated that [^{3}H]-prazosin was binding to two sites. Binding to the high affinity site was saturable and of high affinity (K_D = 0.06 nM). Conversely, the second site had an extremely low affinity for [^{3}H]-prazosin, so low in fact (K_D = 173 nM) that both the high and low affinity sites could not be easily characterized in the same saturation experiments [12].

We have previously shown that [^{3}H]-prazosin binding to the high affinity site has appropriate characteristics for binding to α_1-adrenoceptors [12]. These characteristics were not shown by the low affinity site, since [^{3}H]-prazosin binding could not be displaced by catecholamines at a concentration of 1 mM. Scatchard plots of saturable binding of [^{3}H]-prazosin in rat kidney membranes using (−)-noradrenaline to define non-specific binding revealed only a single high affinity process. The binding site defined by (−)-noradrenaline was identical in nature to the high affinity portion of the [^{3}H]-prazosin binding isotherm observed using phentolamine (1 mM) to define non-specific binding. (−)-Noradrenaline can therefore be used to define non-specific binding in experiments designed to examine α_1-adrenoceptors and to differentiate between α_1-adrenoceptors and the low affinity site labelled by [^{3}H]-prazosin. α-Adrenoceptor antagonists were able to displace [^{3}H]-prazosin binding from the low affinity site, but only at high concentrations. This finding, together with the low K_D for [^{3}H]-prazosin and the extremely high density of binding sites, suggests that the low affinity binding sites are not α-adrenoceptors. In addition the observation that non-adrenoceptor agents displace binding at high concentrations also supports this conclusion. However, it should be stated that some of these compounds (e.g. β-adrenoceptor antagonists and atropine) do possess α-adrenoceptor blocking actions at high concentrations [15, 16].

One possibility to be considered is that during the preparation of membranes by homogenization in isotonic buffers, vesicles are formed which then take up [^{3}H]-prazosin. However, dissociation experiments (results not shown) indicated that [^{3}H]-prazosin dissociated from the low affinity site quite rapidly (<2 sec) suggesting that this is not the case. In addition, other rat tissues have been screened under identical conditions to kidney to see whether two sites can be identified. In cerebral cortex, the Scatchard plot was linear, indicating binding to a single site. These findings support the idea that vesicle formation and subsequent ligand uptake is not a contributing factor to the biphasic Scatchard plots obtained with [^{3}H]-prazosin binding to rat renal cortical membranes. Studies using cerebral cortex also indicate that the biphasic Scatchard plots observed for [^{3}H]-prazosin binding to renal cortex were not a consequence of technical considerations, such as binding of the ligand to glass fibre filters.

The use of high concentrations of phentolamine (1 mM) to define non-specific binding may have had unforeseen, membrane perturbating effects. This phenomenon has been well documented for anaesthetics and tranquilizers [17]. However, it is apparent in the present study that the low affinity site did not result from the use of a high concentration of phentolamine used to define non-specific binding since two sites were also seen with a low concentration (10 μM) of phentolamine.

Since the low affinity binding site for [^{3}H]-prazosin has to date only been found in the kidney, one possibility is that it represents an excretory or reabsorptive site associated with renal handling of weak acids and bases, including many drugs [18, 19]. Prazosin at physiological pH would exist as a cation by virtue of its amino substituent. The binding site could therefore represent a site at which cationic molecules are bound prior to trans-tubular transport. It would be of interest to examine other radioactively-labelled cations or anions to determine whether they, too, identify the same high capacity site.

All other studies characterizing [^{3}H]-prazosin

binding have used Tris buffer with incubations performed at 25°. Our studies indicate that the ionic composition of the incubation media can have important effects on binding characteristics in some tissues. The low affinity site revealed in the present study is both ion- and temperature-sensitive since when incubations were performed at 25° in Tris buffer, no low affinity site could be detected. These studies indicate that when comparisons are made between drug affinities determined by receptor binding techniques or by examination of an end organ response it is important to examine binding in an ionic environment (i.e. in physiological buffers), irrespective of the nature of the ligand, since other potentially important ligand binding sites may be left undetected.

The high affinity binding of [^{3}H]-prazosin to α_1-adrenoceptors in rat renal cortical membranes was insensitive to Ca^{2+} ions. This was shown in experiments in which the saturation of [^{3}H]-prazosin binding was examined in Krebs' phosphate or Tris buffers at 37°. The characteristics of the high affinity site were similar irrespective of the incubation medium. In addition the high affinity site was selectively labelled by low concentrations of [^{3}H]-prazosin, and under these conditions Na^+, K^+, Ca^{2+} and Mg^{2+} had virtually no effect on binding. These findings agree with previous reports which show that antagonist binding to α_1-adrenoceptors is insensitive to cations [4, 11, 20, 21]. The low affinity site however, displayed a marked ion-dependence in particular to Ca^{2+}, which regulated the density of binding sites in a negative fashion. Removal of Ca^{2+} by EGTA unmasked more sites in both phosphate and Krebs' phosphate buffer.

In conclusion, the results of this study suggests that when studied in rat renal cortical membranes prepared and incubated in Krebs' phosphate or sodium phosphate buffers, [^{3}H]-prazosin binds to two sites, a high affinity site having properties of an α-adrenoceptor and analogous to the site observed in Tris buffer at 25° and a low affinity site which is both Ca^{2+} and temperature sensitive and can be differentiated using (−)-noradrenaline to define non-specific binding.

REFERENCES

1. B. B. Hoffman and R. J. Lefkowitz, *Ann. Rev. Pharmac.* **20**, 581 (1980).
2. D. Cambridge, M. J. Davey and R. Massingham, *Br. J. Pharmac.* **59**, 514P (1977).
3. D. C. U'Prichard, M. E. Charness, D. Robertson and S. H. Snyder, *Eur. J. Pharmac.* **50**, 87 (1978).
4. R. Hornung, P. Presek and H. Glossman, *Naunyn Schmiedeberg's Archs Pharmac.* **308**, 223 (1979).
5. P. Miach, J. P. Dausse, A. Cardot and P. Meyer, *Naunyn Schmeideberg's Archs Pharmac.* **312**, 23 (1980).
6. P. Greengrass and R. Bremner, *Eur. J. Pharmac.* **55**, 323 (1979).
7. R. J. Summers, B. Jarrott and W. J. Louis, *Neurosci. Letts.* **20**, 347 (1980).
8. P. J. Barnes, J. S. Karliner, C. Hamilton and C. T. Dollery, *Life Sci.* **25**, 1207 (1979).
9. G. A. McPherson and R. J. Summers, *J. Pharm. Pharmac.* **33**, 189 (1981).
10. J. M. Schmitz, R. M. Graham, A. Sagalowski and W. A. Pettinger, *J. Pharmac. exp. Ther.* **219**, 400 (1981).
11. M. D. Snavely and P. A. Insel, *Molec. Pharmac.* **22**, 532 (1982).
12. G. A. McPherson and R. J. Summers, *Br. J. Pharmac.* **77**, 177 (1982).
13. P. J. Munson and D. Rodbard, *Analyt. Biochem.* **107**, 220 (1980).
14. G. A. McPherson, *Comp. Prog. Biomed.* **17**, 107 (1983).
15. V. N. Heinz and F. Hofferber, *Arzneim-Forsch/Drug Res.* **30**, 2135 (1980).
16. O. D. Gulati, H. M. Parikh and P. R. Raghunati, *Eur. J. Pharmac.* **22**, 196 (1973).
17. S. Abraham, E. H. Cantor and S. Spector, *Life Sci.* **28**, 315 (1981).
18. P. Seeman, *Pharmac. Rev.* **24**, 583 (1972).
19. E. J. Cafuny, *Am. J. Med.* **62**, 490 (1977).
20. B. R. Rennick, *Ann. Rev. Pharmac.* **12**, 141 (1972).
21. D. A. Greenberg, D. C. U'Prichard, P. Sheehan and S. H. Snyder, *Brain Res.* **140**, 378 (1978).