Biochemical Pharmacology, Vol. 33, No. 5, pp. 751–756, 1984.
Printed in Great Britain.

0006-2952/84 $3.00 + 0.00

THE BINDING OF [^{3}H]PRAZOSIN AND [^{3}H]CLONIDINE TO RAT JEJUNAL EPITHELIAL CELL MEMBRANES

DAVID J. COTTERELL, KENNETH A. MUNDAY and JUDITH A. POAT
Department of Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, U.K.

(*Received* 8 *July* 1983; *accepted* 26 *September* 1983)

Abstract—The binding of [^{3}H]prazosin and [^{3}H]clonidine to rat jejunal epithelial cell membranes has been studied. The membrane preparation was enriched in baso-lateral components as determined by Na^+, K^+ ATPase and alkaline phosphatase activities. The membranes possessed two saturable specific binding sites for [^{3}H]prazosin, a high affinity (K_d 0.17 nM) low capacity (B_{max} 27.3 fmole bound per mg protein) and a low affinity (K_d 5.0 nM) high capacity (B_{max} 276 fmole bound per mg protein) site. The specificity of both sites was similar and was related to α_1-adrenoceptors. [^{3}H]Clonidine bound to the membranes in a saturable fashion (K_d 7.3 nM). The specificity of this site was related to α_2-adrenoceptors. The [^{3}H]clonidine binding site was present in the membranes in much lower density (B_{max} 22.8 fmole bound per mg protein) suggesting that α_1-adrenoceptors predominate in this tissue.

There has been an increasing amount of information suggesting that noradrenaline and adrenaline have several effects on intestinal transport. *In vivo* infusion of noradrenaline stimulates net absorption of fluid from closed rat jejunal sacs [1], an effect which is inhibited by α-adrenoceptor antagonists such as phentolamine but not by β-adrenoceptor antagonists. In rabbit ileal mucosa *in vitro* adrenaline and noradrenaline decrease electrical potential difference and short circuit current [2], these effects being due to an increase in the net absorption of Na^+ and Cl^- by an electrically neutral process and net secretion of HCO_3^- being abolished. Again these effects were inhibited by α- but not β-adrenoceptor antagonists [2]. Thus there is general agreement that catecholamines act as antisecretory agents by an adrenoceptor-linked mechanism.

α-Adrenoceptors have been characterized into two groups designated α_1- and α_2 [3, 4]. α_1-Adrenoceptors are postsynaptically located whilst α_2-adrenoceptors are found both pre- and postsynaptically. Recently Chang *et al.* [5] have determined that *in vitro* in rabbit ileum the ion changes produced by adrenaline can be inhibited by yohimbine but not by prazosin, and are mimicked by clonidine. These results suggested that the transport responses to noradrenaline are via α_2-adrenoceptor stimulation. In this study they found no evidence for an α_1-adrenoceptor role in intestinal function. In contrast to this observation net fluid absorption by *in vitro* sacs of rat jejunum *was* stimulated by noradrenaline, an effect which was blocked by α_1-adrenoceptor antagonists [6]. The present study attempts to identify the intestinal adrenoceptor population by direct ligand binding techniques.

MATERIALS AND METHODS

Membrane preparation

Male Wistar rats (200–250 g) were killed by cervical dislocation and a 16–20 cm length of jejunum, distal to the ligament of Trietz, removed. The jejunum was rinsed thoroughly with ice-cold 0.154 M NaCl and filled with a citrate solution containing KCl (1.5 mM), NaCl (96 mM), sodium citrate (27 mM), KH_2PO_4 (8 mM), Na_2HPO_4 (5.6 mM), pH 7.3, and incubated for 15 min at 37°. The citrate solution was discarded and the jejunal sac refilled with a 250 mM sucrose solution containing 10 mM triethanolamine–HCl and 1 mM PMSF, pH 7.6. The sac was gently manipulated to release epithelial cells into the lumen. The cells were collected by centrifugation (300 *g* × 10 min) and washed twice with cold sucrose solution. The final pellet was resuspended in fresh buffer and homogenized in a Polytron PT-10 homogenizer. The 'crude' plasma membrane fraction was prepared from this homogenate by the methods of Murer *et al.* [7] and Scalera *et al.* [8]. The homogenate was centrifuged at 3000 *g* for 15 min and the supernatant centrifuged at 26,000 *g* for 20 min. The resulting dark pellet and supernatant were discarded and the 'fluffy layer' formed above the pellet was collected.

This method essentially prepares functional vesicles used for studies of ion transport. In these experiments the vesicles were lysed by rehomogenization in 10 mM triethanolamine–HCl and 5 mM Tris–HCl. The final pellet was resuspended in the lysis buffer at a concentration of 2–3 mg protein per ml.

Binding assay

Membranes (200 μl containing 300–700 μg protein) were incubated in 800 μl 10mM triethanolamine–HCl containing 50 μl [^{3}H]prazosin (1–2 nM; sp. act. 23 Ci/mole) or [^{3}H]clonidine (8–10 nM; sp. act. 20 Ci/mole) and the volume made up to 2 ml with 50 mM Tris–HCl, pH 7.6. Specific binding was assayed in duplicate tubes by the addition of 1 μM prazosin or 10 μM phentolamine for prazosin binding, and 10 μM clonidine for clonidine binding. Drugs when used were added in 50 μl Tris–HCl buffer, pH 7.6. After incubation at 25° for 16 min binding was stopped by placing the tubes on ice for 1 min and rapidly filtering through Whatman glass fibre (GF/B) filters. The filters were washed twice

with 5 ml Tris–HCl buffer and placed in 1 ml 1% sodium dodecyl sulphate for 12 hr. Tritoscint (8 ml) was added and the tubes were assayed by liquid scintillation spectrometry in an Intertechnique SL40 counter, being corrected for background, quenching and machine efficiency. The results were expressed as fmole bound per mg protein after subtraction of filter blanks, which consisted of identical incubations in the absence of membranes.

Enzyme assays

Alkaline phosphatase. Alkaline phosphatase activity was assessed by the measurement of phosphate released from *p*-nitrophenylphosphate [9]. The validity of the assay was established using alkaline phosphatase (bovine intestine, Sigma) and care was taken to ensure that samples from the cell fractionation procedure were always on the linear portion of the curve.

Sodium–potassium ATPase. Na/K ATPase was estimated by the measurement of phosphate released from ATP. The activity of the Na/K ATPase was taken as that inhibited by the addition of 2 mM ouabain to duplicate assay tubes [10]. Care was taken to ensure that the enzyme activity in the cell samples was on the linear portion of the curve.

Protein was estimated using Coomassie brilliant blue dye with bovine serum albumin as standard [11].

Analysis of data

The number of specific binding sites (B_{max}) and the dissociation constant (K_d) were determined using computer-assisted curve-fitting and line-fitting analysis. Hill plots were used to assess the homogeneity or heterogeneity of binding sites; results were fitted by a least-squares fit to a logistic equation. Where Hill plots were significantly less than one, a two-site analysis was employed.

RESULTS

Purity of the preparations

Ouabain-sensitive Na/K ATPase and alkaline phosphatase were assayed in fractions taken throughout the preparation of the membranes. Table 1 shows the results obtained. In the final membrane preparation there was a 3-fold increase in the activity of the Na/K ATPase and a 30% reduction in the activity of the alkaline phosphatase. Subsequently, [^{3}H]prazosin binding (6.5 nM) was measured in the same fractions and as is shown in the table, the specific binding was greatest in the final membrane preparation. The results suggest that the fraction contained an enrichment of baso-lateral membranes, and was used for subsequent binding studies.

Binding studies

Crude intestinal baso-lateral membranes bound both [^{3}H]prazosin and [^{3}H]clonidine in a specific saturable fashion.

[^{3}H]Prazosin binding

Specific [^{3}H]prazosin binding was saturable in the concentration range 0–10 nM (Fig. 1) while the non-specific binding was linear over this concentration range. Computer-fit saturation analysis of this data gave an equilibrium dissociation constant of 5.01 ± 0.77 nM and a maximal binding capacity (B_{max}) of 276 ± 28 fmole bound per mg protein. The K_d was also assessed by the determination of the rate of association (k_1) and dissociation (k_{-1}) in separate experiments (see Fig. 2). From these separate estimations, the K_d was determined as 1.2 nM, which is in reasonable agreement with the value obtained by saturation analysis. The specificity of this saturable [^{3}H]prazosin binding component was tested by determining the ability of a range of adrenoceptor agonists and antagonists to compete for the binding site. The results, expressed as IC_{50} values, are shown in Table 2. The table shows that the binding site appears to have the characteristics of an α-adrenoceptor site in that (−)-noradrenaline and adrenaline were both active in displacing specific binding while (+)-noradrenaline, atenalol, GABA, acetylcholine and serotonin were all inactive in competing for the binding. The table also demonstrates that antagonists were more active in displacing specific [^{3}H]prazosin binding, the most potent antagonists being those directed at the α_1-adrenoceptor subclass. Thus prazosin, indoramine and WB4101 were all more

Table 1. The assessment of the purity of rat jejunal baso-lateral membranes

		Na^+,K^+ ATPase	Alkaline phosphatase	Specific [^{3}H]prazosin binding
Crude homogenate		100	100	100
↓				
Pellet 1				27.6
Supernatant 1				287.7
↓	Fraction 2	97.8	98	
Pellet 2				23.9
Supernatant 2				23.3
Fluffy layer				
↓				
Crude baso-lateral membranes		324	63	310.6

The results are expressed as percentage activity recorded in the crude homogenate (Na^+,K^+ ATPase 0.287 μmole phosphate produced /min per mg; alkaline phosphatase 2.56 μmole *p*-nitrophenol produced/min per mg) and are mean values of 3–6 determinations in 1–2 experiments.

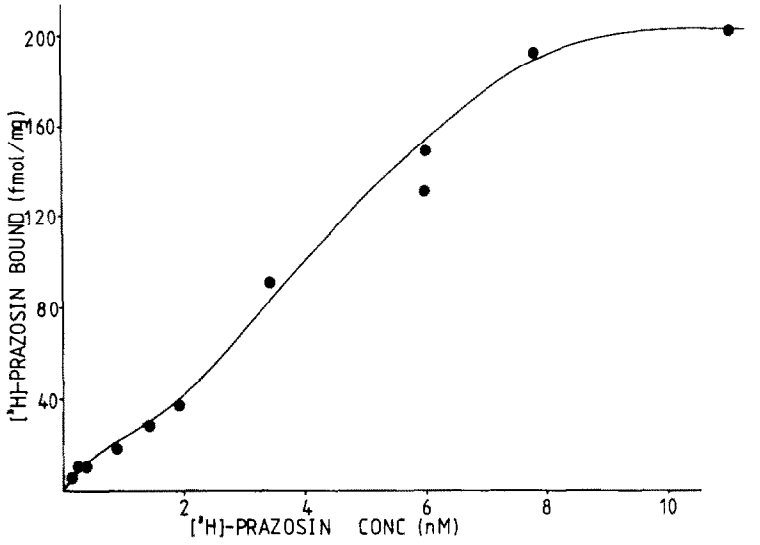

Fig. 1. Specific [3H]prazosin binding to rat jejunal baso-lateral membranes. Membranes (300–700 μg) were incubated for 16 min at 25°. Specific binding was assessed by the addition of 1 μM prazosin to half of the tubes. The points are the means of triplicate determinations from two experiments.

active than yohimbine and rawolscine, prazosin being 300 times more active than yohimbine.

Recently [3H]prazosin has been used to label α_1-adrenoceptor sites in other transporting epithelial tissues, namely the kidney cortex [12]. McPherson and Summers obtained a site of higher affinity than that reported in this study (K_d 0.1 nM). In view of this, a similar site was looked for in baso-lateral-enriched jejunal membranes. In these experiments the protease inhibitor PMSF (phenylmethylsulphonylfluoride) was incorporated into the buffer and 10 μM phentolamine was used to displace specific binding. Under these conditions a binding site with higher affinity was obtained. Figure 3 shows the saturation data obtained in the [3H]prazosin range 0–0.5 nM. Computer-fit saturation analysis gave a value of 0.17 ± 0.04 nM for the K_d and a B_{max} of 27.3 ± 4.2 fmole bound per mg protein. The ability of a selection of the drugs tested on the lower affinity site to compete for the binding was examined (Table 3). The results demonstrate that the rank order of potency was similar to that described for the low affinity site; again the α_1-adrenoceptor antagonist prazosin was 300 times more potent than the α_2-adrenoceptor antagonist yohimbine. Of the drugs tested indoramine and noradrenaline were more potent in displacing specific [3H]prazosin binding from this site than from the site previously described; the other drugs tested had similar IC_{50} values. The potency of prazosin and phentolamine at the site was compared when maximum specific binding was defined with 1 μM prazosin or 10 μM phentolamine. The displacement curves for the two drugs were not similar as shown in Fig. 4, the Hill coefficients of 1.6 for phentolamine and 0.91 for prazosin (phentolamine as displacer of maximum specific binding) emphasizing this difference.

[3H]*Clonidine binding*

Rat jejunal membranes enriched in baso-lateral membranes bound [3H]clonidine in a specific saturable fashion. Saturation analysis gave a K_d of 7.3 ± 2.1 nM and a maximum binding capacity of 22.8 ± 10.6 fmole bound per mg protein (from three experiments). Kinetic analysis from the rate of association and dissociation gave values of K_{obs} 0.331 min^{-1}, k_2 0.237 min^{-1} and k_1 1.67 × 10^7 M resulting in an equilibrium dissociation constant of 14.2 nM, which is in agreement with the results described from saturation analysis. The specificity of the site as determined by the ability of drugs to compete for the specific [3H]clonidine binding was clonidine (IC_{50} 11.2 nM), phentolamine (63 nM), (−)-noradrenaline (127 nM), yohimbine (351 nM), prazosin (3903 nM), phenylephrine (5620 nM), atenolol > 10 μM serotonin, histamine, dopamine and (+)-noradrenaline inactive at concentrations up to 100 μM. Thus this site seems to be related to α_2-adrenoceptors, shows stereospecificity, and agonists displace more potently than antagonists. At this site α_2-adrenoceptor-directed drugs are amongst the most potent, clonidine and yohimbine being more potent than prazosin and phenylephrine. Yohimbine was 11 times more potent than prazosin in displacing the specific [3H]clonidine binding in contrast to the previously described sites.

DISCUSSION

The results demonstrate that rat jejunal epithelial cell membranes enriched in baso-lateral membranes contain specific saturable binding sites for both [3H]-prazosin and [3H]clonidine. The requirements for these specific binding sites as assessed by a range of drugs suggest that they have similar characteristics to α-adrenoceptors. On both binding sites (−)-noradrenaline was much more active in displacing specific binding than (+)-noradrenaline showing stereospecificity of the binding sites [13].

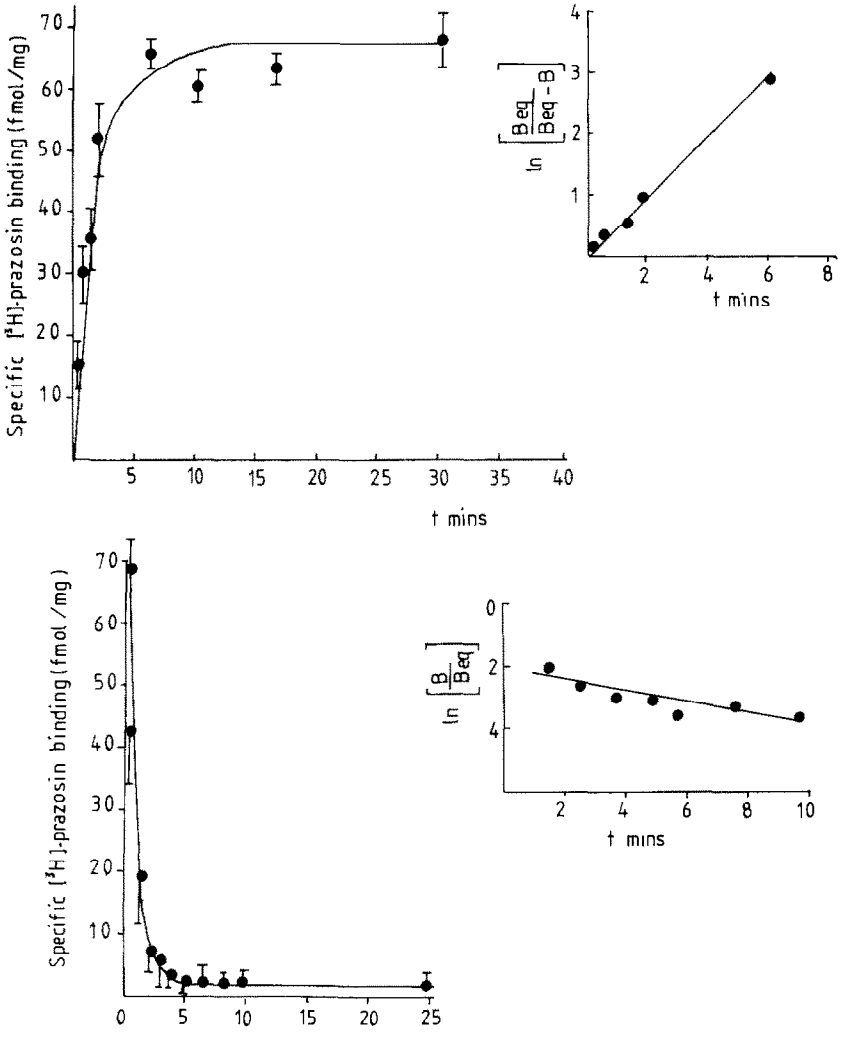

Fig. 2. (a) Determination of the rate of association of [^{3}H]prazosin binding to crude rat jejunal baso-lateral membranes. The rate of association was determined with membranes (300–600 μg) + 4.5 nM [^{3}H]prazosin. Prazosin (1 μM) was added to half of the tubes to define specific binding. Determinations are the mean ± S.E.M. of four determinations. The inset shows the pseudo-first order rate kinetics obtained from association data. From these, K_{obs} was determined as 0.65 min^{-1}. (b). Rate of dissociation of [^{3}H]prazosin from crude baso-lateral membranes. Membranes (300–600 μg) were incubated with 4.5 nM[^{3}H]prazosin for 16 min at 25°. At time zero, 1 μM prazosin was added and the rate of dissociation assessed. Determinations are the mean ± S.E.M. of four determinations. The inset shows the first order kinetic plot of these data; the dissociation constant k_2 was 0.143 min^{-1}.

Using [^{3}H]-prazosin as an α_1-adrenoceptor-directed ligand, two binding sites were identified, one with a K_d of 0.17 nM and a B_{max} of 27 fmole per mg protein and the second with a K_d of 5 nM and a maximal binding capacity of 276 fmole per mg protein. A similar situation has been described with membranes prepared from rat kidney cortex [12] where two sites labelled by [^{3}H]prazosin, K_d 0.15 and 6 nM, were identified. The order of potency of adrenoceptor drugs at both of these sites was essentially the same with the exception that indoramine and noradrenaline had increased potencies for the highest affinity site. The potency ratio of prazosin and yohimbine, which are generally accepted as α_1- and α_2-adrenoceptor antagonists, respectively, remained the same. It is interesting to note that the α_1-agonists phehylephrine and methoxamine were only very weakly active in displacing specific [^{3}H]prazosin binding at either of the sites whereas the α_2-adrenoceptor agonist clonidine was surprisingly active. Again this is similar to results obtained in renal cortex membranes with [^{3}H]prazosin binding, where clonidine was 20 times more active than methoxamine in displacing the binding [12]. This low potency of phenylephrine and methoxamine is interesting in that in a study of transport effects of rabbit ileum the inability of phenylephrine to mimic the noradrenaline effect was interpreted as evidence against an α_1-adrenoceptor involvement. However, this lack of effect may be due to the low efficacy of this drug in transporting epithelia [5].

The Hill coefficients for various drugs on the [^{3}H] prazosin binding sites were very variable and showed no consistent pattern with the agonists and antagon-

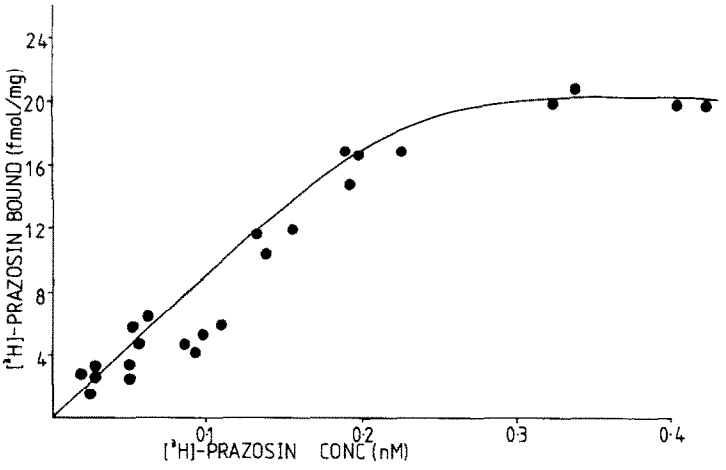

Fig. 3. Specific binding of [3H]prazosin to baso-lateral membranes from rat jejunum. Membranes (200–300 μg) were incubated for 30 min at 25°. Specific binding was assessed by the addition of 10 μM phentolamine to half of the tubes. PMSF was added to the citrate incubation to enhance the integrity of the membranes. The points are means of triplicate determinations of four experiments.

ists. This is very similar to the situation in rat renal membranes [12], with [3H]clonidine binding in the ileum [14] and [3H] clonidine and [3H]prazosin in rat brain [15]. Whether this effect is indicative of co-operativity at the sites or that the ligands are binding to more than one site is unclear.

Again the physiological relevance of the two binding sites for [3H]prazosin is at present difficult to interpret. This could represent two distinct α-adrenoceptor populations or be manifestations of the same site. There remains the possibility that the two sites are a result of the assay systems used in the same way as [3H]clonidine binding is altered depending on whether the assay is in HEPES or Tris–HCl buffer.

Table 2. Effect of drugs on low affinity [3H]prazosin (K_d 5.01 nM, B_{max} 276.6 fmole/mg) binding

	IC_{50} (nM)
Prazosin	14.74
Indoramine	50.68
Phentolamine	1034.00
WB4101	3083.00
(−)-Noradrenaline	20,660.00
Yohimbine	24,940.00
Clonidine	23,271.00
Rauwolscine	36,270.00
Adrenaline	99,180.00
Phenylephrine	496.31 μM
Methoxamine	936.29 μM

Imprimine and histamine were active at 50 μM, serotonin and dopamine at 100 μM. Acetylcholine GABA, (+)-noradrenaline and atenalol were inactive at concentrations less than 100 μM.

Membranes were incubated with 1.5–2.5 nM [3H]prazosin for 17 min at 25° in the absence and presence of varying concentrations of the drugs tested; IC_{50} values are described as the concentration of drug displacing 50% of the maximal displaceable binding as defined by 1 μM prazosin. Each drug was tested at 7–8 concentrations in triplicate and the best line-fit determined by the Keen plot first produced for radioimmunoassay fitted by a least-squares fit to a logistic equation.

In this case, both sites were similar whether prazosin or phentolamine was used as the displacing agent to define specific binding. However, there were differences in the displacement curves, prazosin generally displacing 15% more binding than phentolamine. In other systems where α-adrenoceptors have been studied there are similar discrepancies, thus in platelets where there are no α_1-adrenoceptor binding sites the binding of [3H]dihydrogocryptine is not totally accounted for by [3H]yohimbine, an α_2-adrenoceptor antagonist, and in the intestine [3H]-yohimbine binding has a large component of binding apparently unrelated to an α_2-adrenoceptor [16].

Rat jejunal baso-lateral enriched membranes also contain specific binding sites for the α_2-adrenoceptor agonist [3H]clonidine. The K_d for this site was 7.4 nM by saturation analysis and 14.2 nM from independent measurements of the association and dissociation constants; the B_{max} was 22.8 fmole bound per mg membrane protein. These values correspond well

Table 3. Effect of drugs on high affinity [3H]prazosin (K_d 0.2 nm, B_{max}27.3 fmole per mg) binding site in rat jejunal epithelial cells

	IC_{50} (nM)
Indoramine	0.9
Prazosin	13.1
(−)-Noradrenaline	537.7
Phentolamine	1422.0
Yohimbine	33,780.0
Clonidine	36,890.0
Phenylephrine	> 1.0 mM

Membranes were incubated with 0.5–0.6 nM [3H]prazosin for 30 min in the presence of 100 μM PMSF and in the absence and presence of varying concentrations of the drugs tested; IC_{50} values are described as the concentration of drug displacing 50% of the maximal displaceable binding as defined by 10 μM phentolamine. Each drug was tested at six concentrations in triplicate in three experiments and the best line-fit determined by the Keen plot fitted by a least-squares fit to a logistic equation.

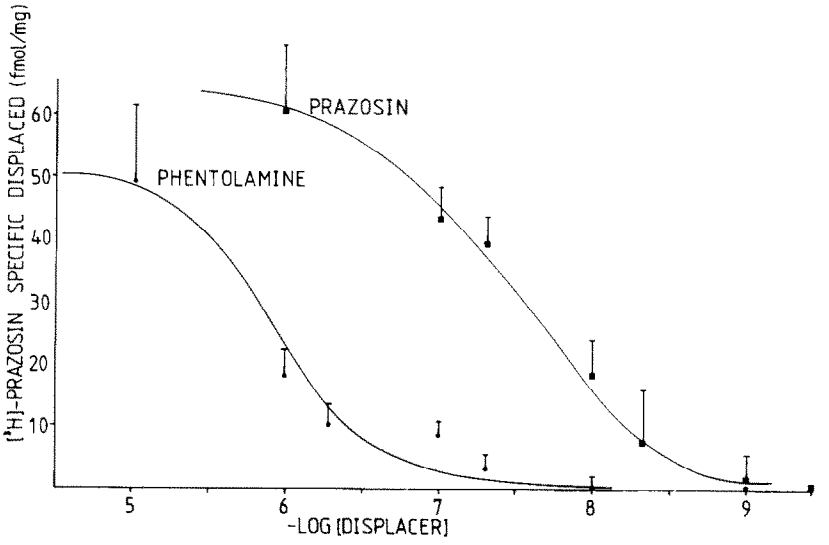

Fig. 4. Displacement of [³H]prazosin binding (1.03 nM) from crude rat jejunal membranes by phentolamine (●) and prazosin (■). The results are the mean ± S.E.M. of triplicate determinations.

with a specific binding site identified in rabbit ileum membranes using *p*-[³H]aminoclonidine. This ligand also displayed specific binding to a site of higher affinity (0.4 nM) which the authors suggested was not of physiological significance. Using [³H]yohimbine as an α_2-adrenoceptor-directed antagonist ligand, two reports have recently identified single specific binding sites in intestinal membranes [16, 17] with K_ds of 6.0 and 28 nM, similar to that reported with [³H]-clonidine in the present study. The specificity of this binding site suggests that it is related to an α_2-adrenoceptor since clonidine was the most potent of the compounds tested in displacing binding, and yohimbine was more potent than prazosin in displacing the specific binding.

The present study has thus identified specific binding sites for both α_1- and α_2-adrenoceptors in basolateral membranes from rat jejunum. There appears to be a greater density of [³H]prazosin binding sites. These data agree well with the ability of α_1-directed adrenoceptor antagonists prazosin, WB4101 and indoramine to inhibit noradrenaline-induced stimulation of net fluid transport in rat jejunal everted sacs [6] compared with yohimbine and rauwolscine, which were very weak antagonists of the response. These observations are somewhat in variance with those of Chang *et al.* [16], who suggested that in rabbit ileum α_2-adrenoceptor antagonists are the most effective in blocking NaCl absorption. This difference may be accounted for by the differences in areas of the intestine and the different species studied. A second possible explanation for the discrepancy is that the study in rabbit intestine was carried out on tissue where secretion was stimulated by administration of theophylline. Thus it is possible to speculate that α_1-adrenoceptors modulate absorptive processes and α_2-adrenoceptors are involved in secretion, the adrenoceptor profile of differing parts of the intestine varying with the role of that particular part of the enterocyte.

In conclusion, this study has demonstrated functional α_1-adrenoceptors on rat jejunal epithelial cells which appear to be involved in the stimulation of net fluid absorption by noradrenaline [5]. These membranes also contained lower numbers of α_2-adrenoceptors which probably have a role in secretion processes, the two intestinal functions being brought about by independent mechanisms.

Acknowledgements—We would like to thank the following companies for gifts of drugs: Pfizer U.K. (prazosin); Wyeth (guanabenz, indoramine); Wellcome U.K. (methoxamine); Boehringer Ingleheim (clonidine); Glaxo/Allenbury (labetalol); Ward Blenkensop (WB4101) and Roth Karlsruhe (rauwolscine).

REFERENCES

1. N. R. Levens, K. A. Munday, B. J. Parsons, J. A. Poat and C. P. Stewart, *J. Physiol.* **286**, 351 (1979).
2. M. Field and I. McColl, *Am. J. Physiol.* **225**, 852 (1973).
3. S. Z. Langer, *Br. J. Pharmac.* **60**, 481 (1977).
4. K. Starke, *Rev. Physiol. biochem. Pharmac.* **77**, 1 (1977).
5. E. B. Chang, M. Field and R. J. Miller, *Am. J. Physiol.* **242**, G237 (1982).
6. D. J. Cotterell, B. J. Parsons, J. A. Poat and P. A. Roberts, *Br. J. Pharmac.* **78**, Proc. Suppl. C112 (1983).
7. H. Murer, U. Hopfer, E. Kinne-Saffran and R. Kinne, *Biochim. biophys. Acta* **345**, 170 (1974).
8. V. Scalera, C. Storelli, C. Storelli-Jass, W. Haase and H. Murer, *Biochem. J.* 177 (1980).
9. J. B. Sumner, *Science* **100**, 413 (1944).
10. L. Jarett and D. W. McKeel, *Archs Biochem. Biophys.* **140**, 362 (1970).
11. M. M. Bradford, *Analyt. Biochem.* **72**, 248 (1976).
12. G. A. McPherson and R. J. Summers, *Br. J. Pharmac.* **77**, 177 (1982).
13. P. N. Patel, D. G. Patel and R. D. Kiell, *J. Pharmac. exp. Ther.* **176**, 622 (1971).
14. T. Tanaka and K. Starke, *Naunyn-Schmiedeberg's Archs Pharmac.* **309**, 207 (1979).
15. H. Glossman and R. Hornung, *Eur. J. Pharmac.* **61**, 407 (1980).
16. E. B. Chang, M. Field and R. J. Miller, *Am. J. Physiol.* **244**, 967 (1983).
17. T. Nakaki, T. Nakadate, S. Yamamoto and R. Kato, *Molec. Pharmac.* **23**, 228 (1982).