Biochemical Pharmacology, Vol. 32, No. 14, pp. 2243–2247, 1983.
Printed in Great Britain.

0006-2952/83 $3.00+0.00

α_2-ADRENERGIC RECEPTORS IN HUMAN POLYMORPHONUCLEAR LEUKOCYTE MEMBRANES*

JEFFREY O. PANOSIAN and GUIDO V. MARINETTI†

Department of Biochemistry, University of Rochester Medical Center, Rochester, NY 14642, U.S.A.

(*Received* 26 *April* 1982; *accepted* 11 *January* 1983)

Abstract—Human polymorphonuclear cell membranes contain α_2-adrenergic receptors which are measured by binding of the α_2-adrenergic antagonist [^{3}H]yohimbine. The α_1-adrenergic antagonist [^{3}H]prazosin showed no specific binding. High and low affinity sites were detected which had K_d values of 2.38 ± 0.4 and 139 ± 12 nM, respectively, and which bound maximally 4.82 ± 0.9 and 81 ± 9 fmoles of [^{3}H]yohimbine/mg membrane protein. The high and low affinity sites were also detected by competition studies with phentolamine, epinephrine and norepinephrine and by dissociation kinetics of bound [^{3}H]yohimbine. [^{3}H]Yohimbine binding was stereospecifically inhibited by (−)- and (+)-epinephrine and norepinephrine. [^{3}H]Yohimbine binding to intact cells showed about 500 high affinity sites per cell (K_d 0.5 nM) and approximately 4000 lower affinity sites per cell (K_d 3–4 nM). Yohimbine enhanced the (−)-norepinephrine stimulation of cAMP production in intact cells.

Polymorphonuclear (PMN) leukocytes play an important role in phagocytosis, inflammation, and acute allergic reactions [1–3]. These responses are dependent in large part on specific receptors on the cell membrane which mediate responses involving chemotaxis, cell adherence, respiratory burst, and release reactions liberating enzymes, prostaglandins, histamine and leukotrienes [1–3]. β-Adrenergic receptors are found in PMN leukocytes [4, 5] where they are coupled to adenylate cyclase and cAMP production. Cyclic AMP influences chemotaxis, phagocytosis and lysosomal enzyme release [6, 7] and also counteracts the inflammatory effects of some prostaglandins and leukotrienes [8]. In some systems, α_2-adrenergic receptors act antagonistically to β-adrenergic receptors [9]. Therefore, it is important to determine whether PMN cells contain α-adrenergic receptors and whether they are of the α_1- or α_2-subtype. A knowledge of the types and levels of α- and β-adrenergic receptors in leukocytes will help in the understanding of the complex biochemical mechanisms which are involved in their biological function.

We have studied the binding of the α_1-antagonist [^{3}H]prazosin and the α_2-antagonist [^{3}H]yohimbine to human PMN cells and cell membranes. Since specific binding was seen only with [^{3}H]yohimbine, the α-adrenergic receptor on PMN membranes is of the α_2-subtype.

METHODS AND MATERIALS

Preparation of PMN cells and membranes

Method A. Starting with 50–75 ml of freshly drawn heparinized human blood, PMN cells were separated from red cells and MN cells by centrifugation through a Ficoll–Hypaque gradient [10]. PMN cells were separated from red cells by dextran sedimentation [10] at 4° and washed once in cold Dulbecco's buffer. The cell pellet was then subjected to one or two hypotonic/hypertonic washes to lyse red cells. Following two more washes in cold Dulbecco's buffer, the cells were washed and resuspended in ice-cold Krebs–Ringer bicarbonate (KRB) buffer, counted, and held at 4°. Calcium was omitted in the KRB buffer to minimize release of lysosomal enzymes.

PMN cells were primarily granulocytes and had 4–6% contamination with MN cells. Red cell contamination in the MN and PMN cell preparations was not significant in that human red cells showed no specific yohimbine binding. PMN cell preparations had less than one to two platelets per PMN cell. Platelets contain 80–100 α_2-adrenergic receptors per cell as determined by yohimbine binding [11] and dihydroergocryptine binding [12]. We also have found 100–115 α_2-adrenergic receptors per platelet using pure preparations of human platelets. The binding of yohimbine to PMN cells was therefore corrected for platelet contamination.

Membranes were prepared by the method of Williams *et al.* [13]. Protein was assayed according to Lowry *et al.* [14].

Method B. Human leukocytes were incubated at 37° for 30 min with iron particles (Technicon lymphocyte separator reagent), and the PMN cells were separated from lymphocytes and platelets by the Ficoll–Hypaque method of Boyum [10] followed by dextran sedimentation at 4°. (The cells were supplied by Dr. George Segel, Department of Pediatrics, University of Rochester.) PMN cells had adhered to the larger iron particles, and a few PMN cells had taken in some small iron particles. The cells ($4–5 \times 10^8$ cells) were suspended in 10 ml of 25 mM Tris buffer pH 7.5, at 0° containing 0.5 mM $MgCl_2$,

* Supported in part by funds from NIH Grants 1P 50A1-15372 and HL 07496 and Biomedical Research Support Grant RR-0-5403.

† Author to whom correspondence should be addressed.

10 mM mannitol, and 5 mM NaCl and were homogenized with ten strokes of a loose pestle and then with eight strokes of a tight pestle using a Dounce homogenizer. The homogenate was transferred to polypropylene test tubes (17 × 100 mm) and spun at 1000 rpm for 10 sec to remove unbroken or aggregated cells. The supernatant fractions were transferred to other test tubes and spun at 2500 rpm for 15 min in a refrigerated International centrifuge to obtain mixed membranes. These membranes contained bound iron and therefore sedimented at this relatively low speed. When cells were used which did not contain bound iron, the membranes sedimented at 30,000 rpm. The membrane pellets were suspended in 8 ml of Tris–mannitol, NaCl, $MgCl_2$ buffer of double strength and vortexed to obtain a suspension of membranes. Aliquots were taken for protein assay and for binding studies. The membranes were frozen rapidly in an ethanol–dry ice bath and stored at −70° until used (within 1–2 weeks).

Ligand binding to cells and membranes

The stored membranes were thawed and homogenized in a Dounce homogenizer using twenty strokes of a loose and ten strokes of a tight pestle. The membranes were adjusted to a concentration of 500 μg protein/0.5 ml. The binding of [^{3}H]yohimbine or [^{3}H]prazosin was carried out using various concentrations of labeled antagonist (1–20 nM) for various time periods (1–30 min) at 37° in a total volume of 1 ml. Specific binding was defined as total binding minus the binding in the presence of 10 μM phentolamine. Binding was determined by rapidly diluting the membranes with 5 ml of ice-cold buffer and immediately filtering the samples on Whatman GF/C glass fiber filters under vacuum. The filters were washed with 10 ml of cold buffer, dried, and placed in glass scintillation vials containing 5 ml of Amersham ACS mixture. PMN cells (2×10^6) (not preloaded with iron) were incubated in polypropylene tubes at 37° for 30 min in 500 μl of KRB buffer, pH 7.4, containing radioactive ligand, and with and without 10 μM phenoxybenzamine. At the end of the incubation, the cells were diluted with 2.0 ml of ice-cold KRB buffer and filtered immediately through glass fiber filters. The incubation tubes were rinsed three times with 2.5-ml portions of ice-cold KRB buffer which were run through the filters. The filters were washed twice with 5-ml aliquots of ice-cold Tris buffer, dried, and counted in 5 ml of Amersham ACS mixture.

Counting was done on a Searle Delta 300 scintillation counter. The counts were corrected for background and converted to dpm. The results are presented as fmoles of ligand bound per mg membrane protein or as sites per cell. Specific binding was 30–40% of the total binding.

cAMP assay

For these studies blood was obtained from donors, and PMN cells were prepared by Method A and used within 1–2 hr. For basal cAMP levels, $1–2 \times 10^6$ cells in 0.4 ml KRB buffer, pH 7.4, containing 5 mM theophylline were placed in polypropylene tubes in a shaking water bath. For hormone-stimulated cAMP levels, another $1–2 \times 10^6$ cells were incubated in 0.4 ml KRB buffer containing 5 mM theophylline and either 10 μM PGE_1 or 100 μM (−)-norepinephrine. Basal, PGE_1- and norepinephrine-treated samples were run with and without 10 μM yohimbine or 10 μM (−)-propranolol. The cells were incubated for 5 min at 37°, then heated at 90° for 5 min, and spun at 1200 *g* for 15 min at 4°. A 0.25-ml sample of the supernatant fractions was added to 0.25 ml of 50 mM acetate buffer, pH 6.2. The samples were acetylated, and cAMP was measured by radioimmunoassay. All samples were analyzed in triplicate.

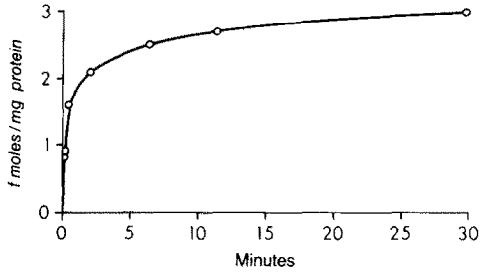

Fig. 1. Time course of binding of [^{3}H]yohimbine to PMN membranes. The specific binding of 5 nM [^{3}H]yohimbine was carried out at 37° for different times as described in the text. Each point is the mean of quadruplicate analyses of one experiment. The percent variability, expressed as S.E.M./mean × 100 for each point, was about 10%.

Materials

Theophylline was obtained from the Sigma Chemical Co., St. Louis, MO; prostaglandin E_1 (PGE_1) was from Upjohn, Kalamazoo, MI; (−)-propranolol from Ayerst Laboratories, New York, NY; phenoxybenzamine from Smith, Kline & French Laboratories, Philadelphia, PA; phentolamine from CIBA Pharmaceutical, Summit, NJ; and ACS mixture from Amersham-Searle, Arlington Heights, IL. The cAMP radioimmunoassay kit was obtained from Collaborative Research Inc., Waltham, MA. Yohimbine[methyl-^{3}H] 84.8 Ci/mmole) and prazosin[furoyl-5-^{3}H] (17 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. (−)-Epinephrine and (−)-norepinephrine were from the Sigma Chemical Co.; (+)-epinephrine and (+)-norepinephrine were from Sterling Winthrop, Rensselaer, NY; and yohimbine hydrochloride was from the Aldrich Chemical Co., Milwaukee, WI.

RESULTS AND DISCUSSION

The time course of binding of 5 nM [^{3}H]yohimbine to PMN membranes at 37° is shown in Fig. 1. Binding reached equilibrium by 30 min. The concentration profile for [^{3}H]yohimbine binding is given in Fig. 2. Two binding sites were observed. Scatchard analysis [15] of three experiments (Fig. 3) gave a K_d of 2.38 ± 0.4 mM for the high affinity site and 139 ± 12 nM for the low affinity site. The high affinity

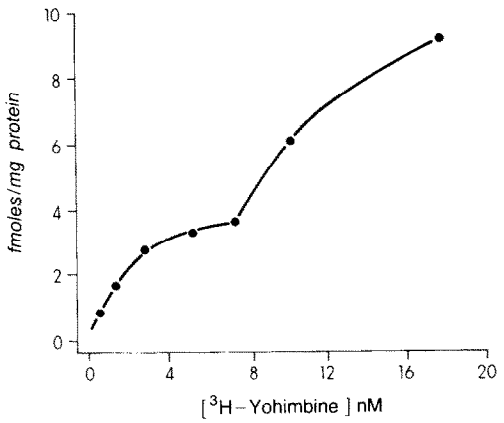

Fig. 2. Concentration profile for binding of [^{3}H]yohimbine to PMN membranes. The specific binding of 1–18 nM [^{3}H]yohimbine was carried out at 37° for 15 min as described in the text. The points represent the mean of quadruplicate analyses of three experiments. The percent variability, expressed as S.E.M./mean × 100 for each point, was about 10%.

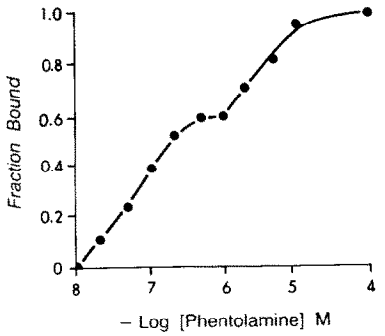

Fig. 4. Inhibition of binding of [^{3}H]yohimbine to PMN membranes by phentolamine. The binding of 5 nM [^{3}H]yohimbine was studied at different concentrations of phentolamine. The fraction bound represented specific binding. Each point is the mean of quadruplicate analyses of one experiment. The percent variability, expressed as S.E.M./mean × 100 for each point, was about 10%.

site had a B_{max} of 4.82 ± 0.9 fmoles/mg protein and the low affinity site had a B_{max} of 81 ± 9 fmoles/mg protein. The K_d values were determined by computer using least squares linear regression analysis.

The inhibition of [^{3}H]yohimbine binding by unlabeled phentolamine is shown in Fig. 4. This inhibition profile also revealed two binding sites. The high affinity site had a K_i of 21.6 nM and the low affinity site had a K_i of 1.4 μM. Similar results were obtained with unlabeled yohimbine. The high affinity site had a K_i of 51 nM and the low affinity site had a K_i of 4.4 μM for inhibition by yohimbine. Thus, the K_i values for unlabeled yohimbine inhibition of [^{3}H]yohimbine binding were higher than the K_d values for the direct binding of [^{3}H]yohimbine. We believe this was due to the difference between unlabeled yohimbine and phenoxybenzamine in their capacities to compete for the binding of [^{3}H]yohimbine.

The binding of [^{3}H]yohimbine was stereospecifically inhibited by norepinephrine (NE) and epinephrine (EPI). Only the profile for NE is shown (Fig. 5). The K_i values were 7.2 μM for (−)-NE and 46 μM for (+)-NE. The K_i values were 2.2 μM for (−)-EPI and 33 μM for (+)-EPI; they were determined by the method of Cheng and Prusoff [16].

The dissociation of bound [^{3}H]yohimbine by phentolamine is shown in Fig. 6. The displacement was biphasic with $T_{1/2}$ values of 11 min and 69 min for the two sites. This supports the finding of two binding sites that was seen in the previous experiments.

The binding of [^{3}H]yohimbine to intact PMN cells (Fig. 7) showed a biphasic profile. Both high affinity sites (approximately 500 sites/cell) with a K_d of about 0.5 nM and low affinity sites (approximately 4000 sites/cell) with a K_d of about 3–4 nM were observed. These studies were carried out on intact PMN cells that had not been preloaded with iron particles and also used phenoxybenzamine rather than phentolam-

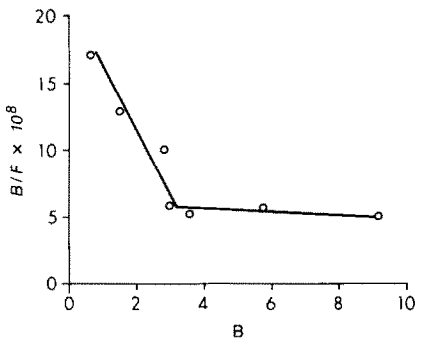

Fig. 3. Scatchard analysis of the binding of [^{3}H]yohimbine to PMN membranes. The binding data in Fig. 2 were analyzed by Scatchard plots [15]. The points represent the mean of three experiments. K_d values and number of binding sites were measured by computer using least squares linear regression analysis.

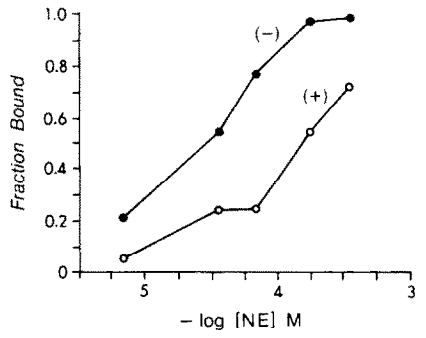

Fig. 5. Stereospecific inhibition of binding of [^{3}H]yohimbine to PMN membranes by norepinephrine. The binding of 5 nM [^{3}H]yohimbine was carried out in the presence of different concentrations of (−)- and (+)-norepinephrine. Each point is the mean of quadruplicate analyses of one experiment. The percent variability, expressed as S.E.M./mean × 100 for each point, was about 10%.

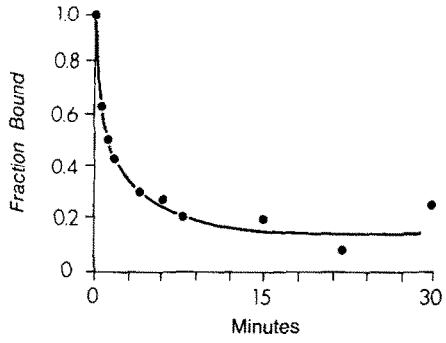

Fig. 6. Dissociation of bound [^{3}H]yohimbine from PMN membranes. Membranes (7.5 mg protein) in 15 ml of Tris–mannitol buffer, pH 7.4, were incubated with 5.5. nM [^{3}H]yohimbine for 15 min at 37°. One-ml aliquots were removed, and the amount of [^{3}H]yohimbine bound at equilibrium was determined. Phentolamine was added to a final concentration of 10 μM, and at various time intervals (45 sec–30 min) one-ml aliquots were removed and the amount of [^{3}H]yohimbine bound was determined. The fraction bound was plotted versus time of dissociation. The fast component had a $T_{1/2}$ of 10.8 min and the slow component had a $T_{1/2}$ of 69 min.

ine to measure specific binding since with intact cells phenoxybenzamine gave higher specific binding than phentolamine. The K_d value for the low affinity sites in cells was close to the K_d value of the higher affinity sites on isolated membranes. It appears that either the treatment of cells with colloidal iron or the process of isolating the membranes induced a loss or alteration of the high affinity sites on intact cells.

To see whether the yohimbine binding sites are representative of α_2-adrenergic receptors, we studied the effect of yohimbine on the norepinephrine and PGE_1 stimulation of cAMP production in intact cells. As seen in Table 1, norepinephrine at a 100 μM concentration increased the level of cAMP, and yohimbine enhanced the norepinephrine effect. The observation is expected if α_2-adrenergic receptors act antagonistically to β_2-adrenergic receptors [9]. Yohimbine had no effect on the PGE_1 stimulation of cAMP production. Propranolol, a β-antagonist, completely blocked the stimulatory effect of norepinephrine. Since PMN cells contain β_2-adrenergic receptors which are linked to adenylate cyclase [4, 5, 17] and we have provided evidence that they also contain α_2-adrenergic receptors, it is appar-

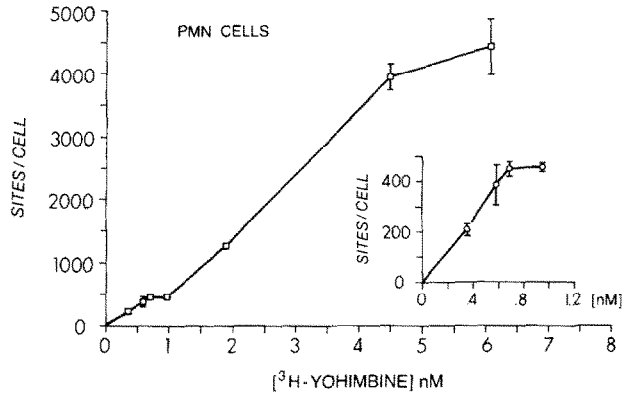

Fig. 7. Concentration profile for the binding of [^{3}H]yohimbine to intact PMN cells. Cells (2×10^6) were incubated in 1 ml of KRB buffer, pH 7.4, for 30 min at 37° with and without 10 μM phenoxybenzamine in order to measure specific binding. The [^{3}H]yohimbine concentration varied from 300 pm to 7 nM. The points represent the mean ± S.E.M. of quadruplicate analyses of one experiment.

Table 1. Effect of yohimbine on norepinephrine and PGE_1 stimulation of cAMP levels in PMN cells*

	cAMP [pmoles · 10^8 cells)$^{-1}$ · min^{-1}]
Basal	6.5 ± 1.3
Plus yohimbine	5.6 ± 1.2
Plus propranolol	3.0 ± 2.2
Plus norepinephrine	12.6 ± 0.1
Plus norepinephrine and yohimbine	19.4 ± 2.3
Plus norepinephrine and propranolol	6.8 ± 1.8
Plus PGE_1	11.0 ± 1.5
Plus PGE_1 and yohimbine	10.5 ± 0.8

* Cells (1×10^6) were incubated with and without 100 μM (−)-norepinephrine or 10 μM PGE_1 in the presence and absence of 10 μM yohimbine or 10 μM (−)-propranolol. cAMP levels were determined as explained in the text. Values are the mean ± S.D. of triplicate analyses of one experiment. The norepinephrine and PGE_1 stimulation of cAMP levels and the yohimbine stimulation of the norepinephrine effect had a P value of <0.05. The yohimbine effect was seen in two separate experiments.

ent that, at the 100 μM level, the norepinephrine interaction with β_2-adrenergic receptors dominates its interaction with α_2-receptors.

Studies with [^{3}H]prazosin demonstrated no specific binding to PMN membranes or cells. Prazosin also was a very weak inhibitor of [^{3}H]yohimbine binding. Thus, our results show that PMN membranes and cells contain high and low affinity binding sites which may represent α_2-adrenergic receptors. Evidence in support of some of these sites being α_2-adrenergic receptors is based on our finding that yohimbine enhanced the norepinephrine-stimulated cAMP production in intact PMN cells. Since yohimbine is an α_2-adrenergic antagonist and since α_2-adrenergic receptors can act antagonistically to β-adrenergic receptors on the adenylate cyclase system, it is expected that yohimbine would enhance the β-adrenergic mediated stimulation of cAMP production. We have yet to determine whether the high or low affinity sites detected by yohimbine binding are correlated with the inhibition of adenylate cyclase.

REFERENCES

1. G. Weissmann, H. M. Korchak, H. D. Perez, J. E. Smolen, I. M. Goldstein and S. T. Hoffstein, in *Advances in Inflammation Research* (Eds. G. Weissmann, R. Paoletti and B. Samuelsson). Raven Press, New York (1979).
2. E. L. Becker, A. S. Simon and K. F. Austen (Eds.), *Biochemistry of the Acute Allergic Reactions*. Alan R. Liss, New York (1981).
3. R. Snyderman and E. J. Goetzl, *Science* **213**, 830 (1981).
4. S. P. Galant and S. J. Allred, *J. Lab. clin. Med.* **96**, 15 (1980).
5. B. H. Dulis and I. B. Wilson, *J. biol. Chem.* **255**, 1043 (1980).
6. G. Weissmann, R. B. Zurier, P. J. Spieler and I. M. Goldstein, *J. exp. Med.* **134**, 149 (1971).
7. J. E. Smolen, J. M. Korchak and G. Weissmann, *J. Cell Biol.* **79**, 208a, (1978).
8. H. E. Claeson, U. Lindberg and C. Malmsten, *Biochem. biophys. Res. Commun.* **99**, 1230 (1981).
9. R. J. Lefkowitz, *Ann. intern. Med.* **91**, 450 (1979).
10. A. Boyum, *Scand J. clin. Lab. Invest.* (Suppl. 97), 77 (1967).
11. M. Daiguji, H. Y. Meltzer and D. C. U'Prichard, *Life Sci.* **28**, 2705 (1981).
12. R. W. Alexander, B. Cooper and R. I. Handin, *J. clin. Invest.* **61**, 1136 (1978).
13. L. T. Williams, R. Snyderman and R. J. Lefkowitz, *J. clin. Invest.* **57**, 149 (1975).
14. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
15. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
16. Y-C. Cheng and W. H. Prusoff, *Biochem. Pharmac.* **22**, 3099 (1973).
17. S. P. Galant and S. Allred, *J. Lab. clin. Med.* **98**, 227 (1981).