Biochemical Pharmacology, Vol. 29, pp. 1361–1365.

0006-2952/80/0515-1361 $02.00/0

STEREOSELECTIVE MUSCARINIC ACETYLCHOLINE AND OPIATE RECEPTORS IN HUMAN PHAGOCYTIC LEUKOCYTES*

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(*Received* 30 *August* 1979; *accepted* 26 *November* 1979)

Abstract—Human granulocytes and monocytes exhibited stereospecific, high-affinity binding to 3-quinuclidinyl benzilate (QNB) and dihydromorphine (DHM), specific ligands for the muscarinic cholinergic and opiate receptors respectively. The stereoselective muscarinic binding occurred in intact, viable cells as well as in cell fragments prepared by sonication. The apparent K_d for the muscarinic binding in granulocytes was 16 nM, and the number of binding sites per cell was on the order of 8×10^4; monocytes contained approximately 3×10^4 binding sites per cell with an apparent K_d of 20 nM. Studies in sonicated and in non-sonicated cell preparations indicate that the affinity of the *l*-isomer is approximately 10 times that of the *d*-isomer. Both cell types also exhibited stereospecific opiate binding with an apparent K_d of 10 nM for granulocytes and 8 nM for monocytes. The number of binding sites per cell was 3000 and 4000 respectively.

Blood leukocytes have been functionally divided into two general categories: the phagocytes and the non-phagocytes concerned with cellular and humoral immunity [1]. The phagocytic leukocytes, the subject of this investigation, can be further subdivided on the basis of nuclear shape into monocytes and granulocytes. The granulocytes, constituting 60–65 per cent of the total leukocyte count, are the most numerous of the white blood cells; the less numerous monocytes make up 3–7 per cent of the total. While the granulocytes are comprised of neutrophils, eosinophils and basophils, the latter two cell types collectively make up less than 6 per cent of the granulocyte population of normal human blood. The present studies, employing highly purified cell preparations, deal primarily with neutrophilic granulocytes and with monocytes.

Until quite recently, studies of membrane receptors in human blood cells were largely directed toward characterization of specific immunological sites and hormone receptor sites. Using techniques developed for work with neural membranes, Abood *et al.* [2] and Aronstam *et al.* [3] demonstrated specific high-affinity binding of opiates and of muscarinic cholinergic agents in human erythrocyte membranes. Previously, opiate receptors had been reported solely in neural membranes [4], while muscarinic receptors had been demonstrated in neural membranes [5], smooth muscle [6], pancreas [7] and heart [8]. Muscarinic binding has since been demonstrated in other types of non-innervated cells, including murine lymphocytes [9] and human neutrophils [10]. Muscarinic binding of monocytes has not been reported previously, nor have there been reports of opiate binding in either granulocytes or monocytes.

Although the *l*-isomer of 3-quinuclidinyl benzilate (QNB) has been shown to have 20 times the affinity of the *d*-isomer for the muscarinic receptors of the central nervous system, stereoselective binding has not been demonstrated previously in peripheral muscarinic systems [11]. Opiate binding, on the other hand, is usually specific for the *l*-isomer.

MATERIALS AND METHODS

The blood used was freshly obtained from healthy donors. Granulocytes, isolated by counterflow centrifugation [12], were centrifuged at 350 *g* for 10 min and resuspended in ice-cold 50 mM Tris buffer, pH 7.5. Monocytes were isolated by counterflow centrifugation in a Beckman JE-6 rotor attached to a Beckman J-21 centrifuge with a modification of the procedure described for granulocytes [12]. Monocytes were harvested after platelet phoresis of human donors with the Haemonetics model 30 cell separator by centrifugation of the platelet bags to obtain residual large cells. Mononuclear cells were isolated from these with Ficoll-Isopaque (Pharmacia Fine Chemicals, Piscataway, NJ) and monocytes were separated from lymphocytes in the Beckman JE-6 rotor (S. M. Hunt, F. J. Lionetti and C. R. Valeri, manuscript in preparation). In some cases, the cells were sonicated with an Ultrasonic micro-tip sonicator probe for 1–2 min at 4° and briefly homogenized prior to use in the binding assays. Plastic ware was used throughout to avoid adhesion of cells to glass surfaces.

Viability of the cells, assessed by the fluorescein diacetate–ethidium bromide (FDA–EB) test described by Lionetti *et al.* [12, 13], was always greater than 98 per cent at the beginning of each experiment. Granulocyte contamination was never greater than 2.5 per cent, erythrocyte contamination

* This research was supported by USPH Grant DA 00464 and a Grant from the Council for Tobacco Research.

5 per cent, and the overall purity of the cells was at least 96 per cent.

Specific binding of *l*-QNB was determined by the filtration assays described by Yamamura and Snyder [6]. A typical incubation medium consisted of 50 mM Tris, pH 7.5, 2 nmoles [^{3}H]*l*-QNB (sp. act. 43 Ci/mmole), and 4×10^6 cells (about 0.5 mg protein) in a volume of 1.0 ml. Unlabeled *l*-QNB was included in some samples to determine specific binding and *d*-QNB in other samples to determine stereospecific binding. Specific binding was the difference in binding in the presence and absence of nonradioactive *l*-QNB. Stereospecific binding was that occurring in the presence of a given concentration of nonradioactive *d*-QNB minus that occurring in the presence of the same concentration of *l*-QNB. The concentrations of nonradioactive ligands in the various experiments are given in the legends to the tables and figures, but the usual concentration was 10^{-5} M. The relative potencies of *d*-QNB and the other muscarinic antagonists, atropine, *N*-methyl-4-piperidylisopentynyl glycolate (MPG) and *N*-methyl-3-piperidyl diphenylisoproprionate (MPP), were determined in a similar manner. An inhibition constant for the binding of oxotremorine to monocytes was obtained by incubating 1.0, 10 or 100 μM oxotremorine with or without 10^{-5} M *l*-QNB in the 1.0 ml volumes containing Tris, 2.5 nM [^{3}H]*l*-QNB and 3×10^6 cells. To correct for [^{3}H]QNB binding to the glass fiber filters, the filtration assay was performed in a similar manner without cells.

All samples were incubated at 0–4° for 40 min, the time needed to reach equilibrium at the lowest concentration of QNB used. They were then rapidly filtered by suction through small glass fiber filters (1.1 cm in diameter) to minimize binding of free muscarinic ligand to the filter. The filters were rinsed twice with 6-ml portions of ice-cold 0.05 M Tris-Cl, pH 7.5, and transferred to Nalgene bags to which 2.0 ml of scintillation fluid were added. Radioactivity was measured after 18 hr in a Delta 300 liquid scintillation counter at a counting efficiency of 35 per cent. The scintillation fluid, consisting of 10 g of 2,5-diphenyloxazole (PPO), 0.5 g of 1,4-bis-[2-(5-phenyloxazolyl)benzene] (POPOP), 2 l. of toluene, and 1 l. of Triton X-100, was purchased from the Eastman Kodak Corp. (Rochester, NY).

Opiate binding was determined in a manner similar to that described elsewhere [2]. Cells suspended in 50 mM Tris, pH 7.5, were pre-incubated for 15 min at 27° with 1.0 μM dextrorphan or levorphanol prior to the addition of 0.1 μCi [^{3}H]dihydromorphine (DHM) with a specific activity of 81 Ci/mmole. After incubation for 15 min longer at 37°, samples were filtered and radioactivity was measured as described above. Each sample contained 3×10^6 cells in a volume of 1.0 ml. As in the muscarinic assays, all work was carried out in plastic tubes, and determinations were done in triplicate. Stereospecific binding is that occurring in the presence of dextrorphan minus that occurring in the presence of levorphanol. Both [^{3}H]QNB and [^{3}H]DHM were obtained from the Amersham/Searle Corp. (Arlington Heights, IL). Levorphanol tartrate and dextrorphan were gifts from Hoffmann-LaRoche (Nutley, NJ). The *l*- and *d*-enantiomers of QNB were provided by Dr. Leo Sternback.

RESULTS AND DISCUSSION

[^{3}H]-*l*-QNB bound specifically to both granulocytes and monocytes. Figure 1 shows a Scatchard analysis of the data for non-sonicated granulocytes. Specific binding is given as the difference in binding of [^{3}H]*l*-QNB in the absence and presence of unlabeled *l*-QNB. Since binding of free QNB to the glass fiber filters represents a signficant source of error at the higher concentrations necessary for these experiments, each point has been corrected for binding to the glass fiber filter.

The general shape of the curve is similar for both cell types. The downward concavity of the Scatchard

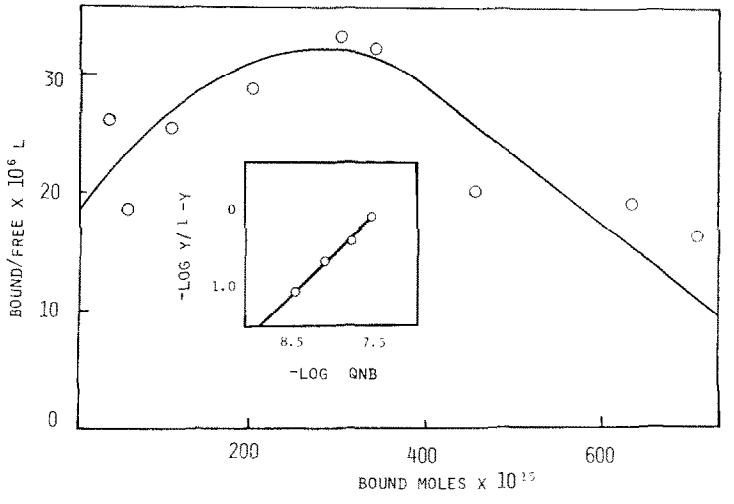

Fig. 1. Scatchard plot of [^{3}H]*l*-QNB binding to intact human granulocytes. [^{3}H]*l*-QNB (1–40 nM) and 5×10^6 cells were incubated with or without 10 μm *l*-QNB. Inset: Hill plot of the data, where *Y* represents fractional occupancy of muscarinic receptors at a given concentration of [^{3}H]*l*-QNB. Data were derived from cells of a single donor and represent a typical experiment. Specific binding (difference in presence and absence of 10^{-5} M nonradioactive *l*-QNB) comprises 70–80 per cent of total binding observed.

plot for granulocytes suggests a positive cooperative interaction of the receptors, manifest at concentrations above 3 nM. A Hill plot, shown in the inset, reveals a slope of 2.28 at concentrations between 3 and 9 nM inclusive. Monocytes exhibit similar binding behaviour with a downward concave trend beginning at concentrations above 2 nM.

The downward concavity might also be attributable to active uptake of the QNB, although the low temperatures at which the experiments were performed would normally inhibit any active uptake systems. Nevertheless, in order to preclude this possibility, the binding of [^{3}H]*l*-QNB in sonicated cell preparations was investigated. While the downward concavity of the plot was diminished in sonicated preparations, the trend never completely disappeared, suggesting than sonication may, in part, disrupt a structural arrangement of the receptors which is necessary for a cooperative binding interaction. Scatchard analysis of the binding of [^{3}H]*l*-QNB to sonicated granulocytes and sonicated monocytes is shown in Figs. 2 and 3 respectively.

Specific binding is reduced by 30–50 per cent in sonicated cells incubated at 0–4°, while the ratio of specific to non-specific binding remains approximately the same as in non-sonicated cells. The decrease may be either due to an alteration in the receptor or the result of a loss of cellular uptake following disruption of the cell. If the latter were correct, then one must conclude that the uptake also requires a specific site and is not energetically driven. The fact that similar results were obtained at 37° with or without sonication (data not presented) is more supportive of the argument that sonication is disrupting the receptor.

On the basis of these studies, the phenomenon of positive cooperative muscarinic binding in granulocytes and monocytes cannot be ruled out. Darfler [14] recently reported a similar type of positive cooperative behaviour in β-adrenergic receptors of both granulocytes and monocytes. For the granulocytes at least, some evidence exists suggesting that

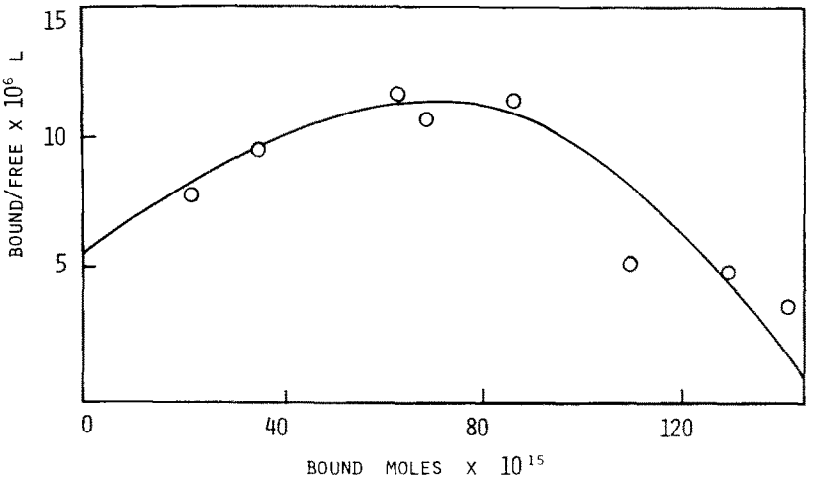

Fig. 2. Scatchard plot of [^{3}H]*l*-QNB binding to sonicated human granulocytes. *l*-QNB (10^{-5} M) was incubated along with [^{3}H]*l*-QNB and 3×10^6 granulocytes for 40 min.

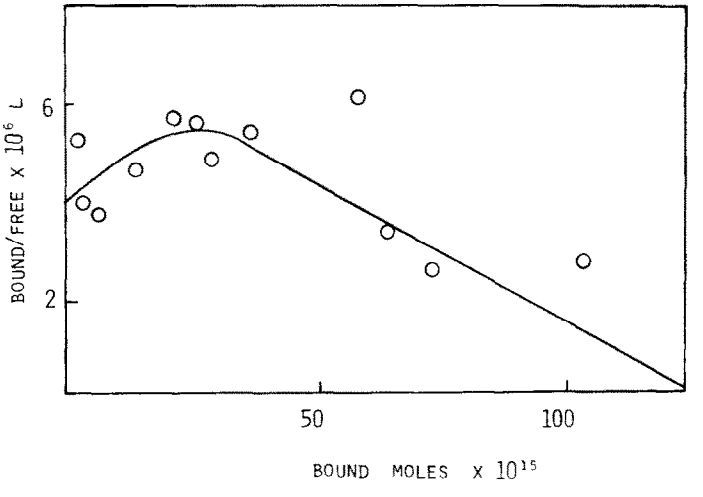

Fig. 3. Scatchard plot of [^{3}H]*l*-QNB binding to sonicated human monocytes. [^{3}H]*l*-QNB (0.5–40 nM) and 4×10^6 cells were incubated with or without 10 μM QNB. Cells were obtained from a single donor and represent a typical experiment.

Table 1. Competition of various antimuscarinic drugs with *l*-QNB binding to granulocytes*

Ligand (10^{-5} M)	% Inhibition of specific [^{3}H]QNB binding	Relative pharmacologic potency	
		Ileum	CNS
Atropine	53	1	0.5
MPP	59	0.01	0.1
MPG	78	0.05	10
d-QNB	84	1	0.5
l-QNB	91	1	50
Oxotremorine†	50		

* The ligand at 10^{-5} was incubated along with 2 nM [^{3}H]*l*-QNB and 3×10^5 granulocytes for 40 min. Data for relative pharmacological potency on acetylcholine induced contractility of rabbit ileum and central nervous system effects were obtained from previous studies [17, 18]. The antispasmodic potency is expressed on the basis of 1 for atropine. CNS potency is based on a battery of behavioral tests in rodents and cats, taking *l*-QNB as arbitrarily 50. Oxotremorine binding was measured in the absence and presence of 10^{-5} M nonradioactive QNB. Abbreviations: MPG = *N*-methyl-4-piperidylisopentynyl glycolate; and MPP = *N*-methyl-3-piperidyl diphenylisoproprionate. The results are an average of two separate experiments agreeing within 7 per cent of the mean.

† $IC_{50} = 0.9 \times 10^{-6}$ M.

the muscarinic cholinergic receptors and β-adrenergic receptors may be functionally linked in a reciprocal fashion. Cholinergic agonists and cGMP have been shown to enhance phagocytosis and lysosomal enzyme release in granulocytes, while epinephrine and cAMP inhibit these functions [10, 15].

The apparent K_d for the binding of [^{3}H]*l*-QNB to intact human granulocytes, calculated from the latter portion of the curve in Fig. 1, is approximately 16 nM, whereas that for the sonicated granulocytes is about 8 nM. The latter K_d is in agreement with that obtained from intact cells prepared by the procedure of Boyum [16] yielding granulocytes which are partially damaged cells, and erythrocyte membranes could account for a lower K_d.

The apparent K_d for the binding of *l*-QNB to monocytes is approximately 20 nM, and the number of binding sites per cell is on the order of 3×10^4. The number of muscarinic binding sites per granulocyte is approximately 8×10^4.

With the use of sonicated granulocytes, five muscarinic antagonists, at a concentration of 10^{-5} M, were compared for their ability to compete with 2.4 nM [^{3}H]*l*-QNB (Table 1). Atropine, which had the lowest binding affinity, was among the most potent in blocking the acetylcholine-induced contraction of rabbit ileum and among the least potent centrally. The assessment of central potency was determined by a composite of behavioral measurements in animals which correlated reasonably well with the psychotomimetic potency [17]. The agent with the highest binding affinity, as well as antispasmodic and central activity, was *l*-QNB. MPG had a relatively high affinity and central activity with comparatively less antispasmodic activity. There was no correlation of binding affinity with antispasmodic activity, although, with the exception of *d*-QNB, there appears to be some relationship between binding affinity and central activity. In the case of rat brain, a good correlation was observed between the relative binding affinity to brain membranes and the pharmacological activity of an extensive series of antimuscarinic agents [11]. At a concentration of 10^{-5} M, oxotremorine, a muscarinic agonist, inhibited QNB binding 50 per cent.

A comparison of the binding affinity of *l*- and *d*-QNB to granulocytes reveals that the *l*-isomer is about 10 times as effective as the *d*-isomer in displacing [^{3}H]*l*-QNB (Fig. 4). The IC_{50} of the *l*-isomer was 8×10^{-8} M as compared to 7×10^{-7} M for the *d*-isomer.

Stereoselective binding in peripheral muscarinic systems has not been demonstrated previously, being absent in erythrocyte membranes [3] and smooth

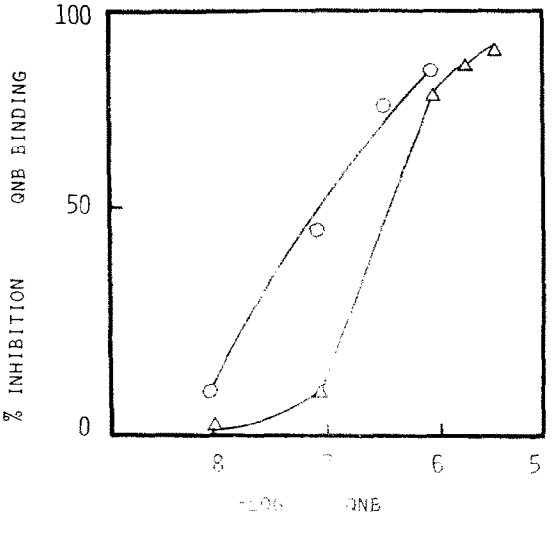

Fig. 4. [^{3}H]*l*-QNB binding to granulocytes as a function of *l*- and *d*-QNB. Two nM [^{3}H]*l*-QNB and 3×10^5 cells were incubated with varying concentrations of either *l*- or *d*-QNB. Key: [illegible] [illegible] QNB.

muscle receptors such as the iris [11]. Muscarinic stereoselectivity appeared to be a phenomenon unique to the central nervous system, where the *l*-isomer had been shown to be 20 times more effective than the *d*-isomer in displacing [^{3}H]*l*-QNB binding [11], and at least 100 times more effective in disturbing operant behaviour in cats [18]. On the other hand, both isomers of QNB are equipotent of smooth muscle cholinergic receptors. The unprecedented appearance of stereoselective binding in a peripheral muscarinic system raises further questions concerning the functional and evolutionary significance of these muscarinic receptors in phagocytic leukocytes. There is, however, a marked difference in K_d, the value for brain receptors being as low as 5×10^{-11} M [19], compared to a value of 8×10^{-9} M for granulocytes. Another difference is that both the granulocytes and monocytes exhibit a marked positive cooperativity, which is entirely absent in the brain receptors. Despite this relatively low-affinity binding of the muscarinic antagonist, QNB, *in vitro* evidence indicates a possible functional role for the muscarinic receptor in granulocytes, as mentioned previously. Both acetylcholine and pilocarpine have been shown to enhance phagocytosis and lysosomal enzyme release in human neutrophils, and several studies have demonstrated blockage or carbachol-potentiated lysosomal enzyme release by muscarinic antagonists [10, 15]. Analogous studies in monocytes have not been reported, but in view of the physiological similarities of the two cell types, a similarity in the receptor-mediated mechanisms is a likely possiblity. The observation of stereoselectivity in both cell receptors would seem to lend further support to the idea that the muscarinic binding sites have some functional capacity; the significance of the stereoselectivity, however, remains unclear.

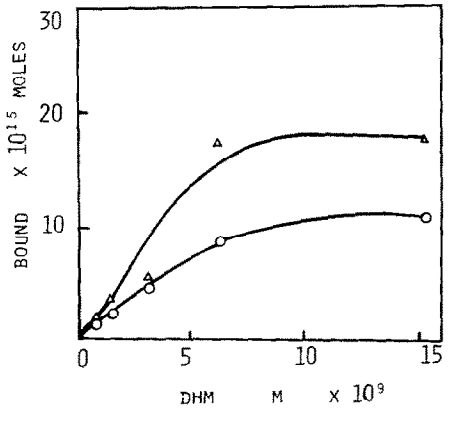

Fig. 5. Binding curve for [^{3}H]DHM to granulocytes and monocytes. Each sample contained 3×10^6 cells and 0.1 μCi [^{3}H]DHM. The data are derived from cells of a single donor and represent a typical experiment. Key: (○) granulocytes; and (△) monocytes.

Stereospecific opiate binding was demonstrable in both granulocytes and monocytes (Fig. 5). A Scatchard plot (not shown) of the data in Fig. 5 yields for granulocytes a K_d of 10 nM and for monocytes, 8 nM. The number of binding sites is approximately 3000 per monocyte and 4000 per granulocyte. The significance of opiate binding sites in leukocytes, as well as erythrocytes [2], is not known. Opioid peptides, such as β-endorphin, are present in plasma and are elevated during stress [20]. It has been reported that the adenylate cyclase activity of neuroblastoma cultured cells [21], as well as brain tissue [22], is inhibited by opiates. It remains to be seen whether endorphins could be another modulator for cAMP-dependent enzymic control in the leukocytes.

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