Immunopharmacology, 16 (1988) 139–149
Elsevier

IMO 00423

Muscarinic cholinergic binding sites on rat lymphocytes*

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(Received 15 April 1988; accepted 29 July 1988)

Abstract: Receptors for neurotransmitters in blood cells could serve as useful markers for the same receptors in solid tissues. Muscarinic receptors have been identified in human, rat and mouse lymphocytes by binding of [^{3}H]quinuclidinyl benzilate (^{3}H-QNB); however, the biochemical and pharmacological characterization of such binding sites has not been complete. Spleen lymphocytes were isolated on a histopaque gradient and incubated in Hank's buffer with ^{3}H-QNB. Binding of ^{3}H-QNB was linear with increasing protein concentrations and was saturable. Binding constants were B_{max} = 111 ± 10.5 fmol/10^6 cells, and K_d = 29.7 ± 3.9 nM (n = 7). An extensive pharmacological analysis of these binding sites indicated that several cholinergic muscarinic drugs were capable of inhibiting ^{3}H-QNB binding. Muscarinic antagonists were more potent than agonists, and lipophilic drugs were more potent than hydrophilic drugs. Several non-cholinergic drugs were also capable of inhibiting ^{3}H-QNB binding; however, they did so also in brain membranes, while a third group of non-cholinergic drugs and neurotransmitters were inactive. Similar results were also obtained in circulating lymphocytes and in lymphocyte membranes. These results suggest that lymphocytes possess muscarinic receptors which share several, although not all, characteristics of the same receptors in brain and other tissues.
Measurement of these binding sites could be useful to monitor the status of muscarinic receptors in solid tissues.

Key words: Muscarinic receptor; Cholinergic system; Lymphocyte

Introduction

Several non-neuronal, non-innervated cells, such as lymphocytes, erythrocytes, platelets and fibroblasts, have been shown to possess receptors for drugs, neurotransmitters and hormones. The presence of these receptors on cells that are easily accessible provides opportunity for ethical investigations, directly in human populations, of their biochemical, pharmacological and functional characteristics, and of their possible modification by genetic, environmental or disease factors. This approach is already being utilized in research on neuropsychiatric diseases (Stahl, 1985), and could also have various applications in occupational and environmental health (Costa, 1987a).

The cholinergic system plays a major role in central and peripheral nervous system functions (Singh et al., 1985), and muscarinic receptors have been identified in the CNS as well as in several peripheral organs (Nathanson, 1987). Alterations of muscarinic receptors have been associated with several diseases and neuropsychiatric disorders (Mash et al., 1985), and with exposure to drugs or toxic chemicals such as metals or pesticides (Nordberg et al., 1980; Costa and Fox, 1983; Costa et al., 1982).

There is repeated evidence that lymphocytes re-

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Abbreviations: ^{3}H-QNB, [^{3}H]quinuclidinyl benzilate; QNX, quinuclidinyl-xanthene-9-carboxylate; GABA, gamma-aminobutyric acid; THIP, tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride; PBS, phosphate-buffered saline; ^{3}H-MS, [^{3}H]methylscopolamine.

* Presented in part at the 38th Annual Meeting of the American Society for Pharmacology and Experimental Therapeutics (Pharmacologist 29, (1987) 181).

spond to cholinergic agonists with increased cyclic-guanosine monophosphate (GMP) levels, enhanced ribonucleic acid and protein synthesis, altered membrane fluidity, and altered immune functions (Strom et al., 1974; Haddock et al., 1975; Masturzo et al., 1985). These findings suggested that functional muscarinic cholinergic receptors are present on lymphocytes. Indeed, by using radioligand binding techniques, several investigations have demonstrated the presence of muscarinic binding sites on splenic or circulating lymphocytes from rats (Maslinski et al., 1983; Strom et al., 1981; Shenkman et al., 1986), mice (Gordon et al., 1978; Atweh et al., 1984; Ado et al., 1986) and humans (Zalcman et al., 1981; Bidart et al., 1983; Adem et al., 1986; Rabey et al., 1986; Bering et al., 1987). Muscarinic receptors have also been labelled with the alkylating agent [^{3}H]propylbenzylcholine mustard in IM-9 lymphocytes (Hootman et al., 1985). Two studies, however, failed to identify muscarinic receptors on murine (Wazer and Rotrosen, 1984) and human (Maloteaux et al., 1982) lymphocytes.

If lymphocytes were to be useful as peripheral markers for the muscarinic system in nervous tissue, the characteristics of muscarinic receptors should be similar, if not identical, to those observed in brain and other solid tissues. One of the most important criteria for identifying and characterizing a binding site as a neurotransmitter receptor is the detailed pharmacology of the binding (Burt, 1985). So far, only a few muscarinic cholinergic drugs have been used to characterize receptors in lymphocytes. One report (Atweh et al., 1984) also describes a muscarinic binding site with mixed muscarinic-nicotinic characteristics. The principal aim of the present study was, therefore, to provide a more comprehensive pharmacological characterization of muscarinic cholinergic binding sites in rat lymphocytes.

Methods

Chemicals

(−)-[^{3}H]Quinuclidinyl benzilate (^{3}H-QNB; 33.1 Ci/mmol) was purchased from New England Nuclear (Boston, MA). SafetySolve was obtained from Research Products International Corporation (Mount Prospect, IL). (−) and (+) QNB, (±) quinuclidinylxanthene-9-carboxylate (QNX), (+)-*cis*-methyldioxolane and naloxone hydrochloride were obtained from Research Biochemicals Incorporation (Natick, MA). Gamma-aminobutyric acid (GABA) was purchased from Calbiochem (La Jolla, CA). The following compounds were generously donated: (+)-nicotine bitartrate (Drs. M. Aceto and E.L. May, Medical College of Virginia, Richmond, VA); mecamylamine hydrochloride and amitriptyline (Merck, Sharp & Dohme, West Point, PA); morphine sulphate (Dr. G. Rosenfeld, Houston, TX); tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol hydrochloride (THIP; Dr. V. Christensen, H. Lundbeck & Co., Copenhagen, Denmark); prazosin hydrochloride (Pfizer Inc., Groton, CT); pirenzepine hydrochloride (Istituto De Angeli, Milano, Italy); diazepam (Hoffman-La Roche Inc., Nutley, NJ). All other compounds were purchased from Sigma Chemical Co. (St. Louis, MO). Scrubbed nylon wool was purchased from Fenwal Laboratories (Morton Grove, IL).

Animals

Male Sprague-Dawley-derived rats (300–400 g; Tyler Laboratories, Bellevue, WA) were used in this study. Animals were killed with carbon dioxide, and their spleens were rapidly removed and placed in 32 ml of Hank's balanced salt solution (without calcium and magnesium). For blood collection rats were anesthetized with ether, and blood (5–7 ml) was drawn by heart puncture into a heparinized syringe and diluted 1:1 (v/v) with Hank's buffer.

Isolation of lymphocytes

The spleen was teased apart with forceps and 8 ml of the cell suspension were layered on 3 ml of Histopaque (specific gravity 1.083) for separation of lymphocytes according to the method of Boyum (1968). After centrifugation at 400 × *g* for 30 min the layer containing lymphocytes was transferred to another plastic centrifuge tube, resuspended in 10 ml of

Hank's buffer, and washed three times by centrifugation (250 × *g* for 10 min) and resuspension in the same buffer. Viability was checked by Trypan blue exclusion and was at least 90%. Cell concentration was usually adjusted to 2×10^6 cells/0.3 ml. Separation of lymphocytes from blood was done similarly, but cells were centrifuged at a lower speed (200 × *g* for 10 min) to remove platelets.

^{3}H-QNB binding in lymphocytes

Binding of ^{3}H-QNB to splenic or circulating lymphocytes was assayed essentially by the method of Yamamura and Snyder (1974). Lymphocytes (2×10^6 cells, equivalent to 70–90 μg of protein) were incubated with ^{3}H-QNB and Hank's buffer (pH 7.5) in a total volume of 0.5 ml in plastic tubes. Following 1 h incubation at 27°C under gentle shaking, the reaction was stopped by addition of 3 ml of ice-cold phosphate buffered saline (PBS). Samples were then filtered through Whatman GF/C filters which were washed three times with 3 ml of ice-cold PBS. Filters were air-dried and counted for radioactivity in 10 ml of SafetySolve in a Packard Tricarb Scintillation Spectrometer at an efficiency of 40%. Atropine (10^{-4} M) was added to half of the tubes for estimation of specific binding, defined as the difference between binding in the absence and in the presence of atropine. Specific binding was approximately 70–85% of total binding. Each sample was assayed in quadruplicate. For saturation binding experiments, increasing concentrations of ^{3}H-QNB (1–60 nM) were used. Values of receptor density (B_{max}) and affinity (defined as the reciprocal of the dissociation constant, K_d) were obtained by linear regression of Scatchard plots (Bennett and Yamamura, 1985) with the aid of the Lundon-I computer program (Lundon Software Inc., Cleveland, OH). In the in vitro inhibition studies, at least six concentrations of the test compound, in quadruplicate, were used for each IC_{50} determination. Values of IC_{50} (concentration of each compound necessary to reduce specific binding by 50%) were estimated by log-probit analysis of the displacement data.

^{3}H-QNB binding in brain membranes

^{3}H-QNB binding in rat brain was performed as previously described (Costa et al., 1982). The forebrain (whole brain minus cerebellum and medulla-pons) was isolated and homogenized in Na^+/K^+-phosphate buffer (0.05 M; pH = 7.4) and centrifuged three times at 49000 × *g* for 10 min. Each time the supernatant was discarded and resuspended in fresh buffer. Binding assays were carried out as described for lymphocytes.

^{3}H-QNB binding to lymphocyte membranes

Lymphocytes were isolated as described, and homogenized with a Polytron homogenizer. After three centrifugations at 49000 × *g* for 10 min, the final pellet was resuspended in Hanks's buffer. An aliquot corresponding to approximately 100 μg of protein was used in the binding assay, which was conducted in Hank's buffer as described for intact lymphocytes and brain tissue.

Separation of B and T lymphocytes

Since different and contrasting results have been found on ^{3}H-QNB binding in T and B lymphocytes (Strom et al., 1981; Atweh et al., 1984) in some experiments we measured muscarinic receptor binding in fractions enriched in T or B cells. These were prepared according to the method of Julius et al. (1973) with slight modifications. Briefly, 0.7 g of nylon wool was placed in a 10 ml syringe and washed 4 times with a total of 40 ml of prewarmed Hank's medium (Hank's buffer containing 5% fetal calf solution, heat-inactivated at 56°C for 30 min). Spleen lymphocytes, isolated on Histopaque as previously described, were then added to the wool. The T-cell fraction was eluted from the column with 30 ml of Hank's medium and collected by centrifugation at 290 × *g* for 10 min. Nylon-wool-adherent B cells were removed by compressing the nylon with the syringe plunger until most of the retained medium was removed. The nylon was then teased with

forceps and after addition of Hank's medium the column was again compressed. This process was repeated until approx. 40 ml of medium had been collected (Handwerger and Schwartz, 1974). Cells were isolated by centrifugation and resuspended in Hank's buffer. Cell concentration was adjusted to 6.7×10^6/ml. Both T- and B-cell-enriched fractions were checked by fluorescence labeling with FITC-goat anti-rat Ig as described by Robles and Pollack (1986). Briefly, single cell suspensions were fixed in 50 μl of 1% paraformaldehyde for 15 min on ice and washed with Hank's buffer containing 10 mM HEPES, 0.2% NaN_3 and 2% fetal calf serum. Five microliters of directly conjugated primary antibody were added to 10^6 cells in the modified Hank's buffer. Following 45 min incubation on ice, cells were washed once with modified Hank's buffer and twice with phosphate-buffered saline and then analysed by using a Leitz fluorescent microscope. B cells were more than 70% in the B cell-enriched fraction and only 8% in the T-cell enriched fraction.

Results

^{3}H-QNB binding to intact lymphocytes

Initial experiments were conducted to assess the linearity and saturability of ^{3}H-QNB binding to rat spleen lymphocytes. Binding increased linearly between 0.5 and 6×10^6 cells (Fig. 1), and 2×10^6 cells (equivalent to approx. 70–90 μg of protein) were normally used in binding assays. Binding of ^{3}H-QNB was saturable; saturation binding data transformed by the method of Scatchard (Fig. 2) indicated a maximal number of binding sites (B_{max}) of 111 ± 10.5 fmol/10^6 cells (2467 ± 234 fmol/mg protein), a dissociation constant, K_d, of 29.7 ± 3.9 nM, and Hill coefficient, n_H, of 0.92 ± 0.02 (values represent the mean ± SEM of 7 determinations). Binding of ^{3}H-QNB to circulating lymphocytes also increased with increasing protein concentration (not shown) and saturation binding parameters were not significantly different from those obtained in splenic lymphocytes: B_{max} was 95 ± 8.9 fmol/10^6 cells, and K_d was 32.7 ± 3.1 nM (mean ± SEM; n = 4).

All experiments described in the present study were performed utilizing a mixed population of lymphocytes. However, since two studies indicated the presence of higher binding in a population en-

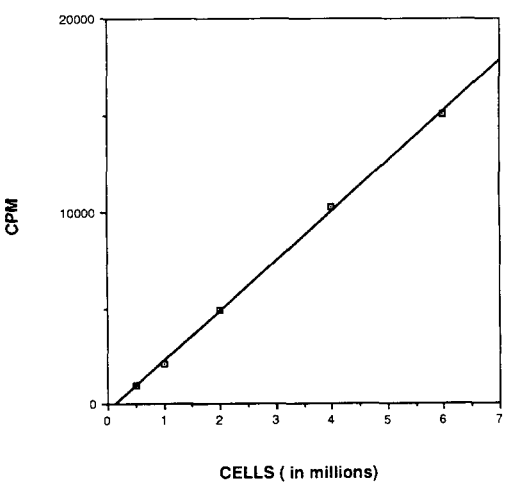

Fig. 1. Effect of increasing cell concentration on ^{3}H-QNB binding to intact splenic lymphocytes. ^{3}H-QNB binding was assayed as described in Methods. Each point represents the mean of two experiments, each done in quadruplicate.

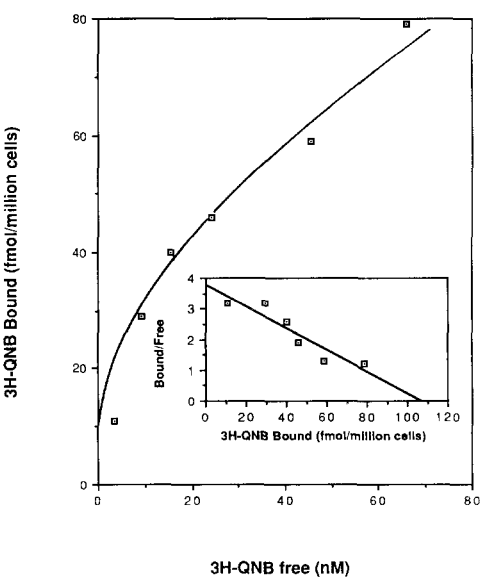

Fig. 2. Saturation curve of ^{3}H-QNB binding to intact splenic lymphocytes. Saturation binding data were transformed as described in Methods to obtain the Scatchard plot shown in the inset. In this particular experiment, maximal number of binding sites, B_{max}, was 106 fmol/10^6 cells and the affinity constant K_d was 27.8 nM.

TABLE I

Inhibition of ^{3}H-QNB binding in rat splenic and circulating lymphocytes by cholinergic muscarinic compounds

	IC_{50} (μM)			
	Splenic lymphocytes		Circulating lymphocytes	
Cholinergic muscarinic antagonists				
Atropine	0.12 ± 0.05	(7)	0.29 ± 0.11	(6)
Scopolamine	6.7 ± 1.4	(3)	4.7 ± 1.98	(3)
Pirenzepine	116.2 ± 49.9	(3)	–	
Methylatropine	215 ± 35	(3)	504 ± 179	(3)
Methylscopolamine	1100 ± 253	(3)	697 ± 241	(3)
QNX	0.15 ± 0.03	(5)	–	
(−)-QNB	0.031 ± 0.011	(5)	–	
(+)-QNB	0.97 ± 0.36	(5)	–	
Cholinergic muscarinic agonists				
Oxotremorine	25.7 ± 5.5	(8)	25.5 ± 8.7	(4)
Carbachol	422 ± 39	(4)	480 ± 76	(3)
Acetylcholine	379 ± 77	(3)	589 ± 131	(3)
cis-Methyldioxolane	> 1000	(2)	–	
Choline	> 1000	(3)	–	

Binding of ^{3}H-QNB in intact lymphocytes was conducted as described in Methods. Concentration of ^{3}H-QNB was 27–30 nM. Values of IC_{50} (concentration of each compound necessary to reduce specific binding by 50%) were estimated by log-probit analysis of the displacement data. At least six concentrations of each compound, in quadruplicate, were used for each IC_{50} determination. Results represent the mean (± SEM) of the number of experiments indicated in parenthesis.

riched in T-cells (Strom et al., 1981) or B-cells (Atweh et al., 1984), we measured ^{3}H-QNB in both T- and B-lymphocytes. Our results agree with those of Atweh et al. (1984) in that we found a higher binding of ^{3}H-QNB (measured at a concentration of 30 nM) in B-lymphocytes (57 ± 8.2 fmol/10^6 cells) than in T-lymphocytes (37 ± 4.4 fmol/10^6 cells).

Pharmacological characterization of ^{3}H-QNB binding

Table I shows the effects of several known muscarinic agonists and antagonists on ^{3}H-QNB binding in rat lymphocytes. The most potent inhibitor of (−)^{3}H-QNB binding was (−)-QNB, with an IC_{50} of 31 nM, similar to the K_d value for the radiolabeled ligand. The binding appeared to be stereospecific, since (+)-QNB was 31-fold less potent than (−)-QNB. QNX and atropine were almost equipotent in inhibiting ^{3}H-QNB binding, while scopolamine, and particularly pirenzepine, were weak inhibitors. Interestingly, the two methyl derivatives of atropine and scopolamine were much less effective than their corresponding tertiary compound. Muscarinic agonists were less potent than muscarinic antagonists. Among the agonists, oxotremorine was the most potent, with an IC_{50} of 26 μM. A number of compounds were also tested in circulating lymphocytes and their potency in inhibiting the binding of ^{3}H-QNB was found to be similar to splenic cells (Table I).

Because of the suggestion by Atweh et al. (1984) of a mixed muscarinic-nicotinic characteristic of ^{3}H-QNB binding site in lymphocytes, we also tested a number of cholinergic nicotinic drugs, as well as several non-cholinergic compounds, for their ability to inhibit ^{3}H-QNB binding to lymphocytes (Table II). Among the cholinergic nicotinic compounds, d-tubocurarine and mecamylamine were capable of inhibiting ^{3}H-QNB binding, with IC_{50} values of 6.3 and 11.0 μM, respectively. Most of the non-cholinergic drugs had no effect, or minimal effect, on

TABLE II

Inhibition of ^{3}H-QNB binding to rat splenic lymphocytes by cholinergic nicotinic compounds and non-cholinergic compounds

	IC_{50} (μM)	
Cholinergic nicotinic compounds		
(−)-Nicotine	503 ± 165	(3)
(+)-Nicotine	> 1000	(3)
Coniine	345	(2)
Hexamethonium	350	(2)
d-Tubocurarine	11.1 ± 2.71	(3)
Decamethonium	> 1000	(2)
Mecamylamine	10.96	(2)
Noncholinergic compounds		
Morphine	18.7 ± 3.1	(3)
Naloxone	640 ± 89	(3)
GABA	> 1000	(2)
THIP	> 1000	(1)
Diazepam	> 1000	(1)
Glutamic acid	> 1000	(1)
Norepinephrine	> 1000	(2)
Yohimbine	2.6 ± 0.8	(3)
Prazosin	4.8 ± 1.6	(3)
Strychnine	40.7 ± 10.0	(3)
Serotonin	> 1000	(1)
Ouabain	> 1000	(2)
Histamine	> 1000	(1)
Diphenylhydantoin	> 1000	(1)
Amitriptyline	0.25 ± 0.07	(3)
Imipramine	4.34 ± 1.03	(3)

See Table I for details.

^{3}H-QNB binding (Table II). However, the opioid morphine, the α_2-adrenergic antagonist yohimbine, the glycine antagonist strychnine and the α_1-adrenergic antagonist prazosin inhibited ^{3}H-QNB binding with IC_{50} values ranging from 2.6 μM to 40.7 μM. In addition, two antidepressant drugs, amitriptyline and imipramine, known to have antimuscarinic properties (Nomura et al., 1987), were rather potent inhibitors of lymphocyte ^{3}H-QNB binding.

In order to determine whether the effects observed with various non-muscarinic drugs were unique to ^{3}H-QNB binding sites in lymphocytes, we examined their ability to interact with the same radioligand in brain membranes. The results of these experiments are summarized in Table III, where the values for lymphocyte binding (derived from Table II) are also listed for comparison. All compounds tested were also capable of inhibiting ^{3}H-QNB in brain tissue, although, with the exception of amitriptyline and imipramine, with somewhat lower potency.

TABLE III

Inhibition of ^{3}H-QNB binding in rat splenic lymphocytes and brain by non-muscarinic compounds

	IC_{50} (M)		Ratio brain lymphocytes
	Lymphocytes	Brain	
(−)-Nicotine	5.0×10^{-4}	2.3×10^{-3}	4.6
d-Tubocurarine	6.4×10^{-6}	2.1×10^{-5}	3.3
Mecamylamine	1.1×10^{-5}	7.2×10^{-4}	65.4
Strychnine	4.1×10^{-5}	1.7×10^{-3}	41.5
Prazosin	4.8×10^{-6}	1.1×10^{-5}	2.3
Yohimbine	2.6×10^{-6}	1.0×10^{-5}	3.8
Amitriptyline	2.5×10^{-7}	1.9×10^{-7}	0.76
Imipramine	4.3×10^{-6}	6.7×10^{-7}	0.16

Values of IC_{50} in intact lymphocytes are derived from Table II. Results represent an average of three determinations. See Table I for details.

TABLE IV

Inhibition of ^{3}H-QNB binding in rat intact lymphocytes, lymphocyte membranes and brain by cholinergic muscarinic compounds

	IC_{50} (M)		
	Intact lymphocytes	Lymphocyte membranes	Brain membranes
Atropine	1.2×10^{-7}	3.4×10^{-7}	1.0×10^{-9}
Scopolamine	6.7×10^{-6}	1.6×10^{-7}	1.2×10^{-9}
Methylatropine	2.1×10^{-4}	1.2×10^{-5}	0.9×10^{-9}
Methylscopolamine	1.1×10^{-3}	7.2×10^{-5}	0.5×10^{-9}
Oxotremorine	2.6×10^{-5}	5.5×10^{-6}	1.9×10^{-6}
Carbachol	4.2×10^{-4}	3.3×10^{-5}	5.4×10^{-5}
Acetylcholine	3.9×10^{-4}	2.0×10^{-4}	1.1×10^{-6}

Values of IC_{50} were obtained as described in Methods. Data for intact lymphocytes are derived from Table I. Results represent the mean of three to eight determinations.

Since a general lower affinity of drugs for muscarinic receptors on lymphocytes had been reported by various investigators, we also compared the potency of muscarinic ligands in lymphocytes and brain tissue. The results, shown in Table IV, indicated that while muscarinic agonists had similar potency in both tissues, albeit slightly lower in lymphocytes, muscarinic antagonists were less effective in lymphocytes by two or more orders of magnitude.

^{3}H-QNB binding to lymphocyte membranes

In some experiments, lymphocytes were lysed and membranes prepared as described in Methods. Binding of ^{3}H-QNB was linear (not shown) and saturable; maximal number of binding sites, B_{max}, was 212.3 ± 28.5 fmol/mg protein and K_d value was 15.1 ± 1.44 nM (mean ± SEM; n = 4). The potency of various muscarinic agonists and antagonists in inhibiting the binding of ^{3}H-QNB to lymphocyte membranes is shown in Table IV. No major differences were found between intact lymphocytes and membrane preparations, with the exception of methylatropine and methylscopolamine, which were 17- and 15-fold more potent, respectively, in inhibiting ^{3}H-QNB binding to membranes (Table IV).

Discussion

The presence of muscarinic binding sites on lymphocytes has been a matter of some controversy. In fact, Maloteaux et al. (1982) observed that the binding of the muscarinic antagonist [^{3}H]dexetimide to human lymphocytes was not saturable and that dexetimide and atropine were 142- and 823-fold less potent, respectively, is displacing [^{3}H]dexetimide from lymphocytes than from brain tissue. Wazer and Rotrosen (1984) found a saturable binding of ^{3}H-QNB to intact murine lymphocytes (K_d = 25 nM) but not on osmotically lysed cells. Both groups of investigators concluded, therefore, that lymphocytes lack defined muscarinic receptors. On the other hand, several other investigators have reported the presence of a saturable ^{3}H-QNB binding in lymphocytes from various species with many of the characteristics of a muscarinic receptor (Gordon et al., 1978; Strom et al., 1981; Zalcman et al., 1981; Bidart et al., 1983; Maslinski et al., 1983; Atweh et al., 1984; Adem et al., 1986a; Shenkman et al., 1986; Bering et al., 1987). Our study confirms and expands these latter findings.

In most studies, values of the affinity constant (K_d) for ^{3}H-QNB were found to range between 1 nM (Gordon et al., 1978) and 67 nM (Zalcman et al., 1981) with a mean of 26.6 ± 5.8 nM. Only one group of investigators found two binding sites for ^{3}H-QNB with much larger K_d values (480 nM and 16 μM; Atweh et al., 1984). In general, there were no significant differences in affinity among species. When binding was measured using lymphocyte membrane preparations slightly lower K_d values were found: 15.1 nM (this study), 15 nM (Adam et al., 1986), 25–40 nM (Bidart et al., 1983). Still, these values are much higher than those reported for ^{3}H-QNB binding in brain, heart or other tissues (less than 0.5 nM). Two exceptions are the rat mast cells and human erythrocyte membranes, where K_d values of 17 nM and 14.8 nM, respectively, have been reported (Masini et al., 1983; Bering and Mueller, 1987). A larger variability has been found with regard to the maximal number of binding sites. For example, B_{max} values vary from 150 receptor/cell (Ado et al., 1986) to 50000 receptors/cell (Zalcman et al., 1981). In the present and in another study (Adem et al., 1986a) direct comparison has been made between intact lymphocytes and membrane preparations. In both studies, the affinity constant, K_d, increased by less than 2-fold, while the B_{max} value decreased by more than 10-fold when binding was assayed in membranes instead of intact cells. This raises the possibility that some of the binding of ^{3}H-QNB observed in intact lymphocytes might be due to trapping of the radioligand within the cell, as recently suggested by Gossuin et al. (1984) in neuroblastoma cells. To circumvent this potential problem, these authors suggested the use of a more hydrophilic ligand such as [^{3}H]methylscopolamine (^{3}H-MS). We have been unable to detect any specific binding of ^{3}H-MS to rat lymphocytes because of

the presence of a specific and saturable binding of ^{3}H-MS to the glass-fiber filters (Costa, 1987b). Recently, however, Bering et al. (1987) have detected binding of ^{3}H-MS to human intact lymphocytes with a B_{max} of 15 fmol/10^6 cells and a K_d of 7 nM. While the absolute number of binding sites is lower than that found in the present study (111 fmol/10^6 cells) or of other studies utilizing ^{3}H-QNB (425–553 fmol/10^6 cells; Shenkman et al., 1986; Rabey et al., 1986), this might be ascribed either to the aforementioned variability among various laboratories, or most probably, to the labeling by ^{3}H-MS of only those muscarinic receptors present on the outer membrane. The more lipophilic ^{3}H-QNB, on the other hand, can also label those receptors which are embedded in the membrane. Indeed, it is interesting to note that even in membrane preparations from rat brain ^{3}H-QNB labels more sites than ^{3}H-MS (Lee and El-Fakahany, 1985).

Previously, only a limited number of muscarinic agonists and antagonists or other compounds had been used to characterize the pharmacological profile of ^{3}H-QNB binding sites in lymphocytes. In the present study, we have found that a large number of muscarinic agonists and antagonists are capable of inhibiting ^{3}H-QNB binding. Muscarinic agonists like carbachol, and particularly oxotremorine, were approximately equipotent in intact lymphocytes, lymphocyte membranes and brain membranes (Tables I and IV). In particular the potency of oxotremorine, which preferentially activates the M_2 subtype of muscarinic receptors, suggests that muscarinic receptors on lymphocytes might be of the M_2 type. Additionally, Bering et al. (1987) found a strong effect of the GTP analogue guanosine 5'-[β,γ-imido]triphosphate on oxotremorine binding, which is seen mainly in tissues rich in M_2 receptors (heart, ileum, brain-stem), and possibly represents a specific response of the M_2 subclass (Vickroy et al., 1983). In addition, stimulation of phosphoinositides turnover, which is thought to be linked primarily to M_1 receptors (Lai et al., 1988), could not be seen in human (Masturzo et al., 1985) or rat (Costa, Kaylor, Murphy, unpublished observation) lymphocytes. However, before any conclusion can be drawn on the particular subtype of muscarinic receptor present on lymphocytes, further studies should be conducted using, for example, recently developed muscarinic antagonists such as AFDX-116. Use of this compound has shown in fact that two subclasses of M_2 muscarinic receptors exist, currently identified as the cardiac and the glandular subtype (Hammer et al., 1986). Further developments in the molecular studies on muscarinic receptors structures will also shed light on the presence of different subtypes in various tissues, including lymphocytes (Peralta et al., 1987).

Muscarinic antagonists were significantly less potent in lymphocytes (either intact cells or membranes) than in brain (Tables I and IV) or in other tissues. Wei and Sulakhe (1982) reported that the affinity of scopolamine and atropine for muscarinic receptors in rat heart membranes was 10-fold lower if the binding assay for ^{3}H-QNB was conducted at 37°C. At this temperature their IC_{50} values were 1.5×10^{-7} M and 5.5×10^{-8} M, respectively, not dissimilar to those found in the present study in lymphocyte membranes (Table IV). However, a lower affinity of muscarinic antagonists in lymphocytes than in brain has been a common finding in all other studies on lymphocyte muscarinic receptors, some of which were conducted at lower incubation temperatures. The reason(s) for this lower affinity of muscarinic antagonists in lymphocytes is not evident; these results are, nevertheless, in agreement with the lower affinity (higher K_d value) of ^{3}H-QNB.

Pirenzepine, an M_1 selective antagonist (Hammer and Giachetti, 1982), was a weak inhibitor of ^{3}H-QNB binding. Together with the evidence discussed above, this is suggestive of the presence of an M_2 subtype of muscarinic receptors on lymphocytes. A surprising finding was that the two quaternary analogues of atropine and scopolamine were very weak inhibitors of ^{3}H-QNB binding. Their potency was somewhat higher in lymphocyte membranes than in intact lymphocytes (Table IV), but still several orders of magnitude lower than in brain tissue. Therefore, in addition to the apparent low affinity of all muscarinic antagonists for the lymphocyte muscarinic receptor, the low potency of the two hydrophilic compounds might be due to the

localization of many such receptors within the lipid bilayer. Brown and Goldstein (1986) suggested that in intact chick heart cells ^{3}H-QNB binds to a subset of receptors which might not participate in physiological responses, but which have all the characteristics of true muscarinic receptors. These low-affinity sites might be a population of receptors sequestered in a hydrophobic compartment within the membrane. Thus, liphophobic compounds, such as methylatropine and methylscopolamine, would have no access to these sites. The different results obtained with ^{3}H-MS and ^{3}H-QNB (see above) could be similarly explained.

One study (Atweh et al., 1984) suggested that QNB binding sites on lymphocytes might be of mixed muscarinic and nicotinic character. This conclusion was based on the finding that d-tubocurarine, a nicotinic blocker, was capable of inhibiting ^{3}H-QNB binding to intact lymphocytes as well as to membranes. We confirmed that d-tubocurarine and also mecamylamine are rather potent inhibitors of lymphocyte ^{3}H-QNB binding (Table II). Both compounds, however, also inhibited binding of ^{3}H-QNB to brain membranes (Table III). Several other nicotinic ligands interacted only weakly with the QNB binding sites. Among the several noncholinergic drugs tested to determine the pharmacological specificity of the sites labelled by ^{3}H-QNB in lymphocytes, most were inactive, or very weak displacers. The two antidepressants amitriptyline and imipramine were rather potent inhibitors, as expected, since both are known to interact with high potency with muscarinic receptors in the nervous system, particularly the M_2 subtype (Nomura et al., 1987). Other compounds such as the α_2- and α_1-adrenoceptor antagonists yohimbine and prazosin were also surprisingly potent in inhibiting ^{3}H-QNB binding in both lymphocytes and brain tissue.

In conclusion, our results indicate the presence of a binding site for ^{3}H-QNB in rat splenic lymphocytes which has been pharmacologically characterized as a muscarinic cholinergic receptor. The only major difference with muscarinic receptors identified in other tissues is a lower affinity for muscarinic antagonists. The presence of a muscarinic receptor on lymphocytes could be exploited for investigating whether alterations of these receptors in other tissues (e.g. brain, heart, lung) is paralleled by a similar alteration in these peripheral cells. Recently, a decrease of ^{3}H-QNB binding in circulating lymphocytes has been found in patients with Alzheimer's disease (Adem et al., 1986b; Rabey et al., 1986). This finding would be in agreement with the decrease in M_2 receptors found in brains from Alzheimer's patients (Mash et al., 1985). Other modifications of muscarinic receptors in solid tissues, due to genetic factors, disease states or exposure to drugs or chemicals, could thus possibly be detected by measuring muscarinic receptors on lymphocytes. In addition, the possible role that lymphocyte muscarinic receptors might have in the immune response and the possible link between the cholinergic nervous and immune systems needs to be explored (Jankovic et al., 1987).

Acknowledgements

This study was supported in part by grant OH-00054 from NIOSH and grants ES-04696 and ES-03424 from NIEHS. We thank Dr. Claire P. Robles for her help in B and T cell separation, and Ms. Lois Harris for secretarial assistance.

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