MOLECULAR PHARMACOLOGY, 15, 28-34

Identification of Muscarinic Binding Sites in Human Neutrophils by Direct Binding

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(Received May 12, 1978)
(Accepted July 28, 1978)

SUMMARY

DULIS, BEVERLY H., GORDON, MICHAEL A., & WILSON, IRWIN B. (1979) Identification of muscarinic binding sites in human neutrophils by direct binding. *Mol. Pharmacol.* 15, 28-34.

Using techniques developed for the study of muscarinic cholinergic receptors in brain homogenate, direct binding to whole live neutrophils has been shown for the potent muscarinic antagonist [^{3}H]quinuclidinyl-benzilate. The specific binding is saturable, proportional to cell concentration, and can be displaced by atropine, oxotremorine, and pilocarpine. The K_d for this system is approximately 8 nM. This whole-cell binding correlates with previously reported effects of cholinergic agents on neutrophil guanosine cyclic 3',5'-monophosphate levels, chemotaxis, and lysosomal enzyme release. Quinuclidinyl-benzilate is shown here to function as a muscarinic antagonist in neutrophils by blocking the carbachol stimulated lysosomal enzyme release *in vitro* from live cells.

INTRODUCTION

Acetylcholine has been shown to mediate cholinergic responses through interaction with cholinergic receptors associated with autonomic ganglia, the spinal cord Renshaw cell, skeletal and smooth muscle, some neurons in the central nervous system, and some secretory cells. Some cholinergic receptors have been described pharmacologically as nicotinic on the basis of their activation by nicotine and blockade by *d*-tubocurarine and hexamethonium. Nicotinic receptors have been described based on these criteria, for example, at mammalian neuromuscular junctions and autonomic ganglia. Some nicotinic sites can be studied by measurement of direct binding of radiolabeled α-bungarotoxin. Other cholinergic receptors have been pharmacologically defined as muscarinic, since they can be activated by muscarine and blocked by atropine (1). Receptors of this type include those of the postganglionic parasympathetic system and vascular smooth muscle. Binding studies of such receptors have utilized high affinity muscarinic agents such as radiolabeled quinuclidinylbenzilate.

The specific, high-affinity binding of the potent muscarinic antagonist QNB[2] has been demonstrated in various innervated systems such as the particulate fractions of brain (2), intestine (3), and heart (4, 5). Analogous binding has been shown in non-innervated cells, specifically erythrocytes (6) and lymphocytes (7). QNB binding in the latter case correlates with demon-

This work was supported by Grants NS07156 and CA21950 from the National Institutes of Health.

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[2] The abbreviations used are: QNB, quinuclidinyl-benzilate; cGMP, guanosine cyclic 3',5'-monophosphate; LDH, lactate dehydrogenase.

0026-895X/79/010028-07$02.00/0

strated responses of lymphocytes to cholinergic agonists, as in augmentation of the cytotoxic response (8) and elevation of cGMP levels (9).

Neutrophils have also been shown to respond to cholinergic agents. Neutrophils constitute roughly 70% of the circulating leukocyte population, and respond to various soluble and particulate factors by chemotaxis and phagocytosis. They can be transformed into secretory cells by exposure to noningestible immune complexes such as an antigen-antibody complex on cartilage, or by pretreatment with inhibitors of microfilament function (the cytochalasins) before incubation with chemotactic or immunological agents (10). Cyclic GMP can mediate this specific release of lysosomal enzymes, which is distinguished from cell lysis by the observation that very little of the cytoplasmic enzyme lactate dehydrogenase is released. Concentrations of the cholinergic agonist carbachol as low as 0.1 nM are capable of more than doubling the intracellular levels of cGMP, and higher concentrations can potentiate lysosomal enzyme release in response to immunological agents by as much as 50% (11). Other agonists, including acetyl β-methylcholine, acetylcholine, and pilocarpine, also increase lysosomal enzyme release in the presence of immunological agents. This increase is prevented by 1 μM atropine, a muscarinic cholinergic antagonist, but not by the same concentration of the nicotinic antagonists hexamethonium or *d*-tubocurarine (12). Carbamylcholine can also stimulate neutrophil chemotaxis in response to chemotactic factors (13) and increases cGMP accumulation caused by those factors (14). From these results it is reasonable to postulate the existence of one or more specific muscarinic binding substances in these cells that may function in a manner similar to that of cholinergic receptors of neural tissue and of smooth muscle.

Direct studies of the binding of muscarinic cholinergic agents in neutrophils have not previously been reported. Because it is surprising to find responses by noninnervated cells to cholinergic agents, it seems important to demonstrate the interaction of these agents with the cells by direct binding studies. Also, these cells offer several unique possibilities for investigation of the muscarinic cholinergic system in that whole live cells can be used, an easily measured physiological response to the agents exists in modulation of lysosomal enzyme release, and a direct dose response has been observed for enzyme release (11). Neutrophils thus provide a good model system for studies of coupling of muscarinic binding to cellular response. For these reasons we decided to investigate some physical and pharmacological properties of muscarinic cholinergic binding in human neutrophils.

MATERIALS AND METHODS

Human neutrophils were isolated from fresh heparinized venous blood, using a Ficoll-Hypaque gradient and hypotonic lysis of erythrocytes (15). The cells were resuspended in Hank's balanced salt solution with 0.25% bovine serum albumin and 0.1% glucose. Viability was determined by exclusion of trypan blue dye, and was always greater than 98%. Final cell preparations contained 96+ % polymorphonuclear leukocytes, with a maximum of 1:1 contamination by erythrocyte ghosts. This contamination should not alter quantitation of QNB binding because of low numbers of sites per cell on erythrocytes (6). All incubations were carried out in plastic tubes.

Specific binding was determined by incubating cells at 22° in Hank's salt solution with [^{3}H]QNB in the presence or absence of 10 μM atropine or 100 μM oxotremorine. Cells were filtered after 40 min, using Whatman GF/B or GF/C filters and a Millipore vacuum filtration apparatus. Cells were diluted with 2 volumes of Hank's solution at room temperature and rapidly filtered by suction. The filters were then rinsed 4 times with 2 ml of Hank's solution, at room temperature, in a manner similar to that described for brain homogenate work (2). The Hank's solution was kept at 22° rather than at 0° to avoid the temperature shock caused by the latter conditions. Incubation volume was 1.0 ml, and except as stated otherwise, incubations contained 3.6×10^6 cells. [^{3}H]QNB, specific activity 16.4 Ci/mmole, was purchased from Amersham Searle Corp. Oxotremorine and pilocarpine were

purchased from Aldrich Chemical Co. Atropine sulfate, aspartic acid, and carbachol were from Sigma Chemical Co.

Flaxedil (gallamine) was purchased from Burroughs Wellcome & Co., histamine from Nutritional Biochemicals Corp., and *d*-tubocurarine chloride from E. R. Squibb & Sons.

Zymosan was purchased from ICN and activated by incubating at 50 mg/ml in human serum for 30 min at 37°. This process fixes complement factors to the surface of the zymosan particles by means of natural serum antibodies to the particles. The zymosan-complement particles were washed thoroughly with Hank's balanced salt solution.

Cytochalasin B was purchased from Aldrich Chemical Co., and added to incubation mixtures at 0.5 μg/ml. In the presence of cytochalasin B, the neutrophils bind but do not engulf the zymosan particles. The lysosomes in this case, instead of fusing with phagosomes, fuse with the plasma membrane to release the lysosomal enzymes into the medium (10).

Neutrophils were preincubated for 5 min at 22° with the cholinergic antagonists, then for 5 min more with carbachol before addition of 5 mg/ml zymosan-complement (16). The incubations were carried out at 37°, shaking at 120 excursions/min. After 60 min, the cells were centrifuged at 4° and the supernatant assayed for lysozyme (17) and LDH (18).

RESULTS

Using the specific muscarinic agonist oxotremorine at 100 μM or the antagonist atropine at 10 μM to displace QNB binding to neutrophils, a saturation curve is obtained as shown in Fig. 1. Specific binding is given by the difference in QNB retention by the cells in the presence and absence of competing agents. One hundred fmoles of QNB-bound corresponds to a difference of approximately 1270 cpm. Nonspecific binding, or QNB retained in the presence of atropine or oxotremorine, was fitted to a straight line by the method of least squares ($r = 0.99$), plotted as a function of QNB concentration. For the saturation curve, nonspecific binding values were read from this line.

The fact that specific binding saturates at relatively low concentrations of ligand, while the nonspecific binding remains linear, indicates that a limited number of high affinity sites are involved. The Scatchard analysis (19) in the insert of Fig. 1 gives a

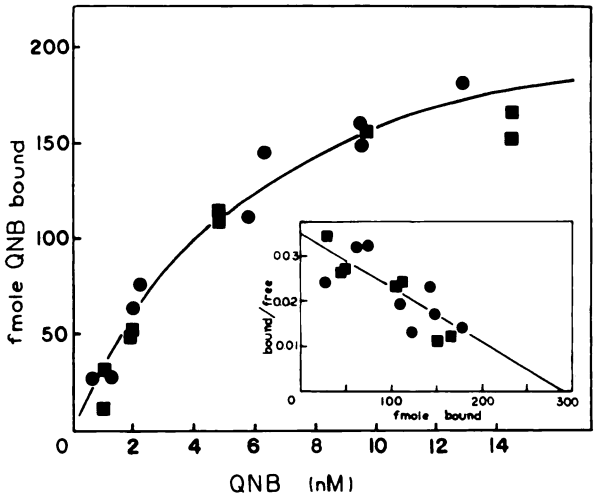

FIG. 1. *Specific QNB binding as a function of QNB concentration.*

3.6×10^6 neutrophils were incubated in 1.0 ml with and without 10 μM atropine (■) or 100 μM oxotremorine (●). Net binding is plotted as a function of total QNB concentration. The differences in QNB binding with and without competitor are statistically different from zero ($P < 0.005$). *Inset.* Scatchard analysis of the binding curve, with the least squares linear regression line giving a K_d of approximately 8 nM.

K_d of approximately 8 nM and on the order of 5×10^4 sites per cell.

The saturation curve shown, a composite of data using cells from a single donor on 3 different days, is representative of the saturation curves obtained with neutrophils from 8 different donors. Specific binding constitutes 30–50% of the total binding.

In another experiment we compared oxotremorine, atropine, and the agonist pilocarpine as displacing agents with QNB at 10 nM. We found no difference in displacement of QNB binding by oxotremorine or pilocarpine at 100 μM, and by atropine at 10 μM, in spite of very different chemical structures (20).

Various nonmuscarinic agents were also checked for their ability to displace QNB binding. Aspartic acid did not decrease binding at 12.5 μM or 125 μM. Histamine, which decreases lysosomal enzyme release in response to zymosan-complement, had no effect on QNB binding at 12.5 μM or 125 μM. The nicotinic agent gallamine also was without effect at 10 and 100 μM. However, *d*-tubocurarine, another nicotinic agent, did displace QNB binding at 100 μM.

Specific QNB binding increases with increasing cell number (Fig. 2). In plotting the mean difference of triplicate determinations with and without atropine, ± SEM, the mean values correlate well with a least squares calculated line ($r = 0.99$).

To check for retention of cells on filters, radioactivity was determined after filtration of identical incubations on both GF/B (pore size 1.0 μ) and GF/C (pore size 1.2 μ) filters. In contrast to tissue homogenate work, with whole cells the retention was essentially complete and identical for the two filters (data not shown). This result was expected because neutrophils have a diameter of 15 μ.

QNB in this system can function as a cholinergic antagonist, as shown in Table 1. Carbachol, a cholinergic agonist, increases lysosomal enzyme release in response to challenge with zymosan-complement particles, as has been shown previously (11, 12). As has also been shown with 1 μM atropine (11, 12), incubation of neutrophils with antagonist alone before exposure to zymosan-complement has no effect on enzyme release, but prior incubation with atropine prevents increased release in the presence of carbachol. We see the same phenomena with QNB at 0.1 μM. In all nine incubations, LDH release, an indication of cell lysis, remained constant. This effect and the specific, saturable binding of QNB to whole cells are consistent with the concept of a kind of muscarinic cholinergic

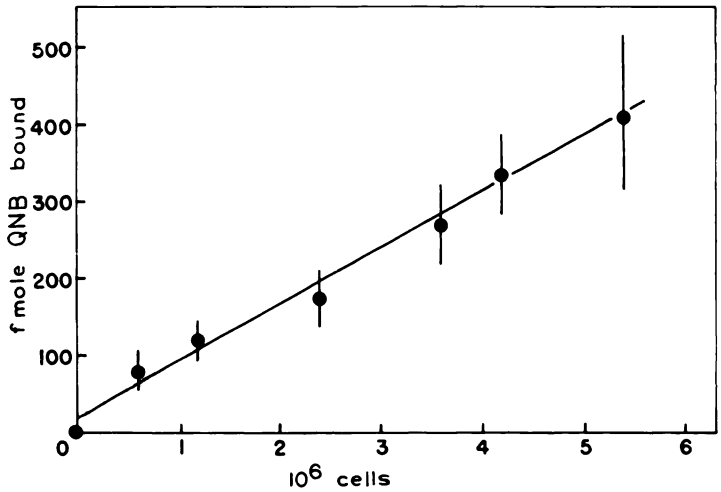

FIG. 2. *Specific QNB binding as a function of cell concentration.*

Values shown are the means ± SEM for the differences between triplicate determinations of QNB binding in the presence and absence of 10 μM atropine, in 1.0 ml incubation volume. The line represents a linear regression least squares analysis of the mean values, with $r = 0.99$.

TABLE 1

Cholinergic modulation of lysosomal enzyme release[a]

Neutrophils were preincubated with the cholinergic agents and cytochalasin B as described in MATERIALS AND METHODS, and the percentage release of total lysozyme and LDH in response to zymosan-complement determined.

Cholinergic agents	% lysozyme	% LDH	% change
1. ——	45	7	——
2. ——	48	7	——
3. 5.0 μM carbachol	57	7	+23
4. 5.0 μM carbachol	57	8	+23
5. 1.0 μM atropine	44	8	−4
6. 5.0 μM carbachol + 1.0 μM atropine	43	8	−6
7. 0.10 μM QNB	46	8	no change
8. 5.0 μM carbachol + 0.10 μM QNB	43	7	−6
9. 5.0 μM carbachol + 0.10 μM QNB	44	7	−4

[a] Total activity/3.75 × 10^6 cells: LDH: −0.618 ΔOD_{340}/min at 37°; lysozyme: −0.056 ΔOD_{450}/min at 37°.

receptor in these cells analogous to receptors described in brain and smooth muscle.

DISCUSSION

Neutrophils *in vivo* mediate inflammation and tissue destruction by a process closely related to that used to induce lysosomal enzyme release *in vitro* (21). Neutrophils bind to immune complexes on noningestible surfaces such as the basement membranes and selectively release lysosomal constituents. These constituents include chemotactic activators, pyrogens, and hydrolytic enzymes.

As indicated earlier, the lysosomal release process can be influenced by cholinergic agents. The identification of muscarinic cholinergic binding sites associated with human neutrophils has been suggested on the basis of this QNB binding assay.

The phenomenon of saturable QNB binding to neutrophils is clearly demonstrated by the difference in QNB retention in the presence and absence of a competing ligand. A second ligand can compete only if a limited number of binding sites are functional. The construction of a saturation curve, however, demands much greater accuracy in measurements. At low concentrations of QNB, the net binding will be small while at higher concentrations as saturation is approached, the specific binding constitutes a continually decreasing fraction of the total binding. We minimized the errors arising from measurement of nonspecific binding by the use of a linear least squares treatment of the data. This procedure is equivalent to using the mean value of a large number of measurements rather than a single measurement. The measurement of muscarinic binding sites, using this competitive assay, is based upon the high potency and specificity of [^{3}H]QNB (22) as well as the specificity of the competing agonists, oxotremorine and pilocarpine, and the competing antagonist atropine. The competing agents are considered classical muscarinic agonists and antagonists.

Flaxedil, histamine, and aspartic acid do not compete for QNB binding sites at high concentrations (125 μM). *d*-Tubocurarine, which at 1 μM does not block carbachol-induced potentiation of lysosomal enzyme release (11, 12), can block QNB binding when used at 100 μM. It has been seen earlier (8) that curare can interact with the lymphocyte cholinergic system in modulating the cytotoxic response, and can at 10 μM completely displace specific QNB binding to erythrocytes (6). The effectiveness of curare in the cholinergic binding system of blood cells may serve to differentiate these binding sites from those of brain homogenate (2) and of smooth muscle (3).

The suggestion that muscarinic binding sites may be functional muscarinic receptors requires support in addition to binding data, even though highly specific cholinergic agents are used in the binding study. Additional criteria are that the cells respond physiologically to a cholinergic agonist and that this *physiological* response can be blocked by cholinergic antagonists, especially QNB, in our case. Lysosomal enzyme release can be considered a true physiological response of neutrophils and cholinergic potentiation of this response is blocked by QNB. The importance of evaluating both the physiological response to appropriate cholinergic agonists and antag-

onists in addition to the cholinergic ligand binding characteristics of the system is emphasized in view of the nonequivalence of α-bungarotoxin binding sites and acetylcholine receptors in some neuronal systems (23, 24).

QNB has been found to exert a dose-response blockage of cholinergic-stimulated secretion in another system, the rat exocrine pancreas (25).

Many studies identify the presence of specific QNB binding in homogenate preparations. While this approach simplifies binding studies by consideration of a more homogeneous membrane fraction, it eliminates the possibility of measuring physiological consequences of such binding. Using whole live neutrophils allows direct correlation of specific QNB binding with a specific physiological response.

There are several disadvantages in using whole cells. Nonspecific binding in the presence of high concentrations of competing muscarinic agents accounts for a sizeable percentage of the total counts. Nonspecific binding in a live cell depends not only on lipid solubility and cytoplasmic concentrations, but possibly also on pinocytosis and exocytosis and on other energy-dependent processes. The result is that nonsystematic errors in the determination of nonspecific binding affect the measured value of specific binding much more than if the competing agent displaced 90% or more of the total retention. We have attempted to minimize these effects by constructing a least squares linear regression line from the experimental nonspecific binding data to use in the saturation data. However, this corrects only half the problems because the same problems arise in determination of total binding. As a result, the Scatchard plot of Fig. 1 reflects the occurrence of sizeable errors arising from the use of whole cells. Also, a greater heterogeneity of specific binding would be expected, due to the heterogeneity of cell organelles. For example, using fractionated brain homogenates, Yamamura and Snyder (2) found significant specific binding to the mitochondrial fraction. Such binding is presumably unrelated to the observed rapid response of neural tissue to cholinergic agents. Similarly our calculated sites per cell may include specific binding sites that are unrelated to the augmentation of lysosomal release.

Our use of whole live cells could also affect the linearity of QNB binding with cell concentration, if binding is related to physiological processes dependent on cell density. We find, however, that the mean values of specific binding as a function of cell number are linear, as with different homogenized tissues. The standard errors of the means are large enough, though, that we can not rule out deviations from linearity.

The observed K_d, or concentration of QNB needed to cause one half the saturable binding, of approximately 8 nM for human neutrophils is considerably higher than that reported for QNB binding to brain homogenate preparation (2). In the case of mammalian heart, muscarinic binding sites have been most recently characterized as having K_d for QNB on the order of 20 pM (26). More recent studies on noninnervated systems have suggested K_d values on the order of 1 nM (6, 7). Recently, however, measurements of the K_d for QNB binding in rat strial brain slices have revealed a K_d of about 3.5 nM, suggesting a considerably lower affinity for binding in slices than in homogenates (27). When results for QNB binding in murine lymphocytes, human neutrophils, and brain slices from rat corpus striatum are compared with brain homogenate preparations, the studies using less disrupted systems suggest a higher apparent K_d.

In spite of some difficulties in studying QNB binding in whole live cells, our results support the idea of specific muscarinic cholinergic binding substances in neutrophils. The high affinity for QNB and the saturability of the specific binding, coupled with direct effects on neutrophil physiology by the ligand used, are consistent with the concept of a muscarinic cholinergic receptor in neutrophils.

ACKNOWLEDGMENTS

We wish to thank Ms. Nannette A. Schwartzman from Dr. Peter Henson's laboratory for invaluable discussion of lysosomal enzyme release techniques.

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