Muscarinic cholinergic receptors in murine lymphocytes: Demonstration by direct binding

([3H]quinuclidinyl benzilate/acetylcholine receptors/atropine)

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ABSTRACT   Using [3H]quinuclidinyl benzilate as a specific cholinergic muscarinic ligand, it has been demonstrated that lymphocytes have muscarinic binding sites. There are approximately 200 sites per cell and the dissociation constant for quinuclidinyl benzilate is approximately 1×10^{-9} M. Quinuclidinyl benzilate receptor binding is blocked by atropine and oxotremorine.

Acetylcholine, released from cholinergic neurons, has been shown to interact with cholinergic receptors in skeletal muscle endplates, in cells of secretory glands, in ganglion cells of the autonomic nervous system, in smooth muscle, and in some cells of the central nervous system (1). Acetylcholine receptors have different properties in different anatomical locations. For example, in mammalian neuromuscular junctions (2, 3), ganglion of the autonomic nervous system (4, 5), and eel electric organ (6, 7), cholinergic receptors have been described as "nicotinic," since they can be activated by nicotine and inhibited by agents such as tubocurarine. Such nicotinic sites have also been studied by chemical measure of direct binding of specific nicotinic ligands such as radiolabeled α-bungarotoxin. Other acetylcholine receptors have been described as "muscarinic" because they can be stimulated selectively by muscarine and blocked by such agents as atropine (1). With approaches similar to those used in nicotinic receptor studies, the presence of high-affinity binding sites for tritiated reversible muscarinic antagonists in smooth muscle and brain have been shown in a number of investigations (8, 9).

Recently, the presence of muscarinic receptors has been demonstrated in erythrocyte membrane by a direct binding assay (10). The possibility of a cholinergic muscarinic receptor associated with another non-neural and noninnervated cell, the lymphocyte, has been suggested by indirect approaches. Augmentation of the cytotoxic response of thymus-derived (T) lymphocytes, increases in cyclic GMP levels, increases in protein and RNA synthesis, as well as enhanced lymphocyte motility in the presence of cholinergic agonists, have been reported (11–14). In most of the above instances, the cholinergic response has been diminished or blocked by the specific muscarinic antagonist, atropine.

However, there has not been a direct binding study to demonstrate the presence of muscarinic cholinergic receptors associated with lymphocytes. The present investigation establishes that murine lymphocytes exhibit high-affinity binding for 3-quinuclidinyl benzilate (QNB), a specific, potent, and longlasting muscarinic agent which has been used previously to demonstrate and characterize cholinergic binding in neural tissue and smooth muscle (15, 16). This muscarinic antagonist

is a quasi-irreversible ligand, in that the dissociation of the QNB–receptor complex is slow (15). Slowness of drug–receptor complex dissociation permits ready separation of unreacted [3H]QNB by rapid filtration and washing and allows quantification of binding sites per cell. The direct demonstration of muscarinic binding sites and estimation of binding sites per cell appears fundamental in the study of cholinergic modulation of lymphocyte function and metabolism.

MATERIALS AND METHODS

[3-3H]Quinuclidinyl benzilate, specific activity 8.4 Ci/mmol, was purchased from the Amersham/Searle Corp. Oxotremorine was purchased from Aldrich Chemical Co. Atropine sulfate was purchased from Sigma Chemical Co. Hanks' balanced salt solution and RPMI 1640 were obtained from Grand Island Biological Co. Whatman 2.4-cm diameter glass fiber filters (6F/B) were purchased from Sargent-Welch Co. and used with a filtration system from the Millipore Corp. These filters were chosen because they have a pore diameter of 1 μm and the cells have diameters of 5–12 μm. Scintillation cocktail was made by adding 26.5 g of diphenyloxazole and 1.59 g of 1,4-bis[2-(4-methyl-5-phenyloxazoly)]benzene to 3.79 liters of toluene (scintillar), which was purchased from Mallinckrodt Chemical Co. Tissue was solubilized with Beckman solubilizer.

Cell suspensions

Male CBA/J mice, 18–22 weeks old, were obtained from the Jackson Laboratory, Bar Harbor, ME. They were killed by cervical dislocation and their spleens were removed. Single cell suspensions in Hanks' balanced salt solution were prepared by tapping the spleens gently on a 100 mesh wire gauze, followed by passage through a 25-gauge needle. Suspensions were centrifuged at $200 \times g$ for 10 min at 4°. The pellet was resuspended in Tris-buffered (pH 7.2) isotonic ammonium chloride and warmed to 37° for 15 min in order to lyse erythrocytes (17). After the mixture was pelleted again in the above manner, the leukocytes were resuspended in Hanks' balanced salt solution and passed through a cylindrical nylon gauze filter to remove large particulate debris. The final cell concentration was always adjusted to 7.5×10^7 cells per ml. Nucleated cells were counted in a hemocytometer. About 1.5×10^8 leukocytes were obtained per mouse spleen.

To obtain adherent cells, we resuspended the leukocytes, after the ammonium chloride treatment and pelleting, in RPMI 1640 and incubated the suspension (CO_2 incubator) in glass petri dishes at 37° for 1 hr. Nonadherent cells were poured off and the dishes were rinsed four times with Hanks' balanced salt solution. The firmly adherent cells were then removed by scraping the dish bottoms with a rubber policeman. These

Abbreviation: QNB, 3-quinuclidinyl benzilate.

Immunology: Gordon *et al.*

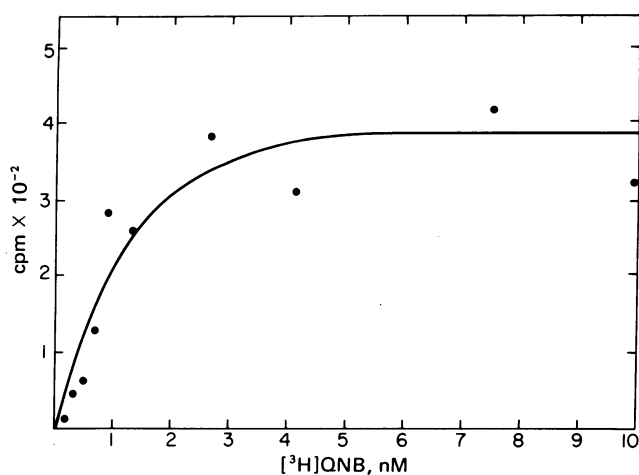*Proc. Natl. Acad. Sci. USA 75 (1978)*   2903

FIG. 1.   Specific binding of [^{3}H]QNB to lymphocytes as a function of the concentration of [^{3}H]QNB. Cells (1.5×10^8) were incubated for 60 min in 2.0 ml of Hanks' balanced salt buffer (pH 7.2) with various concentrations of [^{3}H]QNB. Specific binding was determined as described in the text.

collected cells were suspended in Hanks' balanced salt solution.

Proportions of thymus-derived (T) and bone-marrow-derived (B) lymphocytes were determined by immunofluorescence, with anti-Thy 1.2 and fluoresceinated rabbit anti-mouse immunoglobulin (18). Phagocytic cells were detected by incubating the spleen cell suspension with latex beads and counting the cells that had ingested particles (19).

Specific [^{3}H]QNB binding

To assay specific binding of [^{3}H]QNB, we incubated the ligand with 2 ml of Hanks' balanced salt solution (pH 7.2) containing 1.5×10^8 cells. Another tube contained, in addition to the above, 0.1 mM oxotremorine in order to determine nonspecific binding. All tubes were incubated for 60 min, after which the contents were filtered under reduced pressure through prewetted (phosphate buffer) glass fiber filters (GF/B) positioned over a sintered glass disc. The bowl of the filter makes an impression on the filter and thus outlines the filtering surface. To avoid clogging of the filters, four filters per tube were used. The 2-ml samples were applied in 0.5-ml portions per filter with a 1-ml syringe with a small needle. The 0.5-ml cell suspension was applied slowly (10 sec) under suction so as to form a lens-shaped droplet that did not spread over the entire area of the filter but was slowly pulled into the filter. The approach left a circle with a thin layer of cells and a narrow annulus of filter without many cells. This manner of application allowed easy washing of the cells with five 4-ml portions of ice-cold 50 mM sodium phosphate buffer (pH 7.43). Each portion was added rapidly with a spring-loaded automatic syringe. Total washing time did not exceed 2 min. The filtering centers containing the cells and the annulus were cut out following the imprint of the filtering bowl and the four centers were placed in a glass scintillation vial for determination of radioactivity. Removal of the filter edges, not involved in trapping or washing the cells, reduced the radioactive counts introduced by [^{3}H]QNB binding to the filter. [^{3}H]QNB binding to the filter centers, independent of tissue and after five washings, contributed at the highest [^{3}H]QNB concentration used, 10 nM, 50 cpm above the counting background of approximately 20 cpm. At [^{3}H]QNB concentrations of 5 nM, this contribution was approximately 10 cpm above background levels. At lower [^{3}H]QNB concentrations, there was

Table 1.   Atropine blocks the specific binding of QNB

No. of cells	[QNB], nM	[Atropine], μM	cpm	
			QNB	QNB + atropine
1.5×10^8	0.9	1	984	713
1.5×10^8	2.68	100	1993	1591
1.5×10^8	1.34	100	1116	796
7.5×10^7	1.34	100	541	367

no apparent [^{3}H]QNB binding to the filter center. The filters were placed in 15 ml of scintillant with 0.5 ml of Beckman solubilizer per vial; radioactivity was measured after solubilization had been completed (24 hr). Radioactivity was assayed to better than 0.5% by liquid scintillation spectrometry (Beckman LS 250) at a ^{3}H counting efficiency of 38.5%.

RESULTS AND DISCUSSION

Specific binding of QNB can be measured as the difference in the binding of QNB in the absence and presence of 0.1 mM oxotremorine, a specific muscarinic agonist. This method of measurement is based upon the expected competition of two potent, specific ligands for the muscarinic receptor. Oxotremorine at 0.1 mM should completely block the binding of QNB to specific binding sites when QNB is present at low concentrations. The measurements of specific binding to 1.5×10^8 leukocytes, as a function of QNB concentrations (Fig. 1), display a saturation curve. This curve was constructed from three different cell preparations prepared on different occasions. This saturation curve indicates that a limited number of specific binding sites are involved. That the limit is approached at low concentrations of QNB meets the requirements for a high-affinity muscarinic receptor. The dissociation constant is on the order of 1×10^{-9} M and is comparable to the value reported for erythrocytes (10) but an order of magnitude higher than that for neural tissue (15). As a further test, it was shown that atropine, a specific muscarinic antagonist, 10^{-6} M and 10^{-4} M blocked the specific binding of QNB (Table 1). The differences in the radioactive counts with and without atropine were about the same as obtained with oxotremorine but slightly higher. The specific binding with 1.5×10^8 cells was double what we obtained with 7.5×10^7 cells at 1.34 nM QNB. The first entry in this table (0.9 nM QNB) was obtained with a different cell preparation from the last three entries.

The errors in this type of work may be rather large. Yamamura and Snyder (15) reported an error of about 10% in QNB binding studies using whole brain homogenates. Work with lymphocytes is more difficult in that the density of receptor sites is much lower and there is a relatively high background of uncharacterized counts in the presence of oxotremorine and atropine. The lymphocyte saturation data correspond to only about 200 muscarinic binding sites per cell. Even so, the saturation data have an error of less than 20%. The data are single determinations, except for the point at 1.34 nM QNB, which is the average obtained with two different cell preparations. There is also a duplicate in Table 1. The average of the saturation and near-saturation cpm (QBN $\geq$ 1.3 nM) is 340 cpm, with a standard deviation of 55 cpm and a standard error of the mean of 18 cpm. We also calculated the value of Student's t test for paired observations (QNB binding with and without either oxotremorine or atropine) and obtained a value of 18 for $n = 10$. This corresponds to F much greater than 0.9995 for the existence of a difference between the two groups.

The method used here for determining muscarinic receptors

was essentially the same as that of Yamamura and Snyder (15) except that the present study used whole cells whereas previous work used sonicated tissue.

After the ammonium chloride procedure, the erythrocyte cell contribution to the total leukocyte cell suspension was less than 0.1%, as determined with a hemocytometer. The cellular distribution was: T lymphocytes, 20 ± 3% (SEM); B lymphocytes, 64 ± 3% (SEM); phagocytes, 8 ± 2% (SEM); and "null cells" (cells unidentified as T, B, or phagocytic), 8%.

The lymphocyte-binding data are averages over different types of leukocytes. Therefore, adherent cells, predominantly macrophages, were removed and their binding properties determined. The adherent cells showed specific binding corresponding to about 400 muscarinic receptors per cell at 1.34 nM QNB. Although the number of macrophage-binding sites is higher than that for lymphocytes, the number of macrophages is so small that the saturation value assigned to lymphoctyes is not appreciably changed.

Binding by T lymphocytes has not been distinguished from binding by B lymphocytes, and it is possible that there is a wide difference in the binding capabilities.

The value of approximately 200 binding sites per leukocyte is considerably lower than that observed in a neuroblastoma cell line (NIE 115). With the same [^{3}H]QNB binding assay, about 4000 sites per neuroblastoma cell were observed (20). The value for muscarinic binding sites in lymphocytes is comparable to the estimate for insulin-binding sites in transformed lymphocytes and rat peritoneal macrophages (21).

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