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Further Characterization of Human Red Blood Cell Membrane Cholinergic Receptors

CHARLES R. MANTIONE[1] AND ISRAEL HANIN

Departments of Pharmacology and Psychiatry, University of Pittsburgh Medical School and Western Psychiatric Institute and Clinic, Pittsburgh, Pennsylvania 15261

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SUMMARY

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Human red blood cells bind *d,l*-[^{3}H]quinuclidinyl benzilate, a potent muscarinic cholinergic antagonist, with high affinity (apparent K_D=0.26 nM) and low capacity as determined by equilibrium binding experiments. Nonspecific binding determined as binding in the presence of atropine is hyperbolic, and saturable within the same concentration range as total binding. The *d*- but not the *l*-isomer of benzetimide weakly inhibits ligand binding, which parallels the stereoselectivity but not the potency of drug antagonism to muscarinic receptors in other tissues. While treatment of brain homogenates with Triton X-100 (0.5%) destroys all *d,l*-[^{3}H]quinuclidinyl benzilate binding to that tissue, similar detergent treatment of red blood cell membranes fails to greatly alter either total or certain drug-displaced ligand binding. These results show that the red blood cell membrane binding site for *d,l*-[^{3}H]quinuclidinyl benzilate differs in some important aspects of its binding characteristics, pharmacology, and physical properties from muscarinic receptors similarly studied in other nervous and nonnervous tissue. Further characterization of the red blood cell cholinergic ligand binding site is necessary to establish its identity as either a different form of muscarinic receptor or as an acceptor site on other membrane cholinergic enzymes or carrier proteins.

INTRODUCTION

Human RBC[2] membranes have been postulated to contain several different types of hormone, neurotransmitter, and drug receptors (1–5). Included among them are reports of an acetylcholine receptor. The first data to show that the RBC membrane might have a cholinergic receptor came from electron spin resonance assays of human RBC membrane fluidity (5, 6). Carbamylcholine and muscarine but not nicotine were found to increase the membrane fluidity, and the response could be blocked by atropine but not by curare. Based on these pharmacological observations, the drug-specific RBC membrane site was termed a muscarinic cholinergic receptor.

The binding of a potent radiolabeled muscarinic antagonist, [^{3}H]QNB, has been widely used to directly identify and characterize muscarinic cholinergic receptors on a variety of neuronal and nonneuronal tissues (7, 8). [^{3}H]QNB has also been shown to bind sparsely and with high affinity to human RBC membranes (9). Moreover, pharmacological drug inhibition of this binding has suggested that it represents a muscarinic receptor.

We have examined [^{3}H]QNB binding to human RBC membranes in order to further delineate the nature of this interaction. Since RBCs are not conventionally thought to possesses cholinergic receptors, we have paid specific attention to factors such as binding saturability, kinetics, pharmacology, and physical features in order to establish whether indeed ligand binding in these membranes conforms with properties necessary by definition for receptor labeling in other tissues (10). Our data indicate that the biochemical nature of the muscarinic receptor on RBCs may be different from that of the muscarinic receptor conventionally studied in brain tissue.

METHODS

Materials. Atropine sulfate and choline chloride were obtained from Sigma Chemical Company (St. Louis, Mo.). Unpurified saponin was obtained from Calbiochem–Behring (LaJolla, Calif.). [^{3}H]QNB (29.4 Ci/mmol) was purchased from New England Nuclear Corporation (Boston, Mass.). The stereoisomers of benzetimide were generously supplied by Janssen Pharmaceutica (Bearse, Belgium).

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[2] Abbreviations used: RBC(s), red blood cells(s); [^{3}H]QNB, *d,l*-[^{3}H]quinuclidinyl benzilate; IC_{50}, 50% inhibitory concentration.

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Membrane preparations. Human blood was collected by venipuncture from healthy human volunteers into heparinized glass tubes (Vacutainer, Beckton–Dickinson). Whole blood was centrifuged at 1000 *g* for 30 min at 4–8°C, and the plasma plus buffy coat subsequently discarded. On occasion, and where noted within the text, packed RBCs obtained immediately after their expiration date from a central blood bank were used. Packed RBCs from either source were washed three times in twice their volume with cold isotonic (310 mOsM) Na/K-phosphate buffer at pH 7.4.

Hemoglobin-depleted membranes were prepared by either repeated hypotonic lysis or saponin treatment. Hypotonic lysis of RBCs essentially followed the procedure outlined by Dodge *et al.* (11). RBCs were diluted 1:20 with cold hypotonic (17 mOsM) Na/K-phosphate, pH 7.4, allowed to stand 5 min in ice, then centrifuged at 30,000 *g* for 40 min at 4–8°C. The supernatant was carefully drawn off. The complete lysis procedure was repeated four to six times until a flocculent faint pink membrane pellet was obtained.

An alternative, more rapid and efficient method to remove RBC hemoglobin was to use saponin treatment (12). Crude saponin was dissolved in distilled water (20 mg/ml) and passed through a column (8 × 50 mm) of Dowex 8W-X50 (200–400M) to remove any ionic impurities. The resulting purified saponin was added at a concentration of 0.1 mg/ml to a 10% suspension (v/v) of RBCs in cold isotonic Na/K-phosphate, pH 7.4. After allowing it to stand at room temperature for 15 min, the suspension was centrifuged at 30,000 *g* for 40 min at 4–8°C. The resulting pellet was washed in approximately 20 vol of fresh buffer and recentrifuged to retrieve the RBC membranes.

[^{3}H]QNB binding assay. For receptor studies using brain tissue, female CD-1 mice (Charles River Laboratories, Wilmington, Mass.) weighing 25–30 g were decapitated and the brains rapidly removed. A crude tissue homogenate of whole brain minus cerebellum was prepared and used for binding studies according to the method developed by Yamamura and Snyder (7). A standard assay contained 1 ml of 0.05 M Na/K-phosphate buffer at pH 7.4, per tube, to which the following were added in sequence: homoglobin-depleted RBC membranes (0.5–1 mg protein), test drug or solvent, and finally [^{3}H]QNB. The mixture was vortexed and incubated at 25°C for 60 min. Tissue-bound radioactivity was collected by vacuum filtration over glass fiber filters (GF/B) followed by several washings with cold incubation buffer. The filters were suspended in 10 ml aqueous counting cocktail (Scintiverse, Fisher) and extracted for 24 h. The trapped radioactivity in the filter samples was then measured by liquid scintillation spectrometry.

Membrane protein content was determined by the method of Lowry *et al.* (13) using bovine serum albumin as a standard.

RESULTS

Saturability and kinetics of [^{3}H]QNB binding to RBC membranes. Incubating purified human RBC membranes with increasing concentrations of [^{3}H]QNB resulted in increased binding of radioligand to the tissue until approximately a 5–6 nM final ligand concentration, wherein total binding began to saturate (Fig. 1). Furthermore, in the presence of atropine (100 μM) the total binding of [^{3}H]QNB was reduced by 30–45% at each ligand concentration. The [^{3}H]QNB bound in the presence of atropine also saturated within the same concentration range as the [^{3}H]QNB bound in the absence of any unlabeled drug (Fig. 1).

The difference in [^{3}H]QNB bound in the absence versus the presence of atropine will be referred to as specific [^{3}H]QNB binding. The saturation binding isotherm for specific [^{3}H]QNB binding to human RBC membranes is presented in Fig. 2. Specific muscarinic ligand binding observed in our studies represented very little activity, and saturated around 5–6 nM final ligand concentration. A Scatchard analysis (14) of the data revealed a single small population of [^{3}H]QNB receptors (R_0=3.7 fmol/mg protein) which had a high affinity (apparent K_D=0.26 nm) for the radioligand (Fig. 2).

Effect of stereospecific muscarinic antagonists on [^{3}H]QNB binding. The RBC membrane [^{3}H]QNB receptor has been tested by other investigators for its pharmacological specificity (9). Thus, specific [^{3}H]QNB binding to human RBC membranes has been shown to be inhibited by some muscarinic drugs, but there also has been observed a considerable overlap between the inhibition produced by cholinergic versus noncholinergic drugs. Furthermore, the same study was unable to show any stereoselective drug effects between the isomers of

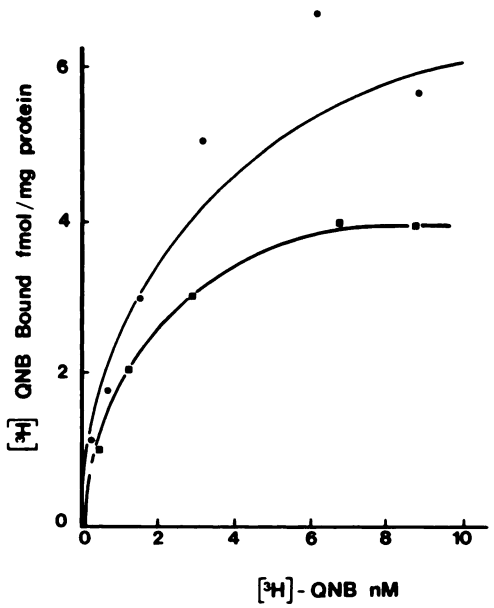

FIG. 1. *Concentration-dependent binding of [^{3}H]QNB to human RBC membranes*

Saponin-purified RBC membranes (0.2 mg protein/ml) were incubated in 1 ml 0.05 M Na/K phosphate, pH 7.4, containing various concentrations of either [^{3}H]QNB or [^{3}H]QNB with 100 μM atropine for 60 min at 25°C. Tissue samples were collected by vacuum filtration over GF/B filters and washed with seven 5-ml volumes of cold incubation buffer. Activity was measured after 24 h of extraction in aqueous counting cocktail. Data represent either the total amount of [^{3}H]QNB bound(●) or the amount bound in the presence of atropine (■). Each value is the mean of triplicate samples which had an average coefficient of variation of approximately 20%.

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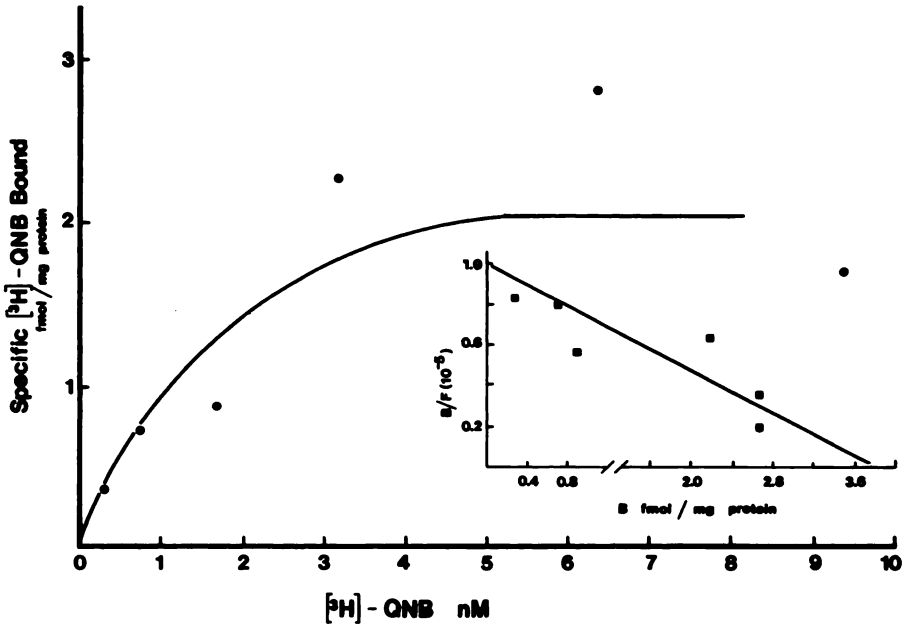

FIG. 2. *Specific [³H]QNB binding to human RBC membranes*

Specific [³H]QNB binding was calculated by subtracting the amount of [³H]QNB bound in the absence, from the amount bound in the presence of atropine (100 μM). *Inset.* Scatchard analysis (14) of specific [³H]QNB binding taken as the fraction of tissue bound ligand (*B*) over the free (*F*) or final [³H]QNB concentration, versus the tissue bound activity. The negative inverse value of the slope yields an apparent K_D = 0.26 nM [³H]QNB, while the abscissa intercept (*B*) indicates a total receptor population (R_0) of 3.7 fmol [³H]QNB/mg protein.

QNB itself, a property of muscarinic receptors identified in other tissues (8, 15, 16).

In this study, therefore, we have continued to probe the pharmacology of the RBC membrane cholinergic receptor by comparing the effect of benzetimide stereoisomers of which the *d*-isomer is a potent physiologically active muscarinic antagonist. Dexetimide, the *d*-isomer, inhibited RBC [³H]QNB binding in a dose-dependent manner with a calculated IC_{50} value of 41 μM (Table 1). The pharmacologically inactive *l*-isomer, levotimide, had little effect (IC_{50}>100 μM). For comparison, dexetimide was an extremely potent antagonist of [³H]QNB binding in brain homogenates, with levotimide being considerably weaker (Table 2).

The magnitude of binding in RBCs compared to brain is noteworthy. It shows the paucity of the RBC [³H]QNB binding sites, which makes their measurement difficult even with currently available high specific-activity tritiated radioligands.

Effect of nonionic detergent on [³H]QNB binding. Membrane receptors in general are complex hydrophobic glycoproteins which most often depend on the structural integrity of the intact membrane for their proper active conformation. Perturbation of the membrane by detergent solution might be expected to destroy the coupling between a receptor binding site and its respective class of ligands. In this study, both mouse brain and human RBC membrane tissue were subjected to nonionic detergent treatment, and [³H]QNB binding was determined. Brain homogenates are rich in muscarinic receptors, and thus readily bound [³H]QNB, which could be almost completely displaced (98%) by atropine (Table 3). As described previously, RBC membranes bound very little [³H]QNB, and atropine was able to reduce this activity only by a third. If [³H]QNB binding was assayed in mouse brain homogenates containing 0.5% Triton X-100, total binding was almost completely abolished. On the other hand, following the same detergent treatment, RBC membranes still bound about 88% of the [³H]QNB previously bound without detergent present. Atropine reduced this activity only by a few percent (5%). By comparison, choline, a weak cholinergic agonist and endogenous constituent of blood, was found to decrease

TABLE 1

Effect of benzetimide stereoisomers on [³H]QNB binding in RBC membranes

Human RBC membranes were prepared by saponin treatment of blood bank RBCs. Various concentrations of each drug were added to 0.28 ml of the membrane pellet in 1-ml total volume of 0.05 M Na/K-phosphate, pH 7.4, and incubated with [³H]QNB (6.8 nM). The reaction was terminated by filtration over GF/B filters followed by three 5-ml volumes of cold incubation buffer. Values represent the mean ± SEM of triplicate samples. The IC_{50} value was determined by linear regression analysis of percentage drug inhibition versus log drug concentration.

Drug	Concentration (μM)	Specific [³H]QNB bound (cpm)	Inhibition (%)
d-Benzetimide[a]	0	583.6 ± 99.8	0
(dexetimide)	0.1	487.5 ± 127.1	17.5
	1.0	382.3 ± 152.4	34.5
	10.0	306.6 ± 63.2	47.5
	100.0	140.2 ± 61.8	76.0
l-Benzetimide[b]	0	221.8 ± 115.0	0
(levotimide)	100.0	183.8 ± 102.4	17.1

[a] IC_{50} = 41 μM.
[b] IC_{50} = >100 μM.

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TABLE 2

Effect of benzetimide stereoisomers on [^{3}H]QNB binding in brain homogenates

Mouse brain tissue homogenates were prepared as described under Methods. Various concentrations of each drug were added to make a total sample of 2.0 ml 0.05 M Na/K-phosphate, pH 7.4, containing 2.5 mg tissue/ml, and incubated with [^{3}H]QNB (1.4 nM). The reaction was terminated by filtration over GF/B filters followed by two 5-ml volumes of cold incubation buffer. Values represent the mean ± SEM of triplicate samples. The IC_{50} values were determined by linear regression analysis of percentage drug inhibition versus log drug concentration.

Drug	Concentration (μM)	Specific [^{3}H]QNB bound (cpm)	Inhibition (%)
d-Benzetimide[a] (dexetimide)	0	11,811.6 ± 334.3	0
	0.0001	9,341.8 ± 161.2	20.9
	0.001	7,283.3 ± 64.1	38.3
	0.01	3,512.8 ± 166.0	70.3
	0.1	927.8 ± 11.6	92.2
l-Benzetimide[b] (levotimide)	0.1	11,742.4 ± 309.9	<1.0
	1.0	10,386.1 ± 183.1	12.1
	10.0	7,072.6 ± 89.9	40.1
	20.0	5,301.0 ± 146.3	55.1

[a] IC_{50} = 0.0019 μM.
[b] IC_{50} = 16 μM.

[^{3}H]QNB binding by 33–34% in both detergent and non-detergent-treated RBC membranes (Table 3).

DISCUSSION

An earlier report by Aronstam *et al.* (9) established that [^{3}H]QNB can bind in a saturable manner to human RBC membranes with an apparent K_D = 1.3 nM and a receptor concentration of 23 fmol/mg protein. Our preparation, however, contained about one-sixth that number of [^{3}H]QNB binding sites; likewise, the dissociation constant was calculated to be about six times lower. This notable difference in total number of binding sites measured in our studies compared to those referred to above probably is a reflection of substantial differences in tissue preparation and binding assay procedures between the two laboratories. Purified RBC membranes were prepared by saponin treatment in our ligand–receptor affinity experiments, whereas Aronstam *et al.* (9) used a hypotonic cell lysis procedure in conjunction with sonication. Also, they studied [^{3}H]QNB binding in a more hypotonic buffer with a chelating agent, which was not included in our assay system. Each of these differences could have resulted in a greater exposure of RBC tissue to radioactive substrate, and could conceivably account for their greater number of measured binding sites.

An important observation in our investigation of RBC membrane cholinergic receptors was the nature of [^{3}H]QNB binding observed in the presence of atropine. Such binding supposedly represents nonspecific binding or background activity, compared with specific [^{3}H]QNB binding, which is often taken as a measure of specific muscarinic receptor activity. Both curves were shown to saturate over the same [^{3}H]QNB concentration range. Usually, nonspecific binding is a linear function of ligand concentration especially in the low (nanomolar) concentration range. This is because nonspecific binding represents heterogeneous interactions of the ligand with the tissue which are too numerous to become saturated over the narrow and low ligand concentration range sufficient enough to saturate the relatively few specific receptor sites. In the case of the RBC membranes, however, this relationship was not upheld since the nonspecific (background) activity was shown to be in the same range as that which is termed specific receptor activity. Such a situation raises the question of whether atropine displaced [^{3}H]QNB bound from solely nonspecific cholinergic tissue sites, or whether such displacement was from specific muscarinic receptors. The increasing knowledge in receptor methodology over the past 10 years has in fact shown that the presence of saturable ligand binding and reasonable kinetics are *not* sufficient conditions to satisfy identification of a true neurotransmitter or hormone receptor (10, 17).

Both a biophysical study (6) and radioreceptor binding study (9) have provided pharmacological evidence to suggest the presence of a muscarinic receptor on RBC membranes. The muscarinic receptor identified in both nervous and smooth muscle tissue has been shown to generally exhibit similar pharmacological properties, including stereoselective inhibition by antagonists (8, 15). In our study with RBC membranes, we have also found that the *d*- but not the *l*-isomer of benzetimide weakly inhibited [^{3}H]QNB binding to the membranes.

However, the available literature on stereospecific binding in RBC membranes is not in agreement. Other investigators (9) have reported a lack of stereospecificity on the part of the RBC membrane cholinergic receptor for the stereoisomers of QNB, and have likened this effect to the lack of muscarinic stereospecificity on mydriasis of the rat iris. On the other hand, Sayers and Burki (18) have shown that there is no correlation between cholinergic activity quantified in pupillary aperture regulation versus other *in vitro* and *in vivo* muscarinic antagonist systems. Finally, recent binding studies have demonstrated that muscarinic cholinergic receptors

TABLE 3

Effect of nonionic detergent treatment on [^{3}H]QNB binding

Mouse brain (female, CD-1) homogenates (2.5 mg tissue/ml) or human RBC membranes prepared by hypotonic lysis of blood bank RBCs were incubated (1 mg tissue/ml) in 0.05 M Na/K-phosphate, pH 7.4, with respectively 1.4 and 6.0 nM [^{3}H]QNB at 25°C for 60 min. Other samples containing either drug, Triton X-100, or drug plus Triton X-100 were incubated under identical conditions. Tissue was collected by filtration over GF/B filters followed by four 4-ml washes with cold incubation buffer. Values are the means ± SEM of triplicate samples.

Preparation	[^{3}H]QNB binding (cpm)		
	Total	Atropine (10^{-4} M)	Choline (10^{-3} M)
Brain homogenate			
+ buffer	7133 ± 181	124 ± 6	2583 ± 127
+ buffer/0.05% Triton X-100	63 ± 18	74 ± 5	99 ± 2
RBC membranes			
+ buffer	415 ± 35	280 ± 17	276 ± 3
+ buffer/0.05% Triton X-100	324 ± 31	307 ± 28	212 ± 12

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in the rabbit iris–ciliary body respond to (−)-QNB 50 times more readily than to (+)-QNB (16).

In this regard, our pharmacological data actually would tend to strengthen the evidence for a muscarinic receptor in RBC membranes. Yet, the IC_{50} (41 μM) for dexetimide is approximately 1/10,000 times less potent than a corresponding value in brain (IC_{50}=0.0019 μM). Contrary to this, radioreceptor binding of other ligands has also been observed to display sophisticated pharmacological specificity, including stereoselectivity, to substrates which are definitively nonspecific (17, 19). This possibility has to be considered in the case of the RBC membrane as well.

We therefore used another approach to characterize the RBC membrane [^{3}H]QNB binding site: the comparison with brain tissue of its susceptibility to degradation by detergent. Membrane receptors have resisted solubilization in active form because of the intrinsic association between receptor protein and membrane lipid which is destroyed following exposure to certain detergents. This is illustrated by the destruction of virtually all [^{3}H]QNB binding sites in mouse brain homogenates, which we have reported here, as a result of exposing brain tissue to Triton X-100. We have also shown, on the other hand, that RBC membranes still substantially bound [^{3}H]QNB in the presence of detergent. While the effectiveness of a strongly hydrophobic drug, atropine, to displace the binding was lost, a strongly ionic drug, choline, equally inhibited [^{3}H]QNB binding by approximately 33% in either detergent of non-detergent-treated membranes. This might be explained by assuming that the detergent–tissue micelles were able to obstruct the action of atropine by internalizing the tissue hydrophobic binding sites in the Triton X-100-treated membranes. At the same time, ionic tissue sites may have still been exposed for choline displacement in both detergent and nondetergent conditions.

Our results show that the RBC membrane [^{3}H]QNB binding site differs in aspects of its binding characteristics, pharmacology, and physical properties from muscarinic receptors similarly studied in other nervous and nonnervous tissue. This suggests that in the RBC [^{3}H]QNB may be labeling a different form of muscarinic receptor. Alternatively, it may be partitioning into a specific membrane subregion characterized by a distinct solubility parameter (20), which may be involved in RBC function. In either case, the presence of a muscarinic component of RBC membranes may have an as yet unrecognized physiological role or clinically useful feature, particularly relevant to the study of RBC membrane function in neuropsychiatric disorders (21). In view of increasing evidence for the potential involvement of cholinergic mechanisms in a variety of neuropsychiatric disorders (22), a clearer understanding of the nature and properties of the RBC cholinergic ligand binding site is warranted.

A preliminary report of this work has been presented (23).

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Send reprint requests to: Dr. Israel Hanin, Western Psychiatric Institute and Clinic, University of Pittsburgh, 3811 O'Hara St., Pittsburgh, Pa. 15261.

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