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Muscarinic Cholinergic Receptor Binding in the Longitudinal Muscle of the Guinea Pig Ileum with [^{3}H]Quinuclidinyl Benzilate

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SUMMARY

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The binding of [^{3}H]quinuclidinyl benzilate (QNB) to homogenates of the longitudinal muscle of guinea pig ileum appears to represent an almost exclusive interaction with muscarinic cholinergic receptor sites. [^{3}H]QNB binds to particulate matter in these preparations in a saturable fashion with respect to [^{3}H]QNB. A variety of muscarinic antagonists and agonists inhibit specific [^{3}H]QNB binding in parallel with their estimated affinity for muscarinic receptors in the guinea pig ileum, based on pharmacological procedures. Numerous nicotinic cholinergic and noncholinergic drugs have negligible affinity for [^{3}H]QNB binding sites. The dissociation constant of the QNB-receptor complex estimated from saturation experiments with [^{3}H]QNB and from inhibition experiments with QNB is about 0.3–0.5 nM at 25°. The bimolecular rate constant of association (4×10^8 M^{-1} min^{-1}) and dissociation (1.2×10^{-2} min^{-1}) were estimated at 35° and indicate a dissociation constant of 0.03 nM. The maximal specific binding of [^{3}H]QNB indicates a concentration of receptors of about 190 pmoles/g of tissue. Specific [^{3}H]QNB binding can also be demonstrated in guinea pig spleen, heart, and lung, but not in guinea pig diaphragm, kidney, or liver.

INTRODUCTION

Direct biochemical studies of neurotransmitter receptors have been described for nicotinic cholinergic receptors in a variety of tissues (1–10), and for the glycine receptor in the mammalian central nervous system (11). A study of atropine binding to the guinea pig ileum suggested that it labeled muscarinic receptors (12, 13). Recently an alkylating agent derived from the muscarinic antagonist benzilylcholine has been utilized to label irreversibly the muscarinic receptors in the guinea pig intestine (14). 3-Quinuclidinyl benzilate is a potent muscarinic antagonist in the central (15, 16) and peripheral nervous system (17). We have employed QNB[3] in a radiolabeled form

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[3] The abbreviation used is: QNB, 3-quinuclidinyl benzilate.

as a simple, sensitive, and specific label to quantify specific muscarinic cholinergic receptor binding in the rat central nervous system (18). In the present study we have employed [^{3}H]QNB to identify and characterize specific muscarinic receptor binding in the longitudinal muscle of the guinea pig ileum.

MATERIALS AND METHODS

QNB was labeled by catalytic tritium exchange at New England Nuclear Corporation. QNB, 50 mg dissolved in 0.3 ml of glacial acetic acid, was mixed with 25 mg of platinum catalyst and 10 Ci of [^{3}H]H_2O. After stirring for 18 hr at 80°, labile ^{3}H was removed under vacuum with methanol as a solvent, and after filtration from the solvent the product was dissolved in 10 ml of methanol. In our laboratory the product was purified by thin-layer chromatography on silica gel with fluorescent indicator No. 6060 (Eastman Kodak) in 1-butanol–glacial acetic acid–water (4:1:1), and the purity of [^{3}H]QNB was checked in three solvent systems. The solvent systems used were heptane–toluene–diethylamine (4:1:1), chloroform–diethylamine (90:10), and 1-butanol–glacial acetic acid–water (4:1:1). Purified [^{3}H]QNB migrated as a single peak with authentic QNB in all three systems. The specific activities of the [^{3}H]QNB were 1.6 and 4.0 Ci/mmole in two batches as determined by comparison with the ultraviolet absorption of standard solutions at 258 nm.

Male guinea pigs (Hartley strain) weighing 300–500 g were killed by a blow on the head, and the entire small intestine was rapidly removed. Longitudinal muscle strips containing adherent (Auerbach's myenteric plexus were obtained by the method of Paton and Zar (19) and then blotted dry, weighed, and homogenized in 10–20 volumes of ice-cold 0.05 M sodium-potassium phosphate buffer (pH 7.4) in a glass homogenizer fitted with a glass pestle. The homogenate, rehomogenized with a Polytron instrument (setting No. 5, 60 sec), was used for [^{3}H]-QNB binding studies.

To assay specific binding of [^{3}H]QNB, 10–50 μl of this preparation were incubated at 25° with 2 ml of 0.05 M sodium-potassium phosphate buffer, pH 7.4, containing [^{3}H]-QNB (0.9 nM). After 60 min of incubation 3 ml of ice-cold sodium-potassium phosphate buffer were added, and the contents were poured onto a glass filter (GF/B) positioned over a vacuum. The filters were rinsed four times with an additional 4 ml of sodium-potassium phosphate buffer. Most determinations of binding were performed in triplicate, together with triplicate samples containing unlabeled QNB (0.01 μM) or oxotremorine (100 μM) to determine nonspecific [^{3}H]QNB binding. The filters were placed in glass vials containing 10 ml of Triton X-100, toluene, 2,5-diphenyloxazole (PPO), and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP), maintained at 25° for 8–12 hr, and the radioactivity then assayed by liquid scintillation spectrometry (Packard Tri-Carb models 3375 and 3385) at a counting efficiency of 40%.

For experiments on acetylcholine antagonism, a strip of muscle from the midregion of the small intestine was suspended in an 8-ml organ bath containing Krebs-Ringer bicarbonate solution at 37°, bubbled with 95% O_2 + 5% CO_2. Isometric contractions elicited by acetylcholine were recorded on a Grass model 79D recorder. An antagonist drug was added immediately after the acetylcholine had been washed out.

Protein was determined by the method of Lowry *et al.* (20), using bovine serum albumin as a standard.

Compounds were obtained as follows: acetylcholine, Eastman Organic Chemicals; acetyl-β-methylcholine and physostigmine, Calbiochem; isopropamide, Smith Kline & French; atropine and neostigmine, Sigma Chemical Company; oxotremorine, scopolamine, and pilocarpine, Aldrich Chemical Company; carbamylcholine, Merck Sharp & Dohme; 3-quinuclidinyl benzilate, Edgewood Arsenal, Md.

RESULTS

Inhibition of [^{3}H]QNB binding by drugs. In typical experiments 2–5 mg of longitudinal muscle of the guinea pig ileum were incubated with about 2000 cpm of [^{3}H]QNB (0.9 nM), and about 300 cpm of total binding occurred. When preparations were first in-

TABLE 1

[³H]QNB binding to longitudinal muscle of guinea pig ileum

Fifty microliters of a 10% homogenate (2.5 mg of tissue) of longitudinal muscle of guinea pig ileum were incubated in 2 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.4) with 5 μl of [³H]QNB (0.9 nM, 2000 cpm) for 60 min at 25°. Simultaneously, separated samples containing unlabeled QNB (0.01 μM) or oxotremorine (100 μM) were incubated to determine nonspecific binding of [³H]QNB. After incubation, the contents were poured through a glass filter (GF/B) positioned over a vacuum, and the filters were washed four times with 3 ml of sodium-potassium phosphate buffer. Negligible binding of [³H]QNB (less than 20 cpm) occurred to the filters when tissue was omitted from the incubation medium. Specific [³H]QNB binding is defined as total [³H]QNB bound minus the binding in the presence of either 0.01 μM unlabeled QNB or 100 μM oxotremorine. Data are presented as radioactivity of individual samples uncorrected for a counting efficiency of 40%.

Incubation conditions	Total [³H]QNB bound			Specific [³H]QNB bound		
	cpm			*cpm*		
[³H]QNB (0.9 nM)	291	281	285			
[³H]QNB (0.9 nM) + oxotremorine (100 μM)	36	30	39	255	250	246
[³H]QNB (0.9 nM) + unlabeled QNB (0.01 μM)	29	26	37	262	255	248

cubated with 100 μM oxotremorine or 0.01 μM unlabeled QNB, binding was reduced to about 30–40 cpm (Table 1). Negligible binding (less than 20 cpm) to filters occurred when tissue was omitted from the incubation procedure. Accordingly, specific [³H]QNB binding was defined as total binding minus the binding in the presence of 0.01 μM unlabeled QNB or 100 μM oxotremorine, both of which provided the same values. The ratio of specific to nonspecific binding under these conditions was about 8:1. About 10% of the [³H]QNB added to the incubation mixture was bound in a specific fashion.

If specific [³H]QNB binding represents primarily an interaction with muscarinic cholinergic receptors, one would expect muscarinic antagonists and agonists to inhibit the binding, while nicotinic and noncholinergic drugs should have negligible affinity. Experiments in which we examined the inhibition of [³H]QNB binding by adding progressively higher concentrations of unlabeled QNB or oxotremorine indicated that the binding of 0.06 nM [³H]QNB was inhibited 50% by about 0.2–0.3 nM nonradioactive QNB and by 0.5–0.8 μM oxotremorine (Fig. 1). Scopolamine appeared to be as potent, and atropine somewhat less potent, than QNB, causing 50% inhibition of [³H]QNB binding at 0.2–0.3 nM and 3–4 nM concentrations, respectively (Table 2). The muscarinic antagonist isopropamide was was about half as potent as atropine, giving 50% displacement at 6–7 nM. Muscarinic agonists were considerably less potent than the antagonists. Of the muscarinic agonists tested, the most potent in inhibiting [³H]-QNB binding were oxotremorine and pilocarpine, which gave 50% inhibition at 0.5–0.8 μM and 0.7–0.9 μM, respectively. Arecoline had about the same potency as acetylcholine and acetyl-β-methylcholine (about 1–4 μM), while carbamylcholine displayed about one-tenth the potency of acetylcholine. For

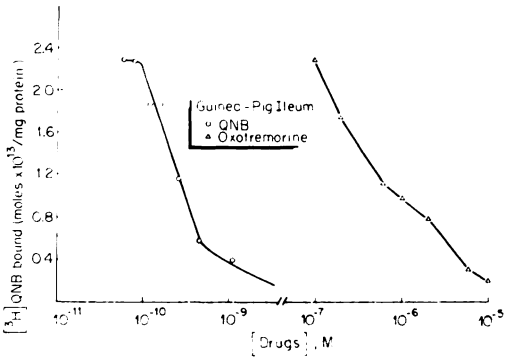

FIG. 1. *Inhibition of specific [³H]QNB binding by various concentrations of unlabeled QNB* (○——○) *and oxotremorine* (△——△).

The incubation medium contained 384 μg of protein of longitudinal muscle homogenate, [³H]-QNB (0.06 nM), and the indicated amounts of unlabeled QNB or oxotremorine in 20 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.4). The mixture was incubated for 60 min at 25°. Specific QNB binding was determined as described in the text. The experiment was replicated three times.

TABLE 2

Relative potencies of drugs in reducing [³H]QNB binding to longitudinal muscle of guinea pig ileum

The following drugs had no effect at 1×10^{-5} M: methylphenidate, γ-aminobutyric acid, glutamic acid, aspartic acid, proline, hemicholinium-3, choline, pempidine, glycine, naloxone, nicotine, hexamethonium, *d*-tubocurarine, dihydro-β-erythroidine, mecamylamine, dimethylphenylpiperizium, diazepam, chlordiazepoxide, and Δ⁹-tetrahydrocannabinol.

Drugs	[³H]QNB binding (ID_{50})[a]	Smooth muscle contraction[b]
	M	*M*
3-Quinuclidinyl benzilate	$2\text{–}3 \times 10^{-10}$	5×10^{-10}[c]
Scopolamine	$2\text{–}3 \times 10^{-10}$	3×10^{-10} (12)
Atropine	$3\text{–}4 \times 10^{-9}$	1×10^{-9} (12)
Methylatropine	$1\text{–}2 \times 10^{-10}$	5×10^{-10} (12)
Isopropamide	$6\text{–}7 \times 10^{-9}$	
Oxotremorine	$5\text{–}8 \times 10^{-7}$	
Pilocarpine	$7\text{–}9 \times 10^{-7}$	$2\text{–}9 \times 10^{-6}$ (21, 22)
Arecoline	$3\text{–}4 \times 10^{-6}$	
Acetylcholine[d]	$2\text{–}4 \times 10^{-6}$	$1\text{–}2 \times 10^{-6}$ (21, 23)
Carbamylcholine	$2\text{–}3 \times 10^{-5}$	2×10^{-5} (21)
Acetyl-β-methylcholine[d]	$2\text{–}3 \times 10^{-6}$	3×10^{-6} (21)

[a] Concentration of drug which inhibited specific [³H]QNB binding by 50% under equilibrium conditions.

[b] Values listed are the dissociation constants (K_D) obtained by pharmacological procedures using intact smooth muscle preparations. The smooth muscle used in refs. 12, 20, and 21 was the longitudinal muscle of guinea pig ileum, while that used in ref. 23 was the circular muscle of the rabbit stomach fundus.

[c] This dose of QNB inhibits the acetylcholine-induced contractions of the longitudinal muscle of the guinea pig ileum 50% after 30 min of exposure to QNB at 37° in oxygenated Krebs-Ringer bicarbonate buffer. Full response to acetylcholine returns 2 hr after removing QNB.

[d] Correction for spontaneous hydrolysis were not made; however, physostigmine (1 μM) was added to the incubation medium to prevent enzymatic (acetylcholinesterase) hydrolysis.

many of the antagonists and agonists tested, the concentrations which inhibited 50% of [³H]QNB binding were very similar to the corresponding dissociation constants of these agents with muscarinic receptors as determined in pharmacological procedures on intact smooth muscle preparations (Table 2). The pharmacological response to a 30-min exposure of QNB (0.3 nM) was long-lived, such that about 60 min were required for 50% recovery at 37° of the contractile response to acetylcholine (1 μM).

Nicotinic cholinergic drugs such as hexamethonium, *d*-tubocurarine, pempidine, nicotine, dihydro-β-erythroidine, mecamylamine, and dimethylphenylpiperizium had little effect on [³H]QNB binding when employed in 10 μM concentrations. A variety of non-cholinergic drugs, including methylphenidate, γ-aminobutyric acid, glutamic acid, aspartic acid, proline, Δ⁹-tetrahydrocannabiol, choline, glycine, naloxone, diazepam, and chloridazepoxide, had negligible effects at 10 μM.

Saturation of [³H]QNB binding. The specific binding of [³H]QNB was saturable with increasing concentrations (Fig. 2). Half-maximal binding occurred at about 0.3 nM. Since about 90% of the [³H]QNB in the medium was in the free form, corrections were not made for the [³H]QNB bound. Nonspecific binding, measured in the pres-

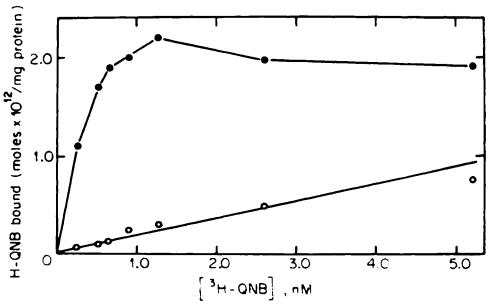

FIG. 2. *Specific binding of [³H]QNB to longitudinal muscle of guinea pig ileum as a function of concentration of QNB*

Tissue (0.14 mg) was incubated at 25° for 60 min in 2.0 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.4) with various concentration of [³H]QNB. Specific (●——●) and nonspecific (○——○) binding of QNB were determined as described in the text. The experiment was replicated three times.

ence of 100 μM oxotremorine, did not saturate and increased linearly with higher concentrations of [^{3}H]QNB. Since half-maximal saturation of specific [^{3}H]QNB binding occurred at about the same concentration as half-maximal displacement with nonradioactive QNB, it appears that the [^{3}H]QNB was biologically equivalent to the nonradioactive drug, in terms of receptor binding. This equivalence also confirms the validity of the determined specific activity of [^{3}H]-QNB.

Maximal inhibition by oxotremorine was the same as that obtained with QNB, which is consistent with their interaction at the same receptor site.

Association and dissociation of [^{3}H]QNB binding. With a reversible ligand such as [^{3}H]QNB the kinetics of association and dissociation can be studied directly. At 35° specific [^{3}H]QNB binding to homogenates of the longitudinal muscle of the guinea pig ileum occurred rapidly. Binding reached half-maximal values in 2.5 min and attained a plateau by about 10–30 min. By contrast, nonspecific QNB binding, measured in the presence of 100 μM oxotremorine, was not time-dependent, and was only about 10% of specific [^{3}H]QNB binding (Fig. 3). The bimolecular rate constant for specific [^{3}H]-QNB-receptor association (k_1) was 4×10^8 M^{-1} $min.^{-1}$

The dissociation of the [^{3}H]QNB receptor complex was examined by incubating ileum homogenates with [^{3}H]QNB at 25° for 60 min. After this period of time the preparation was incubated at 25° with either 100 μM oxotremorine or 0.01 μM nonradioactive QNB, and the decline of bound [^{3}H]QNB was monitored (Fig. 4). In preliminary experiments in which dissociation was examined at 25°, we found the dissociation of [^{3}H]QNB to be very slow, so that all dissociation experiments were performed at 35°. When plotted semilogarithmically, the half-life for dissociation of the [^{3}H]QNB-receptor complex at 35° was about 58 min. The rate constant for dissociation (k_{-1}) at 35° was 1.2×10^{-2} min^{-1}. The dissociation constant (K_D) determined by the ratio k_{-1}/k_1 was 0.03 nM.

[^{3}H]QNB binding in various peripheral tissues. We assayed a variety of peripheral tissues for specific [^{3}H]QNB binding (Table 3). Specific QNB binding, inhibited by 100 μM oxotremorine and 0.01 μM QNB, could be demonstrated in the spleen, heart, and lung. However, no specific [^{3}H]QNB binding was detected in the liver, diaphragm, or

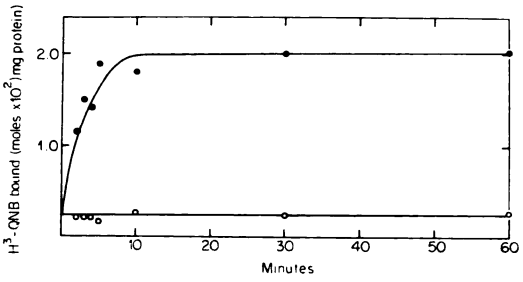

FIG. 3. *Rate of association of [^{3}H]QNB with longitudinal muscle of guinea pig ileum*

The incubation medium contained 140 μg of protein in 2.0 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.4) and 0.9 nM [^{3}H]QNB. Specific and nonspecific binding were determined at various times at 35° as described in the text. The experiment was replicated three times.

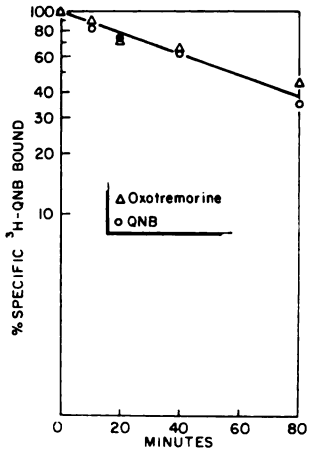

FIG. 4. *Rate of dissociation of [^{3}H]QNB from longitudinal muscle of guinea pig ileum*

The incubation medium contained 140 μg of protein in 2.0 ml of 0.05 M sodium-potassium phosphate buffer (pH 7.4) with 0.9 nM [^{3}H]QNB. After incubation for 60 min at 25°, nonradioactive QNB (0.01 μM) or oxotremorine (100 μM) was rapidly added, and samples were either filtered immediately (zero time) or maintained for various intervals at 35° before rapid cooling and filtration. The experiment was replicated three times.

TABLE 3

Specific [³H]QNB binding to peripheral tissues of guinea pig

Specific [³H]QNB binding was assayed as described in the text and represents total [³H]QNB bound minus binding in the presence of 0.01 μM unlabeled QNB or 100 μM oxotremorine. Specific [³H]QNB binding values represent the means of data obtained in triplicate from three separate experiments.

Peripheral tissue	Specific [³H]QNB bound		
	pmoles/mg protein		
Longitudinal muscle of ileum	1.88	2.08	1.93
Ileum minus longitudinal muscle portion	0.185	0.194	0.131
Heart	0.145	0.133	0.173
Spleen	0.097	0.063	0.020
Lung	0.028	0.049	0.040
Liver	Not detectable		
Kidney	Not detectable		
Diaphragm	Not detectable		

kidney, despite attempts utilizing tissue concentrations ranging from 2 to 20 mg of protein. Specific QNB binding in the guinea pig heart was only about one-tenth of that of the longitudinal muscle of the ileum. Binding in the spleen and lung were only about one-half and one-fourth, respectively, of that in the heart.

The ileum wall, from which the longitudinal muscle is obtained, contains circular muscle and also an abundance of connective tissue, mucosa, and submucosa, and might be expected to be relatively deficient in cholinergic receptors. Specific [³H]QNB binding in the ileum from which the longitudinal muscle had been removed was only about 10% of that in the longitudinal muscle itself (Table 3).

DISCUSSION

A variety of evidence indicates that the specific binding of [³H]QNB to longitudinal muscle homogenates of the guinea pig ileum represents an interaction with postsynaptic muscarinic cholinergic receptors. The relative affinity of a number of muscarinic cholinergic drugs closely parallels the estimated affinity for muscarinic receptors in the guinea pig ileum, based on pharmacological procedures (12, 23). Thus QNB and scopolamine are about 8–10 times more potent than atropine in inhibiting specific [³H]QNB binding. Similarly, among muscarinic agonists there is also a close parallelism between affinity for the QNB binding sites and estimated affinity for muscarinic receptors in the guinea pig ileum, based on pharmacological procedures (12, 21, 23). Oxotremorine, a very potent muscarinic agonist, has about 4 times the affinity of acetylcholine for QNB binding sites. By contrast, a variety of nicotinic cholinergic and noncholinergic drugs have very little affinity for the QNB binding sites.

Specific [³H]QNB binding in the guinea pig ileum resembles QNB binding previously described in homogenates of mammalian brain (18). The regional distribution of specific QNB binding in rat (18) and monkey (24) brain parallels the distribution of choline acetyltransferase. Relative affinities of muscarinic agonists and antagonists are quite similar in the two systems. The affinity constants for QNB determined both by kinetic techniques and by equilibrium data are about the same in both brain and intestine. There is an order-of-magnitude difference between the equilibrium constants for [³H]QNB determined from equilibrium measurements and from kinetic measurements. This may be due to limitations in the technical procedure in measuring rapid association rates.

Of a variety of peripheral tissues examined, specific [³H]QNB binding was detected in the spleen, heart, and the lung, but not in kidney, liver, or diaphragm. The diaphragm represents a tissue in which cholinergic receptors are thought to be wholly nicotinic, which is consistent with our inability to demonstrate muscarinic receptors.

From the direct measurement of specific [³H]QNB binding at saturation, we calculate that 1 g of longitudinal muscle of guinea pig ileum has sufficient receptors to bind 19×10^{-11} mole of [³H]QNB. Assuming that each [³H]QNB molecule interacts with one receptor site, we calculate the number of receptor units in 1 g of ileum longitudinal muscle to be about 10^{14} This figure is about

the same as that determined using alkylating muscarinic antagonists derived from benzilylcholine (13, 25). The calculated number of muscarinic receptors in guinea pig ileum is about 2–3 times the calculated density of muscarinic cholinergic receptors in rat brain as estimated by specific [^{3}H]QNB binding (18), and in the same range as the value obtained for brain muscarinic receptors using other ligands (25–27). It is more than twice the calculated concentration of nicotinic cholinergic receptors in the electric organ of the electric eel, but only about a third the number of such receptors in the electric organ of *Torpedo* (28). The muscarinic receptor density of guinea pig ileum is only slightly higher than the calculated concentration of glycine receptors in rat spinal cord (29) and about 5 times the density of specific opiate receptor binding sites in rat brain (30).

In terms of pharmacological specificity, the apparent muscarinic cholinergic receptor sites labeled by [^{3}H]QNB appear similar to those labeled by a benzilylcholine mustard homologue (14).

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