

[³H]-QUINUCLIDINYL BENZILATE BINDING TO MUSCARINIC RECEPTORS IN RAT BRAIN: COMPARISON OF RESULTS FROM INTACT BRAIN SLICES AND HOMOGENATES

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- 1 The binding of [³H]-(\pm)-quinuclidinyl benzilate ([³H]-(\pm)-QNB) to muscarinic sites in rat brain slice and homogenate preparations was compared.
- 2 Evidence is presented in support of the view that only the (-)-enantiomer of QNB binds with high affinity to muscarinic sites.
- 3 The K_d value for [³H]-(-)-QNB binding in slices was eight times higher than that measured in homogenates.
- 4 Similarly, the potencies of various muscarinic ligands as inhibitors of [³H]-(-)-QNB binding were consistently lower in slices than in homogenates.
- 5 It is proposed that the results may reflect differences in the binding properties of muscarinic receptors in intact tissue slice and homogenate preparations.

Introduction

[³H]-quinuclidinyl benzilate (QNB) has been widely used as a specific ligand for muscarinic acetylcholine receptors in CNS and in peripheral tissues (Yamamura & Snyder, 1974a, b; Strange, Birdsall & Burgen, 1978). Hitherto, most studies with this ligand have involved the use of cell-free membrane fragment preparations, obtained by vigorous homogenization of brain or peripheral tissue, although Yamamura, Kuhar & Snyder (1974) showed that [³H]-QNB could be used to label muscarinic receptors in the intact rat brain after systemic administration *in vivo*. Recent results with another muscarinic ligand, [³H]-propylbenzilylcholine mustard (Burgen, Hiley & Young, 1974), show that the binding characteristics measured in intact intestinal muscle strips differ significantly from those measured in cell-free homogenates (Ward & Young, 1977). In the present study we have explored the possibility of measuring specific binding of [³H]-QNB to muscarinic sites in slices of intact rat brain, and have compared these results with data obtained from cell-free homogenates of the same tissue.

The rat corpus striatum and hippocampus were chosen since they contain relatively high densities of muscarinic binding sites (Yamamura & Snyder, 1974a). Since QNB is optically active, and it is known that the (-)-enantiomer is considerably more potent as an antimuscarinic agent than the (+)-enantiomer (Meyerhoffer, 1972), we have also taken into account the stereoselectivity of the interaction of [³H]-(\pm)-

QNB with CNS binding sites. Our results indicate that only the (-)-enantiomer of the labelled drug contributes significantly to the observed binding, and confirm that there are significant differences in the binding characteristics observed in slices versus brain homogenates.

Methods

[³H]-QNB binding to rat brain homogenates

These experiments were performed essentially as described by Yamamura & Snyder (1974a). Male Wistar rats (100 to 200 g) were decapitated, and their brains rapidly removed and chilled. Corpus striatum and hippocampus were dissected (Glowinski & Iversen, 1966) and homogenized in 10 vol (w/v) of ice-cold 0.32 M sucrose in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged for 10 min at 1000 *g*. The pellet (crude nuclear fraction) was discarded, and the supernatant diluted 40 to 120 fold with 0.05 M sodium-potassium phosphate buffer pH 7.4, or with Krebs-bicarbonate solution pH 7.4. The diluted homogenate was further homogenized with a Polytron (setting No. 5, 60 s), and used for [³H]-QNB binding studies.

Samples of diluted homogenate (2 ml) were incubated at 25°C for 60 min with various concentrations of [³H]-QNB. At the end of the incubation, 3 ml of ice-cold 0.05 M Na-K phosphate buffer pH 7.4, or

Krebs-bicarbonate solution was added, and the samples passed through a glass fibre filter (Whatman GF/B) under vacuum. The filters were washed twice under vacuum with 3 ml of ice-cold buffer. Each determination of binding was performed in triplicate, together with triplicate samples containing 1 μ M atropine to estimate non-specific binding.

Each filter was placed in a scintillation vial, 4 ml of ethoxyethanol added and the vial sealed and left overnight. Then 10 ml of 0.4% butyl-PBD in toluene was added, and the radioactivity counted by liquid scintillation spectrometry.

[³H]-QNB binding to brain slices

The method used was a modification of that described by Hanley & Iversen (1978). Striatal or hippocampal tissue was cross chopped at 250 μ m intervals with a McIlwain tissue chopper. The slices (80 to 100 mg wet weight from one rat brain) were incubated at 37°C in 50 ml Krebs-bicarbonate solution pH 7.4 for 20 to 30 minutes. The supernatant was then decanted and the tissue concentration adjusted to 200 mg/ml. Twenty-five μ l of the suspension (5 mg tissue) was added to a 25 ml conical flask containing 4 ml Krebs-bicarbonate solution and 25 μ l of [³H]-QNB solution was added. For each concentration of [³H]-QNB, triplicate determinations of binding were made, and also triplicate determinations of non-specific binding in the presence of 1 μ M atropine. The flasks were incubated at 37°C with continuous shaking for 30 minutes. At the end of the incubation, the contents of the flasks were filtered rapidly through 25 mm diameter glass fibre filters (Whatman GF/B) under vacuum. The flasks were rinsed with 4 ml Krebs-bicarbonate solution which was passed through the filters. The tissue slices and filters were washed rapidly with 2 \times 4 ml Krebs-bicarbonate solution under vacuum. The filters were prepared for liquid scintillation counting as described for homogenate binding experiments.

Inhibition of [³H]-QNB binding by muscarinic drugs

In order to compare the inhibition of specific [³H]-QNB binding by muscarinic drugs in striatal slices and homogenates, various concentrations of each drug were included in the incubation media (Krebs-bicarbonate solution in each case), together with a fixed concentration of 1.5 nM [³H]-(\pm)-QNB.

Each drug was tested at 4 to 8 different concentrations, and each concentration was tested on triplicate samples. IC₅₀ values were estimated from log/probit analysis of dose-response curves relating percentage inhibition of specific [³H]-QNB binding to drug concentration and the mean values determined

from 3 to 5 separate experiments were used to calculate values for the apparent inhibition constant (K_i) presented in Table 2. Apparent K_i values were calculated from the formula:-

$$K_i = (IC_{50}) / (1 + S/K_d)$$

where S = concentration of [³H]-(-)-QNB = 0.75 nM.

[³H]-(\pm)-QNB (13 Ci/mmol) was obtained in ethanol solution from the Radiochemical Centre, Amersham. [³H]-QNB moved as a single peak with authentic QNB in high voltage electrophoresis (100 V/cm; citric acid/phosphate buffer, pH 3.0). Other drugs were obtained as follows: oxotremorine sesquifumarate from Aldrich Chemical Co. Inc; arecoline hydrobromide, pilocarpine hydrochloride, carbamylcholine hydrochloride (carbachol) and atropine sulphate from Sigma Chemical Co.

Results

Stereoselectivity of [³H]-QNB binding

It has been reported that only the (-)-isomer of (+)-QNB possesses significant antimuscarinic activity *in vivo* (Meyerhoffer, 1972). This has been confirmed in binding studies (Kloog & Sokolovsky, 1977) in which (-)-QNB was found to be approximately 100 times more potent than (+)-QNB in displacing the specific binding of [³H]-N-methyl-4-piperidylbenzilate to muscarinic sites in brain homogenates. It seems likely, therefore, that when [³H]-(\pm)-QNB is used to label muscarinic sites only the (-)-isomer is involved. This hypothesis was supported by the finding that a maximum of approximately 50% of the total [³H]-(\pm)-QNB added was specifically bound when incubations were performed with 0.2 nM [³H]-QNB and various concentrations of striatal tissue homogenate (Figure 1). In calculations of the constants for [³H]-QNB binding to slices and homogenates we have, therefore, assumed that only the active (-)-isomer is involved. This is an important consideration in estimating free ligand concentrations, since under some circumstances when low concentrations of [³H]-(\pm)-QNB are used, a high proportion of the active (-)-isomer may be specifically bound, although the inactive (+)-isomer remains free. We have also assumed that non-specific binding is not stereospecific. However, even if non-specific binding involved only [³H]-(-)-QNB, this would not materially alter our calculations since the amount of non-specific binding in both slice and homogenate experiments was never greater than 10% of the amount of [³H]-(-)-QNB present.

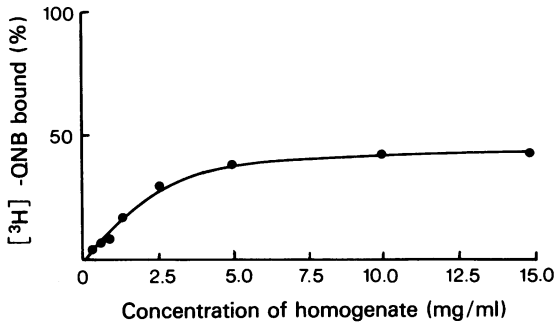


Figure 1 Specific binding of [³H]-(\pm)-quinuclidinyl benzilate ([³H]-(\pm)-QNB) as a function of tissue concentration; % of total label added (0.2 nM [³H]-(\pm)-QNB) bound against tissue concentration (striatal homogenate).

[³H]-QNB binding in homogenates

As described previously (Yamamura & Snyder, 1974a) specific binding of [³H]-QNB in rat striatal and hippocampal homogenates incubated in phosphate buffer represented a major proportion of total binding (Figure 2). Specific binding, but not non-specific binding, was saturable, and Scatchard analysis indicated a K_d for (-)-QNB of 0.042 nM in striatum and 0.025 nM in hippocampus (Table 1). If the same results were used to calculate apparent K_d values for [³H]-(\pm)-QNB the values obtained were considerably higher (0.24 nM in striatum and 0.1 nM in hippocampus). The latter values are similar to the concentrations (0.3 to 0.5 nM) of [³H]-(\pm)-QNB found to cause 50% saturation of specific binding sites in rat brain or guinea-pig ileum by Yamamura & Snyder (1974a, b). Their estimates of the K_d for QNB, however, based on measurements of rates of association and dissociation were 0.03 to 0.06 nM, values which are in close agreement with our estimates of K_d for (-)-QNB from saturation analysis. This result is now explicable, since measurements of association and dissociation presumably reflect only the behaviour of the active (-)-isomer.

As reported by Yamamura & Snyder (1974a) the density of specific QNB binding sites in striatal homogenates was approximately twice that observed in hippocampal homogenates, although the absolute values are higher than found by these authors (Table 1).

When tissue homogenates were incubated in Krebs bicarbonate solution instead of phosphate buffer, the maximum specific binding was unchanged, but the estimated K_d values for (-)-QNB were higher than those observed in phosphate buffer (Table 1), an effect which may be attributed to the difference in ionic strength of the two media (Birdsall, Burgen, Hulme & Wells, 1977).

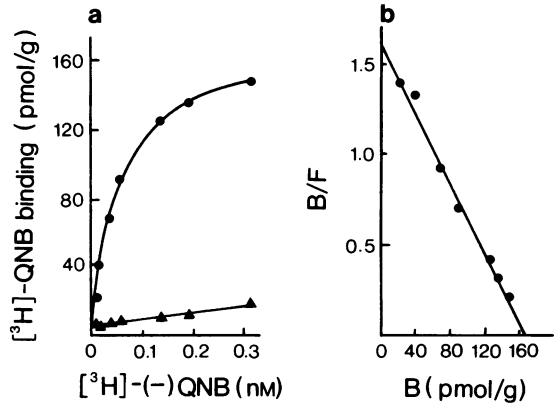


Figure 2 (a) Specific (●) and non-specific (▲) components of [³H]-(-)-quinuclidinyl benzilate ([³H]-(-)-QNB) binding to a homogenate of rat corpus striatum in 0.05 M Na-K phosphate buffer. (b) Scatchard plot of specific binding data. Calculated K_d for (-)-QNB = 0.042 nM.

[³H]-QNB binding in tissue slices

The general characteristics of [³H]-QNB binding to striatal slices have already been described in a previous publication (Hanley & Iversen, 1978). Specific binding of [³H]-QNB occurred rapidly, and reached saturation within 30 min at 37°C. Non-specific binding accounted for a higher proportion of total binding than in homogenate experiments, and furthermore the amount of non-specific binding increased linearly with incubation time (Hanley & Iversen, 1978). Specific binding, measured after a standard incubation of 30 min was at least as great as non-specific binding except at the highest ligand concentrations tested, and at low concentrations of [³H]-QNB accounted for as much as 80% of total binding in striatal slices. Specific binding, but not non-specific binding was saturable (Figure 3) and the estimated K_d values for (-)-QNB were 0.52 nM for striatal tissue and 0.35 nM for hippocampal tissue (Table 1). These values are 7 to 9 times higher than those estimated for (-)-QNB binding in homogenates of these tissues (Table 1).

The maximum density of specific QNB binding sites in striatal slices was approximately 2.5 times higher than in hippocampal slices, and the absolute values estimated from slice binding experiments were similar to those estimated in homogenates (Table 1) when results were expressed per unit weight of brain tissue.

In brain slices, as in homogenates, the specific binding of [³H]-QNB could be described by simple mass action kinetics, since Hill coefficients were close to 1.0.

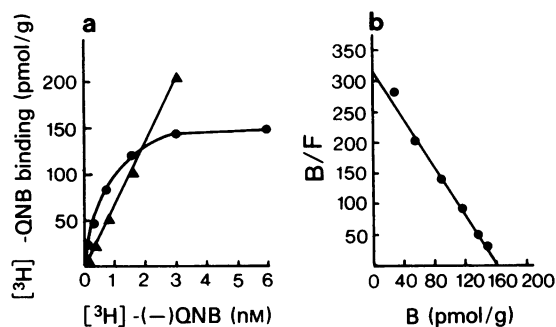


Figure 3 (a) Specific (●) and non-specific (▲) components of [³H]-(-)-quinuclidinyl benzilate ([³H]-(-)-QNB) binding to slices of rat corpus striatum in Krebs-bicarbonate solution. (b) Scatchard plot of specific binding data. Calculated K_d for (-)-QNB = 0.52 nM.

Inhibition of [³H]-QNB binding in striatal slices and homogenates by muscarinic drugs

Five drugs were tested in striatal slice and homogenate preparations for their ability to inhibit specific [³H]-QNB binding. Each drug was tested at several concentrations and IC_{50} values were estimated from the dose-response curves and K_i values calculated as described in Methods. The calculated K_i values (Table 2) for both agonists and antagonists were consistently 5 to 10 times higher in slice experiments than in homogenates.

Discussion

The first experimental approach to ligand binding studies for muscarinic receptors was the now classic study of Paton & Rang (1965) who measured the binding of [³H]-atropine to intact strips of intestinal smooth muscle. Since then a variety of reversible and

irreversible ligands have been used to identify and characterize muscarinic binding sites in peripheral tissues and in CNS (for review see Birdsall & Hulme, 1976). In most cases such studies have been performed with tissue homogenates, although some recent studies have used intact preparations of intestinal smooth muscle (Burgen *et al.*, 1974; Ward & Young, 1977). In a previous publication (Hanley & Iversen, 1978) we described the application of [³H]-QNB binding to studies of muscarinic receptors in intact slices of corpus striatum from rat brain. In the present paper we describe a more detailed comparison of the properties of [³H]-QNB binding in slices and homogenates of two regions of rat brain. In addition we have taken into consideration the stereoselectivity of QNB binding in estimates of its binding parameters. The finding that only approximately half of the added labelled ligand appears to be available for specific binding to striatal homogenates strongly supports the view that only the pharmacologically active (-)-isomer is involved in specific binding to muscarinic sites. This has an important influence on the kinetic constants calculated for saturable QNB binding, and may, for example, explain why previous estimates of K_d values for [³H]-QNB based on saturation analysis and by measurements of association and dissociation rate constants have shown only poor agreement (Yamamura & Snyder, 1974a, b).

As described previously (Hanley & Iversen, 1978), specific binding of [³H]-QNB can be measured readily in intact slices of brain tissue incubated *in vitro*. This is not surprising, since specific binding of [³H]-QNB to muscarinic sites in rat brain can be demonstrated even after administration of the labelled drug *in vivo* (Yamamura *et al.*, 1974), and specific binding of [³H]-propylbenzilylcholine mustard ([³H]-PrBCM) to muscarinic sites in rat brain slices has been demonstrated autoradiographically (Rotter, Birdsall, Burgen, Field & Raisman, 1977).

Table 1 Parameters for specific [³H]-quinuclidinyl benzilate ([³H]-QNB) binding in rat brain slices and homogenates

Tissue and experimental conditions	K_d (nM)	B_{max} (pmol/g wet wt.)
<i>Striatum</i>		
Homogenate-phosphate buffer	0.042 ± 0.004 (3)	161.7 ± 5.0 (3)
Homogenate-Krebs bicarbonate	0.060 (2)	142.7 (2)
Slices-Krebs bicarbonate	0.532 ± 0.055 (3)	170.8 ± 6.7 (3)
<i>Hippocampus</i>		
Homogenate-phosphate buffer	0.025 (2)	67.4 (2)
Homogenate-Krebs bicarbonate	0.050 (1)	77.2 (1)
Slices-Krebs bicarbonate	0.350 (1)	68.0 (1)

Values for dissociation constant (K_d) and maximum specific binding (B_{max}) were derived from Scatchard analysis of saturation curves, as illustrated in Figures 2 and 3. Values are means ± s.e. mean for the number of experimental determinations indicated in parentheses.

Table 2 Comparison of inhibition of [³H]-quinuclidinyl benzilate ([³H]-QNB) binding in slices and homogenates of rat striatum by muscarinic drugs

Drug	n	Calculated K_i value for inhibition of specific [³ H]-QNB binding (nM)			Ratio S/H
		Slices	n	Homogenates	
Atropine	5	3.47 ± 0.62	5	0.42 ± 0.06	8.3
Scopolamine	4	5.78 ± 0.48	3	0.95 ± 0.13	6.1
Oxotremorine	3	8,780 ± 2,380	3	810 ± 230	10.8
Arecoline	4	119,600 ± 55,600	3	11,630 ± 3,440	10.3
Carbachol	3	227,500 ± 136,800	4	43,570 ± 2,420	5.2

Drugs were tested for their ability to inhibit specific [³H]-QNB binding in slices or homogenates of rat striatum incubated with 1.5 nM (±)[³H]-QNB. K_i values were calculated from IC₅₀ data obtained by log-probit analysis of dose-response curves for inhibition of QNB binding, involving triplicate analysis of at least 4 concentrations of each test drug. Results are means ± s.e. mean for number (n) of separate experiments of this type indicated.

Our results are in contrast, however, to those of Strange *et al.* (1978) who were unable to detect saturable binding of [³H]-QNB to intact neuroblastoma cells, although binding to membranes of the same cells could readily be measured.

However, there appeared to be substantial differences between slices and homogenates in the apparent affinity of [³H]-QNB binding sites. In both striatal and hippocampal tissue K_d values in slices were approximately 8 times higher than those estimated in homogenates incubated in Krebs-bicarbonate solution. Furthermore, muscarinic agonists and antagonists were consistently less potent in inhibiting [³H]-QNB binding in striatal slice preparations than in homogenates. Ward & Young (1977) similarly reported that muscarinic drugs were less potent in inhibiting the binding of [³H]-PrBCM to intact intestinal muscle strips than in cell free homogenate preparations. They suggested that differences could probably be explained, at least in part, in terms of access-limitation factors for the penetration of the ligand and the test drugs to muscarinic sites in such intact tissue preparations.

Such factors could possibly account for the differences we have observed between slice and homo-

genate preparations of brain tissue, but we do not feel this to be a very satisfactory explanation, for the following reasons:- (1) In the present studies, unlike those of Ward & Young (1977) who measured the rate of binding of an irreversible ligand, we measured the binding of a reversible ligand under equilibrium conditions. (2) QNB is a lipophilic drug which penetrates readily into mammalian CNS even after systemic administration (Yamamura *et al.*, 1974); thus, it seems unlikely that serious penetration barriers would exist in brain slices of the relatively small dimensions used in the present experiments. (3) The effective exposure of muscarinic sites in brain slices to the muscarinic ligand was confirmed by the finding that the maximum number of binding sites was similar in slices and homogenates.

It seems possible, therefore, that the differences in muscarinic binding obtained with intact brain slices and with homogenates may reflect real differences in the characteristics of such binding in intact tissue versus broken cell preparations.

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