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[^{3}H]QUINUCLIDINYL BENZILATE BINDING TO MUSCARINIC RECEPTORS AND [^{3}H]WB-4101 BINDING TO ALPHA-ADRENERGIC RECEPTORS IN RABBIT IRIS

COMPARISON OF RESULTS IN SLICES AND MICROSOMAL FRACTIONS*

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Abstract—The binding characteristics of [^{3}H]-(*l*)-quinuclidinyl benzilate (QNB) and [^{3}H]WB-4101 to microsomal fractions and slices from rabbit iris muscle were compared. [^{3}H] QNB binding to both microsomal fractions and muscle slices was of high affinity and low capacity and was displaced by muscarinic ligands. The equilibrium dissociation constants (K_D) for [^{3}H]QNB binding to microsomes and slices were 0.069 nM and 1.97 nM, respectively. This shift to a higher value for the K_D of the microsomal fraction compared with that of the slices was also observed for the association rate constants (K_1) and inhibition constants (K_I), but not for the dissociation rate constants (K_{-1}). Kinetic studies on the binding characteristics of [^{3}H]WB-4101 revealed high affinity sites with K_D values of 2.33 and 10.19 nM for microsomal fractions and slices, respectively. The findings of comparable binding patterns for [^{3}H]QNB and [^{3}H]WB-4101 binding to microsomal fractions and intact muscle slices argue against the possibility of alterations in receptor properties following tissue disruption. It is proposed that the differences in receptor-mediated biochemical responses that are seen between intact tissue and cell-free homogenates, such as the 'phosphoinositide effect', are more likely to be due to alterations in receptor function, e.g. changes in ionic permeabilities, rather than to actual changes in receptor properties.

The availability of radioligands of high specific radioactivity has provided the means for identification and characterization of muscarinic and alpha-adrenergic receptors in a variety of tissues. Among these radioligands, the potent cholinergic antagonist [^{3}H]-(*l*)-quinuclidinyl benzilate (QNB) has been used to characterize muscarinic binding sites in membranes from both the central [1, 2] and peripheral [3–7] nervous systems. Similarly, the adrenergic antagonist [^{3}H]WB-4101 has been used to characterize alpha-adrenergic binding sites in membranes from both the central [8–10] and peripheral [11, 12] nervous systems.

The iris muscle of the rabbit is innervated by cholinergic and adrenergic fibers [13], and the presence of both types of receptors has been demonstrated through both pharmacological [14–16] and binding studies [17–19]. Previously we reported that the neurotransmitters acetylcholine [20] and norepinephrine [21] at 0.05 mM and at short time intervals (<10 min) increase significantly the breakdown of triphosphoinositide and the labeling of phosphatidic acid and phosphatidylinositol in rabbit iris muscle that was prelabeled with $^{32}P_i$. These effects are mediated through muscarinic and alpha-adrenergic receptors [20, 21] and are dependent upon the presence of Ca^{2+} in the incubation medium [22]. Kinetic studies on dose–triphosphoinositide responses and dose–contraction responses suggested a close relationship between the biochemical and pharmacological responses [23]. Efforts to demonstrate this phenomenon in iris muscle homogenates were unsuccessful. This observation is supported by the fact that with the exception of synaptosomes [24, 25] there is no experimental evidence to suggest that this phenomenon does occur in a cell-free homogenate. This could be interpreted as follows: (a) that the phosphoinositide effect is coupled to cholinergic muscarinic and alpha-adrenergic receptors through Ca^{2+} as we have suggested previously [21, 22], and that tissue homogenization leads to disruption of this coupling mechanism; or (b) that muscarinic and alpha-adrenergic receptor properties are altered by the homogenization process. Thus, the primary objective of the present study was to compare the binding characteristics of [^{3}H]QNB to muscarinic sites and of [^{3}H]WB-4101 to alpha-adrenergic sites in microsomal fractions and in muscle slices of the albino rabbit iris, to determine whether receptor property alterations have occurred.

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MATERIALS AND METHODS

Chemicals

The *l*-isomer of [^{3}H]QNB (44 Ci/mmole) was obtained from Amersham/Searle (Arlington Heights, IL) and [^{3}H]WB-4101 (25.4 Ci/mmole) from the New England Nuclear Corp. (Boston, MA). Other drugs used in the present study included *l*-epinephrine bitartrate (CalBiochem, La Jolla, CA), oxotremorine (Aldrich Chemical Co., Milwaukee, WI), phentolamine (CIBA Pharmaceuticals, Summit, NJ) and acetylcholine chloride, atropine sulfate, eserine salicylate, *l*-norepinephrine bitartrate, *dl*-propranolol and *d*-tubocurarine chloride from the Sigma Chemical Co. (St. Louis, MO). Unlabeled *dl*-QNB was a gift from Hoffmann-LaRoche, Inc. (Nutley, NJ). All other chemicals were reagent grade.

Preparation of microsomal fractions from rabbit iris

Tissue for the following experiments was obtained from albino rabbits at a local slaughterhouse. Eyes were enucleated shortly after killing and transported to the laboratory packed in ice.

The methods of homogenization, subcellular fractionation, and monitoring of the purity of the preparations by means of electron microscopy and enzyme markers were essentially as was reported previously [26]. Briefly, the rabbit irises (approximately 5 g wet wt) were rinsed with ice-cold buffered saline (pH 7.4). The muscle strips were blotted and immersed in chilled 0.32 M sucrose (pH 7.4), and minced with scissors. The minced tissue was then suspended in 10 vol. of buffered sucrose and homogenized for 4 × 30 sec using a Super Dispax Tissumizer model SDT-182 (Tekmar Co.) at 2/3 maximum speed. The homogenate was filtered through two layers of cheesecloth, and the filtrate was centrifuged at 1200 *g* for 10 min at 4°. The resultant supernatant fraction was spun at 10,000 *g* for 30 min to pellet a mitochondrial fraction. The supernatant fraction obtained was then centrifuged at 100,000 *g* for 90 min to sediment the microsomal fraction. This microsomal pellet was suspended in 50 mM sodium phosphate buffer (pH 7.4) and divided into aliquots for storage at −20°. These aliquots were rehomogenized in a glass homogenizer, diluted, and used in the binding assay individually. No significant differences were observed between fresh and frozen microsomal preparations. Electron microscopic examination of the microsomal fraction revealed a homogeneous preparation of membranes similar to that described in an earlier report [26].

Binding of [^{3}H]*QNB and* [^{3}H]*WB-4101 to microsomal fractions*

The method used to study the binding of [^{3}H]QNB and [^{3}H]WB-4101 was a modification of the method of Yamamura and Snyder [1, 3]. In brief, the standard binding assay was performed in 0.5 ml of 50 mM sodium phosphate buffer (pH 7.4) containing approximately 50 μg of microsomal protein and the labeled ligand. A second set was also prepared containing in addition an excess of competitor, 5 μM atropine or 100 μM phentolamine, for determining specific binding. Other additions were made as indicated. The binding reaction was initiated upon addition of the protein, and incubations were allowed to proceed for 1 hr at 37° for the cholinergic experiments and for 1 hr at 25° for the adrenergic experiments in a shaking water bath. Incubations were terminated with the addition of 2.5 ml buffer, followed by rapid filtration of the mixture through Whatman GF/C glass fiber filters. Each filter was washed three times with 2.5 ml buffer, and then air-dried in scintillation vials. Eight milliliters of scintillation fluid (1000 ml toluene, 150 mg POPOP and 4 g PPO)* was added to the vials and the radioactivity retained on the filters was determined by a Beckman model LS-230 liquid scintillation counter. Each vial was counted for 5 min, and corrections for quenching were made by a quench curve prepared by channel ratios. Specific receptor binding of [^{3}H]QNB or [^{3}H]WB-4101 is defined as the difference between the total binding of the radioligands and the nonspecific binding observed in the presence of 5 μM atropine or 100 μM phentolamine.

Binding of [^{3}H]*QNB and* [^{3}H]*WB-4101 to iris slices*

Binding studies using the slices were performed with irises that were frozen intact at −20°. No discernible differences were observed between experiments run with frozen and fresh tissues. The standard binding assay was essentially the same as described for the microsomal fraction, except that for cholinergic binding the final incubation volume was 2.5 ml instead of 0.5 ml. The incubation medium contained 15–20 mg wet wt of muscle (half of the total iris), the radioligand, and other additions as indicated. After filtration and washing, the tissue was placed in a scintillation vial, air dried, and weighed. The tissue was digested with 30% H_2O_2 at 65° and the ash was dissolved in 0.1 ml of distilled water. Ten milliliters of scintillation fluid (600 ml toluene, 300 ml Triton X-100, 5 g PPO and 100 mg POPOP) was added and the sample was allowed to stand overnight before counting.

In both of the microsomal and iris slice experiments, the reaction components were adjusted such that the amount of radioligand was at least ten times higher than the amount of radioligand binding. This insured that the initial radioligand concentration did not decrease more than 10 per cent during equilibration. Aliquots of incubation media withdrawn from the tissues, both immediately before and after filtration, confirmed that final radioligand concentration was within 10 per cent of the initial levels. Under these experimental conditions, the free ligand can equal total ligand in Scatchard analysis without significantly affecting the results. All experiments were run in quadruplicate. Protein was determined by the method of Lowry *et al.* [27], using bovine serum albumin as standard.

RESULTS

Binding of [^{3}H]*QNB to microsomal fractions and iris muscle slices*

[^{3}H]*QNB binding to microsomal fractions.* In pre-

* POPOP, 1,4-bis-[2-(4-methyl-5-phenylorazolyl] benzene; and PPO, 2,5-diphenylopazole.

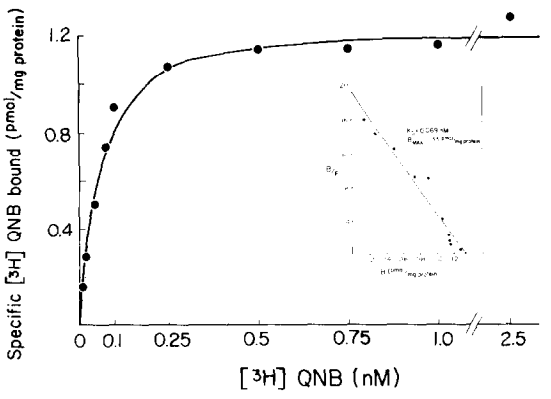

Fig. 1. Specific binding of [^{3}H]QNB at 37° to rabbit iris muscle microsomal fraction as a function of ligand concentration. Microsomal fractions from rabbit iris muscle (about 50 μg) were incubated for 1 hr at 37° with various concentrations of [^{3}H]QNB in a final volume of 0.5 ml. Non-specific binding was measured by addition of 5 μM atropine. Specific binding was determined as the difference between total and non-specific binding at each concentration. Each value is the mean of six to twelve determinations. Inset: Scatchard plot of the specific binding data. The slope obtained by linear regression analysis is −14.45.

liminary experiments on [^{3}H]QNB binding to the crude nuclear supernatant fraction and to the various subcellular fractions of the rabbit iris, we found that the microsomal fraction contains the highest receptor site concentrations. Thus, the microsomal fraction, which contains fragments of plasma membrane, was employed throughout the present study.

[^{3}H]QNB showed two distinct components in its interaction with membrane fragments. The first, non-specific binding, was not displaceable with excess unlabeled atropine and increased linearly with QNB concentration (data not shown). The second component, specific binding, was readily displaced with atropine and was characterized by high affinity, a low number of binding sites, and saturation with increasing radioligand concentration (Fig. 1). As estimated from saturation binding experiments, half-maximal saturation occurred at less than 0.1 nM and involved approximately 1.2 pmole binding sites/mg. The binding of [^{3}H]QNB was overwhelmingly specific in the concentration range studied, with more than 90 per cent bound specifically at 0.1 nM. Scatchard analysis [28] of the binding data gave a straight line (Fig. 1), indicating a single type of ligand–receptor interaction. The K_D for specific [^{3}H]QNB binding was 0.069 nM and the predicted maximum number of binding sites (B_{max}) was 1.33 pmoles/mg. Hill plots of saturation binding experiments gave a straight line with a slope of 0.89 (data not shown), closely approximating 1.0, thus indicating that no cooperativity is involved in the QNB binding.

Kinetic investigation of specific [^{3}H]QNB binding showed a rapid rate of association at 37° (Fig. 2a). Half-maximal binding occurred at approximately 1 min, with complete saturation achieved within 10 min. Non-specific binding was not time dependent. The first-order association constant, k_{obs}, was calculated and used to determine the value for K_1 of $0.14 \times 10^9\,M^{-1}\,min^{-1}$. The reversibility of [^{3}H]QNB binding was readily demonstrated in dissociation experiments. In the presence of excess unlabeled atropine, bound [^{3}H]QNB dissociated steadily with time (Fig. 2b); the $t_{\frac{1}{2}}$ for dissociation was 82 min. This first-order reaction occurred at a rate, K_{-1}, of $0.85 \times 10^{-2}\,min^{-1}$. A kinetic value for

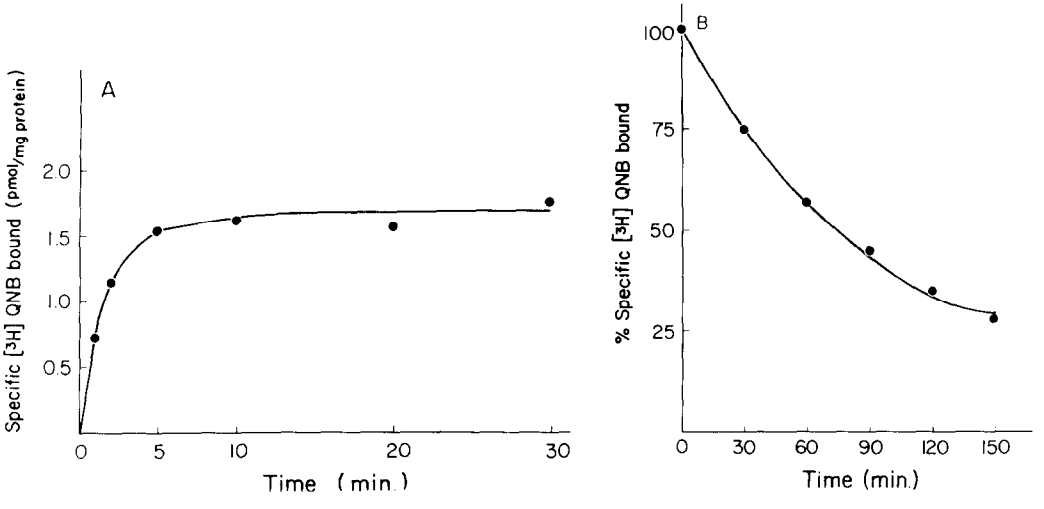

Fig. 2. Time courses of association and dissociation of [^{3}H]QNB binding to iris muscle microsomal fractions. Panel A: Association of [^{3}H]QNB binding. Microsomal fractions were incubated with 0.5 nM [^{3}H]QNB at 37° for various time intervals as described under Materials and Methods. Binding in the absence and presence of 5 μM atropine was measured simultaneously, the difference between these values representing specific binding. Association was begun by addition of microsomes and terminated by rapid filtration. Each value is the mean of six determinations. Panel B: Dissociation of [^{3}H]QNB binding. Microsomal fractions were incubated at 37° in the presence of 0.5 nM [^{3}H]QNB for 1 hr. Dissociation was begun by addition of 5 μM atropine to the reaction mixtures at zero time and terminated by rapid filtration at various time intervals. Each value is the mean of six determinations.

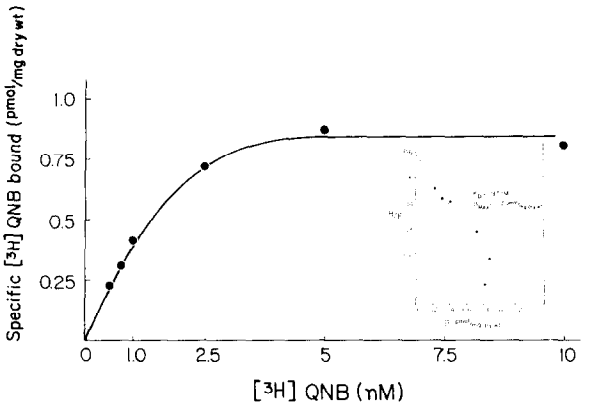

Fig. 3. Specific binding of [^{3}H]QNB at 37° to iris muscle slices as a function of ligand concentration. Conditions of incubation were the same as described under Fig. 1 except that the final volume was 2.5 ml. Each value is the mean of eight determinations. Inset: Scatchard plot of the specific binding data. The slope obtained by linear regression analysis is −0.5064.

K_D, calculated from the K_{-1}/K_1 ratio, was 0.061 nM, which is in excellent agreement with the value derived from Scatchard analysis (Fig. 1).

Binding of [^{3}H]*QNB to iris slices.* Most studies that have been done with radioligands have involved the use of cell-free preparations, obtained by vigorous homogenization of tissues. It was of interest, therefore, to know whether the binding characteristics measured in intact muscle strips differ significantly from those measured in the membrane fragments (Figs. 1 and 2). Employing the same methodology, [^{3}H]QNB binding to intact iris was investigated (Fig. 3). Specific binding was saturable with increasing radioligand concentration, with half-maximal saturation at 1.0 nM. Non-specific binding was linear over the concentration range studied (data now shown). The ratio of specific to non-specific binding was reduced dramatically in the intact muscle experiments, pointing to the high purity of the receptor population in the membrane fraction. Scatchard analysis of the saturation data (Fig. 3) again gave a straight line, indicating a single population of binding sites. The K_D for [^{3}H]QNB binding was 1.97 nM, with an estimated number of binding sites of 1.17 pmoles/mg dry wt. Hill plots of saturation binding experiments (data not shown) gave a straight line with a slope of 0.84, reasonably close to 1.0, which indicates no cooperativity was involved in the [^{3}H]QNB binding.

Kinetic analysis of [^{3}H]QNB binding showed a similar pattern of saturable, time-dependent association of specific binding and time-independent non-specific binding (Fig. 4a). Half-maximal binding occurred at 15 min with complete saturation not evident until after 60 min (Fig. 4). The first-order rate constant, k_{obs}, was calculated and yielded a K_1 of $1.88 \times 10^7\ M^{-1}\ min^{-1}$. The rate of dissociation (Fig. 4b) followed a first-order pattern with K_{-1} of $1.12 \times 10^{-2}\ min^{-1}$. The kinetic K_D, calculated from the K_{-1}/K_1 ratio, was 0.60 nM. This value is significantly less than the 1.97 nM predicted from Scatchard analysis.

Inhibition by muscarinic drugs of [^{3}H]*QNB binding in the microsomal fraction and iris slices.* Four cholinergic drugs were tested in intact iris and membrane fragments for their ability to inhibit specific [^{3}H]QNB binding. Each drug was tested at several concentrations; IC_{50} values were calculated from the dose–response curves and K_I values calculated (Table 1). The K_I values for each drug tested were higher in slices than in microsomes. At 5 μM concentrations, *d*-tubocurarine, propranolol, and bovine serum albumin did not affect [^{3}H]QNB binding (data not shown).

Binding of [^{3}H]*WB-4101 to microsomal fractions and iris slices.* Binding of [^{3}H]WB–4101 (0.1–50 nM) to the microsomal fraction was clearly both specific and non-specific. Non-specific binding increased linearly with concentration and was not displaced by the addition of 100 μM phentolamine (data not

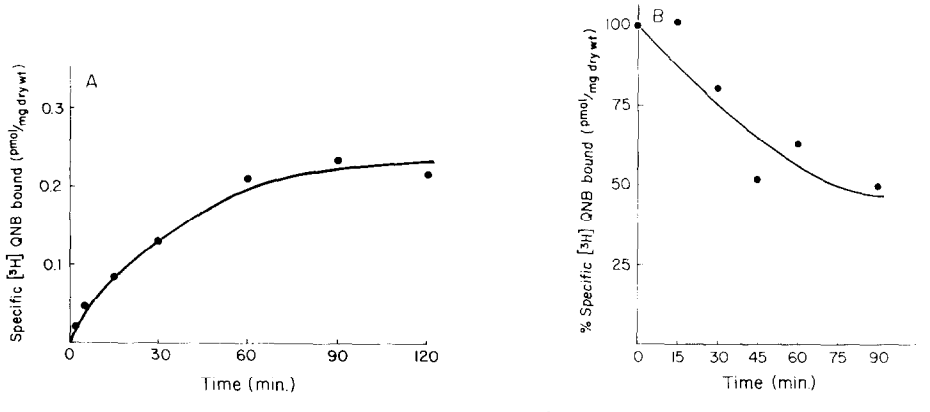

Fig. 4. Time courses of association and dissociation of [^{3}H]QNB binding to iris muscle slices. Panel A: Association of [^{3}H]QNB binding. Conditions of incubation were the same as described under Fig. 2 except that the concentration of [^{3}H]QNB was 1.0 nM and the final volume was 2.5 ml. Each value is the mean of four determinations. Panel B: Dissociation of [^{3}H]QNB binding. Conditions of incubation were the same as described under Fig. 2 except that the concentration of [^{3}H]QNB was 1.0 nM and the final volume was 2.5 ml. Each value is the mean of four determinations.

Table 1. Comparison of inhibition of [^{3}H]QNB binding in microsomal fractions and intact iris slices by muscarinic drugs*

Drug	No. of experiments	IC_{50} and K_I values for inhibition of [^{3}H]QNB binding (nM) Microsomes IC_{50}	Microsomes K_I	Slices IC_{50}	Slices K_I	slices/microsomes
Atropine	4	1.2	0.34	14.6	9.67	28.4
Acetylcholine (+ 1 μM eserine)	4	983	281	5330	3530	12.6
Oxotremorine	4	524	149	35360	23420	157.2
dl-QNB	4	8.2	2.46	6.92	4.58	1.86

* Cholinergic drugs were tested for their ability to inhibit specific [^{3}H]QNB binding in slices or membrane fragments of iris muscle. Radioligand concentration for membrane studies was 0.1 nM (except in the *dl*-QNB experiments in which 0.5 nM [^{3}H]QNB was used), and in the slice experiments 1.0 nM [^{3}H]QNB was used. Each drug was tested at several concentrations and IC_{50} values were calculated from the dose–response curves. K_I values were calculated from the relation $K_I = IC_{50}/(1 + [L]/K_D)$, where $[L]$ is radioligand concentration and K_D is the radioligand equilibrium dissociation constant derived from Scatchard analysis. Results are means for the number of experiments indicated.

shown). Specific binding was displaced by the addition of phentolamine and exhibited an unusual saturation curve, similar to the binding of [^{3}H]WB–4101 to rat cerebral cortical membranes observed by Davis *et al.* [8], when examined over a wide concentration range of the radioligand (Fig. 5). Scatchard analysis of the saturation curve revealed two separate populations of [^{3}H]WB–4101 binding sites, one high affinity and one low affinity (Fig. 5). The high affinity site had a K_D of 2.33 nM with 0.73 pmole binding sites/mg. The low affinity site had a K_D of 55.6 nM and involved 4.56 pmole binding sites/mg. A Hill plot of [^{3}H]WB–4101 saturation data was linear, with a Hill coefficient of 0.71 (data not shown).

Inhibition of [^{3}H]WB–4101 *binding to microsomal fractions by adrenergic drugs.* Four adrenergic drugs were tested in membrane fragments for their ability to inhibit specific [^{3}H]WB–4101 binding. Each drug was tested at several concentrations; IC_{50} values were calculated from the dose–response curves and K_I values were calculated (Table 2). Phenoxybenzamine, phentolamine, epinephrine and norepinephrine effectively displaced [^{3}H]WB–4101 binding in the concentration ranges examined and, in general, the antagonists were more potent than the agonists (Table 2).

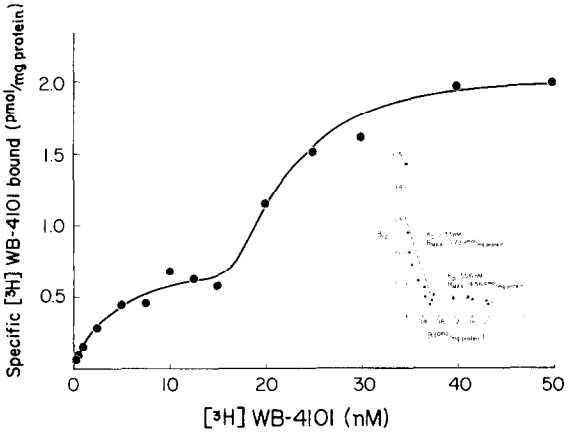

Fig. 5. Specific binding of [^{3}H]WB–4101 at 25° to iris muscle microsomal fraction as a function of ligand concentration. Conditions of incubation were the same as described under Fig. 1 except that the protein concentration was 50–100 μg and non-specific binding was measured by the addition of 100 μM phentolamine. Each value is the mean of six to twelve determinations. Inset: Scatchard plot of the specific binding data. The slopes obtained by linear regression analysis for high affinity and low affinity binding are −0.430 and −0.018, respectively.

Binding of [^{3}H]*WB-4101 to iris muscle.* Binding of [^{3}H]WB–4101 to iris slices was clearly both specific and non-specific. Non-specific binding increased linearly with concentration and was not displaced by 100 μM phentolamine (data not shown). Specific binding was reversed by the addition of phentolamine and, as with the microsomal fraction (Fig. 5), exhibited an unusual saturation curve when examined over a wide range of radioligand concentrations (Fig. 6). Scatchard analysis of this saturation curve revealed two separate populations of [^{3}H]WB–4101 binding sites, one high affinity and the other low affinity (Fig. 6). The high affinity site had a K_D of 10.19 nM and involved approximately 0.414 pmole binding sites/mg dry wt. The low affinity site had a K_D of 57.5 nM and a total of 1.25 pmole binding sites/mg dry wt. A Hill plot of [^{3}H]WB–4101 saturation data was linear, with a Hill coefficient of 0.86 (data not shown).

DISCUSSION

The comparative studies reported in this paper on the characteristics of [^{3}H]QNB and [^{3}H]WB–4101 binding to microsomal fractions and slices of albino rabbit iris are summarized in Table 3. The ligand binding properties are typical of binding to muscarinic cholinergic and alpha-adrenergic receptors. They are saturable, of high affinity, and are reduced by specific antagonists. The suitability of the slices for binding experiments was not unexpected, since [^{3}H]QNB was reported to bind with high affinity and specificity to muscarinic receptors in brain slices [2, 29]. In general, the affinities of both [^{3}H]QNB and [^{3}H]WB–4101 to the microsomal fraction were

Table 2. Inhibition of [^{3}H]WB-4101 binding in microsomal fractions by adrenergic drugs*

Drug	No. of experiments	IC_{50} and K_I values for inhibition of [^{3}H]WB-4101 binding (nM) IC_{50}	K_I
Phenoxybenzamine	4	21.3	14.7
Phentolamine	4	26.1	18.0
Epinephrine	4	480	331
Norepinephrine	4	4880	3366

* Adrenergic drugs were tested for their ability to inhibit [^{3}H]WB-4101 binding in membrane fragments with 0.5 nM [^{3}H]WB-4101. Several concentrations of each drug were tested, and IC_{50} values were calculated from the dose–response curves. K_I values were calculated from the relation $K_I = IC_{50}/(1 + [L]/K_D)$, where $[L]$ is the radioligand concentration and K_D is the radioligand equilibrium dissociation constant derived from Scatchard analysis. Results are means for the number of experiments indicated.

higher than those obtained with the slices (Table 3). Thus, K_D values in slices for [^{3}H]QNB and [^{3}H]WB-4101 were approximately twenty-nine and four times higher than those estimated in the microsomal fraction. Furthermore, atropine was consistently less potent in inhibiting [^{3}H]QNB binding in iris slices than in the microsomal fraction. Gilbert *et al.* [2] similarly reported that antagonists are less potent in inhibiting [^{3}H]QNB binding in brain slices than in homogenates. Ward and Young [30] had previously reported that muscarinic drugs were less potent in inhibiting the binding of [^{3}H]PrBCM* to intact intestinal muscle strips than to cell-free homogenate preparations. The K_D values for [^{3}H]QNB and [^{3}H]WB-4101 in the microsomal fraction and the iris slices are comparable to those obtained with other tissues [2, 8, 10, 19]. Thus, the K_D value for [^{3}H]-(*l*)-QNB binding in the microsomal fraction is 0.069 nM, in good agreement with values reported recently by Gilbert *et al.* [2] for brain tissue; however, these values are an order of magnitude lower than those reported in earlier work using [^{3}H](d*l*)-QNB [3, 5, 6]. The use of the *l*-stereoisomer of QNB in the present study confirms its greater affinity and demonstrates that its use eliminates the many problems that were reported to be associated with the *dl*-isomer [2]. There is an excellent correlation between the Scatchard-derived and the kinetically-derived dissociation constants, which confirms the reliability of our binding assay. It is not clear why Lund-Karlsen [18] was unable to detect saturable binding of [^{3}H]QNB to rabbit iris homogenates, although binding to slices could readily be measured. Kloog *et al.* [19], working with *N*-methyl piperidylbenzilate ([^{3}H]4NMPB) and [^{3}H]QNB, and Page and Neufeld [17], working with [^{3}H]dihydroergocryptine (DHE), demonstrated saturable binding to homogenates of iris muscle of albino rabbits. The finding of dissimilar dissociation constants for [^{3}H]QNB binding in the microsomal fraction and muscle slices should not immediately suggest that muscarinic receptor properties have been altered by tissue homogenization. In fact, our observation that K_{-1} values are similar in the two preparations is compelling evidence that no receptor property alterations occurred. The difference in K_D values seen here is probably attributable to the association (K_1) component. The finding of markedly slower rates of association in intact tissue, compared to the homogenate, is not unexpected because of the diffusional barriers present in intact muscle, which are absent in cell-free preparations. This problem has been discussed by several workers [2, 30]. We suggest that these diffusional barriers are, at least in part, responsible for the difference in K_D values between the microsomal fraction and muscle slices, and that no significant change in receptor properties occurs.

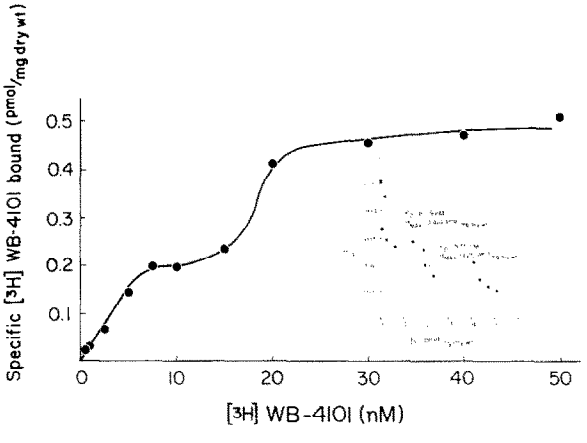

Fig. 6. Specific binding of [^{3}H]WB-4101 at 25° to iris muscle slices as a function of ligand concentration. Conditions of incubation were the same as described under Fig. 3 except that the final volume was 0.5 ml, and non-specific binding was measured by addition of 100 μM phentolamine. Each value is the mean of eight determinations. Inset: Scatchard plot of the specific binding data. The slopes obtained by linear regression analysis for high affinity and low affinity binding are −0.0981 and −0.0174, respectively.

The binding of [^{3}H]WB-4101 to the microsomal fraction and to iris muscle preparations is consistent with its interaction with two classes of sites: a high affinity population with a high specificity for the radioligand and a low affinity population with a greatly decreased specificity for the radioligand (Table 3). Using a variety of alpha-adrenoligands, other investigators have also reported the presence of two subpopulations of alpha-adrenergic receptors. Employing a variety of radioligands, U'Prichard and

* [^{3}H]PrBCM, [^{3}H]propylbenzylylcholine mustard.

Table 3. Summary of muscarinic and alpha-adrenergic binding constants in microsomal membrane fractions and iris muscle slices

	K_D (nM)	B_{max}*	Hill coefficient	K_1 ($M^{-1} min^{-1}$)	K_{-1} (min^{-1})	K_I (atropine) (nM)
[^{3}H]QNB						
Membranes	0.069	1.33	0.89	0.14×10^9	0.85×10^{-2}	0.34
Slices	1.97	1.17	0.84	0.19×10^8	1.12×10^{-2}	9.67
[^{3}H]WB-4101						
Membranes	2.33†	0.73	0.71			
	(55.6)‡	(4.56)				
Slices	10.19	0.414	0.86			
	(57.5)	(1.25)				

* Values for membranes are expressed in pmoles/mg protein; values for slices are expressed in pmoles/mg dry wt.
† High affinity binding.
‡ Low affinity binding.

Snyder [10] reported two distinct receptor types which could be distinguished by their differing patterns of α-adrenoligand binding. Greenberg and Snyder [9], working with rat brain membranes, demonstrated the existence of one α-adrenoreceptor subtype that correlates with [^{3}H]WB-4101 binding and low agonist affinity, and a second that selectively binds [^{3}H]clonidine and has limited antagonist binding capacity. In the present work at higher concentrations [^{3}H]WB-4101 appeared to interact with a second population of sites with a much lower affinity than that found for the high affinity site (Figs. 5 and 6). Similarly, Davis *et al.* [8], working with binding of [^{3}H]WB-4101 to rat cerebral cortical membranes, demonstrated a low affinity binding site, in addition to the high affinity site. It has recently been shown that these α-receptor subtypes could be classified as α_1 or α_2 adrenergic receptors based on the pharmacological profile. This α_1/α_2 model has been used to more closely define complex α-adrenergic binding patterns. [^{3}H]Clonidine, [^{3}H]norepinephrine and [^{3}H]epinephrine have been shown to selectively bind α_2 receptors [10], whereas [^{3}H]WB-4101 has been shown to label α_1 receptors [10, 31]. [^{3}H]DHE reveals a single class of binding sites [9, 32] because it is equipotent to α_1 and α_2 receptors. [^{3}H]WB-4101, however, would not be expected to label a single binding site when analyzed over a wide concentration range because it is not equipotent, but markedly α_1 specific. In the present study the high affinity component of [^{3}H]WB-4101 binding (K_D = 2.33 nM) could correspond to binding of the α_1 site, whereas at higher concentrations (>15 nM) the radioligand interacts in addition with the α_2 site. These components are clearly discernible in both the microsomal fraction and the slices (Figs. 5 and 6, respectively). Further studies using specific α_1/α_2 ligands must be performed before a correlation between the two [^{3}H]WB-4101 binding sites and the α_1/α_2 model system can be confirmed. The existence of two distinct binding sites may explain the finding of Hill coefficients of less than 1.0, although alternative explanations, such as receptor desensitization or negative cooperativity, are conceivable.

The following arguments are set forth to explain the present findings of higher affinities for [^{3}H]QNB and [^{3}H]WB-4101 binding to microsomal fractions as compared to muscle slices: (a) the use of membrane preparations probably allows the attainment of higher receptor site concentrations and may also eliminate some of the drug uptake processes that occur in intact tissue [32]; (b) the differences could probably be explained, at least in part, in terms of access-limitation for the penetration of the ligand and the test drugs to receptor sites in intact tissue preparations [30]; (c) the homogenization process may have separated the receptor from an endogenous factor that may inhibit ligand binding; and (d) the differences in receptor–ligand binding obtained with slices and with microsomes may reflect real differences in the characteristics of such binding in intact tissue versus broken cell preparations [2]. The equal K_{-1} values found in the two preparations argue against alterations in receptor properties. It is possible, therefore, that the loss of the phosphoinositide effect in cell-free homogenates is not due to alterations of muscarinic and α-adrenergic receptors during the homogenization procedure, but rather to disruption of a link (e.g. via cations) between these receptors and the enzyme(s) involved in phosphoinositide metabolism in the intact slice.

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