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DIFFERENCES IN AFFINITIES OF MUSCARINIC ACETYLCHOLINE RECEPTOR ANTAGONISTS FOR BRAIN AND HEART RECEPTORS

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Abstract—The affinities of atropine, scopolamine, 3-quinuclidinyl benzilate and twelve analogues of 3-quinuclidinyl benzilate were determined for the muscarinic acetylcholine receptor (m-AChR) using membrane preparations from caudate/putamen. The affinity constants thus obtained were compared with affinities previously reported for the m-AChR obtained from ventricular muscle. The affinities differed significantly for six of the compounds, the largest difference being 16-fold. Neither solubilization nor variation of physiologically significant salts led to a significant change in the affinity of that compound. These results are interpreted as supporting the subclassification of the muscarinic acetylcholine receptor.

Receptors can be characterized at the molecular level by studying their interactions with radiolabeled agents (usually antagonists) that exhibit high affinity, stereoselective binding where applicable, and high specificity for the class of receptor. Furthermore, the binding of a radioligand to a receptor must be competitively inhibited by agonists and antagonists, for that class of receptors, with affinities in rank order to those obtained from studies of physiological or biochemical response. The radiolabeled antagonist [^{3}H]3-quinuclidinyl benzilate ([^{3}H]QNB) exhibits the desired properties and has been used extensively to study the muscarinic acetylcholine receptor (m-AChR) from a variety of tissues, e.g. heart [1–3], brain [4–6] and pancreas [7–9]. Agonists compete with [^{3}H]QNB (and a variety of other radiolabeled antagonists) for the m-AChR with affinities of 10^{-6} M to 10^{-4} M and exhibit biphasic competition curves [10]. Antagonists, on the other hand, exhibit affinities five orders of magnitude higher than agonists and competition curves concordant with a single class of binding sites when determined using physiologically consistent salts and buffers [11]. Recent pharmacological studies with agonists and antagonists indicate, however, that the m-AChR may exhibit subclasses [12–15] similar to those seen with the adrenergic, dopaminergic, histaminergic and opiate receptor systems. In addition, the anticholinergic drug pirenzepine has been shown to differ in affinity to the m-AChR from atrial and ileal muscle compared to that found in the cerebral cortex and hippocampus [16], and *N*-methyl 4-hydroxypiperidinyl benzilate exhibits different affinities for m-AChR from different regions of mouse brain [17].

To better understand the differences between the putative subclasses of m-AChR, we have synthesized twelve analogues of 3-quinuclidinyl benzilate (QNB) and determined their affinities for m-AChR from ventricular muscle (m_1) and from caudate/putamen (m_2). One of these antagonists exhibited a 16-fold greater affinity for the m_2 receptor than for the m_1 receptor. The results of this study provide additional support for the subclassification of m-AChR and provide initial data on the structure–binding relationships of QNB analogues that define the m_1 and m_2 AChR.

MATERIALS AND METHODS

3-Quinuclidinyl benzilate and the analogues of QNB were synthesized in our laboratories [18]. Purity was determined by reversed phase high performance liquid chromatography (HPLC), and all compounds gave analyses within 0.4% of the theoretical values. Both QNB and 3-quinuclidinyl xanthene-9-carboxylate (QNX, Fig. 1) are racemic mixtures, and the remaining analogues contain all four diastereomers. Atropine and scopolamine were purchased from the Sigma Chemical Co. (St. Louis, MO). [^{3}H]-(−)-QNB (33.1 Ci/mmole) was purchased from the New England Nuclear Corp. (Boston, MA).

Tissue preparation

Hearts were removed immediately upon killing female Sprague–Dawley rats (cervical dislocation under light ether anesthesia). The ventricular muscle was dissected free of atria, major vessels and fat, and minced with scissors. The tissue was homogenized (Brinkman Polytron PC-U) in 20 vol. of ice-cold buffer (buffer constituents varied according to study) containing 10% sucrose. The homogenate was filtered through four layers of cheesecloth and used without further preparation (except in the equi-

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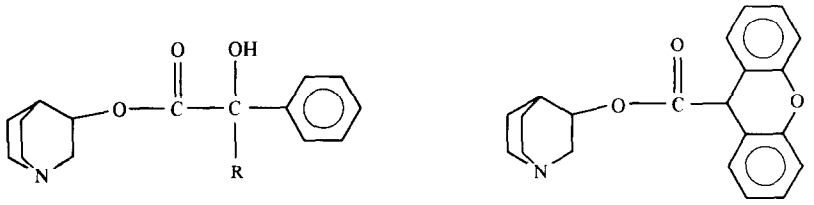

R = C_6H_5; QNB QNX

Fig. 1. Structures of 3-quinuclidinyl benzilate (QNB) and its analogues (see Table 1 for R) and QNX.

librium dialysis studies). The heart of a mongrel dog was removed, and the left ventricular muscle was dissected free of remaining tissue, fat, and vessels, frozen in liquid nitrogen, and stored at −80° until used. One- to two-gram segments from the muscle were prepared as above. Storage for up to 6 months did not change the binding characteristics of the m-AChR. The concentration of m-AChR in such preparations varied from 0.4 to 1.2×10^{-11} M.

The brains of female rabbits (New Zealand Albino, 4–5 lb) were removed immediately upon sacrifice and placed on ice. The caudate nucleus and putamen were dissected free and immediately frozen and stored at −80° until used. Approximately 0.10 to 0.15 g of caudate/putamen (CP) was homogenized in ice-cold buffer as described above and used without further purification. The concentration of m-AChR in these preparations was similar to that obtained using heart.

Equilibrium association constants (K_{VM} *and* K_{CP})

Filtration assay. The equilibrium association constants for the antagonists described herein were determined by competitive ligand binding assay using [^{3}H]QNB as the radiotracer [18]. The compounds were dissolved in 95% EtOH and added to 5 ml of Tris-buffered saline (10 mM Tris, pH 7.4) containing 2×10^{-10} M [^{3}H]QNB. The EtOH was at a final concentration of 0.5% and had no effect on the receptor. Competitive curves were generated using at least ten concentrations of unlabeled compound from 10^{-12} M to 10^{-6} M for (±)-QNB and compounds that exhibited affinities within 5-fold of QNB and from 10^{-11} M to 10^{-5} M for compounds with affinities that differed from that of QNB by greater than 5-fold. Aliquots of 0.1 ml of tissue preparation [prepared as described above in 10 mM Tris-buffered saline (pH 7.4)] were added, and the mixture was vortexed and incubated at room temperature for

Table 1. Equilibrium association constants for muscarinic acetylcholine receptor antagonists for receptor from ventricular muscle and caudate/putamen

Compound No.	R†	N	K* ($\times 10^9$ M^{-1}) VM‡	95%§ Conf. Int.	CP	95% Conf. Int.	CP/VM
1	C_6H_5	25	5.28	(3.7–7.5)	3.62	(2.8–4.7)	0.69
2	4-FC_6H_4	6	3.03	(2.3–3.9)	3.97	(2.5–6.3)	1.3
3	3-FC_6H_4	6	5.03	(3.5–7.2)	4.21	(2.9–6.1)	0.84
4	4-BrC_6H_4	7	0.608	(0.44–0.83)	2.51	(1.7–3.7)	4.1
5	3-BrC_6H_4	5	0.433	(0.32–0.59)	3.05	(2.0–4.6)	7.0
6	2-BrC_6H_4	5	0.401	(0.28–0.57)	0.664	(0.44–1.0)	1.7
7	4-IC_6H_4	6	1.22	(0.87–1.7)	2.37	(1.6–3.5)	1.9
8	3-IC_6H_4	5	0.48	(0.30–0.70)	1.15	(0.07–1.8)	2.4
9	c-C_6H_{11}‖	7	3.51	(2.9–4.3)	2.54	(1.7–3.8)	0.72
10	c-C_5H_9¶	8	8.21	(6.2–11)	3.59	(2.6–5.0)	0.44
11	n-C_4H_9**	5	2.91	(1.8–5.0)	3.44	(2.6–4.5)	1.2
12	HC≡CCH_2	8	0.0303	(0.021–0.043)	0.126	(0.088–0.18)	4.2
QNX††		5	0.225	(0.17–0.30)	3.56	(2.6–4.9)	16
Atropine		9	0.167	(0.11–0.26)	0.744	(0.53–1.0)	4.5
Scopolamine		10	0.135	(0.11–0.17)	0.810	(0.52–1.3)	6.0

* K_{VM} and K_{CP}
† Structure of substituent on m-AChR antagonist; see Fig. 1 for general structure.
‡ Data on ventricular muscle from Ref. 18.
§ 95% Confidence intervals calculated by the method of Munson and Rodbard [20].
‖ Cyclohexyl.
¶ Cyclopentyl.
** *n*-Butyl.
†† For structure, see Fig. 1.

2 hr. Neither continuous agitation nor increased incubation time altered the results. The incubation mixture was rapidly filtered on GF/C filters, washed with 10 ml of ice-cold saline, air dried, placed in ACS (Amersham) liquid scintillation mixture, and counted for 5 min. Data were then analyzed using the LIGAND program of Munson and Rodbard [19] modified for use on an HP 3000 computer and TRS-80 (Radioshack) computer. Confidence intervals were calculated by the method of Munson and Rodbard [20]. The K_{VM} and K_{CP} values in Table 1 were obtained from pooled data from at least five determinations, each in duplicate. The LIGAND program accounts for ligand depletion by receptor. However, we have determined that the concentration of [^{3}H]QNB is reduced by less than 5% at the concentrations of receptor used in these studies.

Equilibrium dialysis. The m-AChR was solubilized using 0.4% digitonin/0.08% cholate as described by Cremo *et al.* [21]. Two grams of ventricular muscle was homogenized in 50 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4) containing 1.5 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. After filtration through cheesecloth, the homogenate was centrifuged at 1000 *g* for 10 min, and the supernatant fraction was discarded. One-tenth volume of the same buffer containing 4% digitonin and 0.8% cholate was added, and the mixture was gently swirled for 5 min at room temperature. The mixture was then centrifuged at 100,000 *g* for 1 hr, and the supernatant fraction was used without further purification. For the CP, 0.15 g of tissue was homogenized in phosphate buffer as above and solubilized without centrifugation. We obtained 80% recovery of receptor from heart, but only 63% recovery from CP. Cremo *et al.* [21] reported 75–98% recoveries from cardiac membranes. Aliquots of 0.3 ml of the solubilized m-AChR were added to 1 cm dialysis tubing and dialyzed for 36 hr at 4° in 20 ml of the same buffer used for the solubilization. Competing ligand was added to the dialysis baths at the same concentrations used in the filtration assay. The concentration of receptor in the dialysis studies was approximately 4×10^{-10} M for both VM and CP and the concentration of [^{3}H]QNB was 2×10^{-10} M. Although the concentration of receptor is considerably higher than that used in the filtration assay, the volume of the dialysis bath provides sufficient radioligand and inhibitor such that ligand deletion is not observed [22]. Specific binding and affinity were determined by the LIGAND program.

RESULTS AND DISCUSSION

The affinities of (±)-QNB, the twelve analogues of QNB, atropine, and scopolamine for the m-AChR from ventricular muscle (VM) and caudate/putamen (CP) are summarized in Table 1. For the structures of the analogues, see Fig. 1 where R is the substituent in column 2 of Table 1. The Hill coefficients for the compounds in Table 1 did not differ from 1 at the 95% confidence level (range from 0.89 to 1.15).

Halogenation of one phenyl ring of the benzilate moiety of QNB by fluorine (compounds 2 and 3) did not alter the affinity of the analogues for the m-AChR from either the VM or the CP. Halogenation by either Br or I reduced the affinity of the QNB derivatives for the m-AChR from the heart (compounds 4–8). (If the intervals provided in Table 1 do not overlap for two compounds being compared, those compounds exhibit different affinities at the 95% confidence level.) Interestingly, substitution by 4-Br, 3-Br, and 4-I (compounds 4, 5, and 7 respectively) did not reduce the affinity of the antagonist for the m-AChR from CP. Compounds 4 and 5 exhibited significantly higher affinity for the m-AChR from the CP than for that obtained from VM. Replacing one phenyl ring with a cyclic aliphatic substituent, cyclohexyl (compound 9) or cyclopentyl (compound 10), or an *n*-butyl group (compound 11) did not alter significantly the affinity of the antagonists for the m-AChR from either tissue source. However, substitution of one phenyl ring by propargyl (compound 12) resulted in a large loss in affinity to the m-AChR from the VM and CP. A differential loss was observed, however, such that compound 12 exhibited a 4-fold higher affinity for the receptor from CP than from that obtained from VM. The planar analogue of QNB, QNX (Fig. 1), exhibited a 23-fold loss in affinity to the m-AChR from VM but was not different in affinity from QNB for the m-AChR from CP. Thus, QNX exhibited the largest CP selectivity of this series of compounds (16-fold). The m-AChR antagonists, atropine and scopolamine, are reported to exhibit different affinities for the receptor from CP and cerebellum [17]. We also found that these antagonists exhibited different affinities for the m-AChR from CP compared to VM.

The above results suggest that the m-AChR from VM and CP may represent two classes of receptor, m_1 and m_2, respectively. Similar data have been presented for the radiolabeled compounds *N*-methyl 4-hydroxypiperidinyl benzilate [17] and pirenzepine [16]. In our hands, the structure–binding relationships suggested that the m-AChR from VM exhibits lower bulk tolerance for the larger halogen substituents and the planar substituent of QNX than seen with receptor from CP. It should be noted that pirenzipine also has a large planar structure which may bind to the m-AChR in the same region as the xanthenyl moiety of QNX. Secondary factors, besides a basic difference in the receptors present in the two tissues, may be responsible for the differences in binding which we and others have observed. One factor which can lead to an artifactual difference in the measured affinities is the binding of inhibitor to non-receptor proteins to an extent which is sufficient to reduce the concentration of inhibitor which can interact with the receptor.

Since the concentration of m-AChR per gram of tissue is approximately 10-fold less in the VM than in the CP, and the heart has contractile proteins which may lead to increased non-receptor binding [23], it is possible that the lower affinity determined using VM compared to CP results from differing degrees of non-receptor binding. In studies such as those presented in Fig. 2, the inhibitor concentration plotted is the total concentration—not the free concentration. Since the inhibitor is not radiolabeled, it is not possible to determine the free concentration of inhibitor. If ligand depletion occurs as the result

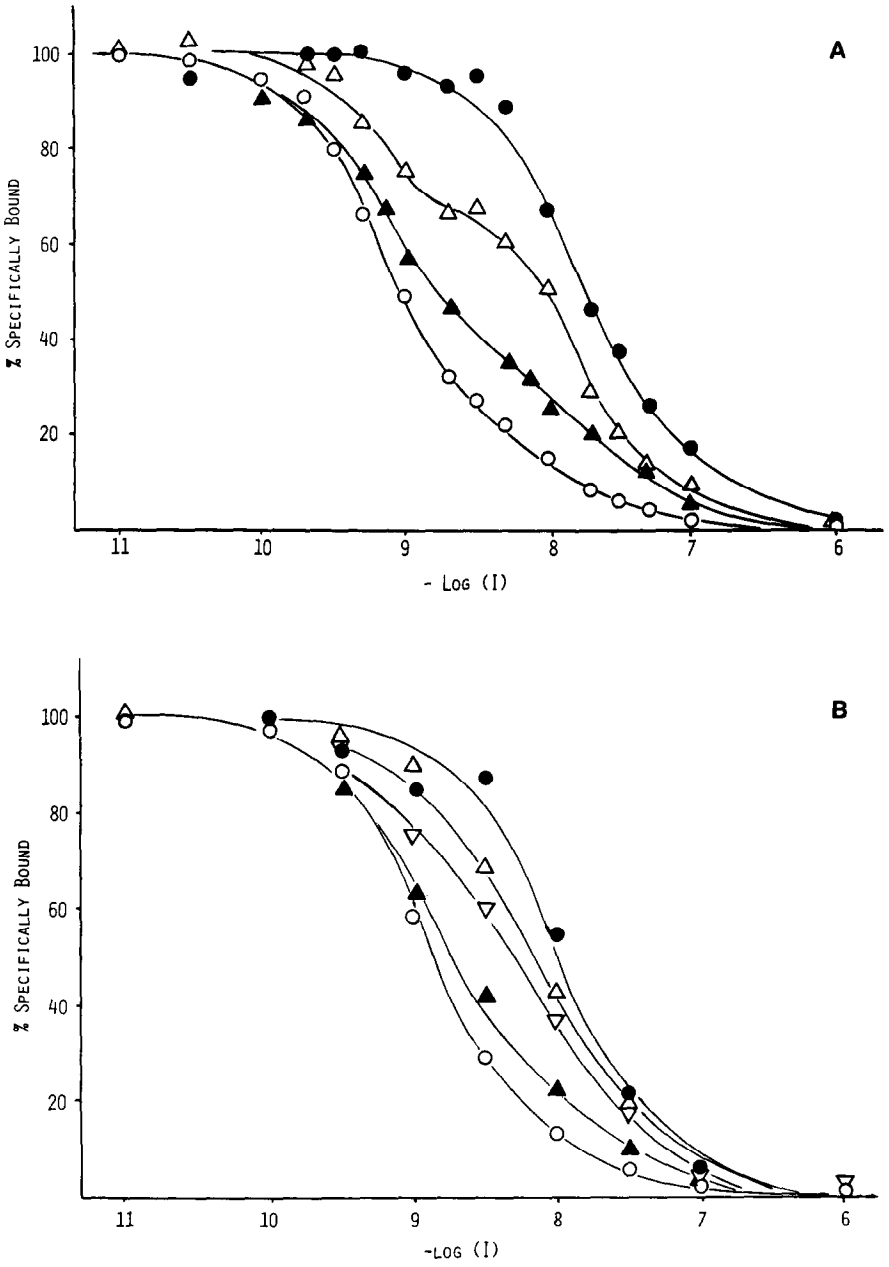

Fig. 2. (A) Inhibition of [^{3}H]QNB binding by QNX (Fig. 1). Key: 100% ventricular muscle (VM) (●), $K_{VM} = 1.24 \times 10^8\,M^{-1}$; 100% caudate/putamen (CP) (○), $K_{CP} = 1.95 \times 10^9\,M^{-1}$; 60/40 VM/CP (△), $K_{VM} = 2.57 \times 10^8\,M^{-1}$ and $K_{CP} = 6.56 \times 10^9\,M^{-1}$; and 30/70 VM/CP (▲), LIGAND converged to unreasonable parameters with limited number of data points. The Hill coefficients and 95% confidence intervals for the respective curves were: 1.02 (0.98–1.06), 1.00 (0.98–1.03), 0.888 (0.85–0.93), and 0.705 (0.68–0.73). (B) Inhibition of [^{3}H]QNB binding by compound 11 (see Table 1). Key: 100% VM (●), $K_{VM} = 2.4 \times 10^7\,M^{-1}$; 100% CP (○), $K_{CP} = 2.4 \times 10^8\,M^{-1}$; 75/25 VM/CP (△), LIGAND converged to unreasonable parameters with limited number of data points; 50/50 VM/CP (▽), $K_{VM} = 2.4 \times 10^7\,M^{-1}$ and $K_{CP} = 2.7 \times 10^8\,M^{-1}$; and 25/75 VM/CP (▲), $K_{VM} = 3.04 \times 10^7\,M^{-1}$ and $K_{CP} = 1.71 \times 10^8\,M^{-1}$. For compound 11 mixed receptor studies, LIGAND analysis was performed with all parameters set as constants *except* K_{VM} and K_{CP}. The Hill coefficients and 95% confidence intervals for the respective curves were: 1.08 (0.79–1.5), 1.03 (0.92–1.1), 1.02 (0.97–1.1), 0.884 (0.79–0.99), and 0.858 (0.82–0.90). For compound 11, data between 15 and 85% inhibition were used to calculate the Hill coefficients.

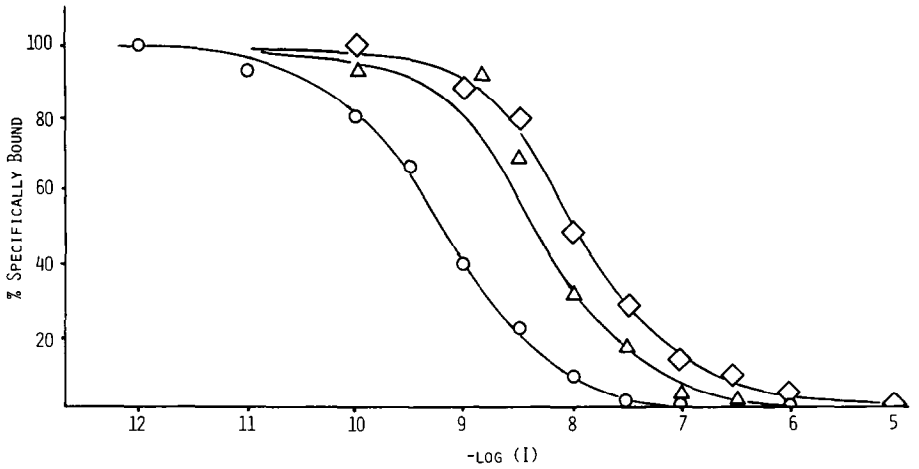

Fig. 3. Effects of buffer and ionic conditions on (±)-QNB and (±)-QNX inhibition of [^{3}H]QNB binding to ventricular muscle m-AChR. Key: (±)-QNB inhibition in either phosphate buffer (pH 7.4) with or without 0.9% NaCl, or 10–50 mM Tris buffer (pH 7.4) with or without 0.9% NaCl, or 50 mM Tris buffer (no saline) with either 1.5 mM $MgCl_2$ or 10^{-5} M GMPPNHP (○); and (±)-QNX inhibition in all the above conditions (◇) *except* 50 mM Tris buffer with no saline or other added components (△).

of non-receptor binding, the concentration of inhibitor which is free to interact with the receptor may be considerably reduced. As a result, the true affinity of the inhibitor will be underestimated. This should not be confused with the depletion of ligand or inhibitor by receptor. The LIGAND program accounts for receptor-mediated depletion of radioligand and inhibitor. In addition, we have found less than 5% depletion of the [^{3}H]QNB concentration in our assays. Although the binding of an unlabeled inhibitor to non-receptor proteins (which is usually related to the lipophilicity of the inhibitor) cannot be easily determined, we can provide the same non-receptor proteins to the CP preparation by mixing the VM and CP preparations.

Using QNX and the propargyl analogue of QNB, we examined the inhibition of [^{3}H]QNB binding to mixtures of VM and CP tissues (Fig. 2, A and B). When only one tissue was present, the resulting inhibition curves clearly represented competition for one class of binding site. The Hill coefficients did not differ from 1 (see figure legend). When the tissues were mixed in various proportions, the resulting curves were biphasic. LIGAND analysis of the biphasic curves [24] indicated that the affinities were the same as those which were obtained from the isolated preparations (see figure legend). Also, the Hill coefficients calculated for the two curves obtained with mixed receptor populations were significantly less than 1. The concentration of m-AChR in VM was 10-fold less than that in the CP. As a result, more tissue was used in the VM preparations to provide equivalent levels of m-AChR in the assay. Since the major fraction of the protein present in

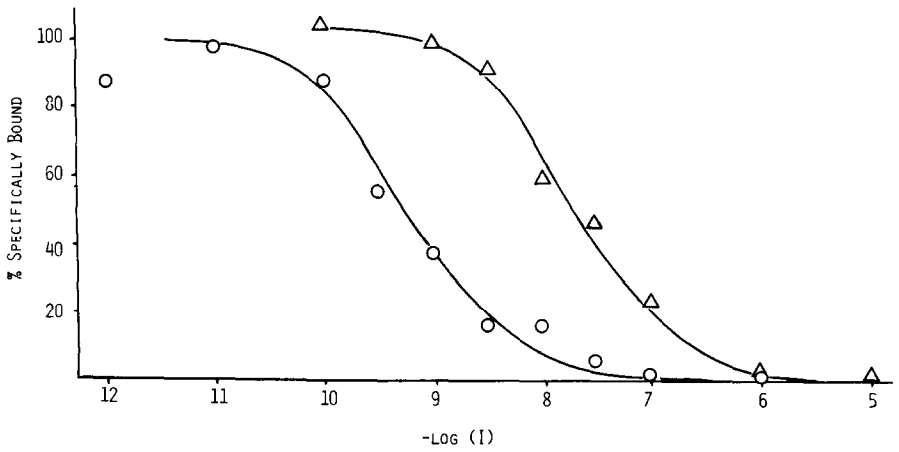

Fig. 4. Equilibrium dialysis: Inhibition of [^{3}H]QNB binding to digitonin/cholate solubilized m-AChR from ventricular muscle by (±)-QNB (○) and (±)-QNX (△). The affinity constants, K_{CP} = 1.96×10^9 M^{-1} and $K_{VM} = 7.28 \times 10^7$ M^{-1}, are within the 95% confidence interval of the values reported in Table 1 for three determinations.

the mixtures was from the heart, inaccurate determination of affinity due to macroscopic lipophilic binding of unlabeled competitor was not likely. Although microscopic effects in the immediate vicinity of the receptor cannot be excluded by this study, the results suggest that the affinity differences seen with QNX in the CP compared to VM were the result of receptor differences and not the result of an artifact.

In addition to the differences in affinity between heart and brain tissues observed for pirenzepine, Hulme *et al.* [11] have reported other differences between the two receptor populations. Receptor heterogeneity is observed in the binding of [^{3}H]*N*-methylscopolamine (NMS) to heart m-AChR in the presence of Tris buffer which was absent in the presence of saline, $MgCL_2$ of the GTP analogue GMPPNHP. Similar studies using receptor from the brain do not exhibit this striking conversion. Although the affinity of [^{3}H]QNB has not been shown to be affected by such conditions, Sastre *et al.* reported an apparent reduction in receptor concentration which is Tris-buffer related and abolished by normal saline [25] or by 10^{-4} M GMPPNHP (personal communication). We have examined the effects of the buffer, GMPPNHP and $MgCl_2$ on the inhibition of [^{3}H]QNB by QNX. We observed a slight shift in affinity (Fig. 3) of 2-fold in the presence of 50 mM Tris which was not seen with phosphate buffer. The addition of normal saline rectified the 2-fold increase in affinity.

The effect of GMPPNHP and $MgCL_2$ on the binding of [^{3}H]NMS (which was not observed in studies on the brain m-AChR) suggests that coupling to the associated cyclase was responsible for the effects observed with heart. Solubilization of the beta-adrenergic receptor has been shown to prevent agonist-induced cyclase activation and the changes in affinity which accompany receptor–cyclase coupling [26]. We therefore solubilized the m-AChR from the VM and CP and examined QNX inhibition to the solubilized preparations (Fig. 4). The solubilization of the m-AChR did not alter the inhibition of [^{3}H]QNB binding by QNX. The affinities of QNX for the receptor from VM and from CP are the same as in the membrane preparations (see figure legend for equilibrium association constants). Although the recovery of receptor from the membrane upon solubilization was not as high with the CP as with the VM, there was no change in the affinity of QNX, which strongly suggests that the population which is extracted by the detergent is representative of the receptor population present in the membranes.

In conclusion, we have demonstrated differences in the affinities of a variety of QNB analogues for the m-AChR when determined using ventricular muscle or caudate/putamen as the source of the receptor. The largest difference was seen with QNX. We have shown that the differences in affinities are not an artifact of ligand depletion and that the differences are maintained upon solubilization of the receptor. Although 50 mM Tris (no saline) caused an increase in affinity of 2-fold, the competition curve was consistent with one class of binding site. In addition, neither GMPPNHP nor solubilization had an effect on the affinity of the ventricular muscle m-AChR, which suggests that we were not observing receptor charges such as those reported by Hulme *et al.* [11] or Sastre *et al.* [25]. Our results are consistent with the proposal that the m-AChR present in the ventricular muscle and caudate/putamen represents subclasses of the m-AChR.

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