tions. Depolymerization can be potentially controlled by protein kinases, calmodulin, or both and can be made to occur at specific sitess in response to events occurring elsewhere in the cell. Calmodulin may therefore function catalytically to depolymerize and reel in microtubules at specific disassembly sites. When chromosomes are attached to cold-stable microtubules, the consequences for control of their anaphase movement by such a disassembly process are obvious (Margolis et al., 1978; Margolis & Wilson, 1981).

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Ligand Interactions with the Solubilized Porcine Atrial Muscarinic Receptor†

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ABSTRACT: Ligand interactions with porcine atrial muscarinic receptor solubilized in a mixed-detergent system (0.4% w/v digitonin and 0.08% w/v cholate) are described. The solubilized receptor interacts with ligands in a stereospecific manner, showing about the same affinity for local anesthetics and antagonists as was found for the membrane-bound protein [Schimerlik, M. I., & Searles, R. P. (1980) *Biochemistry 19*, 3407–3413]. Agonists appear to interact with a single class of noninteracting sites that correspond to the low-affinity agonist sites in the membrane-bound preparation. Kinetic studies of L-[^{3}H]quinuclidinyl benzilate binding to the receptor indicated a two-step mechanism. The first step, in rapid preequilibrium ($K = 5.7 \times 10^{-9}$ M), was followed by a slow conformational change ($k_1 = 4 \times 10^{-3}$ s^{-1}; $k_{-1} = 1.7 \times 10^{-4}$ s^{-1}) in the receptor–ligand complex. The overall dissociation constant calculated from the association kinetics (2.3×10^{-10} M) agreed well with the thermodynamic value for K_{ov} (2.5×10^{-10} M); however, direct determination of k_{-1} gave a value about 4-fold lower (4.0×10^{-5} s^{-1}) than predicted. Possible reasons for this discrepancy are discussed.

The physiological effects of the neurotransmitter acetylcholine on the heart are thought to be mediated by (mAcChR's)[1] (Koelle, 1975). Biochemical studies of this protein have been greatly facilitated by the introduction of the affinity alkylating agent PrBCM (Young et al., 1972) as well as the radiolabeled derivative of the potent muscarinic antagonist L-QNB (Yamamura & Snyder, 1974). This membrane-bound neuroreceptor has been solubilized from brain

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[1] Abbreviations: mAcChR, muscarinic acetylcholine receptor; L-QNB, L isomer of quinuclidinyl benzilate; [^{3}H]-L-QNB, tritiated L isomer of quinuclidinyl benzilate; EDTA, ethylenediaminetetraacetic acid; PrBCM, propylbenzilylcholine mustard; PMSF, phenylmethanesulfonyl fluoride.

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by salt treatment (Alberts & Bartfai, 1976; Carson et al., 1977), digitonin (Aronstam et al., 1978; Gorissen et al., 1978), and lubrol PX (Haga, 1980). A recent communication from this laboratory (Cremo et al., 1981) reported the first solubilization of mAcChR's from atrial microsomes with almost quantitative recovery by using a mixed detergent system consisting of 0.4% w/v digitonin and 0.08% w/v cholate.

The purpose of this paper is to characterize the solubilized mAcChR from porcine atria with respect to its interactions with muscarinic ligands. Data presented below indicate that the solubilized mAcChR interacts in a stereospecific manner, binding the pharmacologically active isomer of benzetimide 2500-fold tighter than the inactive isomer. Antagonists and local anesthetics appear to have about the same affinity for the solubilized mAcChR as was found for the membrane-bound protein while agonists interact with a single population of sites that correspond to the low-affinity class of agonist sites observed in the membrane-bound preparation (Schimerlik & Searles, 1980). Kinetic studies of [^{3}H]-L-QNB binding to the membrane indicated that the kinetic mechanism entails formation of an initial mAcChR–[^{3}H]-L-QNB complex in a rapid preequilibrium which then undergoes a slow conformational change.

Materials and Methods

The mAcChR was solubilized from porcine atrial microsomes in 10 mM sodium phosphate, 1 mM EDTA, 1 mM PMSF, 0.4% w/v digitonin, and 0.08% w/v cholate buffer, pH 7.4, as described by Cremo et al. (1981). The solubilized receptor was quantitated in terms of QNB binding sites (Yamamura & Snyder, 1974) by using the DEAE filter disc assay developed by Schmidt & Raftery (1973) and modified in this laboratory for use in the above-mentioned buffer system (Cremo et al., 1981). Protein concentration was determined by the method of Lowry et al. (1951) as modified by Peterson (1977) with crystalline bovine serum albumin as the standard. Specific activities of the solubilized extract varied between 1 and 5 nmol of QNB sites per g of protein, and recoveries of total QNB sites from the atrial microsomes were between 85 and 98%. The solubilized mAcChR was either used immediately or stored at 0 °C where it appeared to be stable for at least a week (about 20% of the total QNB sites were lost after 8 days at 0 °C).

[^{3}H]-L-QNB (40.2 Ci/mmol), purchased from New England Nuclear, cochromatographed with a nonlabeled standard (the gift of Dr. W. E. Scott, Hoffman-La Roche Inc.) on Merck silica gel 60 plates in $CHCl_3$–MeOH–water–acetic acid (65:25:5:5, R_f 0.58) and acetone–methanol–diethanolamine (10:10:0.3, R_f 0.38) with over 90% of the radioactivity in the QNB spot. Acetylcholine, carbamoylcholine, eserine, scopolamine, tetracaine, and atropine were from Sigma Chemical Co. Pilocarpine and oxotremorine were purchased from Aldrich Chemical Co. Dibucaine, procainamide, lidocaine, and quinidine were purchased from Pfaltz & Bauer, while dexetimide and levetimide were from Janssen Pharmaceutica.

Equilibrium titrations were performed by adding the competing ligand to buffer plus protein followed by addition of [^{3}H]-L-QNB to the desired concentration after about 10 min. Equilibrium was reached after 1–2 h at which time 100-μL aliquots of solutions were applied to 2.4-cm DEAE disks (or 150-μL aliquots to 2.5-cm disks, Whatman). The solution was allowed to soak for 1–3 min, and the disks were washed twice for 10 min each in 10 mM sodium phosphate–1 mM EGTA, pH 7.4, buffer. Disks were then dried and counted (Beckman Model L53133P scintillation counter) in Triton–toluene scintillation fluid. An aliquot of the incubation mixture was also removed and counted to determine the total [^{3}H]-L-QNB concentration. Free ligand concentration was then calculated from total minus bound [^{3}H]-L-QNB concentration. Nonspecifically bound ligand, determined in the presence of 10 μM atropine, was less than 5% of the total label bound at [^{3}H]-L-QNB concentrations up to 15 nM.

Kinetic studies were done by using methodology similar to the equilibrium titrations. Experiments to determine the association rate constant for L-QNB to the mAcChR were done under pseudo-first-order conditions with the ligand in 10-fold excess over binding sites. Identical rates were observed for QNB association whether the reaction mixture was quenched by pipetting onto DEAE filter disks or by addition of a 20-fold excess of unlabeled QNB prior to application to the disk. These results indicated that the reaction was quenched by the disk essentially within the application time for the solution (5–15 s), and therefore, this method was suitable for use in kinetic studies. The rate constant for [^{3}H]-L-QNB dissociation from the solubilized mAcChR was measured by addition of varying concentrations of competing ligand to a solution of mAcChR plus [^{3}H]-L-QNB that had been preequilibrated at room temperature. When the rate constant for L-QNB dissociation was measured by dilution of the mAcChR–[^{3}H]-L-QNB complex, a solution 1 nM in both L-QNB sites and [^{3}H]-L-QNB was equilibrated for 90 min at room temperature, followed by 10-fold dilution. Aliquots (600 μL) were then pipetted onto 2-in. squares of DE81 paper at time t, washed, and counted as described above.

Data Evaluation

Equilibrium titration curves measuring [^{3}H]-L-QNB displacement were analyzed by using weighted least-squares fits to either eq 1 or 2. In eq 1, the value of the $\bar{j}$ function

$$\frac{[I_0]}{[R_0](1 - \overline{RQ}/\overline{RQ_0})} - 1 = \bar{j} = \frac{[Q]}{[RQ]}\frac{K_i}{K} \quad (1)$$

$$\overline{RQ} = \frac{[R_0][Q]}{K(1 + [I_0]/K_i) + [Q]} \quad (2)$$

(Best-Belpomme & Dessen, 1973) was computed from the total concentration of QNB sites ($[R_0]$) and the fractional saturation of QNB sites ($\overline{RQ}$) at total inhibitor concentrations ($[I_0]$) and in the absence of inhibitor ($\overline{RQ_0}$). [Q] and [RQ] were the concentrations of free and specifically bound [^{3}H]-L-QNB at each inhibitor concentration, respectively, and K_i and K are the dissociation constants for inhibitor and L-QNB, respectively. The value for the K_i for a given competitive inhibitor was then calculated from the slope of the plot of $\bar{j}$ vs. [Q]/[RQ] by using the dissociation constant for L-QNB.

Equation 2, adapted from the weighted least-squares fitting programs of Cleland (1967) for use in ligand binding studies, permitted calculation of R_0 concentration, K, and K_i in a single experiment. Comparison of the computed values for $[R_0]$ and K with their previously determined values (e.g., from QNB site assay and Scatchard plot data, respectively) allowed a check on the internal consistency of the experiment. In all experiments where data were fit by using eq 2, significant binding of inhibitor did not occur until the total inhibitor concentration was in large excess over the concentration of L-QNB sites, thus $[I]_{free}$ was essentially equal to $[I_0]$.

Kinetic data were analyzed by a least-squares fit to eq 3 where τ was the relaxation time, $(cpm)_t$ was the radioactivity

$$\ln \frac{|(cpm)_\infty - (cpm)_t|}{|(cpm)_\infty - (cpm)_{NS}|} = -t\tau^{-1} \quad (3)$$

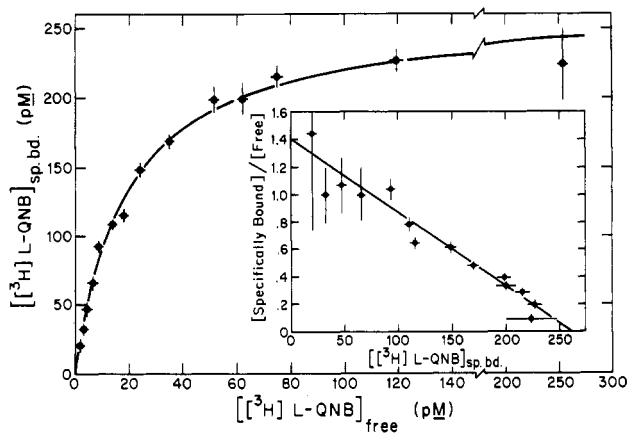

FIGURE 1: [^{3}H]-L-QNB binding to the solubilized mAcChR. Detergent extract, 250 pM in L-QNB sites, in a total volume of 1.2 mL, was equilibrated for 90 min at 25 °C with varying concentrations of [^{3}H]-L-QNB. After removal of triplicate 100-μL aliquots for measurement of total [^{3}H]-L-QNB concentration, triplicate 150-μL samples were pipetted onto 2.5-cm DEAE disks and washed as described under Materials and Methods. After correction for nonspecifically bound label determined in the presence of 10 μM atropine, the data were analyzed in the form of a Scatchard plot (insert). Weighted least-squares analysis gave a dissociation constant, calculated from the reciprocal of the slope, equal to $(1.9 \pm 0.4) \times 10^{-10}$ M and the total concentration of sites, calculated from the abscissa, equal to 262 ± 14 pM. The curve drawn through the data points was computed from the law of mass action by using the computed values for K_{ss} and $[R_0]$.

bound at time t, $(cpm)_\infty$ was the radioactivity bound at equilibrium, and $(cpm)_{NS}$ is the nonspecifically bound [^{3}H]-L-QNB. Control experiments showed that nonspecific binding equilibrated rapidly compared to [^{3}H]QNB association/dissociation with the solubilized mAcChR. Therefore, it was treated as an additive constant to both terms in the numerator of the fraction in eq 3 and cancels.

The fit of the data in Figure 3 to eq 6 in the text was done by the method of weighted least squares where weighting factors were calculated according to Bevington (1969). k_{-1} was first estimated by extrapolation of the plot of τ^{-1} vs. [^{3}H]-L-QNB concentration to zero L-QNB concentration (see eq 5). Using this value for k_{-1}, we calculated k_1 and K from the weighted double-reciprocal fit (Figure 3, insert).

Results

Interactions of [^{3}H]-L-QNB with the Solubilized mAcChR. The equilibrium dissociation constant for L-QNB binding to the mAcChR was determined by using a Scatchard plot (Scatchard, 1949; Figure 1). From the reciprocal of the slope of the weighted least-squares fit to the data (Figure 1, insert), an overall dissociation constant of 190 ± 40 pM was calculated. These data indicate that at equilibrium L-QNB appeared to bind noncooperatively to a single class of sites. The weighted average of four such determinations and values determined from the fit of double-reciprocal plots to eq 2 [(2.50 ± 0.45) × 10^{-10} M, given in Table I] were used to compute the K_i values for inhibitors from eq 1.

Kinetic studies measuring the rate of [^{3}H]-L-QNB association with the mAcChR under pseudo-first-order conditions (Figure 2) showed a single exponential to over 90% of completion of the reaction, again indicating the homogeneity of the solubilized mAcChR preparation with respect to interaction with L-QNB. The calculated intercept of 95 ± 7% indicated that within experimental error all the specifically bound [^{3}H]-L-QNB could be attributed to the single kinetic phase. When the value of τ^{-1} for L-QNB association with the mAcChR was determined as a function of [^{3}H]-L-QNB concentration (the mAcChR concentration was also varied such

Table I: Equilibrium Dissociation Constants for Muscarinic Ligands[a]

classification	compound	dissociation constant	method[b]
antagonist	L-QNB	$(2.5 \pm 0.4) \times 10^{-10}$	II, III
	atropine	$(1.1 \pm 0.1) \times 10^{-9}$	II
	scopolamine	$(4.9 \pm 1.0) \times 10^{-9}$	I
	dexetimide	$(9.0 \pm 1.8) \times 10^{-10}$	I
	levetimide	$(2.1 \pm 0.4) \times 10^{-6}$	I
agonist	acetylcholine[c]	$(1.6 \pm 0.3) \times 10^{-5}$	I
	carbamoylcholine	$(5.7 \pm 1.0) \times 10^{-5}$	I
	oxotremorine	$(3.2 \pm 1.4) \times 10^{-6}$	I
	pilocarpine	$(1.2 \pm 0.7) \times 10^{-6}$	I
local anesthetic	lidocaine	$(1.3 \pm 0.2) \times 10^{-5}$	II
	dibucaine	$(2.5 \pm 0.3) \times 10^{-5}$	II
	tetracaine	$(9.4 \pm 1.0) \times 10^{-6}$	II
	procaine	$(1.9 \pm 0.1) \times 10^{-5}$	II
	quinidine	$(1.5 \pm 0.2) \times 10^{-5}$	II

[a] pH 7.4 in the previously described buffer. [b] Method I was from a fit to eq 1 by using the $\bar{f}$ function; method II, double-reciprocal plots fit to eq 2; method III, a Scatchard plot. [c] In the previously described buffer, pH 6.9, plus 8 μM eserine. At this concentration, eserine did not significantly inhibit [^{3}H]-L-QNB binding.

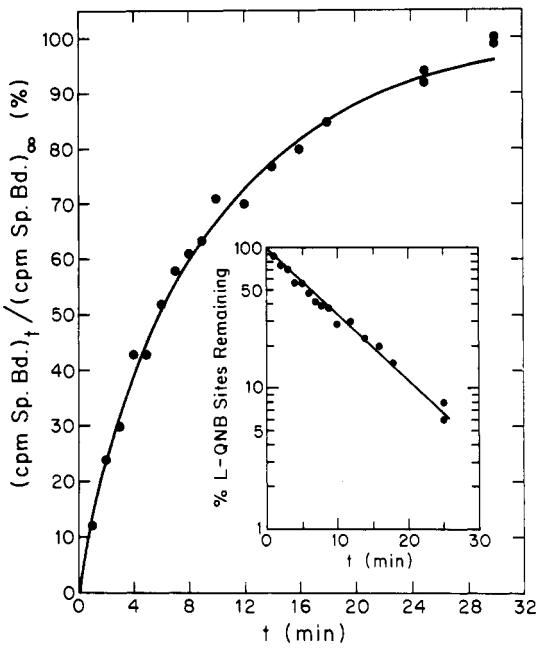

FIGURE 2: Time course for [^{3}H]-L-QNB association with the mAcChR. [^{3}H]-L-QNB (final concentration 2.28 nM) was added to the solubilized mAcChR (final concentration 0.22 nM in L-QNB sites) in a total volume of 4.0 mL (0.24 mg/mL protein) at time zero. At time t, 150-μL aliquots were quenched by pipetting onto 2.5-cm diameter DEAE filter disks. Equilibrium values were measured after 3 h. A least-squares fit of the data to eq 3 gave an intercept value of 95 ± 7% and a value for τ^{-1} calculated from the slope equal to (1.66 ± 0.05) × 10^{-3} s^{-1}. The curve drawn through the data points is the theoretical plot calculated for a single relaxation by using the experimentally determined values.

that the reactions were always measured under pseudo-first-order conditions), it appeared to be nonlinear, leveling off at higher ligand concentrations (Figure 3). The simplest mechanism consistent with these data is given in eq 4. The

$$R + Q \overset{K}{\rightleftharpoons} RQ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RQ' \qquad (4)$$

mAcChR (R) binds L-QNB (Q) in rapid equilibrium to form the RQ complex with $K = [R][Q]/[RQ]$. The RQ complex then undergoes a conformational change to RQ′ with rate constants k_1 and k_{-1} in the forward and reverse directions,

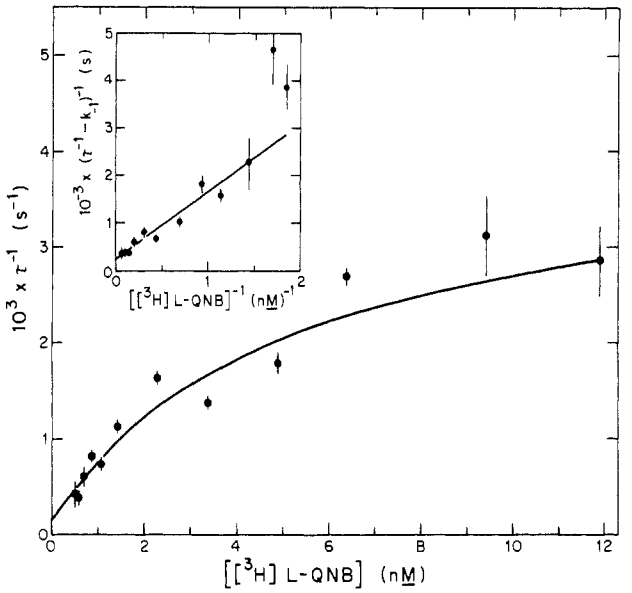

FIGURE 3: Dependence of τ^{-1} on [^{3}H]-L-QNB concentration. The curve through the data was calculated by using $k_{-1} = 1.7 \times 10^{-4}$ s^{-1}, $k_1 = 4.0 \times 10^{-3}$ s^{-1}, and $K = 5.7 \times 10^{-9}$ M. The insert is a weighted least-squares fit to eq 5 that permitted calculation of $k_1 = (4.0 \pm 1.5) \times 10^{-3}$ s^{-1} and $K = (5.7 \pm 2.2) \times 10^{-9}$ M by using the above estimate for k_{-1} obtained by extrapolation to the value for τ^{-1} at zero [^{3}H]-L-QNB concentration.

respectively. In the above mechanism, the first step must be unobservably fast, or two exponentials would be found (Quast et al., 1974). Furthermore, the RQ′ complex must be the exclusive source of bound label since Q rapidly dissociates from RQ during the washing step. The dependence of the relaxation time on ligand concentration for the above mechanism is given in eq 5. The data were computed from a weighted least-

$$\tau^{-1} = k_{-1} + \frac{k_1[Q]}{K + [Q]} \quad (5)$$

squares fit to the reciprocal form of this equation (see Figure 3 and Data Evaluation) after k_{-1} was estimated by extrapolation of the plot of τ^{-1} vs. [^{3}H]-L-QNB concentration to zero ligand concentration.

$$(\tau^{-1} - k_{-1})^{-1} = \frac{K}{k_1}\frac{1}{[Q]} + \frac{1}{k_1} \quad (6)$$

The values obtained from the data evaluation where $k_{-1} = 1.7 \times 10^{-4}$ s^{-1}, $k_1 = (4.05 \pm 1.5) \times 10^{-3}$ s^{-1}, and $K = (5.7 \pm 2.2) \times 10^{-9}$ M. Since $K_1 = k_{-1}/k_1 \ll 1$, the overall dissociation constant for L-QNB from the mAcChR, $K_{ov} = [R][Q]/([RQ] + [RQ'])$, is approximately equal to $KK_1 = (2.3 \pm 1.2) \times 10^{-10}$ M. This value agrees well with the measurements of K_{ov} from either Scatchard plots or fits to eq 2, indicating the consistency of the thermodynamic and kinetic measurements.

The rate constant for [^{3}H]-L-QNB dissociation from the mAcChR–[^{3}H]-L-QNB complex was determined with both antagonists and agonists as displacing ligands or by dilution of the mAcChR–[^{3}H]-L-QNB complex. The data in Figure 4 (10 μM atropine as the displacing ligand) show a single kinetic phase for dissociation when the reaction was followed up to 80% of completion. The oridinate intercept of 100 ± 3%, calculated from a least-squares fit to the data (Figure 4 insert), indicated the absence of any rapid phase in the dissociation kinetics. The observed rate constant for L-QNB dissociation from the receptor, equal to $(4.0 \pm 0.3) \times 10^{-5}$ s^{-1} (weighted average of ten experiments), was a factor of 4-fold slower than that predicted from the analysis of eq 5 and 6. The observed rate constant for L-QNB dissociation and the

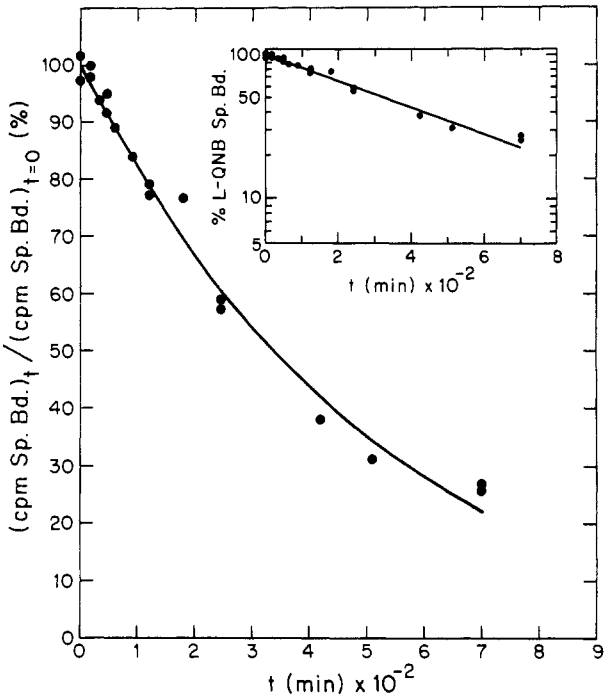

FIGURE 4: Time course for [^{3}H]-L-QNB dissociation. mAcChR (final concentration 0.92 nM in L-QNB sites, 1.0 mg/mL protein) plus 1 nM [^{3}H]-L-QNB were incubated for 1 h at room temperature (total volume 4 mL). At time zero, atropine (final concentration 10 μM) was added, and 100-μL aliquots were withdrawn at time t, pipetted onto 2.4-cm DEASE disks to quench the reaction, and washed as described under Materials and Methods. Data were fit to eq 3 (insert) to give an ordinate intercept of 100 ± 3% and an observed rate constant equal to $(3.5 \pm 0.2) \times 10^{-5}$ s^{-1}. The curve drawn through the data points is the theoretical curve for a single exponential by using the calculated values from the data fit.

number of observed kinetic phases did not vary over atropine concentrations from 10^{-8} to 10^{-5} M, unlabeled DL-QNB concentrations to 1.5×10^{-8} M, 10^{-2} M carbamoylcholine, or 7.5×10^{-5} M oxotremorine. The same value was also found when the rate constant for L-QNB dissociation was measured by 10-fold dilution on the mAcChR–[^{3}H]-L-QNB complex. Thus, the observed rate for L-QNB dissociation appeared to be independent of the concentration and structure of the competing ligand over the concentration range studied.

Equilibrium Titrations. Agonists, antagonists, and local anesthetics appeared to displace [^{3}H]-L-QNB in a competitive manner from a single class of sites. Data obtained from fitting equilibrium titrations to eq 1 and 2 are shown in Figure 5 (for the agonist acetylcholine) and Figure 6 (for the local anesthetic tetracaine), respectively. Dissociation constants determined for all classes of ligands are summarized in Table I.

Discussion

The kinetic studies reported above for L-QNB association with the mAcChR indicate that QNB induces a conformational change in the solubilized mAcChR similar to that previously reported for the membrane-bound atrial mAcChR (Schimerlik & Searles, 1980). The values of $k_{\pm 1}$ obtained for the solubilized mAcChR agree within a factor of 2 with those reported for the membrane-bound mAcChR; however, the prequilibrium constant (K) appears to be about 6-fold higher for the solubilized mAcChR. Thus, the difference in K_{ov} for L-QNB binding to the solubilized mAcChR (2.30×10^{-10} M) compared to the membrane-bound protein (4.1×10^{-11} M)[2]

[2] Computed from the data in Schimerlik & Searles (1980) by assuming that only the L isomer of QNB is active.

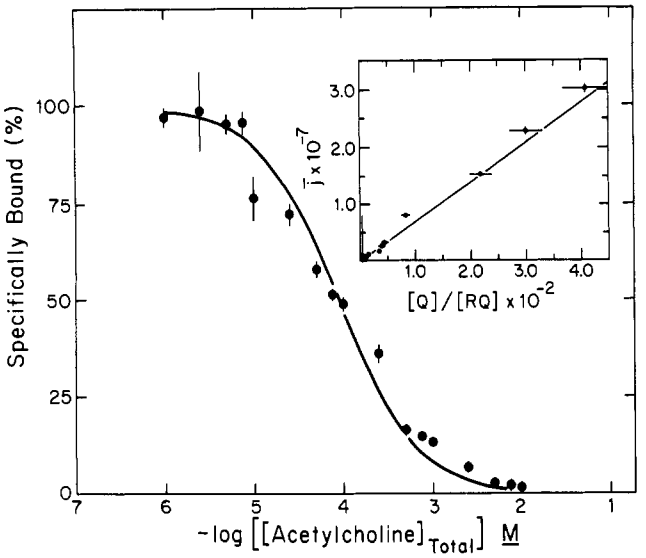

FIGURE 5: Acetylcholine titration of specifically bound [^{3}H]-L-QNB. Titration was performed as described under Materials and Methods except that the pH was adjusted to 6.9 and 8 μM eserine was included. Final concentrations were as follows: 1.05 nM [^{3}H]-L-QNB; 0.33 nM L-QNB sites (0.7 mg/mL protein in 300-μL total volume). Points represent the average of duplicate determinations. The data were evaluated by a weighted least-squares fit to eq 1 (insert) to give a dissociation constant of $(1.6 \pm 0.3) \times 10^{-5}$ M. The curve through the data points was calculated by using this value, a dissociation constant of 2.5×10^{-10} M for L-QNB (Table I), and the experimentally determined free [^{3}H]-L-QNB concentrations.

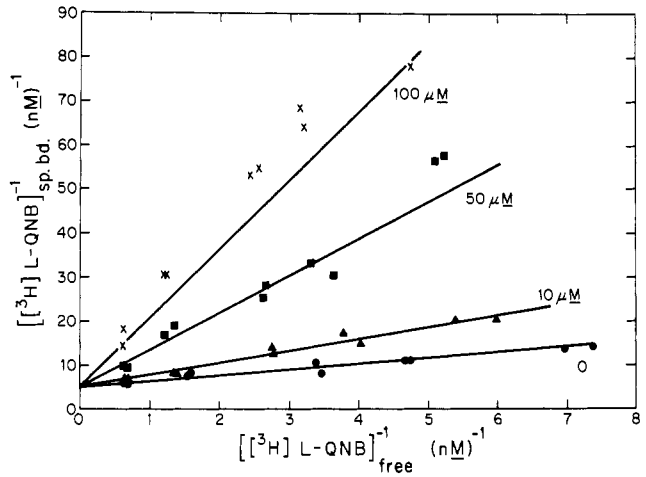

FIGURE 6: Tetracaine titration of specifically bound [^{3}H]-L-QNB. Titration was performed as described under Materials and Methods. Final concentration of L-QNB sites was 0.21 nM in a total volume of 400 μL (0.95 mg/mL protein). The data were evaluated by using a weighted least-squares fit to eq 2, giving values of $K = (2.7 \pm 0.3) \times 10^{-10}$ M for [^{3}H]-L-QNB, $K_i = (9.4 \pm 1.0) \times 10^{-6}$ for tetracaine, and $[R_0] = 0.21 \pm 0.01$ nM. Lines through the data were computed from this fit.

appears to lie in the value of K rather than the rate constants for the conformational change once the antagonist–mAcChR precomplex has been formed.

The data in Figure 3 were also fit to eq 7 which describes the dependence or τ^{-1} on ligand concentration for a simple

$$\tau^{-1} = k_1[Q] + k_{-1} \quad (7)$$

biomolecular association reaction. The values obtained for k_1 (3.7×10^5 M^{-1} s^{-1}) and k_{-1} (3.3×10^{-4}) s^{-1}, give a dissociation constant (k_{-1}/k_1) of 9×10^{-10} M, 4-fold higher than that found in equilibrium measurements. An additional reason for discarding the simple biomolecular association mechanism was that the computed forward rate constant was at least 2 orders of magnitude too slow for a diffusion-controlled association (Hammes, 1978; Bonner et al., 1976). Models involving either two slowly equilibrating receptor forms where only one state binds L-QNB or two-state mechanisms where ligand binding is fast compared to protein isomerizations can also be discarded for reasons identical with those presented for the membrane-bound mAcChR (Schimerlik & Searles, 1980).

Although the values for K_{ov} determined thermodynamically agree with the values determined from the kinetic studies of L-QNB association with the mAcChR, there was a 4-fold difference between the value of k_{-1} determined from the extrapolation of τ^{-1} vs. [^{3}H]-L-QNB concentrations to zero ligand concentration and that determined directly by displacement of the label. The possibility that the mechanism for L-QNB dissociation was via a ternary complex between [^{3}H]-L-QNB, mAcChR, and displacing ligand (suggested in a preliminary report of these data; Herron et al., 1981) was discarded since the dissociation rate constant was the same whether it was measured in the presence of an excess of competitive inhibitor, or directly by dilution of the receptor–[^{3}H]-L-QNB complex. The possiblity that this discrepancy was due to an artifact of the DEAE assay cannot be totally discounted; however, control experiments (Cremo et al., 1981) have shown that the values for K_{ov} and total L-QNB sites determined by this method agree quite well with those determined by equilibrium dialysis. This would not be expected if [^{3}H]-L-QNB was somehow nonspecifically trapped on the disk or one rate constant in the overall mechanism was selectively altered by a factor of 4.

If the data in Figure 3 are refit to eq 6 by using the experimentally determined value for k_{-1} equal to $(4.0 \pm 0.3) \times 10^{-5}$ s^{-1}, one can calculate $K = (8.6 \pm 6.6) \times 10^{-9}$ M, $k_1 = (6.3 \pm 4.8) \times 10^{-3}$ s^{-1}, and $K_{ov} = (5.5 \pm 5.0) \times 10^{-11}$ M. Thus, using the experimentally determined value for k_{-1} results in poorly determined values for k_1, K, and K_{ov}, with K_{ov} being about 4–5-fold lower than that value found from thermodynamic studies.

In an attempt to rationalize the difference in k_{-1} obtained by extrapolation to zero ligand concentration vs. the experimentally determined value, three possibilities were considered. The simplest was that the mechanism given in eq 4 is correct, and the value for K_{ov} determined by using the experimentally determined value for k_{-1} (as opposed to thermodynamic measurements) was an accurate reflection of the limitations (e.g., within a factor of 4–5) of the DEAE assay. The second possibility was that the experimentally observed rate constant for L-QNB dissociation was not equal to the true value for k_{-1} obtained by extrapolation to zero L-QNB concentration. This situation might arise if the [^{3}H]-L-QNB, a hydrophobic ligand, could not freely dissociate from the protein–lipid detergent mixed micelle containing the receptor. In this case, the local concentration of radiolabeled ligand may not be equal to zero, and significant reassociation may be occurring, driving the reaction backward toward RQ′ formation. This would not affect the equilibrium measurements since once the reaction was quenched the DEAE disks were washed in a large volume of 10 mM phosphate buffer, containing no detergent. This could cause micelle disruption followed by dissociation of any weakly bound ligand, leaving only the specifically bound [^{3}H]-L-QNB-mAcChR (RQ′) complex bound to the disk. The final alternative is that the mechanism in eq 4 is not complete, and a more complex mechanism that involves saturation of various preequilibria, and therefore a hyperbolic dependence of τ^{-1} on L-QNB concentration, must be sought. The last possibility must be emphasized in view of previous reports of

anomalous dissociation kinetics in the membrane-bound porcine mAcChR (Schimerlik & Searles, 1980) and digitonin-solubilized mAcChR from bovine (Hurko, 1978) and rat (Gorissen et al., 1978) brain.

Equilibrium titrations showed that agonists, antagonists, and local anesthetics all appeared to displace [^{3}H]-L-QNB in a competitive manner from a single class of binding sites. The affinity of antagonists and local anesthetics for the solubilized mAcChR appeared to agree reasonably well (within factors of 2–5) with those values previously reported for the mAcChR in the membrane-bound state. The fact that stereospecificity in ligand binding was maintained upon solubilization of the mAcChR was shown by the 2500-fold tighter binding of dexetimide (the pharmacologically active isomer of benzetimide) compared to the inactive isomer levetimide.

The behavior of agonists, however, differed from previous results which showed strongly biphasic titration curves (Schimerlik & Searles, 1980) for displacement of [^{3}H]-L-QNB from the membrane-bound mAcChR. Those experiments were analyzed in terms of two noninterconvertible classes of membrane-bound mAcChR's having either low (65–80% of the total L-QNB sites) or high affinity (20–35% of the total number of L-QNB sites) for agonists. The dissociation constants for three of the four agonists studied agreed quite well with those values determined for the low-affinity membrane-bound agonist sites. The value of the dissociation constant for pilocarpine determined above (1×10^{-6} M) fell between those values found for high- (2.2×10^{-7} M) and low-affinity (2×10^{-5} M) sites.

Several possibilities may be considered to explain the loss of biphasicity in the agonist titration data. The first possibility, a selective solubilization of only the low-affinity agonist sites, seemed unlikely since 85–95% of the total L-QNB sites in the atrial microsomes were recovered in the detergent extract. The second possibility was that these are two structurally distinct types of membrane-bound mAcChR in porcine atria, and detergent solubilization affected only the high-affinity agonist sites, converting them to low-affinity sites. While this possibility cannot be discounted, it would certainly be fortuitous that the modified population would interact toward all ligands studied in the same manner as the unmodified population. The third possibility was that the ligand binding units of both populations of mAcChR are similar; however, the high-affinity agonist sites were coupled to a second protein or subunit that serves as either regulator or effector, and detergent solubilization disrupts this interaction. Since agonist binding to the mAcChR in the heart has been shown to activate a guanylate cyclase (George et al., 1970), and agonist affinity has been modulated by both guanyl nucleotides and cations (Berrie et al., 1979; Rosenberger et al., 1980; Sokolovsky et al., 1980), this suggestion, made originally by Birdsall et al. (1979), does not seem unreasonable.

In summary, this paper presents a study of ligand interactions with the mAcChR solubilized from procine atrial microsomes in a mixed detergent system consisting of 0.4% w/v digitonin and 0.08% w/v cholate. Dissociation constants for local anesthetics and antagonists agree fairly well with those found for the membrane-bound mAcChR in porcine atrial microsomes while agonists interact with a single population of L-QNB sites that correspond to the populations of membrane-bound mAcChR's having low affinity for agonists. The kinetic mechanism for L-QNB binding to the solubilized mAcChR appears to be similar to that found for the membrane-bound protein although anomalous dissociation kinetics indicate that a more complicated mechanism may be needed to completely explain the data in both cases.

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