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Muscarinic Sites in the Amphibian Central Nervous System: Characterization and Temperature Studies

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SUMMARY

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Associations of the muscarinic cholinergic antagonist [^{3}H]quinuclidinyl benzilate (^{3}H-QNB) with neural membranes from the frog (*Rana pipiens pipiens*) are described. This ligand interacts with a single set of identical independent sites on these membranes. The set of sites has characteristics suggesting that it represents a population of muscarinic receptor structures. The first thermodynamic association constant of ^{3}H-QNB with the set of sites is $1.7 \pm 0.53 \times 10^9$ M^{-1}. The pseudo-first-order rate constant of association is $2.74 \pm 0.42 \times 10^6$ M^{-1} sec^{-1}. Temperature studies demonstrate several transitions in both the kinetic and thermodynamic properties of association. Finally, when membrane composition is modified by temperature acclimation of the animals, significant changes are observed in both antagonist and agonist interactions with the receptor structure.

NTRODUCTION

In recent years several laboratories have used the nuscarinic cholinergic antagonist [^{3}H]quinuclidinyl benilate (^{3}H-QNB)[2] to characterize a population of muscainic cholinergic receptor structures in neural tissues (1-4), smooth muscle (5) and cardiac tissue (6). Those data lemonstrated that ^{3}H-QNB provides a probe with apparent specificity comparable to the α-neurotoxins used o analyze nicotinic cholinergic receptor populations (7-9). The distribution (10, 11), number (10), and affinity 12) of ^{3}H-QNB binding coincides with expectations deived from physiological data. In addition, other muscainic but not nicotinic ligands significantly interfere with he binding of ^{3}H-QNB to the defined population of sites 12, 13).

In the present report, we have examined several asects of putative muscarinic cholinergic interactions beween cholinergic ligands with membrane fractions derived from neural tissue. The membrane fractions have been derived from the central nervous system of a poikilotherm (i.e., northern grass frogs, *Rana pipiens pipiens*). This tissue has been chosen because these nembranes and the temperature acclimation of these animals provide a flexible biological method of controlling major aspects of membrane structure.

In poikilotherms an inverse relationship is observed between ambient temperature on the one hand and the level of lipid metabolism and the degree of unsaturation in fatty acids on the other hand (14-16). This compensatory change in the degree of unsaturation of fatty acids is seen over a wide range of biological organizations. For example, in crustacean plankton, the iodine number varies inversely with water temperature such that the melting point of membrane lipids is maintained just below the temperature of the environment (14). Similar compensatory changes are noted in earthworms (17), bacteria (15), several types of fish (18, 19) and frogs (16). In the case of frogs (used in this study), a number of changes have been described (16). A decrease in ambient temperature correlates with increases in polyenoic acids such as linoleic (18:2), arachidonic (20:4), and docosatetraenoic (22:4) acids relative to decreases in palmitic (16:0), stearic (18:0), and oleic (18:1) acids. The net effect of these compensatory changes seems to be to maintain some critical level of "fluidity" relative to the ambient temperature.

The significance of membrane fatty acid composition to function is clearly indicated by earlier studies of membrane related systems in *E. coli* auxotrophs deficient in the synthesis of unsaturated fatty acids (20-22). Using this approach numerous membrane associated enzyme

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[2] The abbreviation used is: [^{3}H]QNB, [^{3}H]quinuclidinyl benzilate.

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and transport systems have been shown to be sensitive to the length and degree of unsaturation in membrane lipid fatty acids (e.g., β-galactoside transport, β-glucoside transport, glycerol-3-P acyltransferase, alkaline phosphatase and proline active transport (20–23). Although these studies clearly demonstrate a relationship between fatty acid composition and function, the influences observed are not related simply to general membrane fluidity. Particular changes in membrane composition can increase the transition temperature of one system while decreasing the transition temperature of another system in the same membrane. Different systems can also show greater or lesser response to the same transition. In mycoplasma membranes, NADH oxidase, *p*-nitrophenyl phosphorylase and phosphoenolpyruvate-dependent functions appear to be independent of any transition, while ATPase-dependent functions display different energies of activation above and below the transition temperature (24). If it is assumed that alteration in the characteristics of functional activities with temperature are related to phase separations in the membrane, then the data suggest the existence of different lipid domains associated with different functional activities (20–22).

In this report we have also examined some effects of membrane state on muscarinic interactions.

MATERIALS AND METHODS

Materials were obtained from the following sources: ^{3}H-QNB from New England Nuclear, Boston, Massachusetts (16 Ci/mmole); atropine sulfate, oxotremorine, scopolamine hydrobromide, and pilocarpine from Aldrich, Milwaukee, Wisconsin; acetylcholine chloride, carbamylcholine chloride, carbamyl-β-methylcholine chloride, decamethonium bromide, hexamethonium bromide, DL-homotropine hydrobromide, acetyl-β-methylcholine chloride, DL-muscarine chloride, nicotine, and succinylcholine chloride from Sigma, St. Louis, Missouri. Northern grass frogs (*Rana pipiens pipiens*) were purchased from Mogul Ed, Oshkosh, Wisconsin. During the initial studies the frogs were maintained for 1–5 weeks at 22° after arrival. For the temperature acclimation experiments, the animals were maintained at either 4° or 22° for at least 8 weeks prior to use.

Tissue fractionation. Northern Grass frogs (2.5 to 3.0 in.) were decapitated and their brains rapidly removed and placed in sucrose–phosphate buffer (0.05 M sodium phosphate, pH 7.4, 0.1 M NaCl, 0.32 M sucrose; 4°). The tissue was homogenized in 5 vol (w/v) of the same buffer using a Potter–Elvehjem homogenizer with a Teflon pestle (10 strokes). The homogenate was centrifuged at 1000*g* for 10 min. The supernatant was diluted with sucrose–phosphate buffer to approximately 0.5 mg/ml protein. Protein was determined by the method of Lowery *et al.* (25).

Binding assay. A modification of a previously described method using ^{3}H-QNB was employed in these studies (12). Final incubation buffer was composed of 0.1 M NaCl, 0.16 M sucrose and 0.05 M sodium phosphate, pH 7.4, in a final volume of 0.5 ml. For different experiments, this buffer contained experimental concentrations of membrane proteins, ^{3}H-QNB and various drugs. Bound and free ligand were measured by a continuous flow procedure with Whatman GF/C filters (12). Incubation time and temperature were varied pursuant to experimental protocol.

RESULTS

Thermodynamics. Figure 1 shows the change in "specific" binding of ^{3}H-QNB to receptors in the brain preparation as a function of protein concentration. "Specific" binding is defined in these experiments as that ^{3}H-QNB binding that is blocked by 10^{-4} M scopolamine. These data demonstrate that binding increases linearly with increasing protein concentration in the assay to approximately 150 μg of membrane protein/assay. In all subsequent experiments, less than 150 μg of membrane protein was added to the incubation mixture in order to insure that all experiments could be compared on the basis of moles bound per milligram of membrane protein.

In the next series of experiments, the protein concentration was held constant at approximately 100 μg/assay and a series of concentrations of ^{3}H-QNB was added in the presence or in the absence of 10^{-4} M scopolamine. A linear correction of total binding was developed on the basis of the data in the presence of this concentration of scopolamine. The correction involved using the data in the presence of scopolamine to derive a mathematical expression of nonspecific binding and subtracting this function from the total binding (i.e., in the absence of scopolamine). Development of this method of correction has been described previously (9). Using this correction, the change in "specific" ^{3}H-QNB binding was analyzed. Figure 2 shows the results of five sets of experiments performed over a 6-month period on different membrane preparations. The curves describe the interaction of a homogeneous ligand with a single set of identical, independent sites (i.e., binding increases from 10 to 90% of maximum over 2 log units increase in ligand concentra-

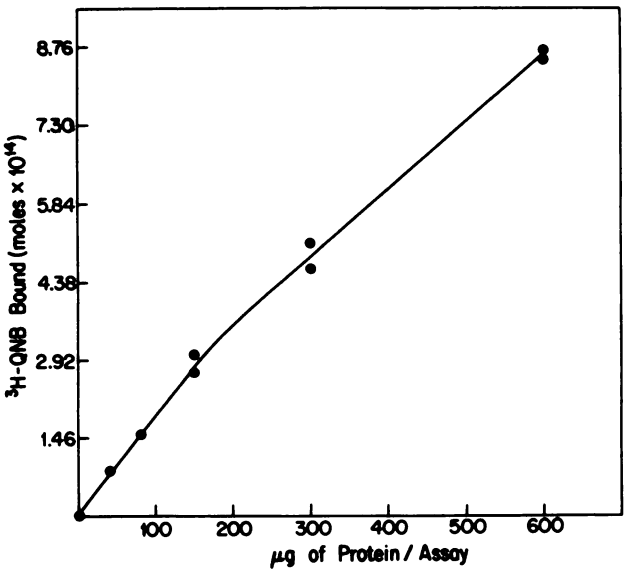

FIG. 1. *A titration of the moles of ^{3}H-QNB "specific" binding as a function of membrane protein*

The ^{3}H-QNB concentration was 8.08 ± 0.2 × 10^{-10} M. "Specific" binding is defined as that which is blocked by 10^{-4} M scopolamine. Assay temperature was 4°.

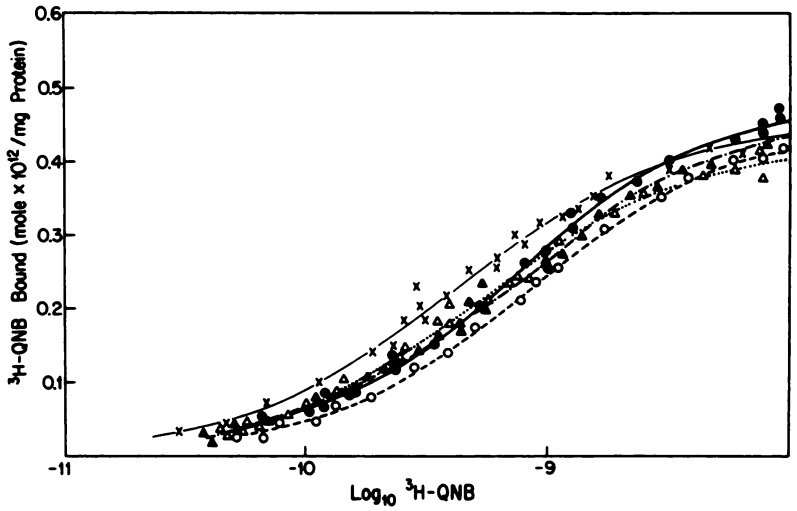

FIG. 2. *The binding of ^{3}H-QNB to membrane fractions from frog neural tissue*
The data are experimentally derived as described under Materials and Methods (4°). The curve is the most appropriate fit of the data to the quation $\nu' = (NK[C])/(1 + K[C])$, where ν' is the amount of ^{3}H-QNB bound; N is the number of available sites; K is the first thermodynamic ssociation constant; and [C] is the concentration of free ^{3}H-QNB. Each curve represents a separate series using different preparations.

ion). The data indicate a first thermodynamic associaion constant (K_A) of (mean ± SD) $1.7 \pm 0.53 \times 10^9$ M^{-1} $K_D = +5.9 \times 10^{-10}$ M). The number of sites in the set lefined as "specific" is $4.48 \pm 0.25 \times 10^{-13}$ mole/mg rotein. No significant difference in affinity (K_A) was bserved between determinations based on multiple exeriments using the same preparation or on experimental oints derived from incubations allowed to reach equilibium in either the forward or reverse direction. The range n K_A (1.22×10^9 to 2.5×10^9 M^{-1}) therefore appears to epresent an intrinsic variation among tissue preparaions. This intrinsic variation is addressed by data preented below.

In the third series of experiments, the effects of 12 holinergic agonists and antagonists on the "specific" inding of ^{3}H-QNB to the membrane protein were exmined (Figs. 3a and b). Protein concentration was held onstant at approximately 100 μg/assay. The ^{3}H-QNB oncentration was 8.8×10^{-10} M and temperature was 4°. The relative potencies of the agents indicated by these data are: DL-homotropine > atropine > scopolamine > oxotremorine > carbamylcholine > muscarone ≅ carbamyl-β-methylcholine ≅ succinylcholine > pilocarpine > acetylcholine ≅ acetyl-β-methylcholine Decamethonium did not have any effect.

Since agonists by definition have a biological effect and antagonists do not, in the next series of experiments we wished to determine if these two classes of compounds interact differently with the putative receptor structure. The experimental approach is based in the effects of these compounds on the binding of ^{3}H-QNB to the defined set of sites. The assumption is that compounds that interact similarly with the receptor structure will be thermodynamically competitive. This analysis has been developed and used previously (9). Figure 4 shows the effect of 7 nM atropine on the binding of ^{3}H-QNB to the defined set of putative receptor sites. These data demonstrate that atropine is a competitive inhibitor of the ^{3}H-QNB binding. Specifically, analysis of the data shows that atropine does not change the character of the ^{3}H-

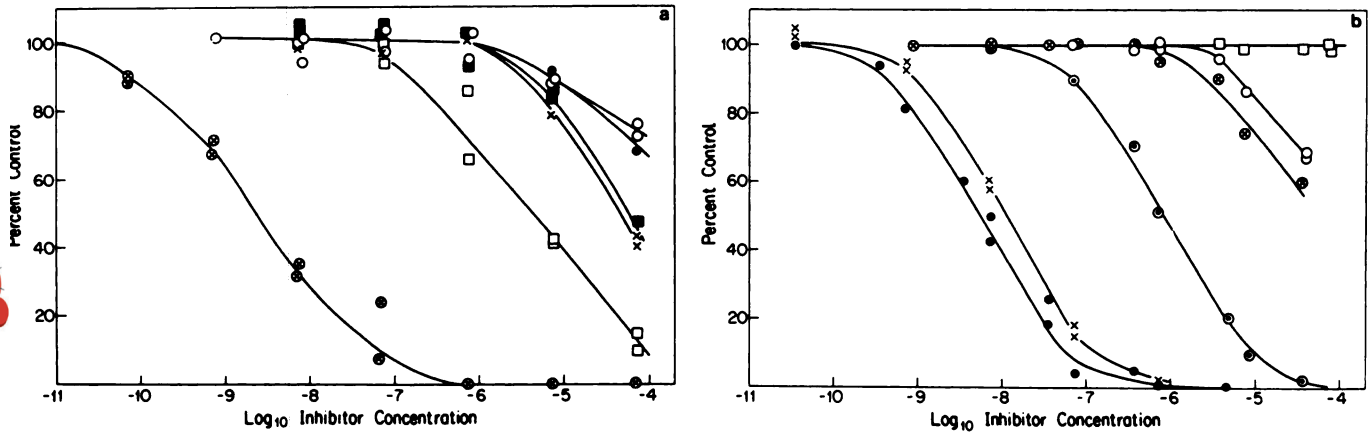

FIG. 3. *The effect of cholinergic ligands on the "specific" binding of ^{3}H-QNB to neural membranes (4°)*
The ^{3}H-QNB concentration was 8.8×10^{-10} M. (a) ⊗, DL-Homotropine; ●, acetylcholine; □, carbamylcholine; ○, Acetyl-β-methylcholine; ×, succinylcholine; ■, carbamyl-β-methylcholine. (b) ⊙, oxotremorine; ●, atropine; ×, scopolamine; ⊗, muscarone; ○, pilocarpine; □, decamethonium.

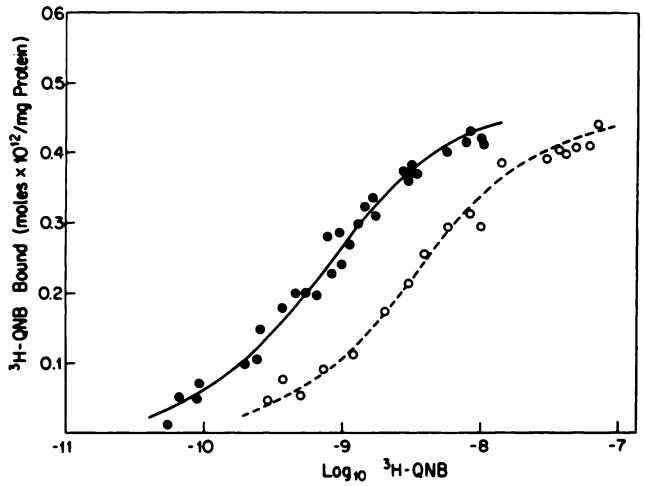

FIG. 4. *A log plot of the binding of ^{3}H-QNB to neural membranes in the absence* (●) *and presence* (○) *of 7 × 10^{-9} M atropine*
The points are experimentally derived and the curve is drawn as described in the legend to Fig. 2.

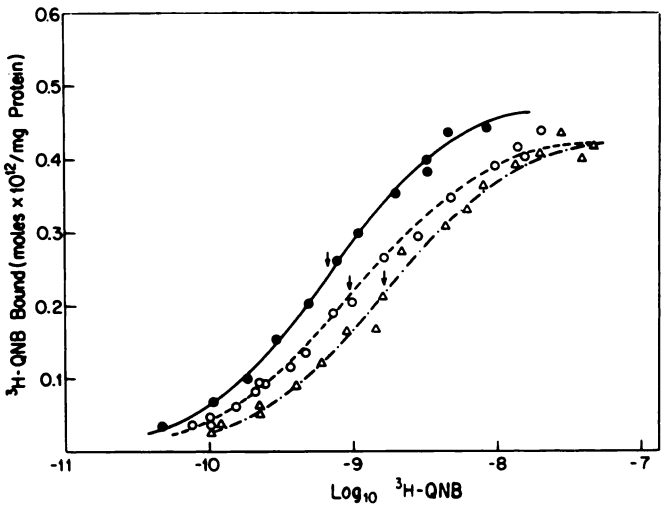

FIG. 6. *A log plot of the binding of ^{3}H-QNB to neural membranes in the absence* (●) *and presence of 2 × 10^{-7} M carbamylcholine* (○) *and 5 × 10^{-7} M carbamylcholine* (△)
The relationship between the experimental points and the drawn curve is the same as described in the legend to Fig. 2.

QNB binding. In both the presence and absence of atropine the interaction of ^{3}H-QNB with the set of sites is a bimolecular reaction of a homogeneous ligand with a single set of identical independent sites. Similarly, the atropine does not alter the number of sites available to the ^{3}H-QNB. The effect of atropine is to lower the apparent affinity of the ^{3}H-QNB binding.

Figure 5 presents data from a similar experiment in which 40 nM scopolamine was added. These data demonstrate that scopolamine is also competitive with QNB. Thus the antagonists, QNB, atropine and scopolamine all interact similarly with the putative receptor structure.

Similar experiments were also conducted using the agonist carbamylcholine. Those data are presented in Fig. 6. Examination of the curve indicates that in both the presence and the absence of carbamylcholine, the interaction of ^{3}H-QNB with the population of sites represents the interaction of a homogeneous ligand with a single set of identical independent sites. The carbamylcholine causes a competitive shift in apparent ^{3}H-QNB binding affinity. However, carbamylcholine also reduces the number of sites available to the ^{3}H-QNB. This result indicates that carbamylcholine either inactivates, or has a much higher affinity for, 10–15% of the population of sites that are identical and independent with respect to QNB, atropine, and scopolamine.

Kinetics. The time course of association of ^{3}H-QNB to the "specific" set of sites was determined at four concentrations of ^{3}H-QNB (approximately: 0.17, 0.38, 0.54, and 1 nM) at each of seven temperatures ranging from 4° to 35°. Figure 7 presents, as an example, the data obtained for the four ^{3}H-QNB concentrations at 35°. The set of time courses for each temperature was analyzed as pseudo-first-order kinetics. The appropriateness of this analysis can be seen by examining Fig. 8. These graphs present the semilogarithmic analysis of the data at 25°. The lines are drawn based on a exponential curve fit.

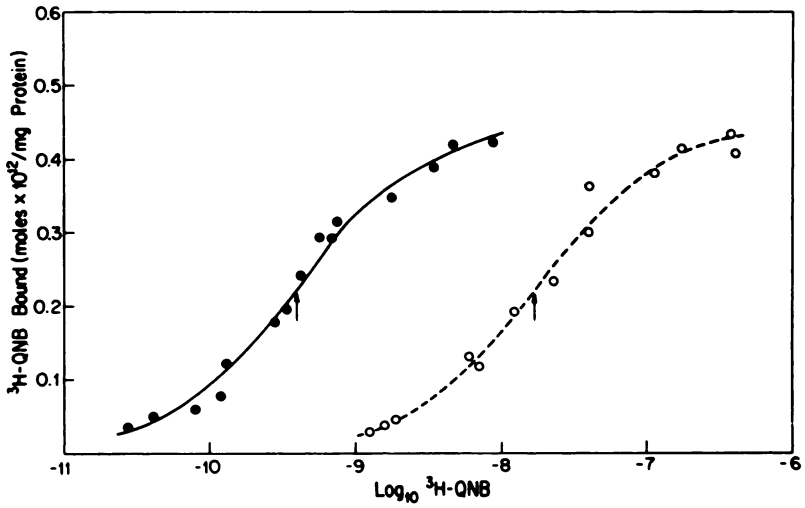

FIG. 5. *A log plot of the binding of ^{3}H-QNB to neural membranes in the absence* (●) *and presence* (○) *of 4 × 10^{-8} M scopolamine (4°)*
The points are experimentally derived and the curve is drawn as described in the legend to Fig. 2.

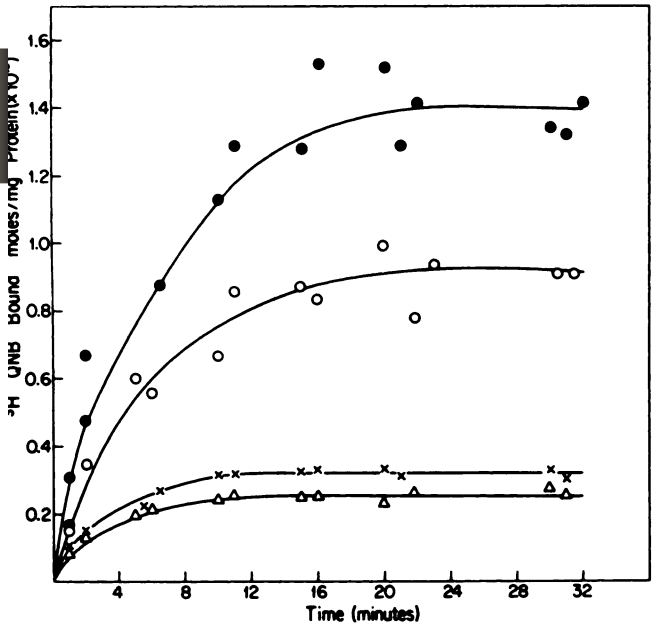

FIG. 7. *A time course of the "specific" binding of ^{3}H-QNB to neural membranes at 35°*

The ^{3}H-QNB concentrations: (●) 1.05×10^{-9} M; (○) 5×10^{-10} M; (×) 1.6×10^{-10} M; and (△) 0.74×10^{-10} M.

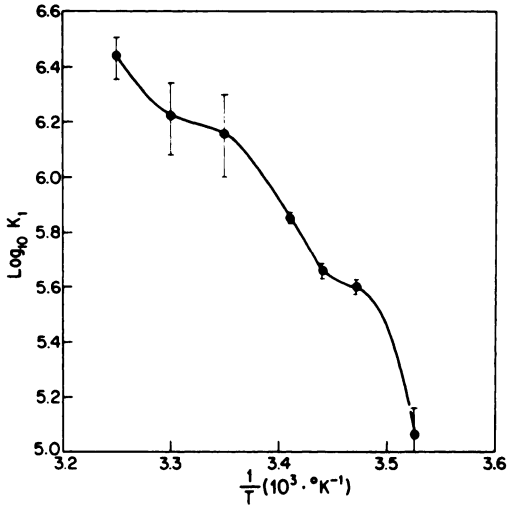

FIG. 9. *An Arrhenius plot demonstrating the effect of temperature on the pseudo-first-order rate constant of the binding of ^{3}H-QNB to neural membranes*

The points are the mean of four independent determinations at four different ^{3}H-QNB concentrations at each temperature. The error bars represent ± one standard deviation of the mean.

These data demonstrate that the pseudo-first-order forward rate constant (K_1) ranges from $2.74 \pm 0.42 \times 10^6$ M^{-1} sec^{-1} at 35° to $1.16 \pm 0.3 \times 10^5$ M^{-1} sec^{-1} at 10°. An Arrhenius plot of this data is presented in Fig. 9. These data demonstrate several transitions in function over a broad temperature range.

Temperature acclimation. In poikilotherms, an inverse relationship has been observed between ambient temperature on the one hand and the level of lipid metabolism and the degree of unsaturation in fatty acids on the other [26]. As an approach to understanding both the intrinsic variation in affinity noted in earlier experiments (Fig. 1) and the role of the membrane in determining receptor properties (Fig. 9) we have conducted a few preliminary experiments exploiting temperature acclimation of these animals. In general, animals received from suppliers are either acclimated to seasonal conditions or are in a transition due to the commercial process. For these experiments, animals were acclimated to either 4° or 22° as described under Materials and Methods. In the first series of experiments, we examined the association of ^{3}H-QNB with membranes from animals acclimated to each temperature. Figure 10 presents the results of one of these experiments. The results demonstrate that when association with the putative receptor structure in the two sets of membranes (4° and 22°) is examined at a constant intermediate temperature (13°), both the number of sites and the binding affinity is higher in the 22° adapted membranes.

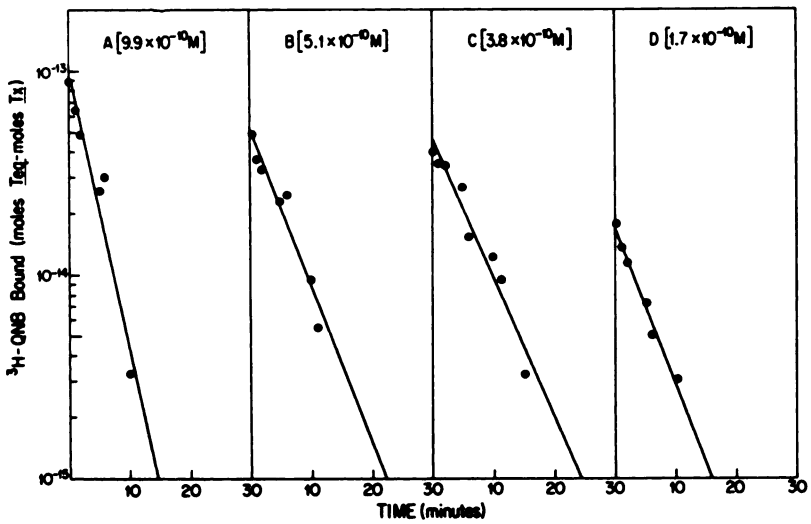

FIG. 8. *Analysis of the time course of the specific binding of ^{3}H-QNB to neural membranes (25°) as pesudo-first order*

The vertical axis represents the log of the difference between the amount bound at time (t_x) and the amount bound at equilibrium for that concentration. The various concentrations are as indicated. The lines were fit to the data by the method of least squares.

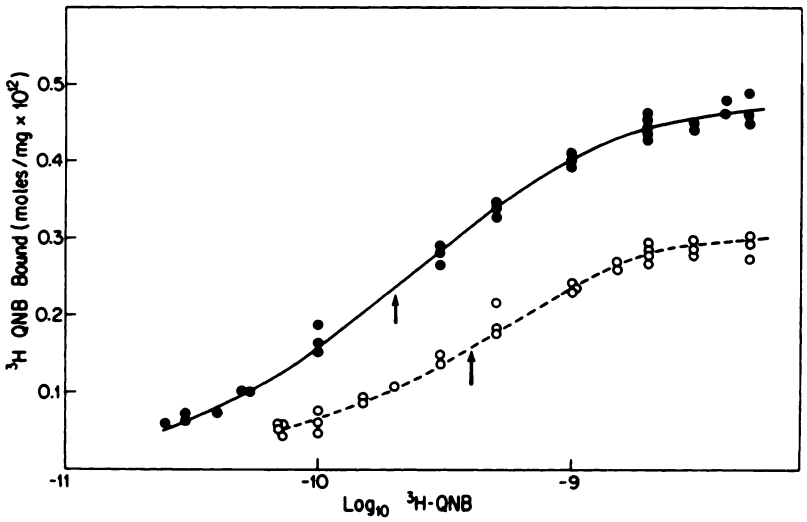

FIG. 10. *A log plot of the specific binding of ^{3}H-QNB to neural membranes from frogs acclimated to 22° (●) and 4° (○)*
The assay temperature was 13°. The relationship between experimental points and the drawn curves is the same as described in the legend to Fig. 2.

Because of the possibility of a more extensive involvement of the membrane in agonist-mediated action than in antagonist-mediated action, we also examined the effect of carbamylcholine on ^{3}H-QNB binding in the two sets of membranes at the intermediate temperature (13°). The results of these experiments are summarized in Fig. 11. The data show that at 13°, carbamylcholine is a more potent inhibitor of ^{3}H-QNB binding to the set of sites in 4° acclimated membranes than it is of the ^{3}H-QNB binding to 22° membranes. This result not only supports the observation of the higher ^{3}H-QNB affinity in the 22° membranes but it also suggests that the acclimation effect is different for the agonist carbamylcholine than for the antagonist QNB. Specifically, if the effects were identical, then no difference should be observed in these experiments since lower QNB affinity should be canceled by lower carbamylcholine affinity. Further experiments analyzing the use of temperature acclimation to study membrane structure–function relationships are currently in progress.

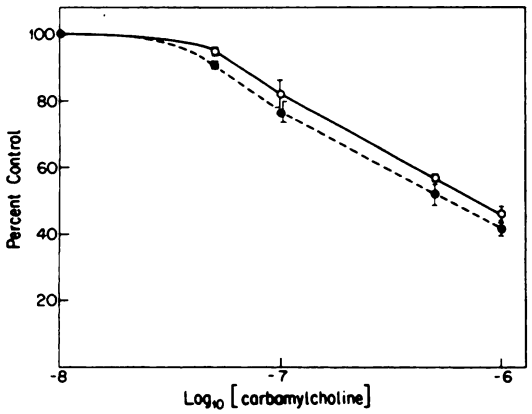

FIG. 11. *The effect of carbamylcholine on the binding of ^{3}H-QNB to neural membranes from frogs acclimated to 22° (●) and 4° (○)*
The assay temperature was 13°. The experimental points represent the mean of four to six determinations. The error bars represent ± one standard deviation of the mean.

DISCUSSION

Reports from several other laboratories have used ^{3}H-QNB to define populations of putative muscarinic cholinergic receptors in a number of mammalian tissues. Values of the thermodynamic association constant for neural tissue range from 8.9×10^8 to 1.67×10^{10} M^{-1} with median values in the area of 10^9 M^{-1}; for heart, values range from 1×10^9 to 8.33×10^9 M^{-1} with median values in the area of $2\text{–}3 \times 10^9$ M^{-1} (2, 6, 27); for smooth muscle, the reported value is 2.38×10^9 M^{-1} (5). In the present report the mean value for neural tissue from unacclimated frogs was $1.\ldots \pm 0.53 \times 10^9$ M^{-1} with a range of 1.22×10^9 to $2.54 \times 10^{\ldots}$ M^{-1}. Present observations are therefore consistent in this respect with previous reports. Similarly, values of the number of sites in neural tissue range from 0.2×10^{-12} mole/mg to 1.05×10^{-12} mole/mg with median values in the area of $0.3\text{–}0.5 \times 10^{-12}$ mole/mg. In the present report the mean value for neural tissue for unacclimated frogs was $0.448 \pm 0.25 \times 10^{-12}$ mole/mg. These observations are also consistent with previous reports. With a few minor exceptions, relative potencies of agonists, antagonists and mixed agonists–antagonists were also found to be comparable to previously reported data (2, 4, 12). Therefore the results indicate that the population of binding sites described in amphibian neural tissue is quite similar to putative muscarinic receptor populations described in other tissues.

In previous studies we have observed that agonists and antagonists interact differently with putative nicotinic cholinergic receptor populations (9). The two antagonists α-bungarotoxin and d-tubocurarine were found to be competitive with each other for a single set of identical binding sites. In contrast, the agonist carbamylcholine demonstrated either multiple or cooperative interactions with this same set of sites. The present report describes similar experiments with the antagonists QNB, atropine and scopolamine, and the agonist carbamylcholine. The results indicate that the three antagonists are all competitive with each other for the same set of identical independent sites. On the other hand, carbamylcholine

is only competitive with a subset of the population of sites defined with ^{3}H-QNB. Whether this represents an intrinsic heterogeneity or a partial inactivation of the population of sites with respect to the antagonist is not clear at present. The relationship of these data to the biological differences between agonist and antagonist action is presently being considered.

Previous reports from other laboratories have demonstrated that a variety of membrane-associated mechanisms undergo transitions in activation energy as a result of temperature-induced membrane phase changes. It has been demonstrated that (NA^+, K^+)-ATPase undergoes a transition in energy of activation in the area of 20° (28). Subsequent work by Kimelberg and Papahadjopoulos demonstrated that following inactivation by deoxycholate and reactivation by dipalmitoylphosphatidylglycerol a transition in activation occurred at about 32° (29). Additional studies demonstrated a close correlation between transitions in the state of the lipid environment and transitions in the activation energy of the ATPase. We have examined the effect of temperature on the forward rate constant of the interaction of ^{3}H-QNB with a defined population of sites. The data suggested that there is a broad range within which transitions take place in the rate constant of the association of ^{3}H-QNB with the muscarinic cholinergic receptor structure in these membranes. Interpretation of these data require consideration of the fact that poikilothermic animals are subject to periodic changes in body temperature concomitant with changes in environmental temperature. The broad transition noted in membrane functions in this system, as opposed to the more discrete transitions noted in tissues from thermal regulating animals, is most likely related to a biological adaptation for flexibility. In addition, the results suggest that the "microenvironments" associated with membrane proteins may be dynamic. Considering the broad range of the transition, the data may indicate that there is an exchange in phospholipids between the "microenvironment" of the protein and the bulk environment in response to temperature. The intent of this exchange may serve to bring more long chain polyunsaturated fatty acids into the "micro-environment" of the protein and thus maintain critical "fluidity" around the functioning protein. A change in phospholipid composition of the receptor microenvironment could be explained without postulating any specialized mechanisms. Phase separations take place with temperature changes in mixed phospholipid membranes. As the higher melting phospholipids reach their transition temperature, there will be a tendency for them to form more crystalline-like regions within the membrane. The formation of these more ordered regions could exclude lower melting phospholipids and the receptor structure. Such a procedure would create a receptor environment that changes relatively rapidly in composition but only slowly in fluidity over a wide temperature range.

The necessary flexibility of amphibian membranes with respect to "fluidity," and the ability of poikilotherms to metabolically respond to long-term temperature stress by changing membrane composition suggest that isothermic acclimation may represent a valuable experimental approach to the manipulation of membrane composition for the purpose of studying the relationship between membrane structure and membrane-mediated function. The present report presents initial studies attempting isothermic acclimation as an approach to the investigation of membrane structure–function relationships. The results demonstrate that temperature acclimation influences both the number of sites and affinity of ^{3}H-QNB binding. Tissue from animals acclimated to 4° have both fewer sites and a lower affinity than tissue from animals acclimated to 22° when studied at a single intermediate temperature (i.e., 13°). The reduction in the number of sites probably represents either a decrease in receptor synthesis or an increase in degradation. On the other hand, the differences in affinity may represent differences in the lipid environment of the receptor. The data suggest that the general increase in polyunsaturated fatty acids with "cold" adaptation reduces the affinity of ^{3}H-QNB for the receptor population. This conclusion is consistent with recent observations reported by Aronstam *et al.* (30). They reported that the exogenous introduction of unsaturated phospholipids into rat neural membranes substantially inhibited the binding of ^{3}H-QNB to a defined muscarinic receptor population in rat neural tissue similar to the one described here in amphibian neural tissue. Although the exact nature of the inhibition was not defined in that report, their data are consistent with a decrease in binding affinity. Since in the present studies, the 4° adapted membranes should be more fluid at 13° than the 22° adapted membranes at 13°, the data suggest that increased fluidity may decrease affinity. An alternative explanation that relates to biological regulation is that the 22° adapted animals are more flexible in their ability to accommodate change. Since 22° is midtemperature range for these animals and 4° is an extreme, it is possible that animals forced to acclimate to an extreme do so at the expense of some flexibility. In either case current studies in progress analyzing the response characteristics of this and other systems in membranes from animals acclimated to several different temperatures should provide additional insight into this and other related questions.

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