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ROLE OF PHOSPHOLIPIDS IN MUSCARINIC BINDING BY NEURAL MEMBRANES*

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Abstract—A role for acidic phospholipids in brain muscarinic receptors is suggested by their effect on the specific binding of a potent muscarinic antagonist, tritiated 3-quinuclidinyl benzilate ([^{3}H]QNB), to neural membranes. The addition of phosphatidylserine (PS) enhances specific muscarinic binding up to 40 per cent, while phosphatidic acid (PA) and phosphatidylinositol (PI) are less effective, and neutral lipids are generally without effect. Unsaturated fatty acids, but neither saturated fatty acids nor the methyl esters of unsaturated fatty acids, inhibit [^{3}H]QNB binding, the amount of inhibition being proportional to the degree of unsaturation. Unsaturation in the phospholipids is also inimical to muscarinic binding. Pretreatment of the membranes with phospholipases A and C decreases binding 100 and 60 per cent respectively. The addition of acidic phospholipids restores some binding activity after phospholipase treatment. Inhibition of [^{3}H]QNB binding by low concentrations of cationic detergents is completely reversible by PS and PA. Ionic, pH and temperature effects on QNB binding indicate that the association of QNB with muscarinic receptors involves primarily hydrophobic interactions.

The role of lipids in membrane structure and function and the nature of protein–lipid interactions are areas of intense research. Much evidence has accumulated which suggests that acidic lipids preferentially associate with membrane proteins. For example, Marinetti and Love [1] have found that, in the red blood cell membrane, phosphatidylserine is five times more likely to be crosslinked to membrane proteins than is phosphatidylethanolamine. Acidic lipids are known to activate enzymes such as Na^+, K^+-ATPase [2], acetylcholinesterase [3], tyrosine hydroxylase [4], and adenyl cyclase [5], and to change the conformation of model proteins [6].

Information on the role of lipids in the activity of neural receptors is limited. Work in this laboratory has indicated a close relationship between phosphatidylserine and the opiate receptor [7, 8]. Eldefrawi *et al.* [9] found that nicotinic receptors from Torpedo electroplax were susceptible to phospholipase C treatment. Bartfai *et al.* [10] found that the binding of muscarinic ligands to a soluble smooth muscle preparation was slightly inhibited by phospholipases C and D. Alberts and Bartfai [11], however, reported that phospholipase C had no effect on atropine binding by a preparation solubilized from rat brain by high salt concentrations. The difficulty in finding solubilizing agents for muscarinic receptors which do not alter or destroy their binding activity may be a reflection of the receptors' intimate association with the lipid membrane environment.

The present studies were carried out in order to determine the importance of lipids for the muscarinic cholinergic receptor of rat brain. A specific antagonist, tritiated 3-quinuclidinyl benzilate ([^{3}H]QNB), was used to measure muscarinic binding [12, 13]. The effects of phospholipases, phospholipids, fatty acids, cationic detergents, ions and temperature on the binding were investigated. The results indicate that acidic phospholipids are components of brain muscarinic receptors.

MATERIALS AND METHODS

Measurement of [^{3}*H*]*QNB binding to brain tissue.* A crude synaptic membrane preparation was obtained by killing 150 g, male, Sprague–Dawley rats with a sharp blow to the lumbar region. The forebrains (everything anterior to the midbrain) were quickly removed and thoroughly homogenized in 20 vol. of 20 mM sodium–potassium buffer, pH 7.4, containing 1 mM EDTA. The homogenate was centrifuged at 3000 *g* for 10 min and the resulting supernatant was centrifuged for 30 min at 40,000 *g*. The pellet was homogenized in 20 mM phosphate buffer, pH 7.4, and used without further treatment. Electron microscopy of the pellet revealed a large amount of disrupted nerve endings and other membranes, but little mitochondrial or nuclear material.

Muscarinic binding was measured by a variation of the method of Yamamura and Snyder [12]. [^{3}H]QNB having a specific activity of 130 mCi/mmole was prepared as previously described [14]. Two ml of a suspension containing 3 mg protein, 20 mM phosphate buffer and [^{3}H]QNB was incubated for 30 min at 35°. The concentration of [^{3}H]QNB in the incubation medium was 10 nM in all experiments, except in the chaotropic ion experiments, where the concentration was 1 or 10 nM, as noted, and in the determination of dissociation constants, where the concentration was varied from 0.1 to 50 nM. Control samples contained a large excess (10^{-5} M) of unla-

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beled QNB. The mixture was filtered by suction through a Whatman GF/B glass fiber filter and washed once with 7 ml of ice-cold buffer. The filters were placed in plastic scintillation vials, and 10 ml of scintillation fluid [10 g of 2,5-diphenyloxazole (PPO), 0.5 g of 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP), 1:1 Triton X-100, 2:1 toluene] was added. The vials were held for at least 12 hr at room temperature before being counted in a Beckman LS-233 counter at an efficiency of 24 per cent. Under these conditions, direct binding of QNB to the filters was negligible (10–20 cpm). Specific binding was defined as the total binding minus the binding in the presence of excess unlabeled QNB.

As a consequence of the limited specific activity of radiolabeled ligands, distortions may be introduced in the determination of very low dissociation constants by the necessity of using relatively high concentrations of receptor [15]. The receptor concentration must be less than one-tenth of the true affinity constant in order for the experimentally derived values to be valid [15]. To obtain such a condition, the receptor concentration was reduced to 20 pM by simultaneously decreasing the amount of protein per assay to 1 mg and increasing the volume of the incubation medium by 10 to 20-fold. Figure 1 depicts the [^{3}H]QNB binding curve at 33.4° with a reduced concentration of receptor. The K_D is 1.9×10^{-10} M, in good agreement with values reported by other investigators [12, 13].

Effect of added lipids on [^{3}H]QNB binding to neural membranes. L-α-Lysophosphatidylcholine (type 1), 1-α-phosphatidylcholine (PC) dioleoyl, dipalmitoyl (grade 1), and from bovine brain (type 111-B), 1-α-phosphatidylethanolamine (PE) dipalmitoyl, cholesterol (Sigma grade), and linoleic (grade 111) and oleic acids were obtained from Sigma Chemical Co. Phosphatidylinositol (PI) from yeast, cerebrosides from bovine brain, and stearic and palmitic acids (puriss) were obtained from Koch-Light Laboratories, Ltd. Arachidonic and docosahexaenoic acids, the methyl esters of all the fatty acids and gangliosides from bovine brain, were obtained from Supelco, Inc. Phosphatidylserine (PS), phosphatidylinositol and phosphatidylethanolamine were prepared from bovine brain by column chromatography by the methods of Rouser *et al.* [16]. All lipids were over 95 per cent pure as estimated by thin-layer chromatography on Silica gel H using chloroform–methanol–acetic acid–water (25:15:4:2).

To study the effect of lipids on muscarinic binding, aliquots of standard solutions of the various lipids in suitable organic solvents were added to Teflon–glass homogenizers and evaporated under a stream of nitrogen. The ratio of protein to added lipid was 6 to 1. A buffered aqueous suspension of neural membranes was added and the mixture was well homogenized. Aliquots of this suspension were then assayed for [^{3}H]QNB binding as described above.

Phospholipase and detergent treatments. Phospholipase A from bee venom, phospholipase C (type 111) from *Bacillus cereus*, and phospholipase D (type 1) from cabbage were obtained from Sigma Chemical Co. Membranes to be treated with a lipase were suspended at a protein concentration of 8 mg/ml in 20 mM phosphate buffer, pH 7.4, containing 3 mM $CaCl_2$ (30 mM calcium in the case of phospholipase D). The phospholipase was then added (0.07 units phospholipase C/mg of protein, 0.7 units phospholipase A/mg of protein, or 0.22 units phospholipase D/mg of protein), and the mixture was incubated at 35° in a water bath. In some experiments, membranes were suspended in buffer containing 7.5 mg/ml of fatty acid-free bovine serum albumin (BSA) (Sigma) during phospholipase A incubation. The reactions were quenched by removing 0.4-ml aliquots to 1.6 ml of ice-cold buffer containing 10 mM EDTA (100 mM EDTA in the case of phospholipase D). The binding assay was carried out as described above except that the incubation was for 1 hr at 0°.

Membranes were treated with cationic detergents (cetylpyridinium chloride and cetyltrimethylammonium bromide from Sigma) by adding small aliquots of concentrated detergent solutions while stirring vigorously. The membrane suspension was allowed to stand at room temperature for 30 min before performing the usual binding assay.

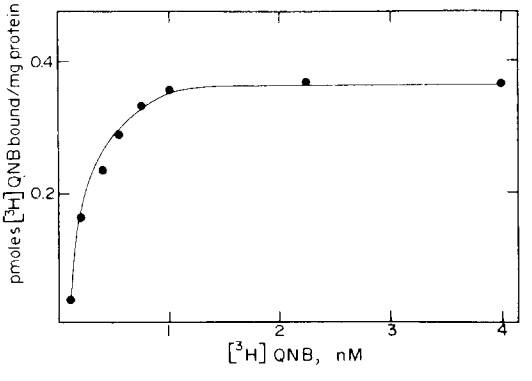

Fig. 1. Specific binding of [^{3}H]QNB to a rat brain membrane preparation. The concentration of receptor was reduced to less than 20 pM by suspending 1 mg protein in 25 ml of incubation media. The tissue was incubated at 33.4° for 30 min and the filter assay was performed as described in Materials and Methods.

RESULTS

Binding of [^{3}H]QNB to neural membranes. From concentration vs binding curves it was observed that saturation of specific binding was attained at a QNB concentration of 1.0×10^{-9} M at 33°. A Lineweaver–Burk plot revealed a K_D value of 1.9×10^{-10} M. The concentration of receptor in the crude synaptic membrane preparation was 0.2 to 0.4 pmole/mg of protein.

Binding constants were determined at several different temperatures. It was found that the dissociation constant decreased with increasing temperature. A plot of the log of the association constant vs $1/T$ reveals a large positive enthalpy change of 13,000 cal/mole associated with QNB binding (Fig. 2). A large positive entropy of 80 cal/mole was also found, so that the free energies of binding were calculated to range from −10,400 cal/mole at 0° to −13,600 cal/mole at 34°.

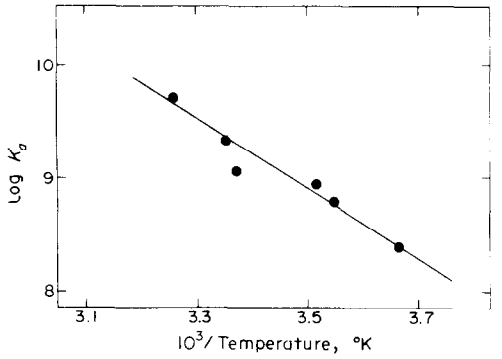

Fig. 2. Influence of temperature on the affinity of [^{3}H]QNB for the muscarinic receptor. Thermodynamic parameters of the binding interaction were obtained from the relationship

$$\log K_a = \frac{-\Delta H}{2.3R}\frac{1}{T} + \frac{\Delta S}{3.3R},$$

as described by Segel [17]. Each point represents the average of two experiments agreeing within 5 per cent.

[^{3}H]QNB binds specifically to brain tissue over a very wide pH range (Fig. 3). Binding is virtually unaffected by H^+ concentration from pH 4 to 9, and significant amounts of binding are seen from pH 2 to 11. Above pH 12, there is a complete inhibition of binding which cannot be reversed by lowering the pH. Ca^{2+} and Mg^{2+} have a slight (10 per cent) inhibitory effect on binding at concentrations of 0.5 mM. Increasing the divalent cation concentration to up to 5.0 mM resulted in no further decrease. EDTA in concentrations up to 100 mM had no effect on the binding.

The presence of chaotropic anions inhibited [^{3}H]QNB binding. NaI decreased binding 10 per cent at 50 mM, while 1 M NaI decreased binding 60 per cent. This inhibition could be completely reversed by increasing the concentration of labeled ligand in the binding assay from 1 to 10 nM, indicating that the loss of binding represents decreased QNB affinity for the receptor rather than destruction or solubilization of the receptor. After the salt was washed away by centrifuging and resuspending the resulting pellet in low ionic strength buffer, the binding potency was completely restored. The strength of various chaotropic ions at 0.5 M in decreasing QNB binding was: $SCN^- > CCl_3COO^- > I^- > NO_3^- > CH_2ClCOO^-$. This series agrees well with that given by Hatefi and Hanstein [19] for the strength of chaotropic ions in most situations.

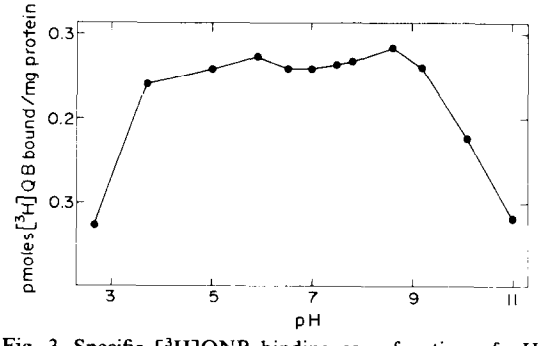

Fig. 3. Specific [^{3}H]QNB binding as a function of pH. The citrate–phosphate–borate buffer of Teorell and Stenhagen [18] was used.

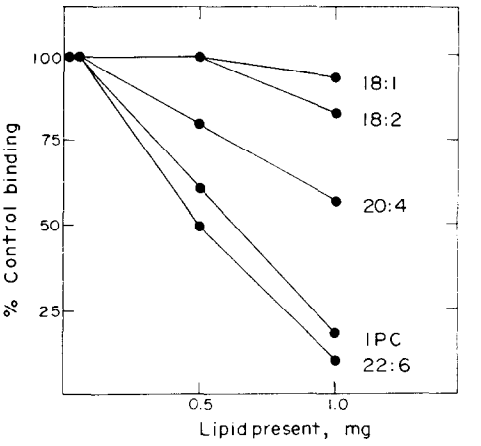

Fig. 4. Effect on specific [^{3}H]QNB binding of lysophosphatidylcholine (lPC) and oleic (18:1), linoleic (18:2), arachidonic (20:4) and docosahexaenoic (22:6) acids. Saturated fatty acids (stearate and palmitate) and the methyl esters of all the fatty acids depicted here had no effect on the binding.

Effect of fatty acids on [^{3}H]QNB binding. Homogenization of neural membranes in the presence of various fatty acids commonly found in brain lipids produced the effects shown in Fig. 4. At high concentrations (0.5 to 1.0 mg fatty acid/3 mg of protein), certain fatty acids inhibited muscarinic binding, the degree of inhibition being proportional to the number of double bonds in the aliphatic chain. Completely saturated fatty acids (palmitate and stearate) and the methyl esters of all of the fatty acids were without effect.

Effect of phospholipids on [^{3}H]QNB binding. The direct addition of certain lipids to neural membranes enhanced high affinity QNB binding, phosphatidylserine and phosphatidic acid being the most active (Table 1). Preincubation of the membranes with 10 mM EDTA doubled the effect of PS, while producing somewhat less activation by certain other lipids. PS and other acidic lipids produced an increased amount of low affinity, non-specific binding of QNB. That the lipid enhancement listed represents specific muscarinic binding can be derived from the fact that PS increases the specific binding of cerebellar and cerebral tissues by amounts proportional to their normal binding, even though the total specific binding in the cerebrum is 5 times greater than in the cerebellum. Evidently the amount of enchancement is related to the concentration of receptor present and not the amount of neural membrane present. It was possible that the routine use of such a high (10^{-5} M) concentration of unlabeled QNB could contribute to artificially high specific binding values by saturating lower affinity sites to which the added lipids may contribute directly. However, lipid enhancement is still observed if the concentration of cold QNB is reduced to only 20–50 times the concentration of labeled ligand, a range in which Scatchard plots reveal no additional binding sites.

Of the lipids tested, cholesterol, PC and brain PI were totally without effect. It cannot be determined

Table 1. Change in specific [^{3}H]QNB binding by the addition of various lipids*

Lipid	Binding when assayed in 20 mM phosphate buffer		Binding when assayed in 20 mM phosphate buffer with 10 mM EDTA	
	[^{3}H]QNB (pmoles bound/mg protein)	Per cent change from control	[^{3}H]QNB (pmoles bound/mg protein)	Per cent change from control
Control, no lipid	0.38		0.38	
Phosphatidylserine				
Whole brain	0.43	+14	0.50	+30
White matter	0.42	+11	0.48	+28
Gray matter	0.39	+3	0.41	+7
Phosphatidylcholine				
Dipalmitoyl	0.37	−3	0.39	+3
Dioleoyl	0.38	0	0.38	0
Brain	0.38	0	0.38	0
Phosphatidylethanolamine				
Dipalmitoyl	0.40	+5	0.43	+12
Brain	0.35	−8	0.36	−5
Phosphatidylinositol				
Yeast	0.37	+3	0.41	+9
Brain	0.38	0	0.38	0
Phosphatidic acid	0.41	+7	0.41	+9
Cerebrosides	0.41	+7	0.41	+8
Gangliosides	0.29	−25	0.29	−25
Cholesterol	0.37	−3	0.36	−4

* Membranes were homogenized in the presence of various lipids, and the muscarinic binding was assayed as described in the text. The ratio of added lipid to membrane protein was 1 to 6 (w/w). The values given are the average of at least three experiments agreeing within 15 per cent.

from these experiments whether the apparent inhibition of binding by gangliosides is real or a reflection of a strong, direct interaction between the lipid and QNB. A water-soluble QNB-ganglioside complex would not be retained by the glass fiber filters, so that this possibility could not be evaluated.

Effect of phospholipases on [^{3}H]QNB binding. The time course of receptor inactivation by phospholipase A is shown in Fig. 5. That the inactivation is enzymatic is shown by the temperature dependence of the inhibition. At 0° the enzyme causes a 15 per cent decrease in binding; at 35° there is a 100 per cent decrease. Also, the inactivation can be largely halted by incubation in 10 mM EDTA which chelates the calcium required for this enzyme. Crude bee venom contains no proteolytic enzymes, so that contamination of this sort may be ruled out [20].

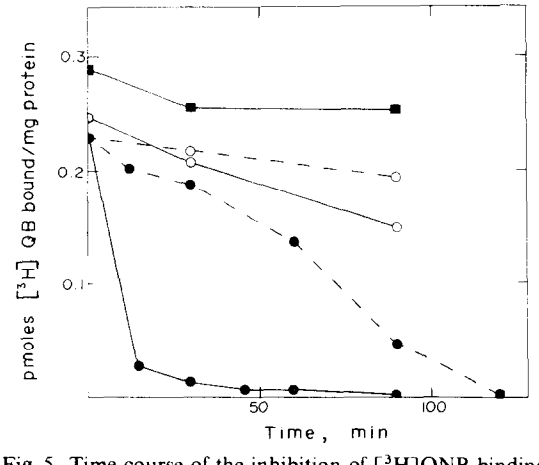

Fig. 5. Time course of the inhibition of [^{3}H]QNB binding by phospholipase A in the presence of Ca^{2+} (●——●); Ca^{2+} and BSA (●– –●); Ca^{2+} and excess EDTA (○——○); and Ca^{2+}, excess EDTA and BSA (○– –○). Samples kept at 35° with no lipase (■——■) served as a control.

Lysophospholipids, which along with fatty acids are the products of phospholipase A action, were inhibitory to QNB binding, the inhibition being linear with increasing concentration of the lipids (Fig. 4).

The addition of fatty acid-free bovine serum albumin at a concentration of 7.5 mg/ml during the incubation of membranes with the enzyme afforded some protection against the enzyme's action (Fig. 5). In an incubated control without lipase, the BSA caused a 10–15 per cent decrease in muscarinic binding. BSA which was preincubated with a mixture of fatty acids was ineffective in mitigating the effects of phospholipase A. In addition, it has been observed in this laboratory that fatty acid-free BSA will not protect another neural receptor, the opiate receptor, from phospholipase A-mediated inhibition. These facts indicate that the receptor protection afforded by the BSA is due to its ability to sequester inhibitory fatty acids and/or lysophospholipids released by the enzyme and is not due to a direct inhibition of the lipase by the albumin.

After membranes were treated with phospholipase A, specific muscarinic binding could be restored by the addition of various lipids (Table 2). PS, PA and to a lesser extent PI were capable of increasing QNB

Table 2. Change in specific [^{3}H]QNB binding by the addition of various lipids to phospholipase A-treated neural membranes*

Condition	Relative binding after lipase treatment in the absence of BSA†	Relative binding after lipase treatment in the presence of BSA‡
Control, no lipase	793	127
Lipase treated:		
No lipid	100	100
PS, whole brain	204	138
PC dipalmitoyl	73	102
PI, brain	170	107
PA dipalmitoyl	282	134
PE, brain	84	90
Cerebrosides	120	101
Cholesterol	93	101

* Membranes (8 mg protein/ml) were treated with phospholipase A (0.7 units/mg of protein) for 45 min at 35°. Aliquots were then homogenized in the presence of various lipids at a lipid to protein ratio of 1 to 6 (w/w) and assayed for muscarinic binding as described in the text. The values listed are the average of two experiments agreeing within 10 per cent.

† 100 = 0.05 pmole [^{3}H]QNB bound/mg of protein.

‡ 100 = 0.3 pmole [^{3}H]QNB bound/mg of protein.

binding after treatment with lipase in the absence of BSA, although the enhanced binding did not approach control levels. In the presence of BSA, PS and PA increased binding 38 and 34 per cent, respectively, to levels which were slightly higher than the no-lipase control levels.

Phospholipase C from *B. cereus* was also inhibitory to [^{3}H]QNB binding. Attempts at regeneration of receptors by the addition of lipids were unsuccessful. PS and PA stimulated binding to a moderate degree but could not elevate binding to lipase-free control levels. Phospholipase D had no effect on QNB binding; however, with the incubation conditions employed, no gross effect of the enzyme on the lipid composition of the membranes could be detected by thin-layer chromatography, performed as described in Materials and Methods, of chloroform–methanol (1:1, v/v) extracts.

Effect of cationic detergents on [^{3}H]QNB binding. Since the lipid activation of muscarinic binding suggests a role for anionic phospholipids in the receptor complex, the effect on QNB binding of cationic detergents, which may become associated with negatively charged lipids in or near the receptor, was investigated. Detergent concentration vs binding curves for cetyltrimethylammonium bromide and cetylpyridinium chloride are shown in Fig. 6. In general, when the detergent concentration was high enough to inhibit binding by more than 60 per cent, the inhibition was irreversible. At physiological pH, there is no solubilization of membrane proteins by the detergents at this concentration. The addition of PS or PA to membranes treated with approximately 10^{-3} M cationic detergent resulted in complete recovery of QNB binding. At this concentration the binding has been reduced about 40 per cent. No other lipids tested (PC, PE, PI, cerebrosides, cholesterol and gangliosides) were effective in restoring binding.

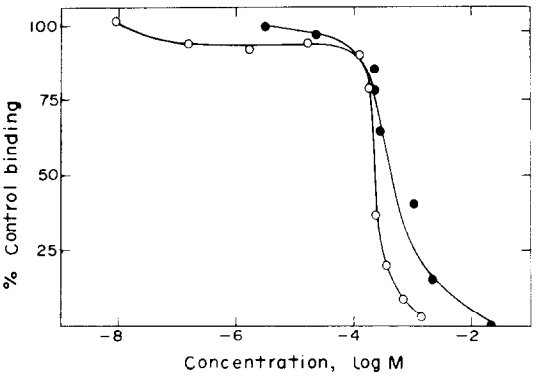

Fig. 6. Inhibition of specific [^{3}H]QNB binding by two cationic detergents, cetyltrimethylammonium bromide (O——O) and cetylpyridinium chloride (●——●).

DISCUSSION

The present results indicate that phospholipids are components of the brain muscarinic receptor insofar as [^{3}H]QNB binding is inhibited by phospholipases and cationic detergents and is stimulated by the addition of certain exogenous phospholipids.

With regard to the inhibitory action of phospholipase A, it is not clear whether the effect is due to inhibition by the reaction products or to the destruction of lipids which are an integral part of the receptor. Both reaction products of phospholipase A, fatty acids and lysophospholipids, inhibit QNB binding when added at high concentrations to neural membranes. It is likely that they can inhibit binding in much smaller amounts when enzymatically released in the immediate vicinity of the receptor. Fatty acid-free bovine serum albumin binds 8 moles fatty acid/mole with a high affinity [21, 22] in addition to binding lysophospholipids; and at concentrations high enough to bind all of the fatty acids present in the membrane lipids, BSA affords significant, but not complete, protection of the receptor from the lipase's action. Since BSA, which was preincubated with a fatty acid mixture, was much less effective in protecting the receptor, the inhibitory action of phospholipase A could be due in part to the reaction products formed. On the other hand, since phosphatidylserine and phosphatidic acid completely restore QNB binding after enzyme treatment, it is also possible that the inhibitory effect is due to the removal of essential phospholipids by the enzyme.

Inhibition of [^{3}H]QNB binding by phospholipase C is incomplete. Increasing the amount of enzyme or the incubation time produces no more than 60 per cent inhibition. There is no restoration of binding after the introduction of any exogenous lipid. The reaction products (phosphoserine, phosphocholine, phosphoinositol, and phosphoethanolamine headgroups and diacyl glycerides) do not inhibit muscarinic binding when directly added to neural membranes. Unlike the products of phospholipase A action, the phosphorylated headgroups and diglycerides formed do not have strong detergent properties which might lead to the destruction of remaining receptor structures. It is possible that the glyceride formed remains adjacent to the receptor, but, lacking the proper headgroup, is unable to participate effectively in muscarinic binding. Since the added phos-

pholipids do not replace or displace the glyceride, binding is not restorable.

Cationic detergents inhibit QNB binding at concentrations which are too low to solubilize any membrane material. This inhibition can be completely reversed by the acidic phospholipids PS and PA. No other lipids tested were effective. The detergents apparently complex with negative charges in or near the receptor, resulting in a direct competition with QNB for the charged binding site or a configurational rearrangement of the receptor which precludes binding. The added acidic lipids either remove the detergent through direct interaction or replace the inactivated charges in the receptor.

Further evidence for phospholipid involvement in the muscarinic receptor comes from the enhancement of [^{3}H]QNB binding when certain phospholipids are added directly to untreated membranes. The polar lipids are especially effective in enhancing QNB binding. Although the extent of the enhancement by the various lipids tended to vary from experiment to experiment, the order of stimulatory strength was constant: PS > PA, PI, cerebrosides, PE (saturated) > PC, cholesterol > PE (brain) > gangliosides. The fact that preincubation of membranes with 10 mM EDTA increases the effects of the lipids suggests that calcium may be involved. Calcium is an intrinsic component of biomembranes, involved in both lipid–lipid and lipid–protein interactions [23, 24]. Treatment of membrane preparations with EDTA may facilitate insertion of exogenous lipids into the membrane as well as allow them to associate with intrinsic proteins which are usually more tightly complexed with adjacent lipids. The lipids are added in concentrations that are much higher than their critical micelle concentrations, so that both monomers and micelles are present. Lipid micelles alone either do not bind [^{3}H]QNB or are not retained by the glass fiber filters used in the binding assay in these experiments. Previous experiments in this laboratory indicate that less than 1 per cent of the exogenous lipid becomes bound to the membranes [7].

The fatty acid composition of phospholipids affects their ability to enhance QNB binding. PS extracted from mammalian gray matter contains a relatively large amount of docosahexaenoic acid and other unsaturated hydrocarbon chains, while PS derived from white matter contains predominately saturated and monoenoic chains [25, 26]. This distribution was confirmed on the bovine PS samples derived from these two sources and used in this experiment. The white matter PS was three times more effective in stimulating muscarinic binding than the gray matter PS. PE derived from brain contains large amounts of unsaturated fatty acid moieties [27] and is somewhat inhibitory to QB binding. Synthetic PE containing only saturated hydrocarbons (palmitate) enhances binding 12 per cent. PI from yeast has 95 per cent saturated or monoenoic fatty acid components [28] and is more stimulatory toward QNB binding than PI from bovine brain, which has a more unsaturated composition and is especially high in arachidonic acid [29]. On the other hand, dipalmitoyl, dioleoyl and brain PC's are equally without effect on QNB binding. When fatty acids are added to membranes, QNB binding is inhibited only by the unsaturated acids, while no fatty acid enhances binding. This inhibition by unsaturated lipids is somewhat surprising in light of the known chemical composition of synaptosomal plasma membranes. The serine and ethanolamine phosphoglycerides of synaptic membranes are characterized by their high degree of unsaturation as compared to those of gray matter, white matter, or myelin plasma membranes [30]. Synaptic sphingomyelin and PC have a saturated composition while phosphatidic acid is only a minor membrane component [30]. The presence of double bonds in lipids may inhibit binding by introducing greater configurational freedom in molecules involved in receptor site functions. The increased conformational possibilities of unsaturated lipids may result in the insertion or absorption of the aliphatic chains to receptor surfaces where they block binding. There is also the possibility that direct ligand–lipid interactions are increased by lipid unsaturation. Nonspecific binding increases when the degree of unsaturation is high.

The thermodynamic, pH and ionic data presented indicate that ionic or polar forces are far less important in QNB binding than are hydrophobic ones. Chaotropic ions, which disrupt water structure and thereby weaken hydrophobic interactions, reversibly decrease the affinity of QNB for the muscarinic receptor. Since binding is constant over a wide range of pH and is unaffected by high ionic strength, electrostatic interactions are only minimally involved in QNB binding. The large positive entropy and enthalpy changes associated with the binding point to the lack of exothermic processes, which would have been expected if hydrogen and ionic binding forces were preponderant. In the bound state, QNB would be held in a more restricted orientation while the large entropy change could result from the release of bound water from the ligand and receptor surfaces upon binding [31, 32]. Alternatively, there could be an increased randomness in the overall membrane receptor complex as a consequence of ligand binding. It is known that the binding of certain drugs to artificial lipid membranes increases membrane fluidity [33].

The nature of QNB binding to the muscarinic receptor is clearly very different from that of acetylcholine, despite the fact that the binding sites for the two appear to at least partially overlap. QNB and acetylcholine are structurally similar, both containing cationic and esteratic sites which are important points of attachment to the receptor. They differ insofar as QNB possesses two aromatic groups capable of strong hydrophobic interaction with lipids and the hydrophobic region of membranes [34]. The arguments favoring the identity of QNB-binding sites with the muscarinic receptor have been presented by Yamamura and Snyder [12] and are partially supported by work done in this laboratory [14]. An understanding of the differences in molecular interactions between QNB and acetylcholine binding is afforded by the concept of accessory hydrophobic receptor areas advanced by Ariëns and Simonis [35]. Adjacent to the muscarinic receptors are areas which interact with the double ring systems commonly found in muscarinic antagonists. The contribution of these hydrophobic forces to antagonist–receptor binding eclipses that of the traditional esteratic and

anionic site interactions, although stereochemical compatibility with these two sites is still necessary for high affinity binding. In this paper, evidence has been presented for the involvement of certain lipids in the muscarinic receptor, as measured by QNB binding. It is possible that the role of lipids is due to their participation in the accessory hydrophobic binding areas.

REFERENCES

1. G. V. Marinetti and R. Love, *Biochem. biophys. Res. Commun.* **61,** 30 (1974).
2. R. Tanaka, in *Biogenic Amines and Physiological Membranes in Drug Therapy, Part A* (Eds. J. H. Biel and L. G. Abood), p. 35. Marcel Dekker, New York (1971).
3. K. Sihotang, *Eur. J. Pharmac.* **63,** 519 (1976).
4. T. Lloyd and S. Kaufman, *Biochem. biophys. Res. Commun.* **59,** 1262 (1974).
5. R. J. Lefkowitz, L. E. Limbird, C. Mukherjee and M. G. Caron, *Biochim. biophys. Acta* **457,** 1 (1976).
6. D. Bach, K. Rosenheck and R. Miller, *Eur. J. Biochem.* **53,** 265 (1975).
7. L. G. Abood and F. Takeda, *Eur. J. Pharmac.* **39,** 71 (1976).
8. L. G. Abood, F. Takeda and N. Salem, in *Alcohol and Opiates* (Ed. K. Blum), p. 283. Academic Press, New York (1977).
9. M. E. Eldefrawi, A. T. Eldefrawi and R. D. O'Brien, *Molec. Pharmac.* **7,** 104 (1971).
10. T. Bartfai, J. Montelius and M. Raftell, *Fedn Proc.* **35,** 1576 (1976).
11. P. Alberts and T. Bartfai, *J. biol. Chem.* **251,** 1543 (1976).
12. H. I. Yamamura and S. H. Snyder, *Proc. natn. Acad. Sci. U.S.A.* **71,** 1725 (1974).
13. S. H. Snyder, K. J. Chang, M. J. Kuhar and H. I. Yamamura, *Fedn Proc.* **34,** 1915 (1975).
14. J. Baumgold, L. G. Abood and R. Aronstam, *Brain Res.* **124,** 331 (1977).
15. K.-J. Chang, S. Jacobs and P. Cuatrecasas, *Biochim. biophys. Acta* **406,** 294 (1975).
16. G. Rouser, G. Kritchevsky and A. Yamamoto, in *Lipid Chromatographic Analysis* (Ed. G. V. Marinetti), Vol. 1, p. 99. Marcel Dekker, New York (1967).
17. I. H. Segel, *Biochemical Calculations*, p. 200. Wiley New York (1976).
18. T. Teorell and E. Stenhagen, *Biochem. Z.* **299,** 416 (1938).
19. Y. Hatefi and W. G. Hanstein, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. XXXI, p. 770. Academic Press, New York (1974).
20. E. Habermann and K.-G. Reiz, *Biochem. Z.* **341,** 451 (1965).
21. A. Jones, *Biochim. biophys. Acta* **427,** 325 (1976).
22. D. S. Goodman, *J. Am. chem. Soc.* **80,** 3892 (1958).
23. D. Papahadjoboulos, G. Poste, B. E. Schaeffer and W. J. Vail, *Biochim. biophys. Acta* **352,** 10 (1974).
24. J. A. Reynolds, *Ann. N.Y. Acad. Sci.* **195,** 75 (1972).
25. J. S. O'Brien, D. L. Fillerup and J. F. Mead, *J. Lipid Res.* **5,** 329 (1964).
26. A. Y. Sun and G. Y. Sun, in *Function and Metabolism of Phospholipids in the Central and Peripheral Nervous Systems* (Eds. L. Amaducci and C. Galli), p. 169. Plenum Press, New York (1975).
27. G. Y. Sun and L. A. Horrocks, *Lipids* **3,** 79 (1968).
28. W. E. Trevelyan, *J. Lipid Res.* **7,** 445 (1966).
29. B. J. Holub, A. Kuksis and W. Thompson, *J. Lipid Res.* **11,** 558 (1970).
30. W. C. Breckenridge, G. Gombos and I. G. Morgan, *Biochim. biophys. Acta* **266,** 695 (1972).
31. B. Belleau, *Ann. N.Y. Acad. Sci.* **144,** 705 (1967).
32. W. A. P. Luck, Top, *Curr. Chem.* **64,** 113 (1976).
33. D. Papahadjoboulos, K. Jacobson, G. Poste and G. Shepherd, *Biochim. biophys. Acta* **394,** 504 (1975).
34. L. G. Abood, in *Drugs Affecting the Central Nervous System* (Ed. A. Burger), p. 127. Marcel Dekker, New York (1968).
35. E. J. Ariëns and A. M. Simonis, *Ann. N.Y. Acad. Sci.* **144,** 842 (1967).