Inactivation of Muscarinic Acetylcholine Receptors in Brain Synaptic Membranes by Free Fatty Acids. Evaluation of the Role of Lipid Phase

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Abstract. Arachidonic, linolenic and linoleic acids decreased the binding of the *m*-cholinergic antagonist [^{3}H] QNB and did not affect the ratio of high to low affinity binding sites to the agonist carbamoylcholine in rat brain synaptic membranes. In the presence of arachidonic acid, SH-reagent N-ethylmaleimide acquired the ability to block QNB binding to receptor. Lipids in the bilayer and annular regions were probed by fluorescence of 1,6-diphenyl-1, 3, 5-hexatriene and pyrene. A microviscosity drop induced by increasing temperature from 10 to 37°C did not affect the level of QNB equilibrium binding, whereas arachidonic acid strongly inhibited the binding at concentrations inducing the same drop in microviscosity as that induced by heating. For various unsaturated fatty acids an equal extent of receptor blocking was reached at quite different degrees of bilayer fluidization, the state of annular lipid being not changed under these conditions. It is suggested that the effect of unsaturated acids is reached through their direct interaction with the receptor, which undergoes a conformational change, rather than by an alteration of the physical state of the lipid phase of the membrane.

Key words: Muscarinic cholinergic receptor — Fatty acids — Membrane microviscosity

Introduction

Several normal and pathophysiological processes in various tissues, including the nervous tissue, are accompanied by an accumulation of free fatty acids in membranes mostly as a result of phospholipase activation. It has been suggested that products of the catalytic activity of phospholipase A_2 (lysophosphatidylcholine and arachidonic acid) are involved in adrenergic reactions (Mallorga et al. 1980) and in the secretion of histamine from mast cells (Buisseret 1982). An increase in the content of free fatty acids is an inherent feature of states such as ischemia (Katz et al. 1976) and hypoxia (Tada et al. 1978).

One of the targets for products of phospholipases might be brain muscarinic acetylcholine receptors since unsaturated fatty acids decrease the specific binding of *m*-cholinergic antagonist quinuclidinyl benzilate (QNB) to synaptic membranes (Aronstam et al. 1977). The mechanism of this phenomenon is, however, unclear. Taking into account that a number of membrane receptors are controlled by the physical state of lipid bilayer (Shinitzky and Souroujon 1979; Heron et al. 1980; 1981), a decrease in the lipid microviscosity observed in various membranes in the presence of fatty acids (Leoni et al. 1982; Prilipko et al. 1983) could be responsible for their effects. Alternatively, fatty acids could interact directly with the muscarinic receptor.

In the present work it was shown that arachidonic, linolenic and linoleic acids block the binding of QNB by acting directly on muscarinic receptors rather than by modifying the lipid bilayer and annular lipid. Moreover, the data obtained suggest that physical properties of lipid have no influence on the interaction between a cholinergic antagonist and muscarinic receptors.

Materials and Methods

Materials

Radiochemicals: *l*-quinuclidinyl[phenyl-4-^{3}H]benzilate (35 Ci/mmol) and iodo [1-^{14}C] acetamide (53 mCi/mmol) (Amersham, England). Iodoacetamide, N-ethylmaleimide, pyrene were obtained from Serva, FRG; 1,6-diphenyl-1, 3, 5-hexatriene, arachidonic acid were from Fluka, Switzerland; linoleic and stearinic acids were from Sigma, USA; linolenic acid was from Koch-Light (England). Dimyristoyllecithin was a gift from the Institute of Bioorganic Chemistry, Acad. Sci. Bel. SSR. Butylated hydroxytoluene (BHT), 2,5-diphenyloxazole (PPO), 1,4-bis (5-phenyloxazol-2-yl) benzene (POPOP) and all other chemicals were of reagent grade (USSR).

Preparation Procedures

Synaptic membranes were isolated from brain hemispheres of albino rats (wt 150—200 g) according to Jones and Matus (1974) on an ultracentrifuge L8-55 (Beckman) with a SW-28 rotor. The sediment was resuspended in distilled water to a protein concentration of 8—10 mg/ml and stored at − 18°C over 5—6 days.

All experiments were carried out in a medium containing 2 mmol/l $CaCl_2$ and 50 mmol/l Tris-HCl buffer, pH 7.4

Measurement of [^{3}H]QNB Binding

Usually, 0.1 ml of [^{3}H]QNB solution (5.18 KBq) was introduced into 0.9 ml of membrane suspension to a final concentration of 2.5×10^{-9} mol/l with subsequent incubation in a shaker for 15 min at 37°C (except where indicated otherwise). Reaction was terminated by the addition of cold incubation medium (5 ml) followed by rapid vacuum filtration through microfiber glass filters GF/F (Whatman) on a Model 25 Ten Place Filter Holder (Bio-Rad Laboratories). The sediment on filters was washed 3 times with 5 ml of cold medium. The specific binding of [^{3}H]QNB to synaptic membranes was

determined as the difference between the total radioactivity bound to membranes and that discovered after incubations with atropine (10^{-6} mol/l). Nonspecific binding did not exceed 4—5 % of total binding.

Treatment of Membranes with Fatty Acids

Stock solutions of fatty acids in ethanol at a concentration of 5 mg/ml were prepared and after respective dilutions with ethanol, 10 μl aliquots were added to 0.89 ml of membrane suspensions. For acids incorporation, they were incubated with membranes (0.2 mg protein) for 30 min at 37°C. Microviscosity tests and [^{3}H]QNB binding assays showed this time to be sufficient to reach a constant level of fatty acid effects. The final concentrations of acids are indicated in the text. At the delution used (1:100) ethanol did not affect the specific binding of [^{3}H]QNB.

Treatment with BHT

Ten μl of BHT ethanol solution were added to membrane suspension (0.88 ml) to a final concentration of 5 μg/ml and incubated for 20 min at 37°C. Arachidonic acid was subsequently added as described above. Membranes preincubated with ethanol without antioxidant, or with antioxidant without acid were used as controls.

Oxidation of Arachidonic Acid with Long-Wave Ultraviolet Light

Ethanol solution of arachidonic acid (5 ml, 1.5 mg/ml) was irradiated by UV-lamp (120 W) through a filter cutting off the short wave ultraviolet (<280 nm), under constant stirring during 7 hours. The accumulation of hydroperoxides from fatty acids under these conditions (Puchkov et al. 1976) was checked with the titanium (IV) reagent (Siggia 1974) and by measuring the optical density at 412 nm. The amount of hydroperoxides formed was about 7×10^{-4} mol/l.

Membrane Treatment with a SH-Reagent N-Ethylmaleimide (NEM)

To 0.8 ml of membrane suspension kept on ice (0.2—0.3 mg protein) and containing arachidonic acid (0.1 mg per mg protein) or no acid (control preparation) $10^{-4} \div 10^{-2}$ mol/l NEM (0.1 ml) were added. The final NEM concentrations are indicated in legends to respective figures. Membranes were preincubated with NEM for 30 min at 37°C under constant stirring, followed by incubation with [^{3}H]QNB.

Labeling of Membranes with SH-Reagent Iodo-(1-^{14}C) Acetamide

Membrane proteins were treated with a mixture of labeled and unlabeled iodoacetamide. The final concentration of the reagent was 2×10^{-3} mol/l (26.6 KBq/ml). After 30 min of incubation with iodoacetamide at 37°C the membranes were centrifuged at $90{,}000 \times g$ for 30 min. The sediment was resuspended in the medium used for QNB binding to concentrations of 0.2—0.3 mg/ml protein and treated with fatty acid as described above. Then it was filtered through GF/F filters.

Radioactivity Assay

The filters with sediments were dried in the air (at 65°C) for 1 h and placed into scintillation liquid PPO + POPOP (6 g/l and 0.075 g/l respectively) in toluene. The radioactivity of ^{3}H and ^{14}C was measured in a Mark III scintillation counter using program 10 for heterogeneous systems.

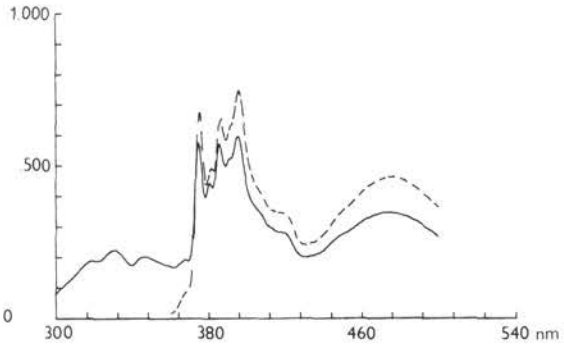

Fig. 1. Luminescence spectra of pyrene incorporated into synaptic membranes. Fluorescence was excited at 280 nm (continuous line) or 330 nm (dotted line). Spectral slit widths were 4 nm and 2 nm for the excitation and emission monochromator, respectively.

Fluorescence Measurements

The fluorescence measurements were carried out on a JY 3CS Jobin-Yvon fluorometer in a cell with an optical length of 1 cm. The optical density of membrane samples did not exceed 0.2 at absorbtion maxima of the dyes. The emission light was recorded at 90° to the excitation beam.

To evaluate lipid microviscosity 1,6-diphenyl-1, 3, 5-hexatriene (DPH) was used (Shinitzky and Barenholz 1978). Stock DPH solution in tetrahydrofuran (2×10^{-3} mol/l) was diluted with incubation medium immediately before the experiment to a concentration of 2×10^{-6} mol/l. Aliqouts of 0.2 ml were added to 1.8 ml membrane suspension (0.2—0.3 mg/ml protein) and incubated at 37°C for 30 min. Rotational mobility of DPH molecules in the lipid environment was determined from fluorescence anisotropy (A):

$$A = \frac{I_{//} - I_{\perp}}{I_{//} + 2I_{\perp}}$$

where $I_{//}$ and $I_{\perp}$ represent the fluorescence intensities measured with the emission analyzer parallel or perpendicular, to the vertical polarization of excitation beam, respectively. The excitation wavelength was centred at 360 nm while that of emission at 430 nm. The fluorescence background was less than 3 %. The spectral width of the slit from the excitation side was about 10 nm.

Pyrene was used for separate probing of microviscosity in both region of the protein environment (annular lipid) and lipid bilayer (Dergunov et al. 1981). Pyrene solution in ethanol (10^{-2} mol/l) was introduced into membrane suspension (0.2—0.3 mg/ml protein) to a final concentration of 10^{-5} mol/l, followed by incubation at 37°C for 20 min and centrifugation at $10{,}000 \times g$ for 20 min. Membrane sediment containing more than 90 % of the pyrene originally added was resuspended in the medium and diluted to the original protein concentration. Fatty acids were thereafter incorporated as described above. Pyrene excitation was carried out in two spectral regions: directly (at 330 nm) and through energy transfer from protein tryptophanyls (at 280 nm) (Fig. 1). In both cases at the dye concentration indicated, the fluorescence spectrum has a band structured in a way typical for excited monomer of pyrene, whereas a second broad structureless band with a maximum at 475 nm reflects emission from excimers formed by association of excited and unexcited pyrene molecules. The ratio of fluorescence intensities of the monomer and excimer states (I_m/I_{ex}) recorded at 375 nm and 475 nm, respectively, was taken as a measure of microviscosity (Dergunov et al. 1981). The rate of excimer formation is

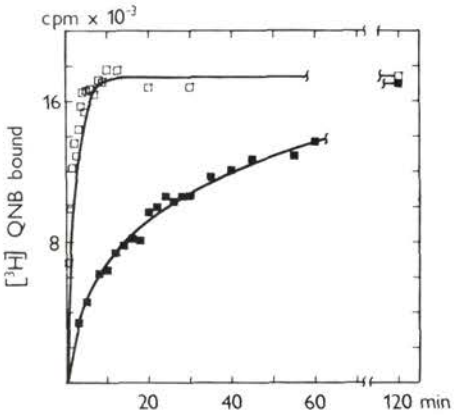

Fig. 2. Kinetics of specific binding of [^{3}H]QNB (2.5×10^{-9} mol/l) by synaptic membranes. Incubation medium: 2 mmol/l $CaCl_2$, 50 mmol/l Tris- HCl buffer, pH 7.4. The concentration of protein was 0.36 mg/ml (1.69×10^{-9} mol/l for the [^{3}H]QNB binding sites); □ – 37°C; ■ — 10°C.

diffusion-controlled and it therefore depends on microviscosity of the environment of the probe molecules. Upon excitation of pyrene at 280 nm through energy transfer there also are contributions from fluorescence of tryptophanyl residues themselves and from the fluorescence of pyrene molecules directly excited at the shortwave end of their absorbtion band. Corrections were therefore made for calculations of true fluorescence intensity of pyrene excited by energy transfer from protein. The contribution from direct excitation of pyrene at 280 nm was determined by recording the excitation spectrum of pyrene incorporated into dimyristoyllecithin liposomes. The contribution of tryptophan emission in the region of monomer and excimer luminescence was evaluated while measuring fluorescence of synaptic membranes in the absence of pyrene.

Pyrene fluorescence parameters at $\lambda_{ex} = 280$ nm and $\lambda_{ex} = 330$ nm were used for microviscosity measurements of boundary lipid and the whole lipid including bilayer, respectively.

Other Methods

Protein was assayed according to Lowry et al. (1951) using crystalline bovine albumin as a standard.

Results

Measurements of Specific Binding of [^{3}H]QNB and Carbamoylcholine

Figure 2 shows the kinetics of interaction of [^{3}H]QNB with synaptic membranes. It may be seen that the binding is a temperature-dependent process. The association rate constant K was calculated from the equation for the second order reaction:

$$\ln \frac{b(a-x)}{a(b-x)} = K.(a-b).t,$$

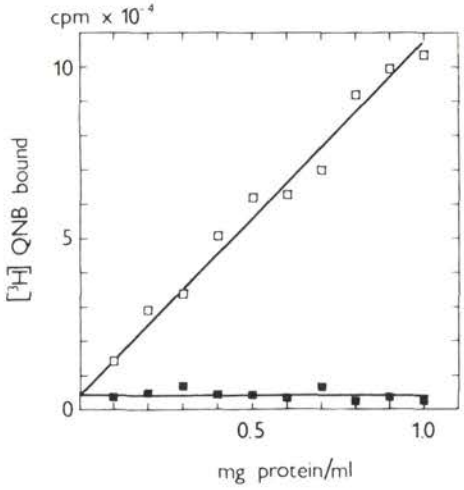

Fig. 3. Binding of [^{3}H]QNB (cpm) by synaptic membranes as a function of the protein concentration in the absence (□) and in the presence (■) of 10^{-6} mol/l atropine. 0.9 ml of membrane suspension was preincubated for 10 min at 37°C with or without atropine and after the addition of 0.1 ml [^{3}H]QNB solution (to a final concentration 2.5×10^{-9} mol/l) the suspension was incubated for further 15 min. The reaction was terminated by the addition of a cold medium (5 ml) with subsequent vacuum filtration through GF/F filters.

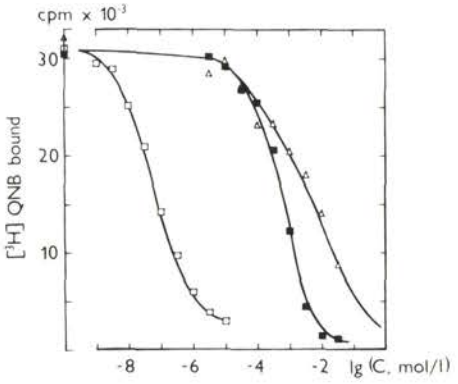

Fig. 4. Competition of [^{3}H]QNB for binding sites with cholinergic antagonist scopolamine (□) and agonists pilocarpine (■) and carbamoylcholine (△). 0.1 ml of incubation medium containing QNB and various cholinergic agents was added to 0.9 ml of membrane suspension (0.4 mg/ml protein). The mixture was incubated for 15 min at 37°C (nonequilibrium conditions). The reaction was stopped by 5 ml of the cold medium with subsequent vacuum filtration. The final concentration of [^{3}H]QNB was 2.5×10^{-9} mol/l while the concentrations of other agents are indicated on the abscissa. Each experimental point represents the mean of 3 determinations.

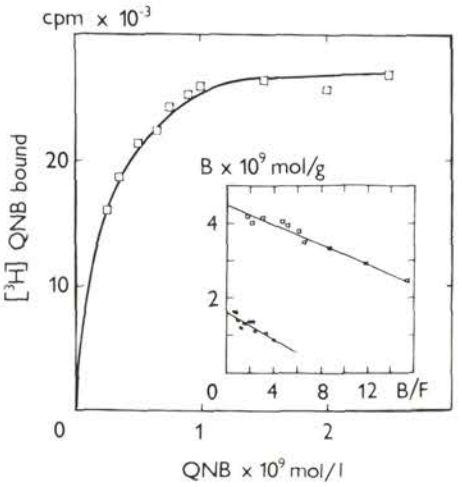

Fig. 5. Specific binding of [³H]QNB (cpm) by synaptic membranes as a function of its concentration. In the insert the same dependence is presented as a Hofstee plot for untreated membranes (□) or in the presence of arachidonic acid (■); B is the amount of [³H]QNB specifically bound per g of membrane protein, and F is the concentration of free [³H]QNB. The concentration of free [³H]QNB was corrected for QNB lost from the solution as a result of binding. 0.1 ml aliquots of [³H]QNB solution in various concentrations ($2.5 \times 10^{-9} \div 2.5 \times 10^{-8}$ mol/l) were added to 0.9 ml samples of synaptic membranes (0.3 mg/ml protein) in the absence or in the presence of arachidonic acid (0.05 mg/mg protein). The suspension was incubated for 60 min at 37°C and the reaction was stopped by the addition of cold medium (5 ml) followed by vacuum filtration.

where a is the initial concentration of [³H]QNB (2.5×10^{-9} mol/l); b is the initial concentration of QNB binding sites determined from Hofstee plots for equilibrium conditions (1.62×10^{-9} mol/l); x is the current concentration of ligand — binding site complexes at time t. The values of K determined by regression analysis as a slope of the plot $\frac{1}{a-b} \ln \frac{b(a-x)}{a(b-x)}$ versus t for 37°C and 10°C were about 0.8×10^8 l.mol^{-1}min^{-1} and 0.03×10^8 l.mol^{-1}min^{-1}, respectively.

The specific (atropine — sensitive) binding of [³H]QNB was in linear relationship with membrane protein up to a concentration of 1 mg/ml (Fig. 3). From Fig. 3 it follows that, under our conditions, the atropine- insensitive binding is not dependent on the protein concentration and that it probably results from QNB adsorbtion to the material of the fiberglass filter. Also, the specificity of QNB interaction was verified in experiments which showed that [³H]QNB binding may be blocked by low concentrations of *m*-antagonist scopolamine while the cholinergic agonists pilocarpine and carbamoylcholine were considerably less effective (Fig. 4). Figure 5 presents a typical relationship between [³H]QNB specific binding and its concentration. The linear Hofstee plot suggests that synaptic membranes

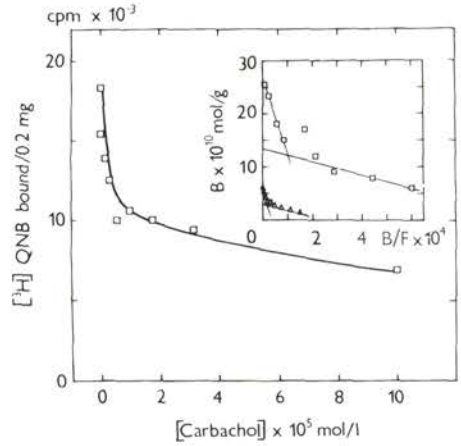

Fig. 6. Specific binding of [³H]QNB by synaptic membranes preincubated with various concentrations of carbamoylcholine. Insert: the same data as a Hofstee plot for untreated membranes (□) or in the presence of arachidonic acid (△) (0.05 mg/mg protein); *B* is the amount of carbamoylcholine bound per g of the membrane protein, calculated from the difference between the amounts of [³H]QNB bound in the absence and in the presence of various concentrations of carbamoylcholine; *F* is the concentration of free carbamoylcholine. The concentration of free carbamoylcholine was corrected for carbamoylcholine lost from the solution as a result of binding. 0.1 ml aliquots of the carbamoylcholine solution ($10^{-5} \div 10^{-3}$ mol/l) were added to 0.8 ml samples of synaptic membranes (0.25 mg protein). The suspension was preincubated for 15 min at 37°C followed by the addition of 0.1 ml of [³H]QNB to a final concentration of 2.5×10^{-9} mol/l and a further incubation for 15 min.

have a single class of binding sites with $K_d = 1.3 \times 10^{-10} \pm 0.09 \times 10^{-10}$ mol/l and $B_{max} = 4.48 \pm 0.12$ nmol/g protein. The amounts of bound [³H]QNB which was added after membrane preincubation with various carbamoylcholine concentrations for 15 min are shown in Fig. 6. Since carbamoylcholine prevents [³H]QNB binding, the difference between these values measured in the absence and in the presence of the agonist permits to calculate the approximate number of [³H]QNB binding sites occupied by carbamoylcholine (Hedlund and Bartfai 1979; Salvaterra 1980). These values were analysed as a function of corrected free concentrations of the agonist in Hofstee plot (Fig. 6, insert). Nonlinearity of the plot shows that carbamoylcholine interacts with two classes of binding sites having high and low affinities. The ratio between the two classes of binding sites was about 1:2, as seen directly from the Hofstee plot.

The Effect of Fatty Acids on the Specific Binding of [³H]QNB and Carbamoylcholine

The influence of a preliminary membrane treatment with fatty acids on antagonist

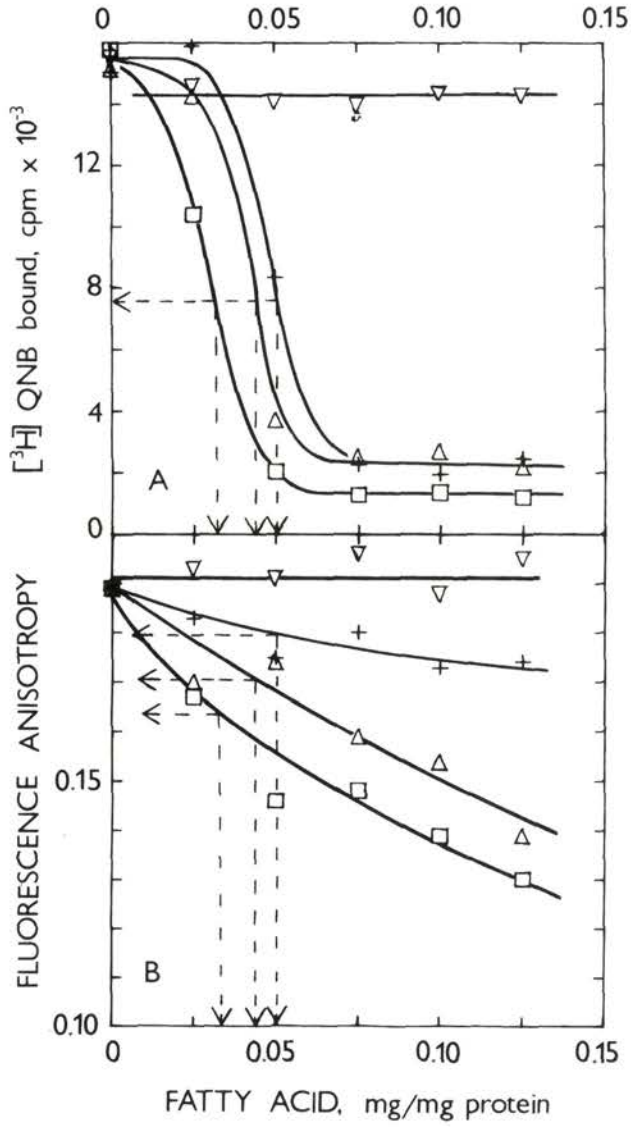

Fig. 7. Binding of [^{3}H]QNB (cpm) by synaptic membranes (*A*) and fluorescence anisotropy of DPH incorporated into the membranes (*B*) as a function of fatty acid concentrations. Vertical broken lines (in *A* and *B*) show the acid concentrations corresponding to the half- maximal inhibition of [^{3}H]QNB binding (horizontal broken line in *A*). Horizontal broken lines in *B* indicates the respective values of fluorescence anisotropy for the same half-maximal acid concentrations. (□), arachidonic acid; (△), linoleic acid; (+), linoleic acid; (▽), stearic acid. For procedures of fatty acid and DPH incorporation into synaptic membranes, see Methods. Final concentrations: membranes protein, 0.2 mg/ml; DPH, 2×10^{-7} mol/l; [^{3}H]QNB, 2.5×10^{-9} mol/l.

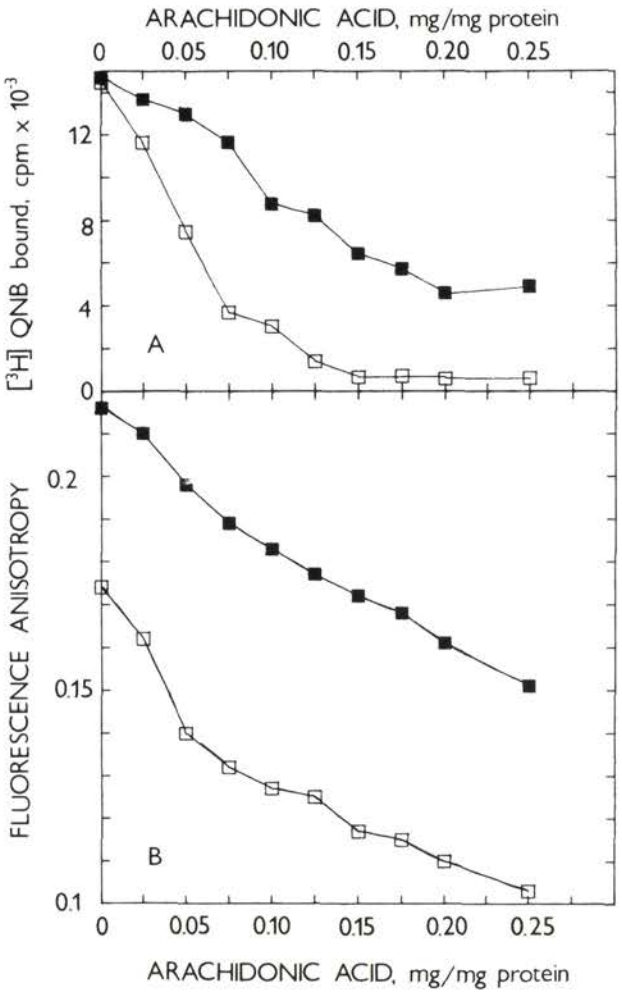

Fig. 8. Binding of [^{3}H]QNB (cpm) by synaptic membranes (*A*) and fluorescence anisotropy of DPH incorporated into the membranes (*B*) as a function of the arachidonic acid concentration. Incorporation of the acid, measurements of [^{3}H]QNB binding and DPH fluorescence were carried out at 10°C (■) or 37°C (□). Durations of incubations at both temperatures were as follows: arachidonic acid, 60 min; [^{3}H]QNB, 120 min; DPH, 30 min. Final concentrations: membrane protein, 0.2 mg/ml; [^{3}H]QNB, 2.5×10^{-9} mol/l; DPH, 2×10^{-7}/mol/l.

binding is shown in Fig. 7. In contrast to saturated stearic acid unsaturated acids block the binding of [^{3}H]QNB. Maximal activities of unsaturated acids were

Table 1. Effect of peroxidation processes on the interaction between arachidonic acid and muscarinic receptors in the synaptic membranes

Additions	[^{3}H]QNB bound, cpm	
	without BHT	with BHT
None	13188 ± 583 (100 %)	14019 ± 311 (100 %)
Arachidonic acid (0.03 mg/mg protein)	7541 ± 393 (57.2 %)	8609 ± 111 (61.4 %)
UV-oxidized arachidonic acid (0.03 mg/mg protein)	7521 ± 433 (54 %)	8218 ± 149 (58.6 %)

The final concentration of [^{3}H]QNB in the membrane suspension (0.2 mg protein/ml) was 2.5×10^{-9} mol/l. Prior to the incorporation of the arachidonic acid, membranes were pretreated with BHT (5 μg/ml). The relative values of [^{3}H]QNB bound are indicated in brackets.

independent on the number of double bonds in the hydrocarbon chains. However, the respective half-maximal blocking concentrations decreased with the increasing unsaturation.

Lowering of temperature resulted in an increase in half maximal blocking concentrations of arachidonic acid while its maximal effect was reduced (Fig. 8). Judging by Hofstee plots, arachidonic acid decreased the density of [^{3}H]QNB binding sites without any change in K_d value (Fig. 5), and it did not affect the ratio between the densities of [^{3}H]QNB binding sites with high and low affinities toward carbamoylcholine (Fig. 6).

To elucidate the possible involvement of peroxidation of unsaturated acids or membrane lipids in inhibition of [^{3}H]QNB binding, following experiments were performed. The action of arachidonic acid was analysed after the incorporation into membranes of antioxidant BHT. Table 1 shows that the blockade of the binding by the acid was practically not affected by the presence of BHT. On the other hand, arachidonic acid preliminarily oxidized by UV-light retained the original level of its inhibiting activity (Table 1).

Decreases in the densities of the [^{3}H]QNB binding sites revealed by the filtration method might also be a consequence of the detergent-like influence of the unsaturated acid, i.e. protein solubilization or membrane fragmentation with improved passage through the filter pores. To evaluate such a mechanism synaptic membranes were labeled with an SH-reagent [^{14}C] iodoacetamide. The membrane preparation treated in that way was incubated with arachidonic acid and filtered through fiber glass filters GF/F. Figure 9 shows that the level of radioactivity retained on the filters was not dependent on the presence of various concentrations of arachidonic acid.

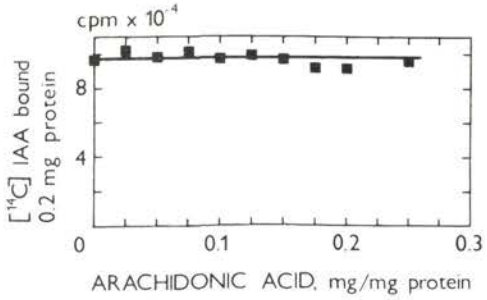

Fig. 9. The effect of arachidonic acid on the integrity of synaptic membranes, determined by radioactivity (cpm) of membranes labeled with [^{14}C]-iodoacetamide ([^{14}C]IAA). The treatment by [^{14}C]IAA and the arachidonic acid is described in Methods. The protein concentration was 0.2 mg/ml.

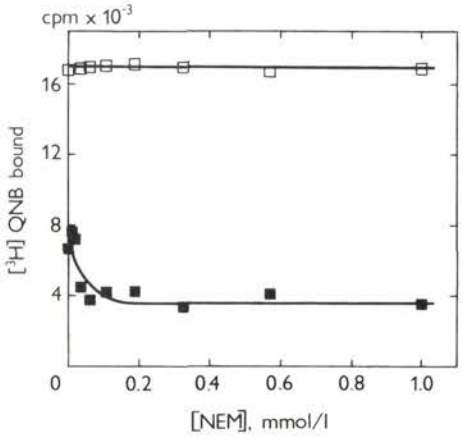

Fig. 10. The effect of N-ethylmaleimide on [^{3}H]QNB binding by synaptic membranes preincubated with (■) or without (□) arachidonic acid. The concentration of arachidonic acid was 0.05 mg/ml protein, and that of [^{3}H]QNB was 2.5×10^{-9} mol/l. For details of the experiments, see Methods.

Change in Sensitivity of [^{3}H]QNB Binding Sites to N-Ethylmaleimide in the Presence of Arachidonic Acid

In control membranes the SH-reagent N-ethylmaleimide had no influence on, or caused a negligible decrease in [^{3}H]QNB binding. However, when added in the presence of arachidonic acid (the latter at a concentration producing half-maximal inhibition of [^{3}H]QNB binding) the efficiency of [^{3}H]QNB binding blockade sharply increased. Maximal effect of N-ethylmaleimide was achieved at concentrations of about 0.1 mmol/l (Fig. 10).

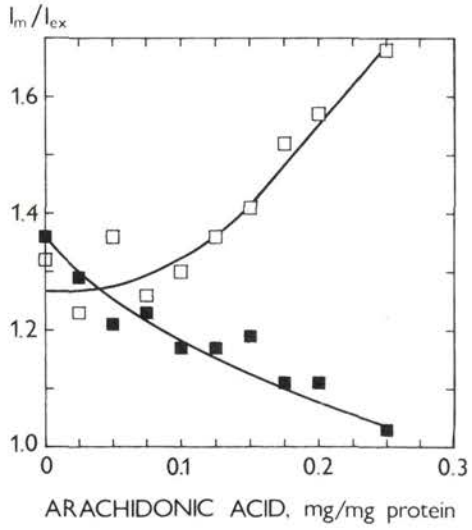

Fig. 11. Values of the monomer to excimer fluorescence intensity ratio for pyrene excited at 280 nm (□) or 330 nm (■) as a function of the concentrations of arachidonic acid added to membrane suspension. For experimental details, see Methods.

Effect of Fatty Acids on Microviscosity of the Membrane Lipid Phase

Anisotropy of DPH fluorescence measured at 37°C was decreased by arachidonic ($C_{20:4}$), linolenic ($C_{18:3}$) and linoleic ($C_{18:2}$) acids in a dose-dependent manner (Fig. 7). The fluidization ability of the acids correlated with the number of double bonds, while saturated stearic acid did not affect the anisotropy values. The action of arachidonic acid was also pronounced at 10°C (Fig. 8).

The use of DPH supplies only averaged information on the physical state of the whole lipid phase (Shinitzky and Barenholz 1978) but it does not permit differentiation between the annular lipid and the bilayer. In contrast, the ratio of intensities of monomer and excimer pyrene fluorescence makes it possible to analyse the two lipid pools separately (Dergunov et al. 1981). Data shown in Fig. 11 indicate that this ratio measured under excitation in the absorbtion band of pyrene (330 nm) decreases over the same range of arachidonic acid concentrations as does anisotropy of DPH fluorescence. However, when pyrene molecules located within the annular lipid were excited through energy transfer from protein tryptophanyls, the ratio of monomer to excimer fluorescence not only did not fall but even increased after fatty acid incorporation into the membranes (Fig. 11).

Discussion

The values of the dissociation constant (1.3×10^{-10} mol/l) and the rate constant of association (0.8×10^{8} l.mol^{-1} mi^{-1}) for [^{3}H]QNB binding at 37°C are in a good agreement with data obtained earlier on neuronal membranes (Yamamura and Snyder 1974). *m*-Cholinergic ligands scopolamine, pilocarpine and carbamoylcholine compete effectively with [^{3}H]QNB for the membrane binding sites (Fig. 4). The existence of a single class of sites for antagonists (Fig. 5) and two classes for agonist (Fig. 6) also agrees with previous information (Hedlund and Bartfai 1979). It may thus be concluded that under our conditions the specific binding of [^{3}H]QNB to synaptic membranes resulted from interaction with muscarinic acetylcholine receptors.

Unsaturated fatty acids (arachidonic, linolenic, linoleic) in contrast to the saturated stearic acid decreased the specific binding of [^{3}H]QNB in accordance with the finding of Aronstam et al. (1978). Since arachidonic acid blocks the binding of both the antagonist QNB and the agonist carbamoylcholine (Fig. 5 and 6) it was important to verify whether solubilization of proteins including muscarinic receptors occurs. Experiments with arachidonic acid added to membranes preliminarily labeled with the SH-reagent [^{14}C]-iodoacetamide showed that, at the concentrations used, the fatty acid was devoid of any significant desintegrating or solubilizing activity (Fig. 9).

As polyunsaturated acids are easily involved in reactions of peroxidation, the intermediate or final products of such reactions might be responsible for the observed effects on [^{3}H]QNB binding. In such a case membrane preincubation with an antioxidant would decrease whereas preliminary oxidation of the acid would amplify the blockade of receptor. Data in Table 1 show, however, that pretreatment with BHT did not prevent the action of arachidonic acid while acid peroxidized by UV-light had the same effectiveness as the unoxidized one. Thus there is no indication pointing to the role of peroxidation reactions, and the "switch off" of *m*-receptors may be connected with their direct inactivation or with modification of the membrane environment (Avakyan et al. 1981). Indeed, it is well known that unsaturated fatty acids are able to increase fluidity of the membrane lipid bilayer (Leoni et al. 1982; Prilipko et al. 1983) and this was confirmed by our measurements of microviscosity using DPH and pyrene fluorescence (Fig. 7*B*, 8*B* and 11). Nevertheless, the following facts practically rule out the possibility that events in the lipid bilayer are responsible for blockage of [^{3}H]QNB binding upon incorporation into membranes of unsaturated fatty acids.

1. The drop in membrane microviscosity induced by temperature rise from 10 to 37°C (see control values in Figure 8*B*) did not result in a decreased [^{3}H]QNB equilibrium binding (Fig. 2 and 8*A*) whereas arachidonic acid (0.15 mg/mg protein) strongly inhibited the binding although at this concentration the acid had

the same fluidization effect as heating of membrane suspension (Fig. 8*A*).

2. An inhibition of the binding by 50 % was reached at quite different degrees of bilayer fluidization for each of the acids used (Fig. 7*B*). Apparently, the physical state of the lipid bilayer does not control the antagonist-receptor interaction. Moreover, as it follows from the data on fluorescence of pyrene located within the annular lipid (Fig. 11), the doses of arachidonic acid sufficient to induce maximal blockade of *m*-receptor did not effect significantly lateral mobility of the probe the latter being even lower at higher acid concentrations. An additional evidence against changes in the nearest environment of *m*-receptors is a constant proportion of two populations of the sites with high and low affinities towards carbamoylcholine (Fig. 6): according to the current concepts it is the difference in environment which determines the heterogeneity of agonist binding sites (Ehlert et al. 1981). Consequently, there are good reasons to suggest a direct influence of unsaturated fatty acids on *m*-cholinergic receptors. Presumably, in the course of such interaction the receptor undergoes conformational change resulting in a blockade of the antagonist binding. This mechanism is consistent with the fact that, at acid concentrations insufficient for inactivation of the whole pool of receptors, a portion of them respond to treatment with the SH-reagent N-ethylmaleimide (Fig. 10) by a further decline in [^{3}H]QNB binding. In the absence of fatty acid N-ethylmaleimide has little or no influence on antagonist binding by the muscarinic receptors as was also observed earlier (Salvaterra 1980; Aronstam et al. 1978). It is possible that the appearence of sensitivity to the SH-reagent upon the accumulation of free fatty acids is a consequence of SH- group exposure, or of change in its reactivity in receptors in which the antagonist binding sites are still intact.

Recently, we have shown that senescence of rats is accompanied by a rise in sensitivity to N-ethylmaleimide in muscarinic receptors of the brain synaptic membranes (Lyskowa et al. 1983). Experiments are now in progress to determine whether free fatty acids accumulate in membranes of old rats.

The data presented here suggest that the physical state of the lipid bilayer has no influence on antagonist binding to muscarinic receptors. However, these receptors are influenced by free unsaturated fatty acids contained in the synaptic membranes, i.e. by phospholipase A products. Their influence goes through direct interaction of fatty acids with the receptors and a change in their conformation.

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