

# Binding of specific ligands to muscarinic receptors alters the fluidity of membrane fragments from rat brain

## A fluorescence polarization study with lipid-specific probes

E.M. Manevich\*, A. Kõiv, J. Järv, J.G. Molotkovsky<sup>+</sup> and L.D. Bergelson<sup>+</sup>

*Tartu State University, Tartu, Estonian SSR, \*Semashko Medical Stomatological Institute, Moscow and <sup>+</sup>Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow, USSR*

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The previously suggested method of following ligand-receptor interactions by measuring ligand-induced changes in membrane fluidity [(1986) FEBS Lett. 194, 313–316] was employed to study the binding of specific ligands of the muscarinic receptor to rat brain membrane fragments containing a fluorescent analogue of phosphatidylcholine (APC) as a membrane probe. Upon addition of carbachol and atropine in low concentrations the fluorescence polarization of the APC-labeled membranes decreased significantly demonstrating that binding of these ligands to the muscarinic receptor increases the fluidity of its lipid environment. The fluidity changes were specific, concentration-dependent and saturable. In comparison with radioligand assays the fluorescent lipid probe method proved to be much more sensitive but the  $K_d$  values obtained by the two methods differed considerably.

Muscarinic receptor; Atropine; Carbachol; Fluorescence; Membrane fluidity

### 1. INTRODUCTION

Recently a new approach to the study of ligand-receptor interaction based on measurement of membrane fluidity changes caused by specific receptor binding was proposed [1]. In the present study we made use of this approach to investigate the interaction of specific ligands with the muscarinic acetylcholine receptor in rat brain. It has been shown that addition of muscarinic agonists to intact lymphocytes results in fluidity changes of the cell membrane [2]. This phenomenon detected by making use of DPH as a

fluorescent probe can have two explanations. Firstly, the binding of ligands with the receptor protein can change direct receptor-membrane interactions. Secondly, the alteration of membrane properties can be brought about by changing some metabolic processes of the intact cell modulated by the muscarinic receptor [3].

In the present study the interrelationship between binding of specific ligands with the muscarinic receptor and changes of fluidity of its lipid environment has been studied in a cell-free system: membrane fragments from rat cerebral cortex. Application of this model system facilitates the interpretation of fluorescence data as compared to living cells. The fluorescent probe APC (1-acyl-2-[12-(9-anthryl)-11-*trans*-dodecenyloyl]-*sn*-glycero-3-phosphocholine), closely related to phosphatidylcholine, the main lipid component of the native membrane, has been used to follow the fluidity changes of the membrane fragments.

*Correspondence address:* L.D. Bergelson, Shemyakin Institute of Bioorganic Chemistry, Ul. Miklukho-Maklaya 16/10, 117871 Moscow, USSR

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## 2. EXPERIMENTAL

Suspensions of membrane fragments from rat cerebral cortex were prepared as described earlier [4]. The concentration of muscarinic receptor sites in the membranes was 0.2 nM as determined by a radioligand binding assay using L-[<sup>3</sup>H]quinuclidinyl benzilate [4]. The fluorescent probe APC was synthesized as described in [5].

Membrane fragments were incubated with APC in 0.05 M K-phosphate buffer for 3 h at 25°C. The molar ratio of APC to membrane phospholipids was 1:100. The final level of fluorescence intensity of the APC-labeled membrane preparation exceeded that of the membrane blank more than 20-fold. All fluorescence measurements were performed on a computerized spectrofluorimeter SFR-100 (Baird, England). The values of fluorescence polarization were determined in the excitation spectrum at excitation and emission slits 2 nm and 5 nm, respectively. The excitation and emission wavelengths were 370 nm and 440 nm, respectively.

In order to measure the fluidizing effect of the muscarinic ligands added to the membrane suspension, the fluorescence polarization was determined before and after incubation of the APC-labeled membranes with different concentrations of carbamoylcholine chloride (Sigma) and of atropine sulfate (Merck) for 10 min at 25°C. All experiments were carried out in triplicate and the mean values were used in the following analysis.

## 3. RESULTS AND DISCUSSION

The lipid fluorescent probe APC effectively incorporated into membrane fragments from rat cerebral cortex. This process can be followed by the increase in fluorescence intensity due to the decay of self-quenching, as the probe molecules entering the lipid bilayer depart from each other. The excitation spectrum of APC incorporated into the membrane fragments is shown in fig.1. The considerable energy transfer from the tryptophanyl groups of membrane proteins lends additional evidence for its localization in the membrane structure (not shown).

Addition of muscarinic ligands to the suspension of APC-labeled membrane fragments led to a considerable decrease in the fluorescence polarization of the probe (fig.2). The effects observed clearly point to fluidization of the probes microenvironment in the cases of both carbachol and atropine. At the same time no changes in the fluorescence polarization occurred when atropine ( $10^{-9}$  M) was added to liposomes prepared by sonication of the total lipids of brain membranes with the APC probe. Thus the alteration of the physical

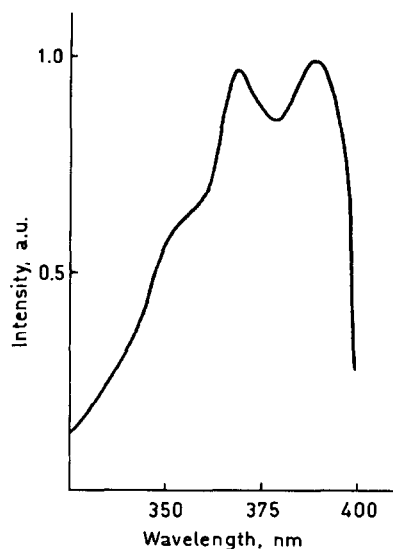


Fig.1. Excitation spectrum of APC incorporated into rat brain membrane fragments.

parameters of the membrane seems to arise from interaction between the ligands and the muscarinic receptor molecule whose conformational changes are transferred to the surrounding lipids. This suggestion is supported by the observation that the fluorescence polarization decreases rapidly within 1–2 min after addition of the ligands and then remains constant for several hours.

The sensitivity of the probes response to ligand binding was high. Statistically significant changes in the fluorescence polarization of APC could be registered already at  $10^{-12}$  M carbachol or atropine, which is about two orders less than the minimal concentration measurable in radioligand binding experiments. At the same time diphenyl-hexatriene and the sphingomyelin analogue of APC [6] introduced into the membrane preparation did not respond to ligand addition at 25°C. These differences indicate that ligand-receptor binding initiates fluidity changes in some lipid domains of the membrane, while others are apparently not affected.

Binding of carbachol with the muscarinic receptor gave a maximal decrease of the fluorescence polarization of APC at nanomolar ligand concentrations and the magnitude of this effect reached up to 22% of the initial polarization value. Further increase in carbachol concentration up to 1 M did

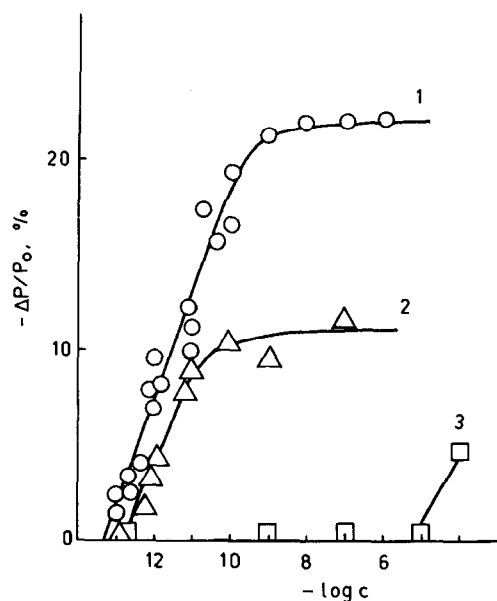


Fig.2. Changes in fluorescence polarization of membrane-incorporated APC after addition of muscarinic ligands to rat brain membrane fragments: curve 1, carbachol; 2, atropine; 3, tetramethylammonium chloride. Incubation time, 10 min.  $\Delta P = P - P_0$  where  $P_0$  and  $P$  are the fluorescence polarization values before and after addition of the ligand.

not lead to additional changes in the fluorescence polarization (fig.2).

Similar fluidization of the lipid environment of APC was detected after addition of the specific muscarinic antagonist atropine to brain membrane fragments. In this case, however, the maximal effect was considerably smaller, comprising only 11% of the initial fluorescence polarization value (fig.2).

Thus the maximal change in polarization of the APC fluorescence brought by the action of an agonist carbachol or an antagonist atropine with the muscarinic receptor was different. We suppose that agonist binding with the receptor results in more significant conformational transition of the protein that causes larger rearrangements in the membrane lipid structure.

The influence of muscarinic ligands on membrane fluidity is a specific effect because tetramethylammonium chloride, a very weak ligand of the muscarinic receptor had no effect on the fluorescence polarization of the probe at ligand concentrations up to  $10^{-6}$  M and only a slight ef-

fect was observed at  $10^{-5}$  M (fig.2). Moreover as can be concluded from the data in fig.3 atropine and carbachol compete for the receptor.

We found that keeping the APC-labeled membrane fragments for several days at  $4^\circ\text{C}$  led to a complete loss of sensitivity of the lipid phase to carbachol or atropine. Under the same conditions radioligand measurements did not reveal any considerable changes in binding of L-[ $^3\text{H}$ ]quinuclidinyl benzilate with the membranes [4]. Thus the membrane fluidization brought about by the muscarinic receptor-ligand interaction appears to be a specific property of the native receptor-membrane complex.

The fluidization effect caused by binding of carbachol or atropine with the muscarinic receptor follows a simple binding isotherm (fig.3) that makes it possible to determine the apparent  $K_d$  values for these ligands. Treatment of these experimental data according to the Hill equation yielded linear dependencies (fig.4). The  $K_d$  values obtained by this method were 3 pM for atropine and 5 pM for carbachol. However, the low value of the Hill coefficient  $n_H = 0.54$  for carbachol points to the presence of heterogeneous binding sites or to negative cooperativity of the binding process.

It is important to emphasize that physiological experiments based on measurement of tissue responses to muscarinic stimulation, such as muscle contraction and inhibition of adenylate cyclase, have yielded  $K_d$  values from 63 nM to 0.12 mM for carbachol and from 0.26 nM to 14  $\mu\text{M}$  for

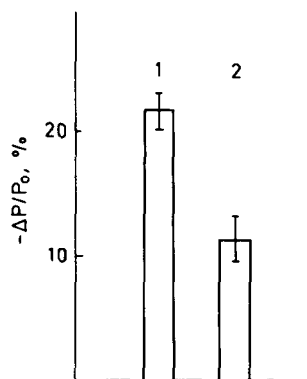


Fig.3. Fluorescence polarization values of membrane-incorporated APC after addition of  $10^{-8}$  M carbachol (1) and  $10^{-8}$  M atropine after carbachol (2).

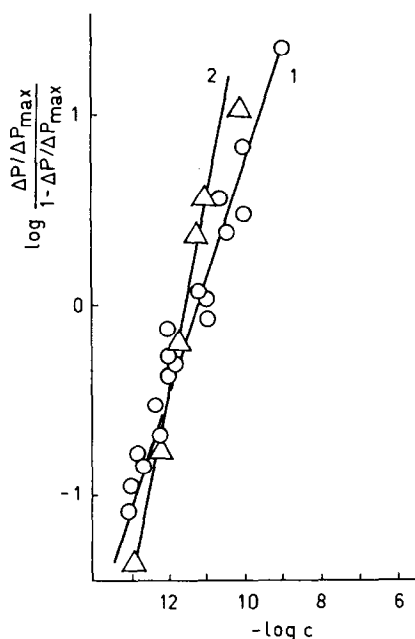


Fig.4. Hill plots of effects of muscarinic ligands on fluorescence polarization of membrane-incorporated APC: curve 1, carbachol; 2, atropine.

atropine [7–16] which differ considerably from the values obtained in the present study. In the particular case of rat brain membranes, measurement of [ $^{32}$ P]phosphate incorporation into phosphatidylinositol and stimulation of [ $^3$ H]inositol phosphate formation gave  $K_d$  values of 12  $\mu$ M [10] and 68  $\mu$ M [7] for carbachol and 1.6 nM [7] for atropine. However, strictly speaking, the latter constants cannot be directly compared with the data obtained from the measurement of membrane responses to the receptor-ligand interaction because the interrelationships between receptor occupancy and physiological or physical phenomena can be different. The present data show that membrane fluidity changes are one of the most sensitive responses to receptor-ligand interaction and that

occupation of only a small part of the receptor sites seems to be sufficient to induce maximal alteration of the lipid organization.

Finally, the difference in ligand concentrations necessary to create physical or physiological effects supports the view that the physical state of biomembranes can be directly regulated by receptor-ligand interaction and that the physiological responses following receptor occupation can be either secondary effects or are not related to alteration of membrane fluidity.

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