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Review Letter

Lipid domain reorganization and receptor events

Results obtained with new fluorescent lipid probes

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Binding of a ligand to the extracellular site of a membrane receptor involves conformational changes not only of the extracellular and cytoplasmic sites but also of the membrane-spanning segments of the receptor protein. The lipids forming the environment of the trans-membrane receptor segments, being flexible and mobile, must follow the shape changes of the receptor molecule to some extent. For this reason ligand–receptor binding itself can be expected to result in changes of the packing of lipid molecules surrounding the receptor. Since lipids are organized in a cooperative manner such changes may affect large areas of the membrane and hence can be registered by physical methods. It has been stated repeatedly that binding of various ligands including hormones, antibodies or lectins to membrane-associated receptors is accompanied by fluidity changes of the target membrane [1–3].

The aim of this article is to summarize recent evidence suggesting that in highly heterogeneous biological membranes containing numerous metastable lipid domains and/or clusters conformational changes of membrane- associated proteins may result not only in global fluidity changes but also in alterations of the supramolecular lipid domain organization. Frequently such changes can be followed by using, as fluorescent probe, analogs of natural membrane phospholipids or glycolipids bearing, at the end of one of the fatty acyl chains, a 9-anthrylvinyl (AV) group (Fig. 1) (reviewed in [4,5]). These fluorescent lipid probes were shown to report truly on the phase behavior of their natural prototypes with the same headgroup [6]. They incorporate spontaneously into membrane preparations and intact cells, and in the latter case may reside for a relatively long time in the plasma membrane without internalization [7].

In comparison to most other fluorophores used in membrane research, the AV group displays important advantages. It causes little disturbance because it is nonpolar, flat, and relatively compact. When attached to the fatty acyl chain at an appropriate distance from the head group the AV-fluorophores reside uniformly and exclusively in the center of the bilayer where the host lipids are packed most loosely, and affect neither the mobility nor the orientation and conformation of the major part of the molecules of the surrounding host lipids including their head groups [8]. The AV-group is characterized also by high quantum yields and very short fluorescence lifetimes [9]. The latter property makes the AV-fluorophore suitable for fluorescence polarization measurements. Moreover, in phospholipid bilayers the restricted rotational diffusion of the AV-labeled lipid molecules is of the same time scale as the fluorescence decay. Therefore even subtle changes in the fluidity of the probes microenvironment produce considerable changes in the polarization of fluorescence. The fluorescent parameters of the AV-group (λ_{exc} 365 nm, λ_{em} 430 nm; high extinction coefficient) make it also a highly efficient acceptor for resonance energy transfer (RET) from nearby tryptophans. Since the distance that RET can occur over is as long as 5 nm while RET efficiency depends on the sixth power of the donor–acceptor distance, RET measurements with AV-labeled lipids permits the following of even extremely small changes in the relative localization of proteins and lipids.

When located in the same environment, AV-labeled phospholipids with different head groups show very similar fluorescence parameters. However, in heterogeneous lipid matrixes where macro- or microscopic

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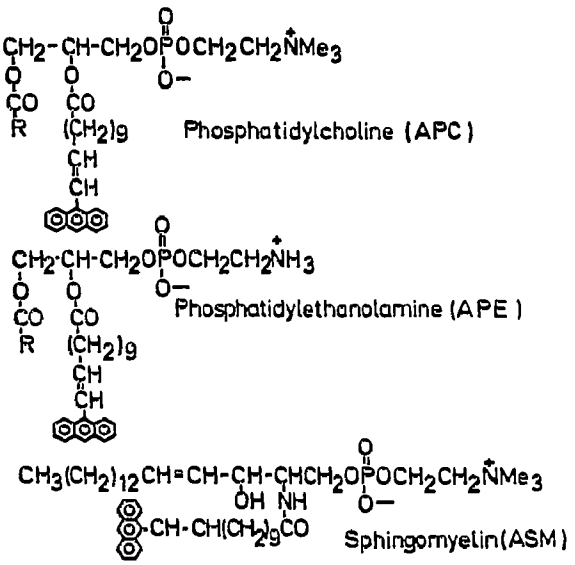

Fig. 1. Anthrylvinyl-labeled phospholipids.

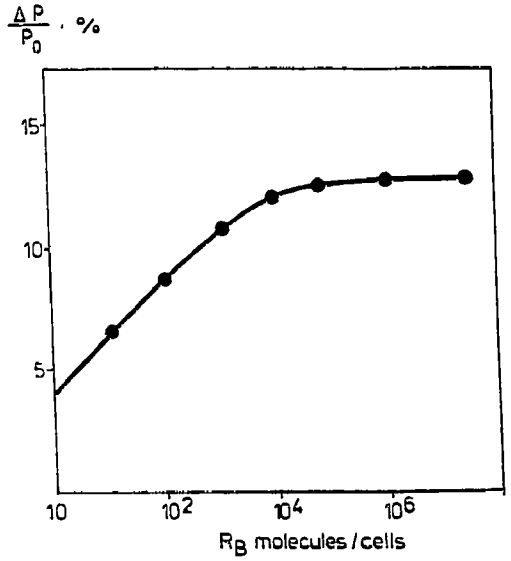

Fig. 2. Changes in fluorescence polarization of ASM-labeled Burkitt lymphoma cells induced by the binding subunit of ricin (R_B). $\Delta P - P_o$, where P and P_o are values of the fluorescence anisotropy before and after addition of R_B.

phase seggregations are possible, these values may differ significantly because the AV-labeled lipids tend to partition between different domains localizing predominantly together with their natural prototypes having the same head group [5,6]. Accordingly we suggested the following approach to visualize changes in lipid domain organization [5]. Membranes or cells are labeled separately with two or more AV-lipids identical or very similar in all aspects except the polar head groups. Substantial differences in the probes fluorescent parameters suggest that the host lipids distribute non-randomly in the membrane and that the various AV-lipids probe different domains with a certain selectivity. If in such systems different probes respond differently to an extrinsic stimulus (e.g. to binding of a ligand), this indicates that the fluidity of various domains probed by the AV-lipids has changed differentially, i.e. that some rearrangement of the lipid matrix must have taken place. By this 'multi probe approach' any disturbance induced by the probes themselves is thus largely removed.

The possibility of using AV-labeled phospolipids to monitor specific ligand–receptor binding was first tested with a simple model, the interaction of the binding unit of the plant toxin ricin (R_B) with Burkitt lymphoma cells doped with AV-labeled sphingomyelin (ASM) or phosphatidylcholine (APC) [10]. The peptide R_B is known to bind to any glycoprotein or glycolipid having an accessible terminal galactose. The fluorescence polarization changes induced by binding of R_B to the cells prelabeled with ASM or APC (Fig. 2) proved to be specific and reversible because they were abolished by excess galactose (but not by glucose) and because the toxic subunit of ricin, R_B, which is known to bind in an unspecific manner, did not induce any response. The sensitivity of the fluorescence polarization response was strikingly high. The lowest R_B concentration detectable by the fluorescence method was 10^{-11} M, which corresponds to a few R_B molecules per cell. Remarkably, the fluorescence polarization values of anthrylvinyl-labeled phosphatidylcholine and sphingomyelin shifted in different directions upon binding of R_B. This lends additional support to the supposition that ligand–receptor binding may be accompanied by a reorganization of the supramolecular domain structure of the lipids surrounding the receptor. At the same time no significant changes could be observed when using diphenylhexatriene as a probe, presumably because the latter distributes less specifically between different lipid domains.

The ricin-induced fluorescence polarization changes of the lipid probe ASM were concentration-dependent, saturable and followed simple Michaelis–Menten kinetics (Fig. 2). In an attempt to derive the association constant from fluorescence polarization changes we assumed that the latter are proportional to the number of occupied binding sites. If that assumption is true the corresponding Hill plot should have a linear regression.

This was indeed found to be the case for low concentrations of R_D. The apparent K_D obtained from the fluorescence polarization data was in reasonable agreement with that derived from radioligand measurements using ^{125}I-labeled R_B [10]. However, the latter method revealed the presence of an additional binding site of lower affinity which was not sensed by the fluorescent lipid probes. Neither did the anthrylvinyl labeled lipids respond to binding of R_B to glycolipids present on the cell surface [11]. It thus appears that while binding of the peptide to its proteinaceous receptor induces notice-

able changes in the domain organization of surrounding lipids, binding of the same ligand to glycolipids is not able to initiate such changes.

In the same manner fluidity changes of the lipid environment were detected after addition of specific muscarinic ligands to rat brain membrane fragments (Fig. 3) [12] prelabeled with APC. In these experiments the muscarinic agonist, carbachol, and the antagonist, atropine, appeared to compete for the same receptor; however, the maximal effect of atropine was considerably smaller. Apparantly agonist binding to the receptor resulted in a more significant conformational change of the protein that caused larger rearrangements in the membrane lipid structure.

The fluidization effect caused by binding of carbachol or atropine to the muscarinic receptor followed a simple binding isotherm (Fig. 3). Treatment of these experimental data according to the Hill equation yielded linear dependencies. The apparent K_D values obtained in this way were 3 pM for atropine and 5 pM for carbachol. The low value of the Hill coefficent, $N_H = 0.54$, for carbachol pointed to the presence of heterogeneous binding sites or to negative cooperativity of the binding process.

The picomolar K_D values obtained in the above study were in marked contrast to the micromolar constants obtained by measurement of tissue responses to muscarinic stimulation (see [13–15] for some recent references). Certainly, this is not surprizing since one could hardly expect a direct interrelation between ligand-induced fluidity changes at 25°C and biochemical or physiological responses at 37°C.

We also attempted to use the fluorescent lipid probes to study the binding of cells to antibodies [16]. When poly- or monoclonal antibodies, raised against the light chains of human Ig, were added to Burkitt lymphoma

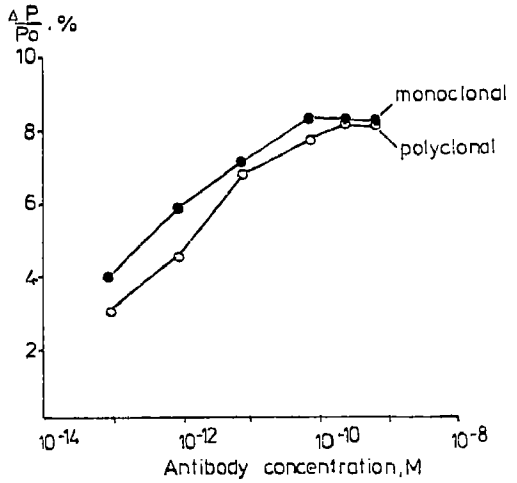

Fig. 4. Dependence of fluorescence polarization changes of ASM-labeled Burkitt lymphoma cells on the concentration of poly- and monoclonal antibodies against the L-chain of human Ig; $\Delta P = P - P_o$, where P and P_o are fluorescence polarization values in the presence or absence of antibody.

cells prelabeled with ASM, binding of even a few antibody molecules clearly could be detected by fluorescence polarization changes (Fig. 4) of the lipid probe, although the dissociation constants differed substantially from those derived from radioligand studies with iodinated antibodies.

In a similar way it was shown that binding of prostaglandins to erythrocytes or of platelet activation factor (PAF) to platelets prelabeled with AV-lipid probes, resulted in lipid rearrangements detectable by changes in the fluorescence polarization of the probes [17,18]. In both cases the response appeared to be specific (the effects of prostaglandin E1 and PAF were not paralleled by prostaglandin E2 and chemically modified non-ac-

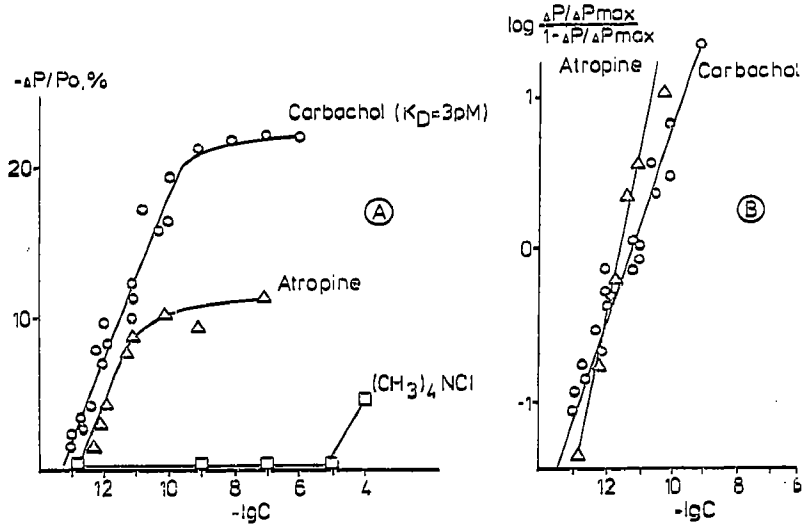

Fig. 3. (A) Changes in fluorescence polarization of APC-labeled rat brain membranes after addition of muscarinic ligands at 25°C. (B) Hill plots of effects of muscarinic ligands on the fluorescence polarization. $\Delta P = P - P_o$, where P and P_o are fluorescence polarization values before and after addition of the ligands.

tive PAF analogs, respectively) and extremely sensitive: even a few ligand molecules per cell induced measurable changes in the *P*-values of the AV-labeled lipid probes. Here again, AV-labeled lipids with different polar head groups proved to respond differently to one and the same ligand, indicative of rearrangements of lipid domain organization in response to ligand–receptor interaction.

Summarizing, it can be said that the results of fluorescent lipid-based binding assays may or may not coincide quantitatively with those of radioligand and other studies. Nevertheless the fluorescence method has at least two merits. First, it appears to be much more sensitive than traditional radioligand measurements because it takes advantage of the fact that conformational changes of one receptor molecule can be transferred to large numbers of lipids organized in a cooperative manner. Second, one observes apparently only specific interactions. Besides this, the fluorescence method is fast, simple and requires no prelabeling of the ligand or the receptor. It thus may become an additional tool in certain areas of receptor research, e.g. in studies of drug- agonists and antagonists competition. A more general and highly provocative question posed by the above findings is whether reorganizations of the lipid matrix induced by ligand–receptor binding are able to influence functional activities of the membrane and to trigger biochemical events inside the cell. This problem appears to deserve further investigation.

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