CO-PRESIDENTIAL ADDRESS

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Lipid Domains in Biological Membranes

Their Structural and Functional Perturbation by Free Fatty Acids and the Regulation of Receptor Mobility

Co-Presidential Address

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We have studied the interaction of free fatty acids (FFAs) with cell membranes and lipid bilayers by monitoring changes in the emission polarization of the fluorescent probes diphenylhexatriene (DPH) and anilino-naphthalene sulfonate (ANS). We found that the FFAs readily intercalate into membranes and produce significant changes in the packing of the lipid molecules. The membrane alterations could be divided into two patterns: the *cis*-unsaturated FFAs (designated Group A) disorder the membranes' interior (as reported by DPH) and order the head group region (as reported by ANS); the *trans*-unsaturated or saturated FFAs (Group B) do not alter the bilayer interior but also order the head group region. Using solution theory, the shift in transition midpoint temperatures as a function of fatty acid type was used to infer that the Group A FFAs partition into fluid domains, while Group B FFAs partition preferentially into gel-like domains. These results are explained in terms of a domain model of membrane lipid structure. Low concentrations of Group A FFAs inhibit the capping of surface immunoglobulin (Ig), whereas no effect was seen with Group B FFAs. The capping inhibition caused by Group A FFAs was reversible with increasing doses of extracellular calcium. Fluorescence photobleaching recovery showed that the Group A FFAs do not inhibit receptor immobilization associated with patch formation but rather inhibit the final energy-dependent movement of the patched receptors into a cap. We have also shown that the Group A FFAs cause a shift in membrane-bound calcium to the lipid phase from probably protein calcium-binding sites. The data have generated a model of receptor mobility invoking a *trans*-membrane, calcium-

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binding, receptor-anchoring protein, linked to the cytoskeleton. Inhibition of capping by Group A FFAs is postulated to be due to perturbation of specific lipid domains associated with this protein, such perturbation leading to conformational changes in the protein, and consequent intramembranous calcium sequestration in the lipid phase, rendering the calcium unavailable for activation of the cytoskeleton. (Am J Pathol 97:211-222, 1979)

As Co-President of the AAP, it is an honor to be able to give you an overview of some of the work undertaken in my laboratory over the past year or so. Whilst attending to the Byzantine affairs of the AAP, my friends and colleagues have borne the brunt of doing the work. At least they have had fun! I am deeply indebted to them. They are: Dr. Richard D. Klausner, Dr. Deepak K. Bhalla, and Dr. Richard L. Hoover. I am also indebted to Dr. Paul Dragsten for collaboration with the fluorescence photobleaching recovery experiments, and Dr. Alan Kleinfeld for his most helpful advice and for the insights provided by his lifetime heterogeneity analysis.

As some of you might know, our laboratory over the years has been interested in the mechanisms underlying various cell surface phenomena which relate to some physiological and pathological processes. These include cell adhesion,[1] eg, the sticking of polymorphonuclear leucocytes to endothelium in the acute inflammatory response [2]; phagocytosis [3,4]; and the nature of tumor,[5] endothelial, and arterial smooth-muscle cell surfaces.[6] One class of probe we have used for perturbing function and structure at the cell surface has been free fatty acids (FFAs), some of which have profound biological effects when incorporated into the surface membrane. These functional effects include alteration of membrane-bound enzyme activity,[7] platelet aggregation,[8] lymphocyte mitogenesis,[9] surface-receptor capping,[10] and cell–cell and cell substrate adhesion.[1] We realized that these biological and functional perturbations would be purely phenomenologic in nature unless they were explicable in terms of the effects of FFAs on membrane structure. In this latter regard it became apparent as the work progressed that FFAs are potent probes of membrane structure, particularly in regard to lipid organization and lipid-protein interactions.[11] It should also be noted that the effect of FFAs on cell surface membranes and cell-surface–related cellular functions is not without relevance to pathological entities such as atherosclerosis, diabetes, and so on.

According to the familiar Singer–Nicolson [12] model of membrane structure, membrane proteins are embedded in a sea of fluid, relatively homogeneous lipid. The proteins are capable of lateral diffusion and movement unless restrained, presumptively, by interaction with the cytoskeleton. Is the sea of lipid truly homogeneous? As far as the lipid is concerned, it has

been suggested, variously, that perturbation of the lipid by protein does not extend beyond a single lipid thickness [13,14] or, alternatively, that long-range lipid-protein interactions may induce specific lipid domains.[9,11,15]

Free Fatty Acids as Probes of Membrane Structure

We have looked specifically at this question of lipid structure, using FFAs as probes for relating the structure of the membrane to biological events: it emerges that FFAs provide a mode for looking at structural functional changes in the lipid phase; and, more particularly, we have been able to obtain evidence for the existence of lipid-domain structures in membranes,[11] the perturbation of which leads to functional changes.[10] An example of the latter relating to surface-receptor mobility will be given.

We have used the technique of fluorescence polarization as a measure of the effects of FFAs on membrane lipid and artificial vesicle lipid structure. In this technique the fluorophores, introduced into the bilayer, are excited with polarized light, and will emit polarized light at a characteristic angle to the plane of excitation. The freedom of rotation of the probe determines the degree of polarization, and thus the degree of polarization reflects the local environmental constraints on the rotation of the probe during its excited-state lifetime. The steady-state polarization is given by

$$p = \frac{I_{11} - I_1}{I_{11} + I_1}$$ [9]

in which I_{11} and I_1 are the components of the fluorescent intensity parallel and perpendicular to the polarized excitation beam. Suitable corrections are made for contributions due to scattering.

The rotational correlation time (ρ) of the probe in cell membranes is determined according to the Perrin equation:

$$\frac{1}{p} - \frac{1}{3} = \left(\frac{1}{p_0} - \frac{1}{3}\right)\left(1 + \frac{3\tau}{\rho}\right)$$

in which p_0 is the polarization observed in a frozen solution at the exciting wavelength. Fluorescence polarization of two different probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) and 8-anilino-1-naphthalene sulfonate (ANS) were used to monitor structural changes due to the introduction of a series of FFAs into biological and model membranes. It is well established that ANS is located at the polar–nonpolar interface with its negative charge oriented toward the polar domain,[16] whereas there is evidence that DPH is localized in the lipid interior.[17] In our experiments we were

able to establish that ANS was essentially reporting on lipid head-group packing and not on binding to protein cationic sites.

Under the conditions of our experiments the FFAs intercalated rapidly (within minutes) into the membranes and vesicles, rapidly reaching saturation, and exist essentially as FFAs almost exclusively in the membranes. With time, incorporation into phospholipid occurs, but the effects of lipid structure are then quite different.[11]

When lymphocytes or isolated plasma membranes were treated with low concentrations of fatty acids—well below the critical micelle concentration—the following became apparent: *cis*-unsaturated fatty acids (Group A) caused an increase in ANS polarization and a decrease in DPH polarization, whereas *trans*-unsaturated and saturated fatty acids (Group B) caused a similar increase in ANS polarization but no change in DPH polarization. It is of interest that several biological activities, such as cell adhesion,[1] cell growth,[1] morphology,[1] and receptor mobility [10] are also differentially affected by the two classes of FFAs.

As the two probes used in this study are reporting on different depths of the bilayer, our results also clearly show that a single perturbing molecule (eg, of Group A FFAs) may not alter the local membrane packing identically at all depths in the bilayer—in fact, the Group A FFAs change the packing in opposite directions. Therefore their effects cannot be described in terms of a single global parameter such as "fluidity" or "microviscosity," as is frequently done, based on DPH polarization values alone.

Binding studies with ^{14}C-labeled FFAs showed that Group A and B FFAs, respectively, partitioned equally well into the membrane from the aqueous phase. We could then ask the question, Are the two types of FFAs inserting into identical or different regions of the membrane? We postulated that we could explain the differences in the polarization effects of the two types of FFAs if a) there were structurally different domains in the membrane and b) there was differential partitioning of the different classes of FFAs into different types of domains.

Partitioning Studies: Evidence for Lipid Domains

Our findings in plasma membranes could not be reproduced in single-phase lipid (gel or fluid) vesicles, but in mixed phase vesicles the polarization measurements were closely imitative of the results in plasma membranes. Furthermore, the results in these model membranes suggested that the Group B FFAs were inserting into the gel phase and the Group A FFAs into the fluid phase.

Differential partitioning is hard to test. However, we used colligative

solution properties to test whether the classes of FFAs preferentially partitioned into either fluid or condensed (gel) lipid phases.[18] We were able to derive a formula which enabled us to use the direction of change in melting temperature to determine whether a FFA preferentially partitions into a gel- or fluid-phase lipid. In simple terms, we used the basic thermodynamic principle that certain molecules inserted into a fluid phase would decrease the melting temperature. (Antifreeze dissolving in water and lowering the freezing point is a good example.) Molecules dissolving in a gel phase would conversely raise the melting temperature. (By analogy, there are certain substances which dissolve in ice and raise the freezing point.)

When FFAs were inserted into dimyristoyl phosphatidyl choline vesicles the shifts in the melting curves were as predicted: Group A FFAs lowered the melting temperatures, and Group B raised the melting temperatures. These partitioning studies provided a firm thermodynamic basis for the concept that there are different lipid domains into which the FFAs are differentially inserting: Group A into fluid phases and Group B into less fluid phases. Our results agree with previous observations of the effects of different FFAs upon endothermic phase transitions,[19] and with the observations that *trans*, but not *cis*, parinaric acid preferentially partitions into gel phases.[20]

We thus postulate that there are discrete lipid domains in cell membranes of relatively gel- or fluid-like nature. A variety of other studies have suggested structural inhomogeneity in the lipid of membranes, including ESR,[13] NMR,[21] electron-diffraction,[22] and electron-microscopic studies.[23]

The modeling of the interaction of FFAs with membranes provides only circumstantial evidence for heterogeneity of membrane lipid and the presence of lipid domains. Direct evidence has been provided through DPH lifetime heterogeneity studies carried out with Dr. Alan Kleinfeld.[11] These will not be discussed here. Suffice it to say that the presence of heterogeneous DPH lifetimes in the membrane strongly supports the idea of the membrane consisting of different lipid domains, some of which are more gel-like and some more fluid.

We postulate that the lipid domain structure may be a consequence of the interaction of certain intramembranous proteins with the lipid. Thus, the ability to selectively perturb one domain with the appropriate FFA may provide a way to probe the functional roles of lipid domains in relation to specific membrane protein functions. Indeed, just such perturbation of a variety of functions has been reported by us [10] and by oth-

ers.[7-9] I wish to deal now specifically with cell-surface-receptor lateral mobility and movement, using as a model immunoglobulin (Ig) capping on murine B lymphocytes.[10]

Effects of Free Fatty Acids on Receptor Mobility

We found that the Group A FFAs, at the same doses, and over the same time course as produced the ANS and DPH polarization changes given above, markedly inhibited capping of Ig, whereas the Group B FFAs had no effect. The cells remained viable, and the inhibition of capping was not due to auto-oxidation of the FFAs, nor due to prostaglandin synthesis.

Because capping involves the movements of receptors laterally in the membrane,[24] it was of interest to see whether the motional characteristics of surface Ig were altered by the Group A FFAs. In collaboration with Dr. Paul Dragsten, fluorescent photobleaching recovery measurements[25,26] were made. With this technique the cell is labeled with fluorescence anti-Ig antibody, a small area is bleached with a laser beam, and the rate of diffusion of unbleached antibody into the bleached area is measured. Fluorescent monovalent anti-Ig Fab fragments will tag surface Ig without inducing patch or cap formation—and thus can be used to measure the lateral diffusion constant of the receptors. When divalent fluorescent $F(ab)_2$ anti-Ig is used, patching and then capping occur: the former is a passive non-energy-dependent step; the latter requires metabolic energy, is inhibited by azide, and is thought to be dependent on the contractile properties of the cytoskeleton. Photobleaching obviously cannot be done on capping cells: it is performed, therefore, under capping conditions (using cross-linking antibody), in the presence of azide, when only patching occurs. Under these only patching conditions, the cross-linked receptors were found to be completely immobilized. When Group A FFAs are used, azide is not necessary: only patching occurs, and the patches are likewise immobile. With Group B FFAs azide is required, but the patches are also immobile.

Thus, Group A FFAs inhibit capping, but not patch formation or receptor immobilization: they inhibit the final, energy-dependent step in capping—that step which invokes a transductive linkage between the patches and the contractile apparatus, activation of the latter, and the consequent energy-dependent sweeping of the patches into a cap at one pole of the cell.

The inhibition of capping by the Group A FFAs could be reversed in a dose-dependent fashion by the addition of extracellular Ca^{++}, but not by Mg^{++}. Ca^{++} additions did not extract the FFAs from the membrane.

Addition of Ca^{++} not only reversed capping inhibition but also reversed the increase in ANS polarization caused by Group A FFAs. This reversal followed the same Ca^{++} dose dependency as did the capping inhibition reversal. The DPH polarization changes caused by the Group A FFAs were not reversed by Ca^{++}.

The particular structural change caused by the Group A FFAs is a knotty issue: the altered head group packing reported by the ANS polarization is a likely candidate, because external Ca^{++} specifically reverses both the head group disturbance and the capping inhibition, both induced by FFAs. However, Group B FFAs also affect head group packing in a similar way, but do not inhibit capping. On the basis of the lipid domain model, and the differential partitioning of Group A and Group B FFAs into different lipid domains, we postulate that Group A FFAs partition into specific lipid domains, disturb those domains, and thus affect specific proteins contained in those domains. Such proteins would be necessary for the machinery of capping, possibly linking patches to the contractile system.

Although Group A FFAs slightly increased Ca^{++} flux into the cell, this was not reversed by high external Ca^{++} and was therefore considered to be trivial. Of greater moment were the effects of FFAs on intramembranous Ca^{++} binding. Membrane-bound Ca^{++} was measured with the fluorescent probe chlorotetracycline (CT), which markedly increases its quantum efficiency in a hydrophobic (eg lipid) environment when it chelates Ca^{++}.[27] Previous studies have shown that CT reports on phospholipid-associated Ca^{++} but not on membrane-protein–associated Ca^{++}.[28]

CT-labeled cells showed a marked increase in CT intensity when treated with Group A, but not Group B, FFAs: the cells were held in a Ca^{++}-free medium. Artificial lipid vesicles did not show such an enhancement. Isolated lymphocyte membranes, prepared in Ca^{++}-free medium, did show such an enhanced CT intensity with Group A, but not Group B, FFAs. Since there was no external Ca^{++} in this latter experiment, the increased CT fluorescence with Group A FFAs is ascribed to a shift in intramembrane Ca^{++} from protein-binding sites to the lipid phase, where the Ca^{++} can be sequestered by the lipid-bound CT.

A Model of Capping

We are able to fit these observations into a heuristic model of capping. Receptors are free to diffuse laterally in the membrane. Cross-linking of Ig receptors forms patches: the patches are immobilized by an as yet unchar-

acterized interaction with a postulated transmembrane anchoring protein, analogous to the X protein proposed by Singer.[29] This anchoring protein immobilizes the patch by some form of interaction with a component of the cytoskeleton. Furthermore, in our model the anchoring protein is a Ca^{++}-binding protein. We postulate therefore that the receptor–ligand complexes not only bind to the anchoring protein but cause a conformational change, $p^o \longrightarrow p^x$, which results in a local submembranous release of Ca^{++}, initiating a link to, and activation of, contractile elements, leading to the sweeping of the patches into a cap.

How would Group A FFAs inhibit capping? Basing our idea on this model, we would propose that the anchoring, Ca^{++}-binding protein sits in specifically more fluid lipid domains, into which the Group A, but not the Group B, FFAs partition. This protein is sensitive to head-group packing in the lipid domain: disturbance of this, as revealed by the ANS polarization changes, leads to a conformational change in the protein, $p^o \longrightarrow p^y$. This conformational change allows for the release of Ca^{++}, which is now sequestered in the lipid region; thus initiation of linkage to, and activation of, the contractile elements does not occur, and the patches remain immobilized and uncapped.

The reversal of the inhibition of capping caused by Group A FFAs can be explained by a relaxation of the altered head-group packing (as was indeed observed with ANS): this allows the calcium-binding protein to return to its Ca^{++}-binding state, release Ca^{++} submembranously, and activate the cytoskeleton.

Falling out of this model would be the possibility of demonstrating in the membrane the postulated Ca^{++}-binding protein. One might also suspect that upon treatment of lymphocytes with Group A FFAs changes in the distribution of elements of the cytoskeleton might be detected. In collaboration with K. Fujiwara we have indeed found this to be the case: utilizing immunocytochemistry, we have found dramatic changes in the distribution of tubulin, myosin, and other components of the cytoskeleton.

Lastly, we believe that we have shown that FFAs, as probes of membrane structure and receptor physiology, should prove useful in elucidating membrane-based mechanisms underlying normal and pathological states.

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