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# Environmental influences on signal transduction through membranes: a retrospective mini-review

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**Abstract**

This mini-review is addressed to the question how the membranous environment may affect traffic of receptors and signalling from membrane-anchored receptors on the outside of cells to transducers and targets on the inside. Signal transduction by membrane-anchored receptors to the interior of the cell and eventually to the genome is a central issue in cellular regulation. In this context the role of membrane fluidity and of the cytoskeleton in restricting the mobility of proteins are discussed and the evidence for a structural order in membranes which could limit the mobility of proteins is scrutinised.


*Keywords:* Receptor mobility; Membrane fluidity; Cytoskeleton; Lipidrafts; Fluorescence photobleaching recovery; Fluorescence resonance energy transfer; Fluorescence correlation spectroscopy; Single particle tracking; Green fluorescent protein

## 1. Introduction

A signalling molecule is sensed by a receptor attached to the membrane of a cell and the receptor is activated. Receptor–ligand interactions at the cell surface are the first steps in cellular signalling. Membrane-bound receptors respond to many and diverse extracellular signals. As diversified as the signals are the receptors which respond to them. But, in each case binding of a signalling molecule converts the dormant receptor to an active state. De-activation is often accompanied by removal of the receptor from the membrane by endocytosis. Because receptor activation and de-activation are initiated in the plasma membrane, it is likely that the membranous environment affects receptor activation and signal propagation and the processes involved in receptor de-activation. Membrane lipids could influence these processes either through global effects on the physical state of the membrane matrix, such as by microviscosity changes or by specific chemical interactions of boundary lipids with receptor proteins and transmitters. Old and new data on the effects of the membranous environment on receptor traffic and receptor-mediated signalling shall be reviewed.

## 2. Membrane fluidity

The membrane bilayer viscosity may be estimated from measurements of rotational and trans-

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lational diffusion of lipid probes. In biological membranes the lateral diffusion coefficients of lipid probes are in the range of $10^{-8}$ cm$^2$ s$^{-1}$ [1].[1] This is consistent with measurements of rotational diffusion of lipid probes by fluorescence depolarisation or magnetic resonance spectroscopy.

A variety of procedures are at hand to change fatty acids of phospholipids and alter the phospholipid-content of membranes of eukaryotic cells. This allows the investigator to study the effects of such changes on the function of membrane-bound proteins. Such manipulations include supplementation of tissue culture cells with fatty acids and cholesterol, the use of mutants defective in lipid or sterol biosynthesis, introduction of synthetic phospholipids into membranes by exchange or fusion with lipid vesicles and the maintenance of animals on diets enriched or deficient in essential fatty acids. Moreover, membrane structure has been perturbed by interaction with filipin, mellitin and other substances that preferably target cholesterol. Finally, membrane viscosity changes have also been induced by temperature changes (for a review see Helmreich and Elson [2]).

### *2.1. Adaptation of membrane fluidity to temperature changes*

Studies of temperature adaptation of bulk fluidity in membranes of eukaryotes in general and in long-term thermally acclimated fishes in particular have pointed to the operation of powerful compensatory mechanisms to maintain fluidity over a wide range of temperatures. These mechanisms of temperature adaptation are shared by most eukaryotes. Such compensatory mechanisms were studied in cells in response to changes in the fatty acid composition of the membrane. For example [3], plasma membranes enriched with linoleate (18:2) showed a compensatory decrease of the membrane's own oleate (18:1) content. This adjustment was independent of the nature of the polar phospholipid head groups to which these fatty acids were attached. The consequence of adaptation was that changes in the membrane's fatty acids were tolerated and did not lead to changes in membrane viscosity with changes in temperature. This was deduced from fluorescence polarisation measurements with 1,6-diphenyl-1,3,5-hexatriene. In other experiments [4], liver plasma membranes were compared with liver membranes from rats fed the same diet, but supplemented with 5% corn oil as a source of unsaturated fatty acids. Again a compensatory decrease occurred in indigenous 16:1 and 18:1 unsaturated fatty acids in the membranes of the animals, fed unsaturated fatty acids. Moreover, in order to fluidise the membranes [5], turkey erythrocytes were enriched with *cis*-vaccenic acid, but this manipulation did not affect the lateral diffusion of the probe 4-nitrobenzo-2-oxa-1,3-diazolyl-phosphatidylethanolamine (NBD-PE).

Thus, these and other data provide evidence for the existence of powerful compensatory mechanisms in living cells to prevent adverse effects of changes in membrane microviscosity. Obviously, nature has optimised the hydrocarbon chain composition of all phospholipids to maintain membranes fluid in the physiological temperature range [6]. Thus, to invoke a biologically meaningful role for changes of membrane fluidity would imply that such a control is exerted on a membrane whose fatty acid composition has already rendered it optimally fluid and stable at physiological temperatures.

Therefore, the lipid bilayer of an eukaryotic membrane is expected to be fluid at physiological temperatures. Hence the simplest dynamic membrane model would predict that membrane

[1] According to Saffman and Delbrück the translational diffusion coefficient, $D_T$ , for a freely moving protein should only weakly depend on molecular size. This is in contrast to rotational diffusion. The weaker dependence means that $D_T$ is not as sensitive an indicator of aggregation as is the rotational diffusion constant. $D_T = k\ T/4\pi\mu h\ \{\log\ \mu h/\mu' a - \gamma\}$ where $k$ = Boltzmann's constant, $T$ = absolute temperature, $\mu$ = viscosity of the lipid bilayer, $\mu'$ = lenght of the cylindrical particle, (thickness of the bilayer), $a$ = radius of the cylindrical particle and $\gamma = 0.5722$ (Euler's constant). The rate of rotational diffusion depends sensitively on the size of the diffusing molecule as well on the viscosity of the lipid bilayer in which it is embedded. For example treating the protein as a cylinder with its axis normal to the plane of the membrane, the diffusion coefficient for rotation, ($D_R$), about the cylindrical axis is: $D_R = k\ T/4\pi\mu a^2 h$; where $a$ = the radius of the cylinder, $h$ = the length of the cylinder immersed in the membrane, $\mu$ = the membrane viscosity, $k$ = Boltzmann's constant, $T$ = the absolute temperature. The equation assumes that the resistance to rotation offered by the relatively low viscosity of the water surrounding the membrane is negligible compared to that of the membrane bilayer.

proteins are free to move laterally over the cell surface at rates limited only by the bilayer viscosity. This assumption is implicit in the fluid mosaic model of biological membranes, proposed by Singer and Nicholson [7], which visualised the lipid bilayer of the membrane as a homogenous, two-dimensional fluid matrix. This assumption will now be critically examined in light of old and new experimental data.

## 3. Diffusion of membrane proteins

Encounters of randomly dispersed receptors on membranes of cells of the immune system with cell-bound antigen–major histocompatibility complex (MHC)-complexes play a central role in eliciting immune responses. Although cell-bound antibody-like receptors recognise antigens or haptens, just like antibodies circulating in the blood, in some cases the antigen to which the receptor binds is not soluble but is bound to another cell. Thus, a characteristic feature of the immune response are cell–cell interactions. A simple theoretical analysis of rates of encounter of cell-bound surface molecules has been provided by Bell [8]. Bell compared interactions of antigen and antibody in solution and bound to membranes and found that the rate constants were considerably smaller when antibodies and antigens were both membrane-bound, as when the reactants were in solution, e.g. $D_m \approx 10^{-10}$ cm$^2$ s$^{-1}$ in the former as compared with $D_s \approx 5\times10^{-8}$ cm$^2$ s$^{-1}$ in the latter case. A quantitative analysis of the role of diffusion in interactions of membrane-bound reactants in the immune system was carried out by Mc Connell and colleagues and published in a series of papers [9]. They studied the kinetics of binding the cell-bound antibody to spin-labelled haptens, presented in lipid vesicles. According to their model, the faster rate of binding of the haptens in fluid dimyristoylphosphatidylcholine (DMPC)-vesicles as compared with haptens in solid dipalmitoyl-phosphatidylcholine (DPPC)-vesicles results from the faster rate of diffusion of the haptens in the fluid vesicles. This leads to a higher frequency of collisions with the cell-bound antibody. In other experiments [10], it was shown that coupling of cell-bound IgE-receptors with haptens results in the formation of membrane patches, containing immobilised cell-bound IgE–hapten complexes. Thus, productive interactions of a membrane-bound receptor with its ligand and complex formation may be accompanied, in some cases, with immobilisation of the receptor–ligand complex.

### *3.1. Restraints on mobility of proteins in membranes*

Reasonable estimates of molecular dimensions lead to the conclusion that the lateral diffusion coefficients of proteins in fluid membranes should be in the range of $10^{-9}$ cm$^2$ s$^{-1}$ or larger. However, experimental results obtained with the fluorescence photobleaching recovery (FPR) technique, show that protein diffusion coefficients are at least one order of magnitude slower, a typical value being near $10^{-10}$ cm$^2$ s$^{-1}$, with many examples having $D<10^{-10}$ cm$^2$ s$^{-1}$. Furthermore, a variable but significant fraction of membrane proteins is immobile on the usual experimental time scale with typical $D$ values of $D<5\times10^{-12}$ cm$^2$ s$^{-1}$. Elson and Reidler [11], have attributed high- and low-affinity binding to undefined sites on components of membrane proteins. High-affinity binding sites were made responsible for immobilising and anchoring that fraction of membrane proteins which are not recovered in FPR experiments. Low-affinity sites were assumed to be responsible for reducing the mobility of membrane proteins, resulting in slow diffusion of the mobile fraction of such proteins.

### *3.2. Hindered diffusion and immobile fractions of membrane proteins in FPR experiments* [12][2]

More recent results obtained by single particle tracking (SPT) by means of computer-enhanced confocal video microscopy with colloidal gold- and fluorescent-tagged proteins require a new interpretation of FPR experiments. FPR data have been interpreted assuming random Brownian diffusion of a mobile fraction of a membrane protein with another fraction of variable size being immo-

[2] I am greatly indebted to one of the reviewers for bringing these data to my attention.

bile. Webb and his laboratory have introduced a constrained diffusion model for the interpretation of FPR data which takes anomalous 'subdiffusion' of membrane proteins into account. The model rests on SPT-experiments, which have shown that a single species of a protein on the cell surface can exhibit different types of motion; directed motion, Brownian diffusion and anomalous subdiffusion (for a review, see Saxton and Jacobson [13]). According to this model, the apparently immobile fraction in FPR experiments represents molecules, exhibiting anomalous subdiffusion. These proteins are not immobile. They diffuse very slowly, as a consequence of undefined interactions. This is expressed in a range of diverse diffusion coefficients. The Webb laboratory [12], has compared FPR and SPT measurements of the IgE[3] receptor on leukaemia cells. Whereas 30% of the IgE receptor was immobile in FPR, only 4% was immobile in SPT-measurements. They concluded that the apparently immobile fraction, reported by FPR, represents a subpopulation of IgE receptor molecules undergoing constrained percolative diffusion [12,13].

These data suggest that constraints on the mobility of proteins exist in membranes, in addition to viscosity. These constraints retard the lateral diffusion of at least some membrane proteins, including receptors.

A membrane infrastructure interacting with a diffusing molecule could result in varying time- and space-dependent constraints (for more information addressing these problems, see Jacobson and co-workers [14,15]). But, granted that such restraints exist, what is their nature? There is no single answer. In the following, a few possibilities shall be mentioned.

### *3.3. Complexes between proteins and lipids*

An example how a membranous phospholipid surface could immobilise proteins, attached to it, is the assembly of blood clotting factors. In this case, the membrane surface is like a scaffold, helping to orient and align the proteins, a process which is necessary for the conversion of the inactive precursors, the zymogens, into active serine proteases. The assembly on this two-dimensional platform facilitates by juxtaposition, their mutual interactions and increases greatly their activity [16]. The kinetics of immobilisation in the course of the assembly of proteases on the membrane surface has to my knowledge not yet been studied quantitatively. On the other hand, in the case of the band 3 protein of erythrocytes, restraints of the mobility of this membrane protein have clearly been demonstrated [17].

### *3.4. Connections between the cell surface and the cytoskeleton*

The band 3 protein with a molecular mass of 90 kDa comprises approximately 25% of the total protein in erythrocyte membranes. It was estimated from rotational measurements that approximately 60% of band 3 protein can rotate freely, but that the remainder is immobile on the experimental time scale. Measurements of translational diffusion by FPR yielded a value of $D < 4 \times 10^{-11}$ cm$^2$ s$^{-1}$ for the immobile fraction. Biochemical studies have provided a structural explanation for the immobility of a large fraction of the band 3 protein: The band 3 protein binds to spectrin which is linked via ankyrin to the erythrocyte cytoskeleton. Fowler and Bennet [18] showed that the membrane attachment site of spectrin is responsible for the impediment of the lateral mobility of the band 3 protein. Furthermore, as was shown by Sheetz et al. [19], when spectrin was partially removed from the erythrocyte membrane, the lateral diffusion coefficient, $D_{L,}$ of the band 3 protein increased from $4 \times 10^{-11}$ to $2 \times 10^{-10}$ cm$^2$ s$^{-1}$ and so did $R_f$, the fractional recovery of the protein. Steck [20] proposed a model where integral membrane proteins, such as band 3 proteins are entrapped in a net, where they can rotate freely, while their translational movement is restricted by links to the spectrin/ankyrin–cytoskeleton network.

Interactions of the cell membrane with the cytoskeleton in other cells, for example epithelial cells, are more complex than in erythrocytes. In these cells the cytoskeleton is composed of several fibre systems with a large number of accessory

[3] IgE, Immunoglobulin E, a class of immunoglobulins.

proteins, some of which interact directly with the cell surface.

An approach to find links between the cell surface and the cytoskeleton that limit the lateral mobility of membrane proteins is to measure diffusion of proteins in 'blebs', that is in regions of membranes which have been detached from underlying structures by lifting the plasma membrane from the cytoskeleton [21]. Such measurements have been carried out with Con A[4] receptors on rat myoblasts [22]. All the Con A receptors became mobile in the 'blebs'. The same applies to the mobility of acetylcholine receptors in 'blebs' of neuromuscular endplates, or of IgE receptors in 'blebs' of rat basophil leukaemia cells or of low-density lipoprotein, LDL receptors in 'blebs' of human fibroblasts. The experiments with 'blebs' have indicated that the slow diffusion rates of some membrane-anchored receptors are probably a consequence of interactions of surface proteins with the cytoskeleton which are detached in 'blebs'. This is supported by experiments with synthetic cytoskeleton-free vesicles [23].

Another approach to study restraints on protein mobility in membranes was based on work of Edelman [24], showing that cross-linking of membrane-bound antigen and antibody was prevented by Con A. This phenomenon was named 'anchorage modulation' and was studied in detail with B lymphocytes. The prevention of patch formation of antigen–antibody complexes by Con A was interpreted as a consequence of an interference by the Con A receptor aggregates with the diffusion of antigens to the immunoglobulin receptors on the surface of these cells. This is a prerequisite for the formation of antigen–antibody complexes and subsequent cross-linking.

### *3.5. Connections between the plasma membrane and the extracellular matrix (ECM)*

Membrane-integrins are diverse, heterodimeric, αβ-structures which bind to components of the extracellular matrix, such as proteoglycans, fibronectin and collagen. Integrin-mediated interactions regulate also the organisation of the intracellular actin cytoskeleton and control cell–cell adhesion [25], cell shape, locomotion and spreading of cells. As the name implies, integrins are integrators. They are receptor-like cell surface proteins, linking the extracellular matrix (ECM) with the cell membrane and the latter with the cytoskeleton. But, whether interactions of cells with the ECM affect the mobility of membrane proteins is rather doubtful. There are no large differences in protein mobility in adherent cells that do interact with the ECM and cells that have no ECM-anchorage.

### *3.6. To sum up*

There is evidence that links of the membrane to the cytoskeleton are responsible, at least in part, for the immobility and/or the slower than expected lateral diffusion of cellular proteins. The molecules establishing these links remain to be clarified, but it is reasonable to expect that these interactions must be highly regulated, in order to permit the complex dynamics of cells, their polarity, deformability, elasticity, rheological properties and shape changes, in response to signals.

Old and new data implicate in particular a role of the actin cytoskeleton in receptor location and function. Moreover, the cytoskeleton (and phosphorylation), seem to control endocytic sorting of particular G protein-coupled receptor (GPCR). But how such influences, if they exist, can limit the motion of membrane proteins remains an open question.

### *3.7. Is there a structural order in membranes which could limit the mobility of proteins?*

The question is whether a structural order of the plasma membrane itself, imposed by lipids and proteins may hinder the random mobility of receptors. The experiments which are discussed were mainly carried out with β-adrenergic receptors. These receptors are a prototype of G protein-coupled, heptahelical, or serpentine receptors. They transmit adrenaline signals. G protein-coupled receptors belong to the largest family of membrane-bound receptors. They bind to many important water-soluble hormones, notably catecholamines, adrenaline, noradrenaline, serotonine

[4] Concanavalin A is a lectin.

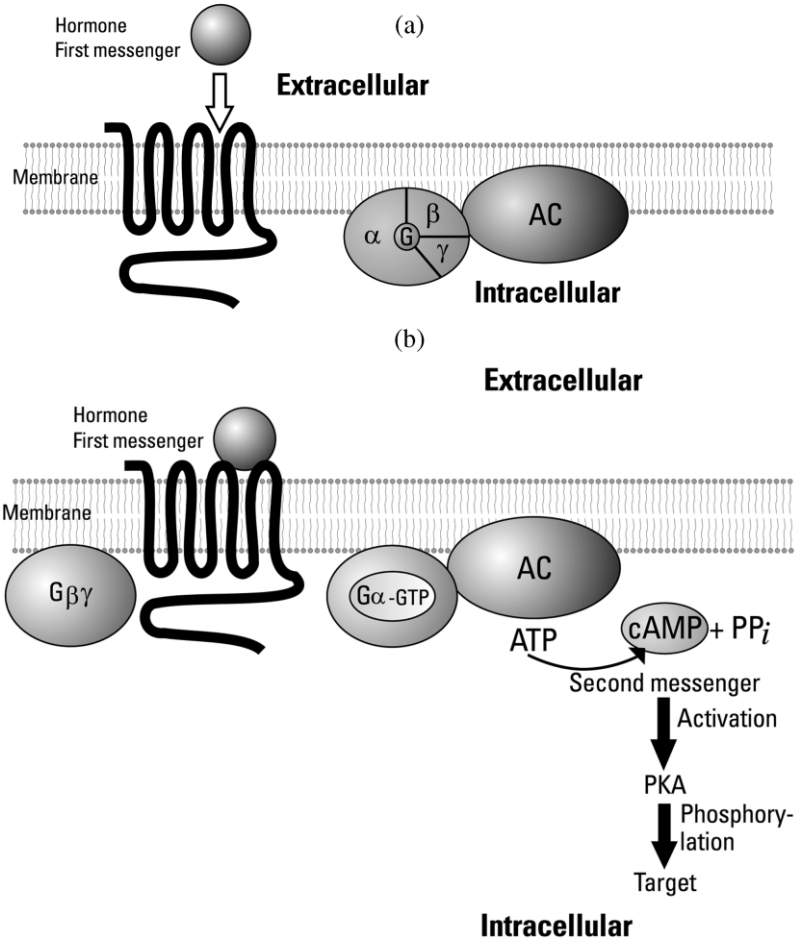


Fig. 1. (a,b) Signal transduction from a G protein-coupled heptahelical receptor to a target. The situation in (a) is before and in (b) after the receptor was activated by the hormone and has made contact with the transducer, a heterodimeric, α,β,γ-G protein. After coupling to the receptor and activation, the α,β,γ-G protein dissociates into the β,γ-subunits and the α-subunit, which is now in the active, GTP-bound, 'on' state. The active G-α transducer finds the adenylyl cyclase in the two-dimensional space of the membrane by collision coupling. The activated adenylyl cyclase forms the second messenger cAMP from ATP, which in turn activates the cAMP-dependent protein kinase (PKA) and downstream targets (from Helmreich [35], p. 79, Fig. 5.3).

and many others. They also accept and transmit sensory, light, odour and taste signals.

## 4. Receptor activation and signal transfer in membranes

Recognition of the ligand occurs at the outer surface of the membrane, but transmission of the signal by heterotrimeric G proteins to the target, for example to the enzyme adenylylcyclase, occurs at the cytosolic phase of the membrane.

Based on the topology shown in Fig. 1a,b, it would seem reasonable to expect that the physical state and the constituents of the plasma membrane bilayer which create the microenvironment, where signalling occurs, affect any of the steps involved in receptor signalling. In an early study [26], we have tried to perturb the membrane of pigeon erythrocytes which contain a highly catecholamine-responsive β-adrenergic G protein-coupled receptor–adenylyl cyclase system with the cyclic macrolide filipin, because it forms defined com-

plexes with cholesterol. Moreover, the amount of cholesterol–filipin complexes formed in membranes can be quantitatively assessed by recording changes in the absorbency ratio: 320:356 nm. The filipin-induced perturbation was probed with the amphipathic 8-anilino-1-naphtalene sulfonate (ANS) and the hydrophobic probe perylene. Treatment of membranes with filipin and the formation of cholesterol–filipin complexes neither affected hormone binding to the receptor on the outside nor the catalytic activity of the target enzyme, adenylylcyclase, located on the inside of the plasma membrane. (In the latter measurements, adenylylcyclase was activated chemically, by $AlF_4^-$, rather than by the G protein-coupled receptor.) However, the filipin-induced membrane perturbation severely interfered with the formation of the active GTP-bound form of the transducer, the GTP-bound α subunit, which arises together with the βγ subunits from heterotrimeric α,β,γ-G proteins on replacement of GDP by GTP, an exchange reaction catalysed by the activated receptor. The fact that perylene sensed the membrane perturbation, resulting from the cholesterol–filipin interaction, suggested that the changes in the membrane responsible for the intervention with collision-coupling of the receptor and the G protein occurred mainly in the hydrophobic core of the lipid matrix, because it is accessible to perylene. Accordingly, changes detectable with the amphipathic ANS were minor. (The membrane regions accessible to and probed by ANS are regions with access to water.) Since inhomogeneities in membranes, resulting from filipin–cholesterol clustering had been observed before [27], we have interpreted our observations to mean that cholesterol and the structural order of the lipid matrix is an element of prime importance for effective signal-transfer, involving membrane-bound, multi-component receptor-signalling assemblies.

We have also studied signal transfer from G protein-coupled β-adrenergic receptors to the target enzyme adenylylcyclase in secondary human liver cells, Chang liver cells [28]. The content of membrane phospholipids in these cells was altered by fusion with liposomes made up of dimyristoylphosphatidyl choline (DMPC) or dioleyl-phosphatidylcholine (DOPC) or dipalmitoylphosphatidylcholine (DPPC). The rate of transfer of the phospholipids was high, resulting in an enrichment of the plasma membrane of the liver cells with 20–30 mol% of the phospholipid offered. In the DOPC-enriched membranes the mobility of pyrenedecanoic acid, an excimer forming fluorescent probe was increased and correspondingly the broad thermotropic phase transition between 20 and 30 °C, observable in membranes from normal, non-enriched cells by electron paramagnetic resonance measurements with 5-nitroxystearic acid disappeared in the DOPC-enriched membranes. These changes in the physical state were accompanied at physiological temperatures in the fluidised membranes by a drastic reduction in the number of β-receptors, titratable with ligand. The number of receptor sites was reduced by 40–60%, compared with the corresponding numbers in normal cells or in cells enriched with DPPC. However, the same number of β-receptors as in normal cells or in cells enriched with DPPC was titrated in the fluidised, DOPC–DMPC-enriched membranes at 17 °C, a temperature below the thermotropic phase transition. The loss of β receptor–ligand binding sites in the fluid DOPC–DMPC-enriched membranes at 37 °C was accompanied by down-regulation of the hormone- and G protein-dependent adenylylcyclase activity, whereas the basal and chemically ($AlF_4^-$)-stimulated adenylylcyclase activities were not affected.

The analysis of the physical state of the DMPC- and DOPC-enriched membranes with spin- and fluorescent-probes verified that introduction of DMPC or DOPC made the membranes of these liver cells more fluid in a random fashion at physiological temperatures, above the thermotropic phase transition. Thus, it would have been expected that receptor mobility is increased in the fluidised membranes, leading to a greater frequency of collisions with G protein and target and consequently resulting in a greater receptor-mediated activation of adenylylcyclase. But the opposite was found. Contrary to expectations, the membrane made more fluid actually hindered rather than facilitated receptor coupling with G protein and activation. Actually, a possibly greater mobility of the receptors resulted in their disappearance from the surface. This led us to conclude that in

normal cells the phospholipids in the membranes are apparently organised in a way that prevents random disorder and dislocation of membrane-anchored receptors. Apparently, order must be maintained to guarantee their function.

To get more information we have measured in collaboration with Henis et al. [29], the lateral mobility and distribution of β adrenergic-receptors in the same cells, human Chang liver cells by FPR and video intensification microscopy. The β receptors were tagged and made visible by non-covalent binding of a fluorescent, non-active ligand, 7-(2-allylphenoxy)-2,2-dimethyl-6-hydroxy-1-(4-nitrobenzo-2-oxa-1,3-diazolyl)-1,4-diazaheptane (ALP–NBD). Of the fluorescent staining 60–75% was specific, e.g. displaceable by non-fluorescent β receptor-specific ligands. The rather high and variable percentage of unspecific binding was criticised by Rademaker et al. [30]. It is probably due to the hydrophobicity of the fluorescent ALP–NBD ligand. To eliminate this source of artefacts, we have since synthesised and introduced the fluorescent β-adrenergic antagonist, BODIPY–CGP[5] [31]. In the FPR study with ALP–NBD most of the non-activated β-receptors were found to be immobile. This immobility was correlated with a clustered, inhomogenous distribution of the fluorescent receptor ALP–NBD complexes. Visible patches were also observed under the microscope. Thus, membrane-bound β adrenoreceptors in the dormant, non-active state appear to be aggregated and immobile. Although, short pre-incubation at 37 °C with a powerful β agonist, the receptor activator (−)-isoproterenol, (prior to the addition of the fluorescent label, ALP–NBD and the FPR-measurements), induced a gradual release of the β receptors from their membrane anchorage, resulting in a more homogenous distribution of the receptors on the cell surface, but the time course of the agonist-induced mobilisation of β receptors suggested that the mobilisation of the β receptors was not due to receptor activation. More likely, the slow mobilisation was related to the second step, following activation, e.g. the desensitisation of the receptor and its removal from the membrane. Incidentally, receptor mobilisation was not due to an increase in membrane fluidity, as verified with a suitable lipid probe. Reports that dormant antagonist-labelled β receptors on L 84 myoblasts are in patches [32], and arguments [33], against random distribution of β receptors in vesicles prepared from S 49 cell membranes, suggest that the immobility of this class of receptors and their location in patches is not unique to Chang human liver cells. There are several possibilities to explain the receptor immobility. For example, de-sensitised receptors might be temporarily immobilised in chlathrin-coated pits on the way from the membrane to intracellular endosomes. It should be remembered that whereas the inactive β-receptors are pre-aggregated and immobile, the receptors for insulin and epidermal growth factors are initially mobile but become immobile on activation [34]. This difference may reflect the fundamentally different mechanisms of signal transduction by these different receptor types [35].

The immobility and the patched distribution of the β-adrenergic receptor might be a property not shared by all GPCRs. For example, another GPCR, rhodopsin, in the rod outer segment membranes of the photoreceptor cells in the eye is freely mobile. Whether the mobility of rhodopsin is due to special properties of the very fluid retinal membrane remains open [36]. Recent data from Hofmann's laboratory [37], are of interest in this context. They show by illumination of rod disc membranes with light at 520 nm that nearly complete activation of rhodopsin, as indicated by formation of a large amount of the active Meta II state of rhodopsin, is accompanied by a drastic reorganisation of the boundary lipids of rhodopsin with release of phosphatidylserine. It would be interesting to see how this change in the rhodopsin–lipid complex on activation affects the mobility of rhodopsin in the disc membrane.

### *4.1. New advances and prospects*

Today, imaging of receptors on intact cells can be done by linking by gene fusion receptors with a fluorescent protein, such as green fluorescent protein (GFP), and expressing the fusion product

[5] BODIPY, borodifluoropyrrhomethene, 3,5′-dimethyl-2,2′-pyrromethene-1,1′-borodifluoro-5 propionic acid succinimidylester. CGP, CGP12177 is Ciba Geigy product 12 177.

in cells [38]. Advantages of the new technique are that they at least ameliorate undesired effects of the fluorescent ligand which has to be introduced in order to make the object to be scrutinised, detectable in the cell.

In addition, technical refinements, such as two-photon counting and fluorescence correlation spectroscopy,[6] with single-molecule detection have opened up new experimental possibilities to study receptor activation, de-activation and relocation in cells. Fluorescence correlation spectroscopy (FCS) with single molecule sensitivity allows to deal with the often extremely low concentrations of receptors on cell membranes; problems that have greatly vexed investigators before. Schwille et al. [39] could show that FCS analysis of the diffusion of fluorescent molecules in membranes is possible with single-molecule sensitivity. The method detects, like SPT, diverse molecular diffusion behaviour of proteins in membrane environments, but is much easier to handle and less time consuming than SPT. FCS measurements can be comfortably performed at nanomolar concentrations in volume elements of approximately $10^{-16}$ l.

## 5. De-sensitisation and removal of GPCRs from membranes

De-sensitization and receptor recycling are complicated processes which require mobility. Since Roth and Porter [40] first recognised coated membrane vesicles in eukaryotic cells, evidence has been accumulating that these structures are the gate through which cell surface receptors, heptahelical receptors but also insulin, EGF and many other receptors must pass through, when they leave the membrane. The first step in the removal pathway is the entry of the receptors into coated pits, where the clathrin coat of these structures acts as a molecular filter that sorts out protein molecules. Stages that follow the initial sorting, when the coated pits finally bud off to form coated vesicles, have first been visualised by Heuser with his deep etch technique [41].

Agonist-induced trafficking, sequestration and down-regulation of a GFP-tagged $\beta_2$-adrenergic receptor has been followed in intact cells [42,43]. The recent studies of GPCRs, particularly the $\beta_2$-adrenoceptor, have provided evidence for additional, unexpected roles of endocytosis in receptor regulation [44]. Rapid receptor desensitisation involves ligand-dependent phosphorylation of receptors, followed by binding of proteins, called arrestins, which disrupt the interaction between the receptor and the G protein [45]. Desensitisation of GPCRs is associated with a physical redistribution of receptors and movement from the plasma membrane to intracellular membranes [46]. Although there are differences between mammalian and avian β adrenoceptors. Two β1 adrenoceptor forms in turkey erythrocytes with molecular masses of approximate 50 and 40 kDa have been described [47]. The larger form was now shown to be due to the excision of an intron in the avian (turkey) β1 adrenoreceptor gene. This intron is missing in the mammalian β-adrenoceptor genes, which are intronless. When this intron is excised, a region coding for 59 carboxyterminal amino acids is added to the gene and when this spliced gene is transcribed, a larger form of the turkey β1 receptor is expressed that cannot undergo endocytosis. Only, the smaller form without the C terminal addition can enter the endocytosis pathway. Differences in detergent solubility between the large and the small receptor variant led Wang and Ross [48] to speculate that the carboxy-terminal extension in the larger form may anchor the receptor to the cytoskeleton and forestall its movement to coated pits and endocytosis. Internalisation is thought to promote dephosphorylation of receptors by bringing them to an endosome-associated phos-

[6] FCS analyses the diffusion times of molecules and the changes when they interact. This is done by fluctuation analysis of fluorescent labelled molecules within a well-defined volume element or within a cell. The volume element is the confocal volume, defined by the excitation spot of a focused laser beam and by the emission region, defined by the aligned pinhole of the detection optics. The fluctuations are analysed by treating the measured photon counts with the mathematical method of correlation functions. If the two interacting molecules are of different size only the smaller one needs to be labelled with a fluorescent tag. This method is called autocorrelation spectroscopy. If the two interacting molecules are of same or similar size, both have to be labelled. This method is called cross-correlation spectroscopy. If FCS is combined with powerful light microscopy, such as with confocal laser-scanning microscopy (LSM), the method is called fluorescence correlation microscopy (FCM).

phatase [49]. After dephosphorylation, the receptor is returned to the plasma membrane and is again responsive to signals. On the other hand, there is an alternative path a receptor can take. This was visualised by fluorescence microscopy with the δ-opioid receptor, a GPCR [50]. Receptors move via clathrin-coated vesicles [51], eventually to lysosomes [52], where they are degraded. Another important and consequential finding [53,54], deserves to be mentioned, namely the realisation that receptor endocytosis of certain GPCRs might establish connections with mitogenic kinase cascades, perhaps in membrane ''rafts'', although these links are not yet clearly defined.

### *5.1. Are protein–protein interactions responsible for immobilisation of GPCRs?*

There is an increasing amount of data in the literature on heterologous and homologous interactions of GPCRs and on the role of scaffolding proteins for GPCRs (for a review see Milligan and White [55]). Association in membranes would be expected to affect the mode of diffusion of GPCRs. Pertinent information on that point is not yet available, as far as I am aware.

### *5.2. To sum up*

The behaviour of receptors in the membrane suggests a dynamic membrane structure. The results of our own experiments with the β-adrenergic receptor and more recent studies, discussed above [56], are compatible with some kind of order in the membrane which prevents random mobilisation. A membrane 'infrastructure' varying both in time and space could be formed by complexes of membrane proteins with lipids. The idea of a microstructure of cell membranes has received support from a quite different line of evidence, namely from experiments demonstrating differential miscibility in detergent of lipids and cholesterol from different entities of plasma membranes.

## 6. The role of microdomains

How differences in lipid composition between various membranes in the cell, for example between endosomal and plasma membranes, but also between the cytosolic and the surface layer of the plasma membrane are established and maintained is a main problem in the study of biomembranes. Research addressed to this problem led to the discovery of several lipid translocators [57]. One aspect of this problem is the question how differences in lipid composition of membranes may arise which are assumed to cause the formation of microdomains and 'lipid rafts' [58]. In a retrospective review it is appropriate to remember that phase separations in lipid mixtures which could form microdomains have already been described 20 years ago, mainly by Mc Connell and his laboratory [59]. Another point is a biological role of lipid rafts. It is speculated that formation of these microdomains might be related to signal transduction through cell membranes (for comments see Edidin [60]). For example, it was reported [61,62], that the aggregates formed between cell-anchored receptors and cell-bound antigens in stimulated T lymphocytes induce fusion of small lipid–cholesterol complexes and formation of large rafts. Moreover, lipid rafts and the cytoskeleton may be connected, because the membrane-bound integrins associate with the membrane protein caveolin [63] (see Fig. 2a,b).

Although little is yet known about these interactions, they could assist in forming integrin clusters at the membrane which might serve as a scaffold for the assembly of multi-component signalling-structures, such as the G protein-coupled β receptor system or the cytokin receptor-linked signalling arrays. These scaffolds might be connected with lipid rafts, because caveolin also binds cholesterol and glycosphingolipids, which are believed to be the main components of membrane 'rafts' [64]. However, the nature of these microdomains, their size, composition, organisation, their physiological role and even their very existence, are still matters of discussion. One reason is that small lipid rafts in native membranes may be transient and difficult to detect. On the other hand, larger lipid domains have been detected by microscopy [65,66]. Membrane cholesterol–sphingolipid rafts are characterised by their insolubility in the non-ionic detergent Triton X-100 in the cold. For example, in the apical plasma membrane of epi-

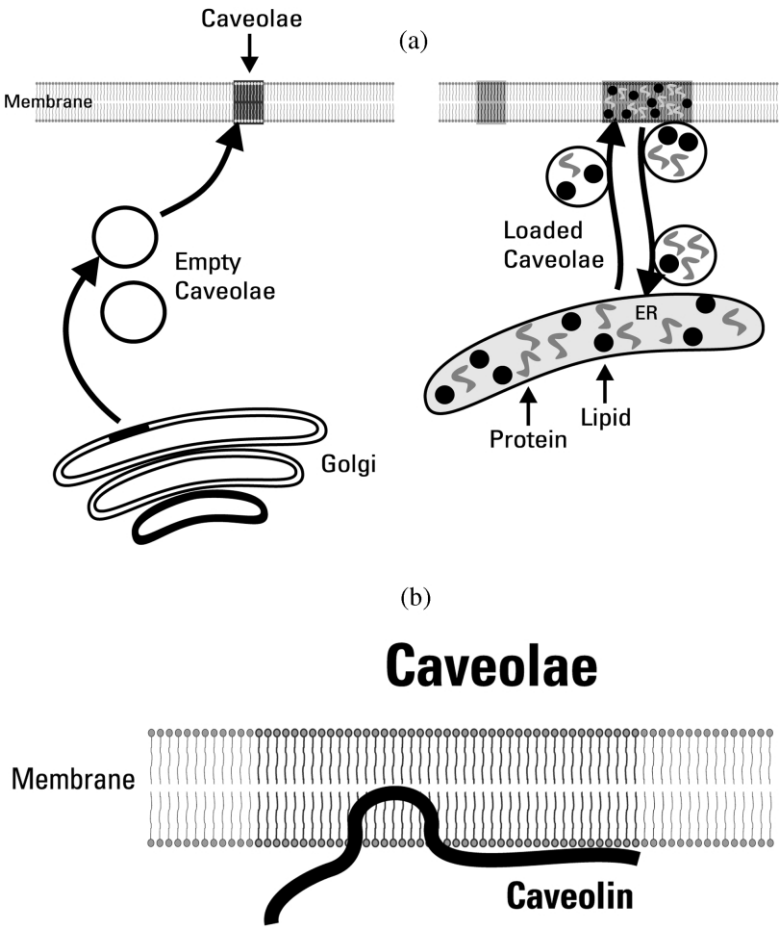


Fig. 2. (a) The function of 'caveolae'. On the left is shown that caveolae transport cholesterol and other lipids from the endoplasmic reticulum (Golgi), to the plasma membrane where they are integrated. On the right is shown back-transport of loaded caveolae from the plasma membrane to the ER (from Helmreich [35], p. 143, Fig. 8.4, originally reproduced from Fig. 3 in R.G. Anderson, 'The caveolae membrane system', Annu. Rev. Biochem. 67 (1998) 199–225, with permission of the author and Annu. Rev. Biochem). (b) Interaction of caveolins with caveolae. Caveolins are oligomeric proteins and the main constituents of caveolae. Caveolins are bound to membranes by palmitoyl residues, attached to their carboxyl-terminal aminoacids (not shown). (The scheme is based on the information in Parton [76].)

thelial cells, the co-existence of different lipid rafts with different protein contents which do not intermix, were inferred from differences in their solubility in detergent [67]. Moreover, attempts have been made to estimate the size of membrane rafts by determining the distance between raft components by means of fluorescence resonance energy transfer (FRET). However, such measurements are difficult to interpret. One reason is that FRET between suitable donor–acceptor molecules decays as the sixth power of the distance between them [68]. Therefore the distance within which FRET can still be detected is limited to approximately 10 nm. Another difficulty was pointed out by Kenworthy and Edidin [69], who have directed attention to the fact that even in a population of randomly distributed, unclustered molecules some of them will be within FRET distance if their concentration is high enough. Therefore, if only a small number of these molecules would be in rafts,

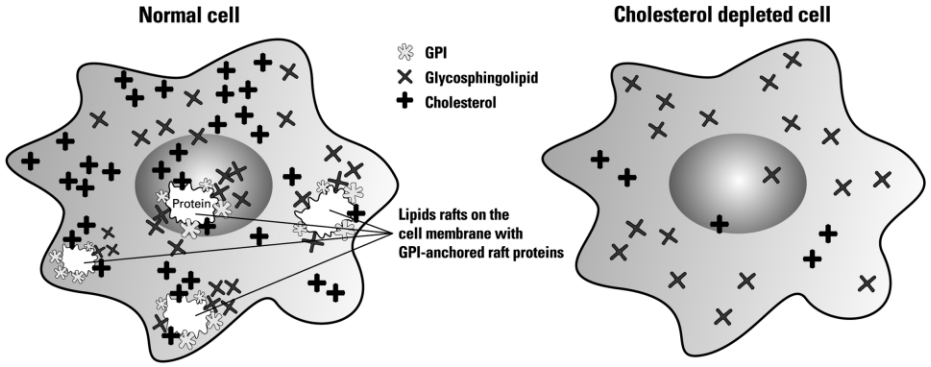


Fig. 3. Lipid rafts in the cell membrane. Large lipid rafts with proteins are visible by microscopy. They are shown on the left and compared with small lipid rafts in cholesterol-depleted cells on the right. Small lipid rafts are invisible under the microscope. (This scheme was reproduced from Edidin [77], with permission of the author and STKE-Science.)

the FRET signal of this tiny fraction would be swamped by the accidental signals of the excess of free, randomly distributed molecules outside of rafts. Pralle et al. [70], have measured the local viscous drag of raft proteins in membranes of intact cells (fibroblasts).[7] The positional fluctuations of the raft-protein complexes, confined by a laser trap to a small region with a diameter of $\leq 100$ nm, were measured. The diffusion of proteins anchored to membrane rafts was compared with that of proteins never associated with rafts. The viscous drag of proteins anchored to rafts was significantly larger than that of the non-raft proteins, regardless of the type of anchor which connects them with the rafts. When the rafts were dissolved by cholesterol depletion, the viscous drag of the proteins which were originally in these rafts decreased to that of non-raft proteins. Since the results suggested that rafts diffuse as stable structures, it became possible to estimate with the Saffman–Delbrück equation [1] from the local viscous drag measurements the size of the rafts. Rafts were estimated to have an average area of approximately 2.100 nm$^2$ with a mean radius of $26 \pm 13$ nm. From that one can predict that a single raft contains approximately 3.500 lipid molecules and less than 60 proteins. Hence such small rafts, consisting of only a few thousand lipid molecules, have a very limited capacity to accommodate proteins. Therefore in order to accommodate enough proteins to form signalling assemblies, small rafts must fuse and form larger rafts [60,61].

### *6.1. The role of cholesterol*

A special role has been attributed to the cholesterol content of lipid domains. Actually, cholesterol was proposed by Simons [71], as the driving force in raft formation (see Fig. 3).

Cholesterol can form small, reversible complexes with lipids containing long saturated acyl chains, such as glycosphingolipids, which are found in rafts [72]. When membrane cholesterol increases, more cholesterol molecules may be sequestered in the form of cholesterol–lipid complexes. Increases in cholesterol concentrations are therefore thought to drive the fusion of small rafts into structures hundreds of nanometers in diameter [73]. With increasing concentrations, cholesterol will eventually interact with proteins, a process that may help in the formation and stabilisation of rafts. (However, large scale sequestration of cholesterol could affect its availability for other cholesterol-dependent functions [74].)

Here, I wish to recall a review by Konrad Bloch [75], written in 1983, where he interpreted the role

[7] Raft-proteins were expressed in cells and made detectable by reaction with antibody-linked fluorescent latex beads. An optical trap was devised in an inverted microscope, using a near infrared laser that excites the fluorophores inside the trapped microsphere via a two-photon process.

of cholesterol in membranes with amazing foresight and vision. I quote verbatim: p. 47:

> 'The sterol pathway terminates with cholesterol, a molecule designed to optimise attractive van der Waal's interactions with phospholipid acylchains in the membrane bilayer'

And on p. 51:

> 'Sterol-induced changes in membrane fluidity or solute permeability are widely viewed as meaningful indices of sterol function in vivo. Much experimental evidence in support of this notion exists but it should be kept in mind that the commonly used methods monitor only bulk fluidity. There may be localised membrane regions or domains of substantially lower or higher sterol content differing in the fluidity from the bulk phase'.

## 7. Conclusions

The central question addressed in this retrospective essay is; how do membranes affect the mobility of proteins, such as receptors in a way to allow them to optimally perform their function and transmit signals across the membrane into the cell. Signal transfer requires coupling with transducer molecules. From my own experiments with a typical membranous hormone receptor system, the G protein-coupled β adrenergic receptor system, and from experiments by others, discussed above, I have arrived at the following conclusions:

1. Temperature-induced phase transitions do not play a physiological role in the modulation of receptor signalling across the cell membrane.
2. In multi-component signalling assemblies, such as in the case of heptahelical receptors coupled to G proteins, the receptor in the membrane need not obligatorily be laterally mobile over macroscopic distances in order to ensure rapid interaction with the transducer. On the other hand, relocation of de-activated receptor–ligand complexes and recycling of receptors requires mobility of the receptor.
3. Much data point to restraints in the membrane on the mobility of proteins. Experimental perturbation and enrichment of membranes with phospholipids have drastic effects on membrane-bound receptors. These experiments, carried out quite some time ago, did already point to an order in the lipid organisation of membranes, necessary for optimal signalling. Such an order was not considered in the fluid mosaic model of biological membranes by Singer and Nicholson who took note of the evidence that some proteins can move unhindered laterally over the cell surface.
4. In some cases, interactions with the cytoskeleton may be responsible for constraints. In others, microdomains, lipid rafts, in membranes could be responsible for the order in membranes. But whether microdomains play a role in signal transfer from membrane-bound receptors and are like scaffolds for the organisation of signalling-competent multi-component assemblies, are still open questions.
5. Although there is a consensus of opinion that the membranous environment may in many ways affect receptor activation, signal transduction through membranes and receptor recycling, the assortment of experiments, some old and some new, which I have presented shows how much needs still to be learned about the mobility of proteins in membranes and its constraints, before we shall understand how signals are transduced effectively across cellular membranes. There are, however, reasons to be optimistic and I conclude therefore my short, retrospective review of the role of receptor mobility with confidence. This optimism is vindicated by new methods, introduced lately, which have already contributed much to the study of receptor traffic. Therefore, we can anticipate many new and surprising findings in the future.

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This essay was dedicated with my respects and regards to Professor John T. Edsall at the occasion of his birthday.

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