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Review Letter

Lateral mobility of proteins and lipids in the red cell membrane and the activation of adenylate cyclase by β-adrenergic receptors

Reiner Peters

Max-Planck-Institut für Biophysik, Kennedy-Allee 70, 6000 Frankfurt 70, FRG

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Models of β-adrenergic signal transduction in red blood cell membranes frequently assume that at least one of the membrane-bound components is laterally mobile and distributes the hormonal signal in the membrane plane. However, direct measurements reveal that protein lateral mobility in the red cell membrane is severely restricted. Furthermore, the spectrin-actin skeleton compartmentalizes the cytoplasmic face of the red cell membrane into a regular array of small elementary areas. These considerations support models in which the β-adrenergic signal is spread in the membrane plane by a molecule which has binding sites on the membrane but diffuses in the aqueous compartment.

β-Adrenergic receptor; G-protein; Adenylate cyclase; Lateral mobility; Photobleaching; (Red blood cell)

1. INTRODUCTION

The plasma membrane has been described as a thin film made up of a fluid lipid bilayer containing a diversity of bilayer-spanning and surface-attached proteins [1]. In that description the bilayer has a moderate viscosity, similar to that of olive oil, so that both lipids and proteins are quite mobile in the plane of the membrane. The concept has given rise to a number of brilliant speculations on the role of lateral mobility in membrane-mediated functions. An early and influential example of such thinking relates to hormone receptors. In his 'general two-step fluidity hypothesis for the mechanism of modulation of adenylate cyclase activity of cell membranes by hormones' Cuatrecasas [2] suggested that both hormone receptor and adenylate cyclase move freely in the plane of the membrane and that the cyclase is activated by collision with hormone-receptor complexes. Ever since the mobile receptor hypothesis was formulated lateral mobility has played a pivotal role in models of membrane-mediated hormonal signal transduction. However, when direct measurements of lateral mobility in membranes became feasible [3] it was necessary to adopt a more differentiated picture of both membrane structure and lateral mobility [4,5]. The few direct measurements of β-adrenergic receptor lateral mobility [6,7] suggested, for instance, that the receptor is immobile.

For these reasons we [8] were left for some time with a paradox: How can models of hormonal signal transduction implying long-range lateral diffusion be reconciled with a membrane organization severely restricting the lateral mobility of membrane proteins? Recent progress, shining new light on this question, has triggered off a vivid discussion. Guanidine-nucleotide-binding proteins (G-proteins) have been found to be involved in a diverse number of signal transduction processes including hormonal, neuronal, sensorial and immunological responses. For the case of the photoreceptor disc membrane the cyclic dissocia-

Correspondence address: R. Peters, Max-Planck-Institut für Biophysik, Kennedy-Allee 70, 6000 Frankfurt 70, FRG

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tion of the G-protein transducin into membrane-bound and water-soluble subunits has been fully elucidated [9]. For the β-adrenergic system Gilman and colleagues [10] have suggested a 'G-subunit dissociation model' which has many analogies to the photoreceptor case. Chabre [11] argued on basic thermodynamic principles that specific protein-protein interactions among β-adrenergic components have to take place on the membrane surface and not in the hydrophobic phase of the bilayer.

The present article contributes to the current discussion from the point of view of lateral mobility. The article focusses on the red blood cell (RBC). This restriction may appear somewhat arbitrary but is actually advantageous because: (i) models of hormonal signal transduction are largely based on RBC studies; (ii) the molecular architecture of the RBC membrane is well known; and (iii) the lateral mobility of lipids and proteins in the RBC membrane has been intensively studied. The RBC offers an additional, rather singular advantage: its membrane represents a limiting case among cellular membranes with regard to both architecture and mobility. As we will show in the course of the article the pronounced specialization of the RBC membrane enables one to draw particularly simple conclusions.

2. CURRENT MODELS OF THE INTERACTION BETWEEN β-ADRENERGIC RECEPTORS AND ADENYLATE CYCLASE

At the level of the plasma membrane five distinct proteins are involved in the transduction of β-adrenergic signals: the stimulatory receptor, Rs; the stimulatory G-protein, Gs; the adenylate cyclase, AC; the inhibitory receptor, Ri; and the inhibitory G-protein, Gi. The following focusses on components of the stimulatory pathway which have been studied in greatest detail. Rs is a bilayer-spanning glycoprotein which has been cloned and sequenced from a number of species and tissues [12]. Rs has a hormone binding site at the external and a G-binding site at the cytoplasmic membrane surface. Gs [13] is a hetero-trimer consisting of an α-, β- and γ-subunit of 42–45, 35 and 8 kDa, respectively. The primary structure of these subunits, as determined by cDNA cloning, contains no sequences which could serve as a bilayer-spanning anchor. This classifies Gs as extrinsic although usually detergent is necessary to keep the protein in solution. However, upon binding of GTP the α-subunit dissociates from the β,γ-complex and becomes soluble [14]. AC has been purified from heart [15] and other tissue. AC is probably a bilayer-spanning protein. Its catalytic site is located at the cytoplasmic surface of the membrane.

Interactions between Rs, Gs and AC can be summarized in general terms. Upon binding of hormone (H) Rs undergoes a conformational change which transmits the hormonal signal from the external to the cytoplasmic membrane surface. On the cytoplasmic membrane surface hormone-activated Rs interacts with Gs such that GDP is released from and GTP bound to Gs_α, the α-subunit of Gs. Gs_α then interacts with AC transforming it into the active enzyme. Hydrolysis of the Gs_α-bound GTP to GDP terminates activation of AC. Gs_α is then free to enter a new cycle of GDP-GTP exchange and concomitant Rs-AC interactions.

Models of the interactions between H, Rs, Gs and AC have to account for two basic observations: (i) one common cellular pool of AC molecules can be activated by different hormones. In the turkey RBC, for instance, adenosine or catecholamine activates the same AC pool [16]; (ii) one Rs molecule can activate several AC molecules. For instance, if the Rs population of turkey RBCs is partially inactivated by an irreversible antagonist the maximum specific activity of AC is not reduced; 5% of the receptor population still activates the total AC pool [17]. For these and other reasons most of the published models assume that at least one of the membrane-bound components is laterally mobile so that the hormonal signal can be distributed over the entire membrane surface and be transmitted from one Rs to several ACs or from different Rs-species to the same ACs. Open questions concern the state of association between Rs, Gs, and AC and the identity of the laterally mobile signal carrier. The 'collision coupling mechanism' [17] assumes that Rs is mobile and that Gs and AC form a permanent complex. Alternatively, it has been proposed that Gs functions as a 'shuttle' between Rs and AC [18–20]. The quoted 'G-subunit dissociation model' [10]

Table 1

Lateral mobility of proteins and lipids in the red cell membrane

Preparation	Method	T (°C)	Other parameters	D (10^{-10} cm$^2 \cdot$s^{-1})	R (%)	Ref.
Band 3						
Human RBC ghost	fm	23		0.03	nd	3
Human RBC	fusion	0	fresh blood	immobile	nd	21
		23		0.06		
		30		0.3		
		37		0.4		
		0	aged blood	immobile		
		23		0.02		
		30		0.01		
		37		0.2		
Human RBC	fusion	30	control	0.1	nd	36
			4×10^{-6} M 72 kDa-fragment of ankyrin	0.2		
Human RBC	FRAF	30	control	0.22	nd	22
			12.5 mM ATP	0.88		
			12.5 mM 2,3-P_2-glyc.	0.55		
			0.6 mM neomycin	0.002		
			0.6 mM spermine	0.002		
Mouse RBC ghost	fm	24	control	0.45	nd	33
			spectrin deficient	25		
Human RBC ghost	fm	21	46 mM Na phosphate	0.43	11	34
			26 mM Na phosphate	0.70	38	
			21 mM Na phosphate	0.82	36	
			18 mM Na phosphate	0.87	37	
			13 mM Na phosphate	0.67	34	
			9 mM Na phosphate	0.95	51	
			5 mM Na phosphate	0.50	72	
Human RBC	fm, FRAF	37		0.38	88	25
Human RBC ghost	fm	37	control	0.21–0.95	nd	37
			10^{-6} M TPI	0.94–44		
Human RBC	fusion	30	control	0.30	nd	38
			spectrin cross-linked	0.18		
Human RBC ghost	fm	22	control	0.01	nd	35
			10 ng trypsin	1.0	nd	
			20 ng trypsin	4.2	90	
			40 ng trypsin	4.8	90	
			100 ng trypsin	5.7	100	
Lipid probes						
Human RBC ghost	fm	7		9	nd	47
		40		75		
Human RBC	fm	−3	control	9.5	nd	48
			cholesterol depleted	6.0		
		40	control	23		
			cholesterol depleted	23		
Mouse RBC ghost	fm	24	control	140	nd	40
			spectrin deficient	150		
Turkey RBC ghost	fm	5	control	12	50	49
		25		50	90	
		45		190	90	
		25	0.33 μM cis-vaccinic acid/mg protein	70	90	
Human RBC	fm	25		84	nd	39
Mouse RBC				130		
Frog RBC				53		

D, lateral diffusion coefficient; R, mobile fraction; RBC, red blood cell; fm, fluorescence microphotolysis; FRAF, fluorescence redistribution after fusion, a fusion method which quantitates redistribution by microfluorimetry; nd, not determined

assumes that Gs dissociates upon interaction with hormone-activated Rs into the α- and β,γ-subunits and that the α-subunit alone interacts with AC.

3. LATERAL MOBILITY OF PROTEINS AND LIPIDS IN THE RBC MEMBRANE

Two methods have been used to determine the lateral mobility of proteins in the erythrocyte membrane, cell fusion [21,22] and fluorescence microphotolysis [23,24]. It was shown [25] that both methods yield identical results. Among RBC membrane proteins only the mobility of band 3 has been measured so far. Lipid mobility has been studied by insertion of various different fluorescent lipid analogs into the RBC membrane.

The results of mobility measurements, collected in table 1, lead to the following general conclusions: (i) the apparent lateral diffusion coefficient D of band 3 in the normal RBC membrane is exceptionally small, i.e. about 0.2×10^{-10} cm$^2 \cdot$s^{-1} at 30°C (this value may be compared with the diffusion coefficient of integral membrane proteins in a variety of other cells which is in the range of 5×10^{-10} cm$^2 \cdot$s^{-1} [4,5] or in reconstituted bilayers which is $1-5 \times 10^{-8}$ cm$^2 \cdot$s^{-1} [26]); (ii) band 3 mobility is strongly temperature dependent with D values of $0.03-0.06 \times 10^{-10}$ cm$^2 \cdot$s^{-1} at 23°C and 0.4×10^{-10} cm$^2 \cdot$s^{-1} at 37°C, respectively; (iii) the mobile fraction R of band 3 in the RBC membrane is 60–90% (similar R values have been reported for many integral membrane proteins); (iv) lipid mobility, in sharp contrast to band 3 mobility, is quite large with D values of $10-200 \times 10^{-10}$ cm$^2 \cdot$s^{-1} which is average for lipids in cell membranes.

4. THE STRUCTURE OF THE RBC MEMBRANE AND ITS RELATION TO LATERAL MOBILITY

The human RBC membrane consists of two layers which differ widely in function, dynamics and mechanical properties [27]. The same holds also for avian RBCs [28]. A fragile lipoprotein bilayer interfaces the cell with its environment. The bilayer contains, for instance, hormone receptors, immunologic determinants and transport proteins. The most abundant protein of the lipoprotein bilayer is the band 3 protein. It occurs at a frequency of about 10^6 molecules per RBC. The monomer consists of a single peptide chain of 100 kDa mass which traverses the lipid bilayer several times [29]. In addition to the bilayer spanning part the band 3 protein has a large 45-kDa fragment exposed at the cytoplasmic membrane surface. The membrane skeleton, a protein network, is attached to the bilayer on its cytoplasmic surface. It provides for mechanical properties and for long-range order in the membrane plane. It is composed of spectrin, actin, band 4.1 and several minor components. Spectrin occurs at a frequency of about 2×10^5 copies per RBC and actin at about 5×10^5 copies per RBC. Spectrin consists of 2 peptide chains, the α- and β-subunits, having a mass of 240 and 225 kDa, respectively. The α- and β-subunits form heterodimers which have the shape of highly flexible rods with a length of about 100 nm in the maximally extended configuration. Spectrin dimers combine to yield tetramers and higher oligomers. RBC actin occurs in the form of short filaments of about 15 monomers. Each actin filament can bind several spectrin tetramers and thus serves as nodal point in the spectrin-actin meshwork. Recently, this meshwork has been directly visualized by electron microscopy [30,31].

Membrane skeleton and lipoprotein bilayer are fused together by two specific linkage proteins: ankyrin (10^5 copies per RBC) and band 4.1 (2×10^5 copies per RBC). Ankyrin is a 210 kDa protein with one binding site for the β-subunit of spectrin and one for the band 3 protein. The ankyrin binding site of the β-subunit resides in a specific domain of the molecule, close to the middle of the peptide chain. Band 4.1 does not bind to a specific segment of the α- or β-subunit of spectrin but seems to require both subunits for stable attachment. Band 4.1 has a binding site for the cytoplasmic 35-amino acid residue of glycophorin A. However, binding between glycophorin A and band 4.1 occurs only if glycophorin is associated with a specific phospholipid, namely triphosphoinositide (TPI) [32]. In the absence of TPI band 4.1 binds to band 3.

The very small lateral mobility of band 3 in the lipoprotein bilayer of normal RBCs is clearly determined by the membrane skeleton. In spectrin-deficient variants of mouse RBCs the diffusion coefficient was about 50-fold larger than in normal cells [33]. Reduction of ionic strength and eleva-

tion of temperature destabilize the membrane skeleton. Both measures lead to a strong increase in band 3 lateral mobility [34]. Spectrin can be degraded in a controlled and gradual manner by sealing small amounts of trypsin into RBC ghosts. Degradation of spectrin, as analysed by gel electrophoresis, is accompanied by a very large increase in the lateral mobility of band 3 [35]. A 72 kDa fragment of ankyrin, if added to permeable RBC ghosts in large excess, increases the lateral diffusion coefficient of the band 3 about two-fold [36]. TPI increases band 3 mobility to a comparable extent [37]. On the other hand, cross-linking of spectrin by diamide reduces band 3 mobility [38]. There is little if any influence of the membrane skeleton on lipid mobility. The D values of lipid probes in normal RBC membranes are quite high [39]. In spectrin-deficient mouse RBC membranes lipid probe diffusion is not larger than in normal cells [40].

An obvious parameter restricting lateral mobility of band 3 is the linkage of band 3 to spectrin via ankyrin. However, the stoichiometry of ankyrin and band 3 reveals that maximally about 10% of band 3 is directly attached to the membrane skeleton. Dimerization or dynamic oligomerization of band 3 could indirectly link a further fraction of band 3 to the skeleton. Anisotropy decay measurements [44] showed that the rotational mobility of band 3 in the RBC membrane, in contrast to lateral mobility, is quite large. Two fractions of band 3, a slowly and a fast rotating one, were observed. The slowly rotating fraction decreased and the fast rotating increased correspondingly when a cytoplasmic fragment of band 3 was split off by trypsin digestion. From these results it was concluded that up to 40% of band 3 are linked to the membrane skeleton. In measurements of lateral mobility an immobile band 3 fraction of comparable magnitude (10–40%) was observed (table 1). From these considerations the idea emerged [40,42] that the lateral mobility of band 3 is restricted by two different mechanisms. A fraction of band 3 (up to 40%) is immobilized by attachment to the membrane skeleton. The larger fraction of band 3 is trapped by means of the cytoplasmic fragment in the interstices of the skeleton. Within the free areas of the skeleton lateral and rotational diffusion of band 3 are restricted slightly. However, passage from one area to another is only possible when the barrier (a spectrin oligomer) is broken or when the contact points between skeleton and lipoprotein bilayer (ankyrin, band 4.1) are unloosened. The small but finite lateral mobility of band 3 indicates that breakage or detachment of the skeleton does occur in the normal RBC membrane, although at a relatively low frequency.

5. A HYPOTHESIS

Recent electron microscopic studies have demonstrated that the cytoplasmic surface of the RBC membrane is compartmentalized by the spectrin-actin membrane skeleton into a continuous array of predominantly triangular fields [30,31]. We will refer to these fields as elementary areas. Our basic assumption is that the topographic compartmentation of the RBC membrane is also a functional one, although only with respect to that category of membrane proteins exposing a substantial fragment at the cytoplasmic membrane surface. Rs, Gs and AC, all are members of that category. We assume that in the elementary areas these proteins can move in a comparatively unrestricted manner. The exchange between different areas, however, may be a relatively rare event. Thus, long-range diffusion should suffice, for instance, to distribute at random integral proteins over the membrane surface on the time scale of hours. Long-range diffusion would be too slow, however, to be effective on the time scale of seconds and minutes as typical for the activation of adenylate cyclase. For instance, in the framework of the collision coupling mechanism the Rs of turkey RBC membranes should have a minimum lateral diffusion coefficient of $0.4–9 \times 10^{-10}$ $cm^2 \cdot s^{-1}$ at 25°C [43]. This value holds for the case in which every collision between Rs and AC is successful in activating AC. If the efficiency of AC activation is smaller than unity, which can be reasonably assumed, the diffusion coefficient has to be larger by an equivalent proportion. The lateral diffusion coefficient of band 3 is about 0.05×10^{-10} $cm^2 \cdot s^{-1}$ at 25°C (table 1), i.e. well below the lower limit set by the collision coupling mechanism for Rs. Within one elementary area the mobility of Rs and AC and hence their collision frequency would be quite large. Provided, however, that Rs and AC are randomly distributed

in the membrane plane an Rs and an AC molecule will hardly ever be found in the same elementary area. There are approximately 1000 molecules of each, Rs and AC, per RBC [47] which corresponds to a density of $4/\mu m^2$. The membrane skeleton, however, has about 125–500 elementary areas per μm^2 [30]. It may be recalled that the abundance of G is much larger than that of Rs and AC so that many of the elementary areas may contain at least one G.

If our contention should be correct that neither Rs, Gs nor AC can serve as laterally mobile carrier of the β-adrenergic signal in the RBC membrane, then other possibilities have to be considered. These are: (i) a carrier which diffuses in the lipid bilayer, or (ii) a carrier which diffuses in the aqueous compartment. Although the association of Rs with glycolipids has been demonstrated [45] there is no strong evidence suggesting that a glycolipid or another lipid molecule carries the hormonal signal from Rs to AC. On the other hand, the 'G-subunit dissociation model' [10] suggests that Gs_α is the laterally mobile signal carrier and recent experiments have shown that Gs_α is indeed soluble in water [14]. Let us therefore briefly discuss what can be expected for a ligand which partitions between an aqueous compartment and a membrane carrying quasi-immobile binding sites. According to Elson and colleagues [23,46] and Crank et al. [50] two cases are to be discriminated depending on whether the relaxation time of ligand binding is much shorter or much longer, respectively, than the characteristic diffusion time. In the former case the mobility measurement will yield two classes, namely a mobile fraction characterized by D_w, the diffusion coefficient in water, and an immobile fraction (diffusion coefficient on the membrane $D_m = 0$). In the latter case a single effective diffusion coefficient D will be measured which is given by:

$$D = D_w \cdot f_w + D_m \cdot f_m \quad (1)$$

where f_w and f_m are the fractions of the ligand in the aqueous compartment and bound to the membrane, respectively ($f_w + f_m = 1$). f_w is given by:

$$f_w = 1/(1 + K \cdot B) \quad (2)$$

where K is the bimolecular association constant of ligand and binding site and B is the concentration of binding sites. Inserting some plausible values into eqns 1 and 2 yields the range of values given in table 2. The result suggests that, due to the particular conditions in the RBC, apparently contradictory properties such as a small ligand fraction in the aqueous compartment and a sufficiently large effective lateral diffusion coefficient are perfectly compatible. Therefore we complete the article by suggesting that the interaction of Gs with R on the one hand – i.e. exchange of GDP by GTP, dissociation of Gs_{GTP} into $Gs_{\alpha GTP}$ and $G_{\beta\gamma}$, association of $Gs_{\alpha GDP}$ and $G_{\beta\gamma}$ to Gs_{GDP} – and of Gs_α with AC on the other – i.e. association of $Gs_{\alpha GTP}$ with AC, hydrolysis of GTP, dissociation of $Gs_{\alpha GDP}$ from AC – are localized in different elementary areas of the RBC membrane. Gs_α quickly moves by diffusion in the aqueous phase and thus couples the different functional domains. The inhibitory pathway can be included into the scheme by recruiting additional elementary areas carrying Ri and Gi.

Table 2

Partition and mobility of a water-soluble ligand for which immobile binding sites exist on a membrane

K (M^{-1})	f_w (%)	f_m (%)	D (10^{-10} $cm^2 \cdot s^{-1}$)
6×10^8	10	90	200
5×10^9	1	99	20
5×10^{10}	0.1	99.9	2

Calculated according to eqns 1 and 2 with: number of binding sites per RBC = 1000; volume of RBC = 100 fl; and $D_w = 2 \times 10^{-7}$ $cm^2 \cdot s^{-1}$. K, bimolecular association constant of ligand and binding site; f_w, fraction of ligand in the aqueous compartment; f_m, fraction of ligand bound to membrane; D, effective lateral diffusion coefficient of ligand

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