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Cholesterol affects spectrin–phospholipid interactions in a manner different from changes resulting from alterations in membrane fluidity due to fatty acyl chain composition

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Abstract

We previously showed that erythrocyte and brain spectrins bind phospholipid vesicles and monolayers prepared from phosphatidylethanolamine and phosphatidylserine and their mixtures with phosphatidylcholine (Review: A.F. Sikorski, B. Hanus-Lorenz, A. Jezierski, A. R. Dluzewski, Interaction of membrane skeletal proteins with membrane lipid domain, Acta Biochim. Polon. 47 (2000) 565). Here, we show how changes in the fluidity of the phospholipid monolayer affect spectrin–phospholipid interaction. The presence of up to 10%–20% cholesterol in the PE/PC monolayer facilitates the penetration of the monolayer by both types of spectrin. For monolayers constructed from mixtures of PI/PC and cholesterol, the effect of spectrins was characterised by the presence of two maxima (at 5 and 30% cholesterol) of surface pressure for erythroid spectrin, and a single maximum (at 20% cholesterol) for brain spectrin. The binding assay results indicated a small but easily detectable decrease in the affinity of erythrocyte spectrin for FAT-liposomes prepared from a PE/PC mixture containing cholesterol, and a 2- to 5-fold increase in maximal binding capacity (B_{max}) depending on the cholesterol content. On the other hand, the results from experiments with a monolayer constructed from homogenous synthetic phospholipids indicated an increase in $\Delta\pi$ change with the increase in the fatty acyl chain length of the phospholipids used to prepare the monolayer. This was confirmed by the results of a pelleting experiment. Adding spectrins into the subphase of raft-like monolayers constructed from DOPC, SM and cholesterol (1/1/1) induced an increase in surface pressure. The $\Delta\pi$ change values were, however, much smaller than those observed in the case of a natural PE/PC (6/4) monolayer. An increased binding capacity for spectrins of liposomes prepared from a "raft-like" mixture of lipids could also be concluded from the pelleting assay. In conclusion, we suggest that the effect of membrane lipid fluidity on spectrin–phospholipid interactions is not simple but depends on how it is regulated, i.e., by cholesterol content or by the chemical structure of the membrane lipids.

Keywords: Spectrin; Erythroid and non-erythroid spectrin; Membrane skeleton; Membrane fluidity; Lipid raft; Cholesterol

1. Introduction

The membrane skeleton is a network of spectrin tetramers cross-linked by actin filaments associated with the plasma membrane via a variety of protein receptors, i.e. ankyrin or protein 4.1. Erythrocyte spectrin and its non-erythroid analogues can also interact directly and independently with the lipid domain in natural membranes or in model systems [2–10], review: ref. [1]. It was found that both spectrins bind directly to liposomes containing the aminophospholipids PE and PS, and

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid; DMSO, dimethyl sulfoxide; DMPE, 1,2-dimyristoyl-phosphatidylethanolamine; DPPE, 1,2-dipalmitoyl-phosphatidylethanolamine; DSPE, 1,2-distearoyl-phosphatidylethanolamine; DMPC, 1,2-dimyristoyl-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-phosphatidylcholine; DSPC, 1,2-distearoyl-phosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SM, sphingomyelin; DOPC, 1,2-dioleoyl-phosphatidylcholine; DOPE, 1,2-dioleoyl-phosphatidylethanolamine

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penetrate monolayers made up of these phospholipid mixtures, inducing an increase in monolayer surface pressure [1]. We previously demonstrated the interaction of erythrocyte and brain spectrin with a class of membrane sites (present in erythrocyte and synaptic plasma membranes) that proved resistant to extraction with NaOH and protease treatment. Moreover, this binding was competitively inhibited by liposomes prepared from membrane lipids [10]. The data from recent studies concerning direct interactions of erythroid and non-erythroid spectrins with phospholipids, in particular PS, imply that multiple binding sites for lipids exist in both subunits (α and β) of erythroid and non-erythroid spectrins [11,12]. The physiological significance of these interactions remains unclear. On the other hand, the data from our laboratory indicate that one of the lipid-binding sites, located in the region of carboxyl terminus of the β-spectrin, proved sensitive to inhibition by purified erythrocyte ankyrin [10,13,14]. This indicates that the 15th repeat of the β-subunit, which is an ankyrin-binding domain, plays an important role in the interaction with membrane lipids. One of the possible roles of this interaction would be to serve as an alternative binding site when there is not enough functional ankyrin to accommodate all the spectrin tetramers in the membrane, or when ankyrin's spectrin-binding affinity is reduced, e.g., upon its phosphorylation [1].

Many authors attempted to determine the mechanism of spectrin–lipid interaction and the phospholipid selectivity of spectrins, but the results did not allow for full understanding of the mechanism of interaction (Review: [1]). Therefore, we decided to address the question whether changes in the fluidity of the phospholipid monolayer affect spectrin–phospholipid interaction. Another important issue is the occurrence of lipid rafts—specialized domains into which most cellular plasma membranes are organised [15,16]. Therefore, the interaction of both types of spectrin with monolayers prepared from a "raft-like" lipid mixture was analysed. The results presented here suggest that the effect of membrane fluidity on the interactions with erythrocyte or brain spectrin also depends on the way this parameter is regulated.

2. Materials and methods

2.1. Materials

Sephacryl S-500-HR, DEAE-Sephacel, Sepharose 4-B, DTT, EGTA, PI, DMPE, DPPE, PSPE and rhodamine isothiocyanate were obtained from Sigma Chemical Co. (St. Louis, MO). Egg PC, egg PE, DMPC, DPPC and DSPC were from Lipids Products (Nutfield, UK). Cholesterol and DMSO were from Merck (Darmstadt, Germany). DOPC and DOPE were from Northern Lipids Inc. (Vancouver, Canada). The other reagents were from ICN.

2.2. Methods

Brain (tetrameric) and erythrocyte (dimeric) spectrins were purified and their purity was assessed as described previously ([8] and references therein).

Monolayer measurements were performed by using a Teflon trough (surface area of 24 cm^2) and a Nima tensiometer ST 9000 (Nima Technology, Coventry UK), at room temperature (20 °C), and filter paper plates were used. A subphase buffer (25 ml), containing 5 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, 150 mM NaCl, 0.5 mM DTT and 1 mM NaN_3, was stirred with a small stirrer bar (5 × 2 mm). Monolayers were formed by injecting a chloroform solution of a phospholipid mixture with a Hamilton syringe into the subphase buffer. After stabilisation (10–20 min) of the monolayer at the desired initial surface pressure (8–14 mN/m), aliquots (5 μl) of brain or red blood cell spectrin dialysed against the above-mentioned buffer were injected into the subphase, and surface pressure (π) measurements were taken. The data points represent the average values of several independent experiments with an average variation not larger than 10%. It should be noted that this method, although fast and useful, gives only comparative results demonstrating the interaction of the protein with the lipid, as the measurements were performed at low initial surface pressure values, far from the 30–35 mN/m suggested as appropriate for comparison with natural membranes [17].

For the pelleting assays, purified erythrocyte spectrin was labelled with rhodamine isothiocyanate. The protein was dialysed against a 0.1 M sodium carbonate buffer, pH 9.0, and a DMSO solution of the rhodamine isothiocyanate (1 mg/ml; 50 μl for each 1 ml of protein solution) was added. After overnight incubation in the dark at 4 °C, Tris was added to the mixture (to a final concentration of 100 mM), and after a 2-h incubation at 4 °C, glycerol and sucrose were added (to respective final concentrations of 5% and 0.5 M). The mixture was dialysed against the buffer described above as the subphase buffer for 48 h through 3–4 buffer changes. After such a procedure, less than 1% of the fluorescence could be measured in the supernatant after protein precipitation with 10% trichloroacetic acid. Also, the binding of labelled spectrin was inhibited by unlabelled protein: at 10-fold excess, ~70% inhibition of binding was observed.

Frozen and thawed (FAT) liposomes were prepared according to [18] using the "subphase" buffer containing 20% Dextran T40, and the spectrin binding assay was carried out as described previously [9]. The diameter (~400 nm, as measured by Photon Correlation Spectrometry) of the liposomes did not change significantly within even weeks of storage at 4 °C. The obtained pellets were dissolved in 2 ml 1% SDS and fluorescence measurements were taken in a Kontron spectrofluorimeter at excitation and emission wavelengths of 553 and 575 nm, respectively.

3. Results

To test whether changes in fluidity have an effect on the interaction of erythrocyte and brain spectrin with phospholipid monolayers, we used two different methods of changing monolayer fluidity: (1) we changed the cholesterol concentration in the mixtures of natural PE/PC (3:2) and PI/PC (7:3) phospholipids (cholesterol is well known to increase the fluidity of a gel phase and to decrease it in a liquid crystal phase phospholipid mono- and bilayers, i.e., below T_m it increases the fraction of gauche rotamers while opposite is seen above T_m) and 2) we used synthetic phospholipid mixtures—DMPE/DMPC, DPPE/DPPC, DSPE/DSPC and DOPE/DOPC—to form a monolayer.

The dependence of the surface pressure changes of a natural, egg PE/PC monolayer containing various amounts of cholesterol on erythrocyte spectrin (dimeric form) concentration in the subphase is shown in Fig. 1A. It should be noted that highly hydrated egg PE/PC (3:2) mixture has its transition temperature below the temperature of measurements (20 °C) [19]. Thus, addition of cholesterol decreased its fluidity. To facilitate comparison, the values observed for each cholesterol content in this monolayer at the chosen spectrin concentration of 1.56 nM are shown in Fig. 1B. The baseline in this case is the $\Delta\pi$ value obtained for pure PE/PC monolayer. The addition of erythrocyte spectrin to the subphase buffer induced a larger increase in the surface pressure ($\Delta\pi$) of PE/PC monolayers containing 5% and 10% cholesterol than seen for monolayers without cholesterol. The values for other cholesterol concentrations

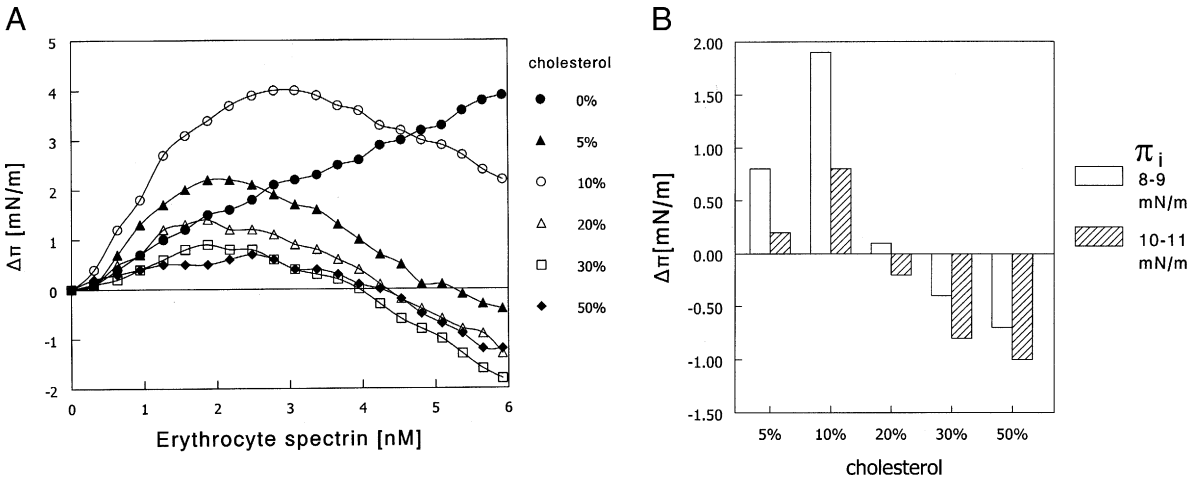

Fig. 1. Changes in the surface pressure ($\Delta\pi$) of the PE/PC 3/2 monolayer containing increasing concentrations of cholesterol after the addition of erythrocyte spectrin to the subphase. (A) Dependence on erythrocyte spectrin concentration at initial surface pressure (π_i): 8–9 mN/m. The values of the surface pressure change induced by the protein without a lipid monolayer were subtracted from $\Delta\pi$. (B) Dependence on cholesterol content at an erythrocyte spectrin concentration of 1.56 nM in the subphase at indicated initial surface pressure (π_i) values. The value of $\Delta\pi$ change for the monolayer without cholesterol is considered 0.

between 20 and 50% were close to or smaller than those observed for monolayers without cholesterol (Fig. 1B). Another characteristic feature of this interaction is that for monolayers containing cholesterol, the $\Delta\pi$ increases only at low concentrations of erythrocyte spectrin (up to 2–2.5 nM), and then decreases well below the values observed for the spectrin without a lipid monolayer (Fig. 1A). These results were similar both for smaller (8–9 mN/m) and for larger (10–11 mN/m) π_i values (see Fig. 1B).

Similar experiments performed on brain spectrin (Fig. 2) indicate that the presence of cholesterol modifies the interaction of brain spectrin with a given monolayer to a greater extent than observed for erythroid spectrin (compare Figs. 1 and 2). The values observed for each cholesterol content in this monolayer at the chosen brain spectrin concentration of 3.95 nM (brain spectrin always occurs as a tetramer) are shown in Fig. 2B. For both smaller and larger values of π_i, an increase in the surface pressure change of a monolayer prepared from PE/PC with cholesterol was larger than that for a monolayer without cholesterol; a relatively symmetrical peak was obtained around 20% cholesterol. The exceptions are the values for 5 and 50% cholesterol at a π_i of 10–11 mN/m; these were identical to the control values (Fig. 2B). It also seems that the change in this parameter in the case of erythroid spectrin is less sensitive to the value of π_i (compare data from Figs. 1B and 2B).

The changes in surface pressure of the PI/PC (7/3) monolayer containing variable concentrations of cholesterol upon the increase of the concentrations erythrocyte or brain spectrin in the subphase are shown in Figs. 3A and 4A, respectively. As the data are presented in a similar format to Figs. 1B and 2B, it can clearly be seen that in the case of erythroid spectrin, there was a double peak for the effect at 5% and 30% cholesterol, while in the case of non-erythroid

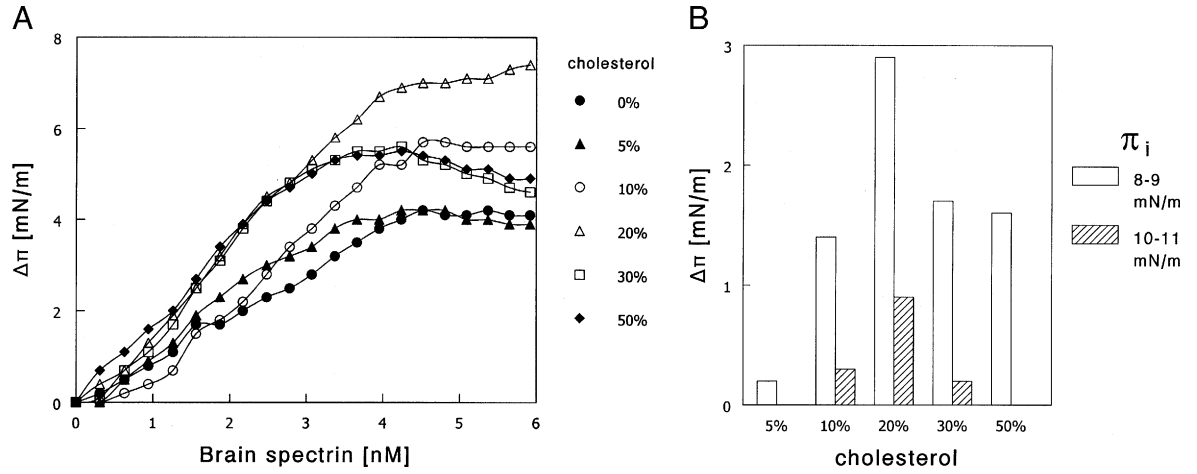

Fig. 2. Changes in the surface pressure ($\Delta\pi$) of the PE/PC 3/2 monolayer containing increasing concentrations of cholesterol after the addition of brain spectrin to the subphase. (A) Dependence on brain spectrin concentration at initial surface pressure (π_i): 8–9 mN/m. The values of the surface pressure change induced by the protein without a lipid monolayer were subtracted from $\Delta\pi$. (B) Dependence on cholesterol content at a brain spectrin concentration of 3.95 nM in the subphase at indicated initial surface pressure (π_i) values. The value of $\Delta\pi$ change for the monolayer without cholesterol is considered 0.

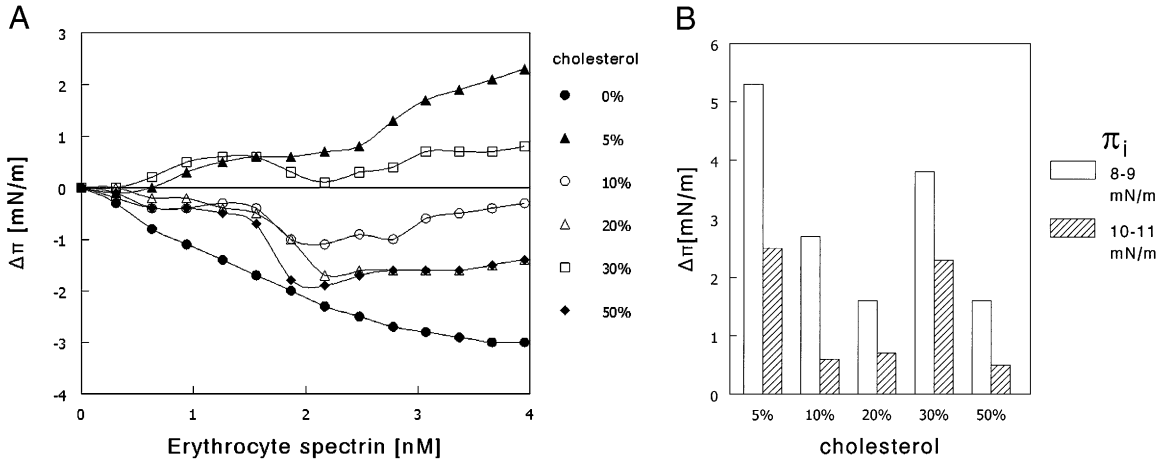

Fig. 3. The effect of cholesterol on erythrocyte spectrin interaction with the PI/PC 7/3 monolayer. (A) Dependence of $\Delta\pi$ on erythrocyte spectrin concentration at a π_i of 8–9 mN/m. The values of the surface pressure change induced by the protein without a lipid monolayer were subtracted from $\Delta\pi$. (B) Dependence of the $\Delta\pi$ value on cholesterol content in the monolayer at an erythrocyte spectrin concentration of 3.95 nM in the subphase at the indicated initial surface pressure (π_i) values.

spectrin, a single peak at 20–30% cholesterol was observed (Figs. 3B and 4B). This is similar to the effect of cholesterol on the PE/PC monolayer (see above). All the $\Delta\pi$ change values observed for erythroid spectrin were larger in the presence of cholesterol than in its absence (Fig. 3B), while only the peak values of $\Delta\pi$ change (at 20–30% cholesterol) were larger when brain spectrin was added to the subphase (Fig. 4B).

In order to test whether the observed changes depend on fluidity or rather on lateral phase heterogeneity, we performed a small set of experiments to test the interaction of red blood cell and brain spectrin with phospholipid monolayers containing PE and PC at a 3:2 ratio, composed of homogenous synthetic lipids containing C14, C16 and C18 saturated, and C18 unsaturated fatty acyl chains, namely: DMPE/DMPC, DPPE/DPPC, DSPE/DSPC and DOPE/DOPC. The results of these experiments performed similarly to those presented above (Figs. 1–4) are shown in Figs. 5 and 6. For clarity, only the values of $\Delta\pi$ at one spectrin concentration, 3 nM for erythroid spectrin (Fig. 5) and 2.8 nM for brain spectrin (Fig. 6), are shown (plateau level). The sequence of bars reflects an increasing transition temperature of particular phospholipid mixtures. In general, the injection of erythrocyte spectrin into the subphase buffer induced a smaller increase in the surface pressure of the above-mentioned monolayers compared to the results obtained for the natural PE/PC monolayers (see Fig. 5). The smallest $\Delta\pi$ change was observed for the DMPE/DMPC monolayer film at $\pi_i \sim 8$ and for the DOPC/DOPE monolayer across the whole range of π_i values. Larger changes were observed for the DPPE/DPPC mixture, but for the DSPE/DSPC (6/4) monolayer, the obtained values were similar to or even higher than those for the natural PE/PC monolayer (note the data obtained for higher initial surface pressures, Fig. 5). When brain spectrin was injected into the subphase, the surface pressure change is proportional to the transition temperature being the largest in the case of DSPE/DSPC monolayers (see Fig. 6).

Taking into consideration the results presented above, it can be concluded that the effect of the changes in monolayer fluidity on spectrin–monolayer interactions induced by the presence of cholesterol differs from the effects of those

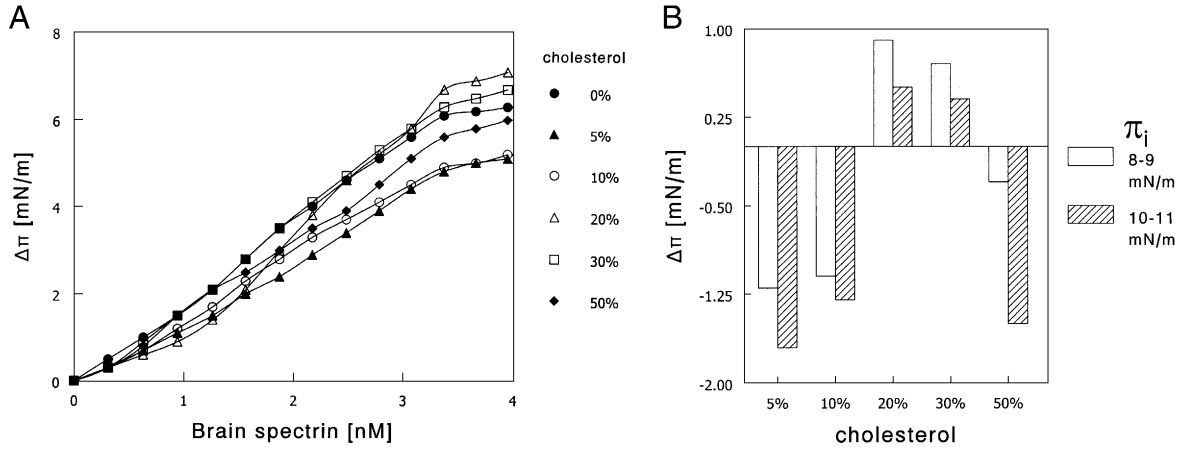

Fig. 4. The effect of cholesterol on brain spectrin interaction with the PI/PC 7/3 monolayer. (A) Dependence of $\Delta\pi$ on brain spectrin concentration at a π_i of 8–9 mN/m. The values of the surface pressure change induced by the protein without a lipid monolayer were subtracted from $\Delta\pi$. (B) Dependence of the $\Delta\pi$ value on cholesterol content in the monolayer at a spectrin concentration of 3.95 nM in the subphase at indicated initial surface pressure (π_i) values.

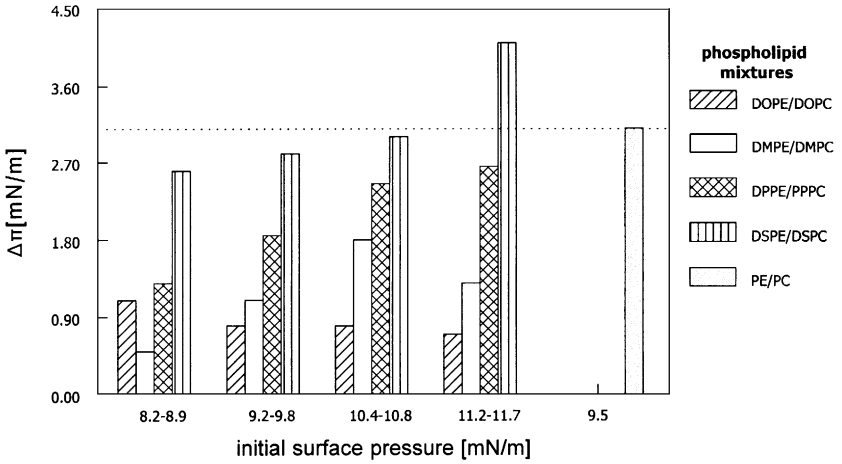

Fig. 5. Change in the surface pressure ($\Delta\pi$) of the synthetic phospholipid mixture monolayers DMPE/DMPC (3/2), DPPE/DPPC (3/2), DSPE/DSPC (3/2) and DOPE/DOPC (3/2) after the addition of erythrocyte spectrin to the subphase, at the indicated initial surface pressure (π_i) values, in comparison with natural PE/PC (3/2) monolayers at π_i=9.5. The values of the surface pressure change induced by the protein without a lipid monolayer were subtracted from $\Delta\pi$. Red blood cell spectrin concentration in the subphase: 3 nM.

induced by the change in fatty acyl chain length and the degree of saturation of the phospholipids forming the monolayer.

In the context of the results of experiments addressing the effect of the fluidity of lipids forming the monolayer, it is interesting to test the interaction of spectrins with monolayers composed from a lipid mixture resembling that of membrane rafts. Fig. 7 presents the dependence of the surface pressure change on the initial surface pressure of a monolayer composed from a "raft-like" lipid mixture: SM, DOPC and cholesterol at a 1:1:1 weight ratio [14,15]. An increase in $\Delta\pi$ was observed after the addition of erythrocyte spectrin into the subphase, but the obtained values ($\Delta\pi$ change max. 2–2.5 mN/m over the value of the protein alone level) were much smaller than those observed for the natural PE/PC monolayer, i.e. 6–7 mN/m above the level for protein alone [10]. A similar dependence of $\Delta\pi$ change on π_i could be observed for brain spectrin (Fig. 8). After the addition of brain spectrin into the subphase buffer, the observed $\Delta\pi$ change was max. 2–3 mN/m compared to max. 6–7 mN for the natural PE/PC monolayer [10].

In order to test whether the above results can be compared with the results obtained in the pelleting assays, we performed binding assays using rhodamine isothiocyanate-labelled erythrocyte spectrin and FAT liposomes (see Materials and methods) prepared from DMPE/DMPC (6/4), DPPE/DPPC (6/4), DSPE/DSPC (6/4) (Fig. 9A) and cholesterol/SM/DOPC (1:1:1) (Fig. 9B), and from a natural PE/PC (6/4) mixture containing 0, 10, 20 and 50% cholesterol (Fig. 9C). The binding isotherms at pH 7.5 for the phospholipid liposomes described above are shown in Fig. 9A–C. Saturable binding of erythrocyte spectrin to FAT liposomes with the equilibrium dissociation constants in the nanomolar to submicromolar range was observed in all cases (Fig. 9). The presented data indicate that the differences in $\Delta\pi$ changes observed for the monolayer experiment are rather connected with the changes in the maximal binding capacity, as the changes in equilib-

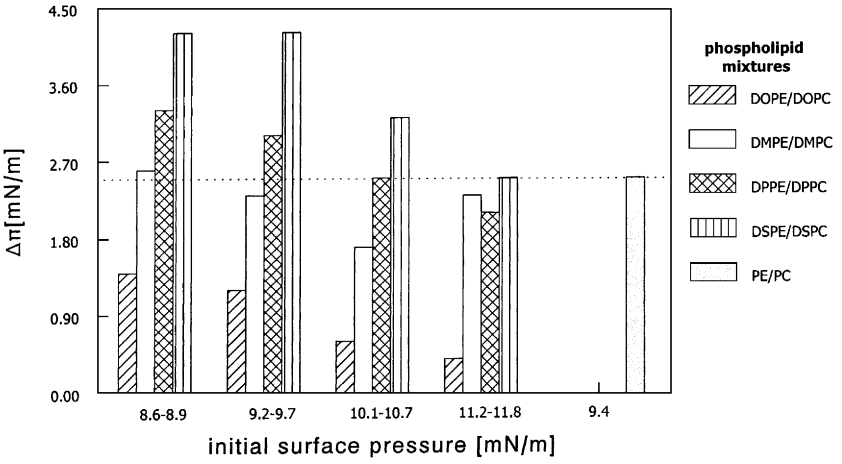

Fig. 6. Change in the surface pressure ($\Delta\pi$) of the synthetic phospholipid mixture monolayers DMPE/DMPC (3/2), DPPE/DPPC (3/2), DSPE/DSPC (3/2) and DOPE/DOPC (3/2) after the addition of brain spectrin to the subphase, at indicated initial surface pressure (π_i) values, in comparison with natural PE/PC (3/2) monolayers at π_i=9.4. The values of the surface pressure change induced by the protein without a lipid monolayer were subtracted from $\Delta\pi$. Brain spectrin concentration in the subphase: 2.78 nM.

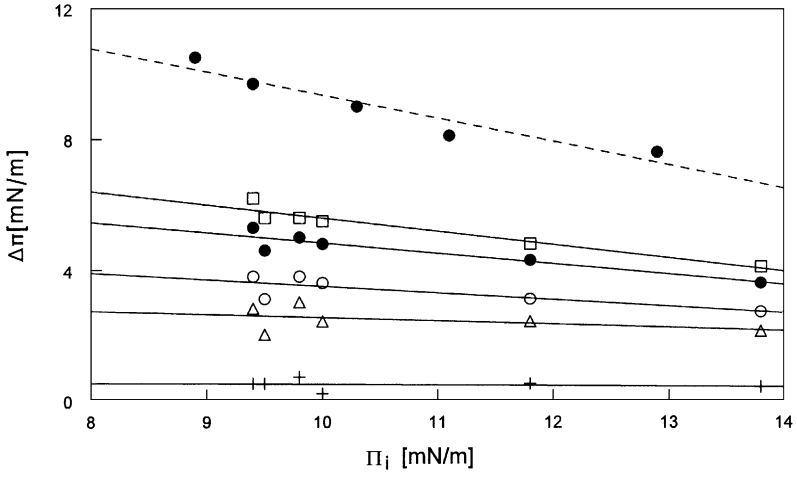

Fig. 7. Change in the surface pressure ($\Delta\pi$) of monolayers formed from a SM/DOPC/cholesterol (1/1/1) mixture as a function of the initial surface pressure (π_i) after the addition of erythrocyte spectrin into the subphase; for comparison, the $\Delta\pi$ changes for PE/PC (3/2) monolayers (dashed lines) are inserted. Erythrocyte spectrin concentrations in the subphase are indicated by the following symbols: 0.64 (+), 1.9 (△), 2.52 (○), 3.74 (●), 4.96 (□) nM.

rium dissociation constants (K_D) upon changes in membrane fluidity (Fig. 9A and B) were not substantial. On the other hand, when cholesterol was included in the lipid mixture, a change between 0 and 10% was significant (~5-fold increase in K_D value), while the differences in K_D observed for the various cholesterol contents were rather moderate (within one order of magnitude). It is interesting that a high affinity (~54 nM) was observed for FAT liposomes prepared from the "raft-like" mixture of lipids.

4. Discussion

Our results indicate that increasing concentrations of cholesterol in the monolayer facilitate the penetration of the monolayer by spectrins: in the case of erythroid spectrin, the maximum is observed at 10% and for brain spectrin, the maximum is at 20% cholesterol. For comparison, we performed assays on erythrocyte spectrin binding FAT liposomes prepared from a PE/PC mixture containing 10, 20 or 50% cholesterol (Fig. 9C). It seems that the different cholesterol contents of the liposomes primarily affected the maximal binding capacity (B_{max}) while the affinities remained within one order of magnitude the same. The only substantial difference in K_D observed for 0 and 10% cholesterol in liposomal membrane.

It was previously reported that the effect of brain spectrin on monolayers composed from anionic phospholipids and their mixtures with PC was much larger than that observed in the case of erythrocyte spectrin [20]. The results presented above indicate that the inclusion of cholesterol into a PI/PC mixture also changes the effect of each spectrin on the monolayer surface pressure. Moreover, the maximum of effect of the brain spectrin is at 20–30% cholesterol, while for the erythrocyte spectrin, two maxima (at 5 and at 30% cholesterol) can be observed. The above data, in particular for brain spectrin, concur with the physiological concentration of the cholesterol in the membrane, which varies between 24% and 29%. According to Radhakrishnan et al. [21,22] three coexisting liquid phases could be

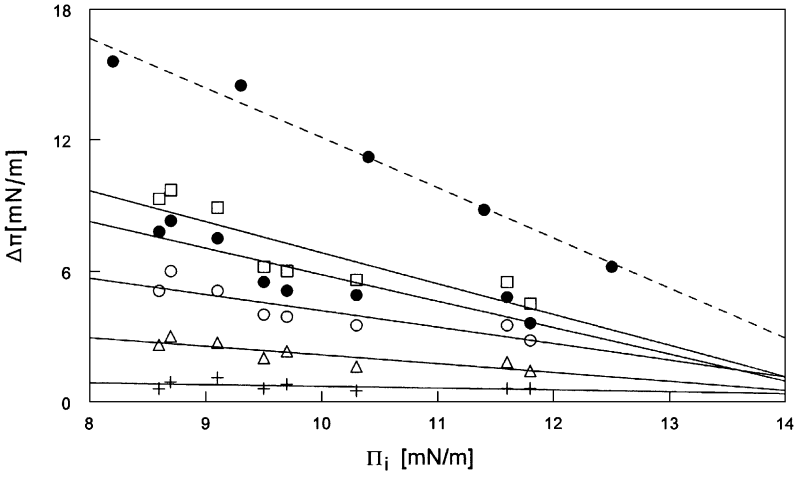

Fig. 8. Change in the surface pressure ($\Delta\pi$) of monolayers formed from a SM/DOPC/cholesterol (1/1/1) mixture as a function of the initial surface pressure (π_i) after the addition of brain spectrin into the subphase; for comparison, the $\Delta\pi$ changes for PE/PC (3/2) monolayers (dashed lines) are inserted. Brain spectrin concentrations in the subphase are indicated by the following symbols: 0.63 (+), 1.56 (△), 2.78 (○), 3.95 (●), 4.81 (□) nM.

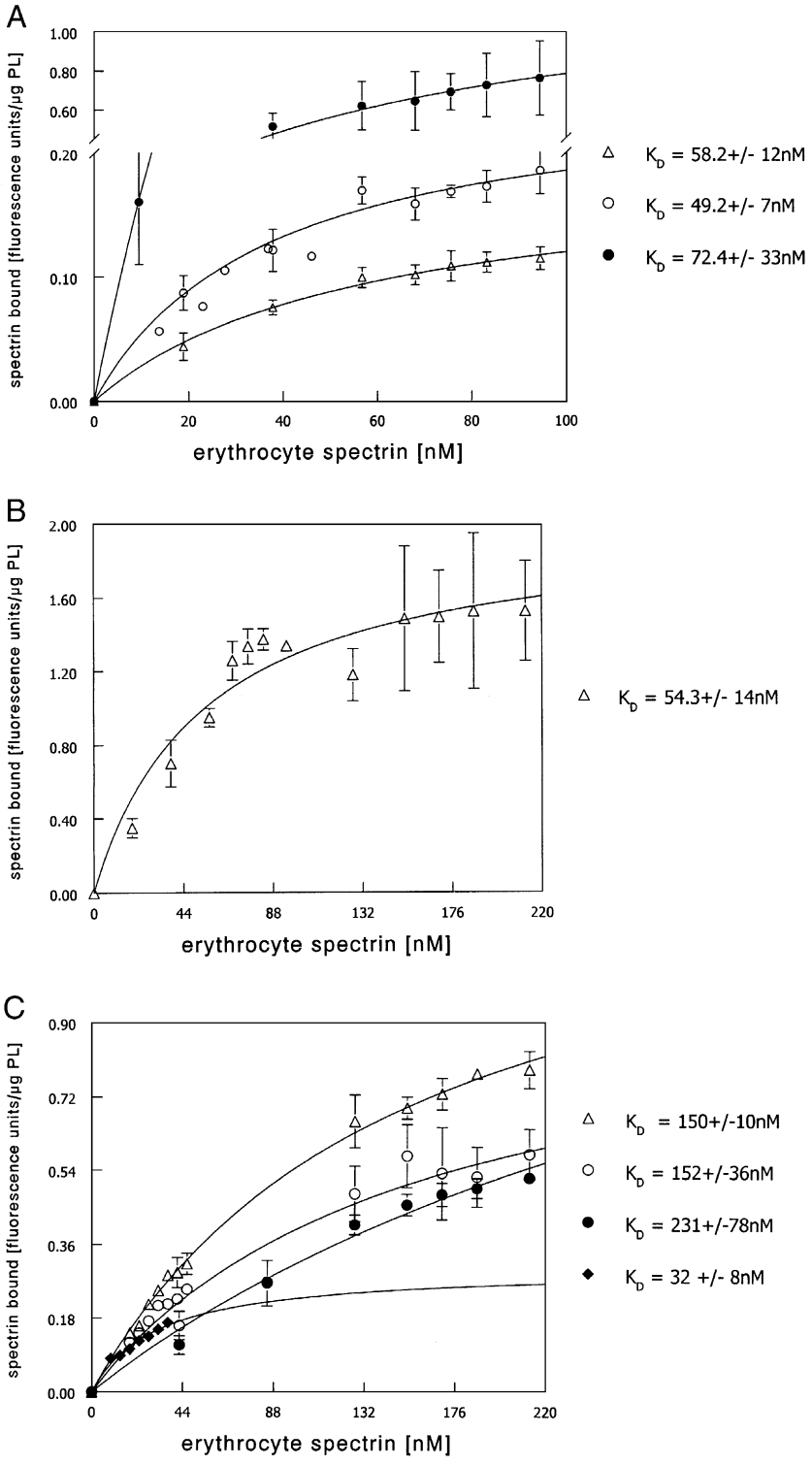

Fig. 9. Binding of increasing concentrations of rhodamine isothiocyanate-labelled erythrocyte spectrin by FAT-liposomes prepared from: (A) DMPE/DMPC 3/2 (△), DPPE/DPPC 3/2 (○), DSPE/DSPC 3/2 (●); (B) Cholesterol/SM/DOPC 1/1/1 (△); (C) A PE/PC 3/2 mixture containing 10% cholesterol (△), 20% cholesterol (○), 50% cholesterol (●) and a PE/PC 3/2 mixture without cholesterol (♦). Binding assay carried out at pH 7.5. Error bar=±S.D. Equilibrium dissociation constants calculated by nonlinear regression are shown in the legend. Other details in Materials and methods.

anticipated in a monolayer composed from cholesterol and phospholipids with long, saturated acyl chains: a phospholipid-rich phase, a condensed cholesterol/phospholipid complex-rich phase and a cholesterol-rich phase [22]. The existence of a cholesterol/phospholipid complex is indicated by the presence of two upper miscibility points [23]. Although the phase diagrams of the monolayer prepared from specific phospholipid mixtures with cholesterol are not available, we may anticipate that the cholesterol-facilitated ability of spectrin to penetrate the monolayer could be a result of the segregation of lipids into

small domains (or phases) [22]; therefore, the phase borders could function as "binding sites" for spectrins. The presence of the maxima at similar cholesterol contents could substantiate this assumption. In such a case, each spectrin could "sense" different kind of defects in the monolayer. This in particular concerns the charged phospholipid monolayer, which was reported to yield more complex phase diagrams [23].

The conclusion that the above effect of cholesterol is not simply the result of the change in membrane fluidity is supported by the results of experiments with monolayers constructed from homogenous synthetic phospholipids, which indicated an increase in the $\Delta\pi$ change correlating with the increased acyl chain length of the phospholipids used to prepare the monolayer (Figs. 5 and 6). The obtained results indicate that the effect of erythrocyte spectrin on the monolayer containing homogenous fatty acyl chains was generally smaller (with the exception of the DSPE/DSPC monolayer at π_i of 11 mN/m) than that on the natural PE/PC monolayer. In particular, it is interesting that for the synthetic lipid monolayer, the $\Delta\pi$ increased with increasing π_i, unlike in the case of brain spectrin, for which decreasing values of $\Delta\pi$ change with increasing π_i were observed. Again, the results from the pelleting assays carried out for erythroid spectrin confirm that the changes concern maximal binding capacity rather than affinity (Fig. 9A). All synthetic phospholipids with the exception of DOPE/DOPC were in the gel phase at the temperature of the measurements. The surface pressure changes induced by both spectrins were much smaller for DOPE/DOPC than for monolayers prepared from other mixtures phospholipids used in the experiments. (Figs. 5 and 6). In the case of monolayers prepared from synthetic phospholipids, our data show a clear dependence of the surface pressure change ($\Delta\pi$) on the lipids' acyl chain length and saturation, implying that the higher the transition point, the larger the effect of either spectrin. Although the monolayer experiments could not mimic the natural membrane bilayer, as the measurements were performed at low surface pressure values, it showed the changes in binding capacities for spectrins depended on the way the fluidity of the monolayer is changed. The data seem to concur with that obtained via the pelleting assay using liposomes which better mimic the natural membrane bilayer.

Differences in structural and physical properties between sphingolipids and phospholipids in their ability to compress hydrophobic chains could promote the phase separation in the membrane. The presence of cholesterol, in particular its condensed complexes with SM [22], facilitates the formation of membrane domains called rafts, characterised by the occurrence of highly ordered acyl chains. It remains unclear whether a raft's lipids interact directly with spectrin, or if raft proteins are needed. There is evidence that spectrin, actin, protein 4.1 and protein 4.2 could partially associate with lipid rafts [24], but this is a rather weak association [25]. On the other hand, it is likely that the membrane-associated skeleton plays a major role both in the regulation of the size and in the lateral positioning of the rafts [26]. In experiments with raft-like monolayers constructed from DOPC, SM and cholesterol (1/1/1), we observed an increase in the surface pressure after the addition of erythrocyte and brain spectrin into the subphase. However, the obtained values of $\Delta\pi$ change were much smaller than those observed in the case of the natural PE/PC (6/4) monolayer. On the other hand, the results of the pelleting assays indicate that red blood cell spectrin binds FAT liposomes prepared from these lipids in a saturable manner with a K_D of 54+14 nM. This could further support the view that the presence of domains and domain borders would promote interaction with spectrin.

Some laboratories reported that the formation of lipid rafts in the outer leaflet of the bilayer could have an effect on the behaviour of lipids in the inner leaflet of the same bilayer [22,27]. It is known that the presence of PE, the main component of the inner leaflet, in the phospholipid mixture could promote the formation of rafts, even within one monolayer [28]. This result for the first time indicates an interaction of spectrins with membranes prepared from a raft-like mixture of lipids.

In conclusion, it can be supposed that the effect of membrane lipid layer fluidity is not simple but depends on the way of its regulation, i.e., the cholesterol content or the chemical structure of the membrane lipids.

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References

[1] A.F. Sikorski, B. Hanus-Lorenz, A. Jezierski, A.R. Dluzewski, Interaction of membrane skeletal proteins with membrane lipid domain, Acta Biochim. Pol. 47 (2000) 565–578.
[2] A.F. Sikorski, M. Kuczek, Labelling of erythrocyte spectrin in situ with phenyllisothiocyanate, Biochim. Biophys. Acta 820 (1985) 147–153.
[3] C. DeWolf, P. McCauley, J.C. Pinder, Regulation of the mechanical properties of the red blood cell membrane by protein–protein and protein–lipid interactions, Cell. Mol. Biol. Lett. 1 (1996) 89–96.
[4] R.I. MacDonald, Temperature and ionic effects on the interaction of erythroid spectrin with phosphatidylserine membranes, Biochemistry 32 (1993) 6957–6964.
[5] P.J. O'Toole, I.E.G. Morrison, R.J. Cherry, Investigations of spectrin–lipid interactions using fluoresceinphosphatidylethanolamine as a membrane probe, Biochim. Biophys. Acta 1466 (2000) 39–46.
[6] A.F. Sikorski, K. Michalak, M. Bobrowska, Interaction of spectrin with phospholipids. Quenching of spectrin intrinsic fluorescence by phospholipids suspension, Biochim. Biophys. Acta 904 (1987) 655–660.
[7] S. Ray, A. Chakrabarti, Membrane interaction of erythroid spectrin: surface-density-dependent high-affinity binding to phosphatidylethanolamine, Mol. Membr. Biol. 21 (2004) 93–100.
[8] W. Diakowski, J. Szopa, A.F. Sikorski, Occurrence of lipid receptors inferred from brain and erythrocyte spectrums binding NaOH-extracted and protease-treated neuronal and erythrocyte membranes, Biochim. Biophys. Acta 1611 (2003) 115–122.
[9] W. Diakowski, A.F. Sikorski, Interaction of brain spectrin (fodrin) with phospholipids, Biochemistry 34 (1995) 13252–13258.
[10] W. Diakowski, A. Prychidny, M. Świstak, M. Nietubyć, K. Białkowska, J. Szopa, A.F. Sikorski, Brain spectrin (fodrin) interacts with phospholipids as revealed by intrinsic fluorescence quenching and monolayers experiments, Biochem. J. 338 (1999) 83–90.

[11] X. An, X. Guo, H. Sum, J. Morrow, W. Gratzer, N. Mohandas, Phosphatidylserine binding sites in erythroid spectrin: location and implications for membrane stability, Biochemistry 43 (2004) 310–315.
[12] X. An, X. Guo, W. Gratzer, N. Mohandas, Phospholipid binding by proteins of the spectrin family: a comparative study, Biochem. Biophys. Res. Commun. 327 (2005) 794–800.
[13] K. Białkowska, A. Zembroń, A.F. Sikorski, Ankyrin inhibits binding of erythrocyte spectrin to phospholipid vesicles, Biochim. Biophys. Acta 1191 (1994) 21–26.
[14] A. Hryniewicz-Jankowska, E. Bok, P. Dubielecka, A. Chorzalska, W. Diakowski, A. Jezierski, M. Lisowski, A.F. Sikorski, Mapping of an ankyrin-sensitive, phoshpatidylethanolamine/phosphatidylcholine mono- and bilayer binding site in erythroid β-spectrin, Biochem. J. 382 (2004) 677–685.
[15] A.V. Samsonov, I. Mihalyov, F.S. Cohen, Characterization of cholesterol–sphingomyelin domains and their dynamics in bilayer membranes, Biophys. J. 81 (2001) 1486–1500.
[16] C. Yuan, J. Furlong, P. Burgos, L.J. Johnston, The size of lipid rafts: an atomic force microscopy study of ganglioside GM1 domains in sphingomyelin/DOPC/cholesterol membranes, Biophys. J. 82 (2002) 2526–2535.
[17] D. Marsh, Lateral pressure in membranes, Biochim. Biophys. Acta 1286 (1996) 182–223.
[18] M.J. Hope, M.B. Bally, L.D. Mayer, A.S. Janoff, P.R. Cullis, Generation of multilamellar phospholipids vesicles, Chem. Phys. Lipids 40 (1986) 89–95.
[19] R. Koynova, M. Caffrey, Phases and phase transitions of the phosphatidylcholines, Biochim. Biophys Acta 1376 (1998) 91–145.
[20] W. Diakowski, A.F. Sikorski, Brain spectrin exerts much stronger effect on anionic phospholipids monolayers than erythroid spectrin, Biochim. Biophys. Acta 1564 (2002) 403–411.
[21] A. Radhkakrishnan, T.G. Anderson, H.M. McConnell, Condensated complexes, rafts, and the chemical activity of cholesterol in membranes, Proc. Natl. Acad. Sci. U. S. A. 97 (2000) 12422–12427.
[22] A. Radhakrishnan, H.M. McConnell, Condensed complexes of cholesterol and phospholipids, Biophys. J. 77 (1999) 1507–1517.
[23] A. Radhakrishnan, H.M. McConnell, Critical points in charged membranes containing cholesterol, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 13391–13396.
[24] U. Salzer, R. Prohaska, Stomatin, flotilin-1, and flotilin-2 are major integral proteins of erythrocyte lipid rafts, Blood 97 (2001) 1141–1143.
[25] B.U. Samuel, N. Mohandas, T. Harrison, H. McManus, W. Rosse, M. Reid, K. Haldar, The role of cholesterol and glycosylphophosphatidylinositol-anchored proteins of erythrocyte rafts in regulating raft protein content and malarial infection, J. Biol. Chem. 276 (2001) 29319–29329.
[26] K. Jacobson, C. Dietrich, Looking at lipid rafts? Trends Cell Biol. 9 (1999) 87–91.
[27] P.W. Janes, S.C. Ley, A.I. Magee, Aggregation of lipid rafts accompanies signalling via the T cell antigen receptor, J. Cell Biol. 147 (1999) 447–461.
[28] D.A. Brown, E. London, Structure and function of sphingolipid- and cholesterol-rich membrane rafts, J. Biol. Chem. 275 (2000) 17221–17224.