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Brain spectrin exerts much stronger effect on anionic phospholipid monolayers than erythroid spectrin

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Abstract

Red blood cell spectrin and its nonerythroid analogues are linked to integral proteins of the membrane by several skeletal protein receptors, such as ankyrin and protein 4.1 together with p55. However, there are also many reasons for believing that they are insufficient to engender all the properties that characterise the native membrane. Therefore, we are concerned with the mechanism by which brain spectrin interacts with phospholipids of the membrane bilayer. Brain and erythrocyte spectrin were shown previously to bind phospholipid vesicles as well as monolayers prepared from aminophospholipids: phosphatidylethanolamine and phosphatidylserine and their mixtures with phosphatidylcholine (PC). In the present study, it is shown that brain spectrin binds to monolayers prepared from anionic phospholipids, such as phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidyl glycerol, diphosphatidylglycerol, and their mixtures with PC. Brain spectrin injected into the subphase to reach nanomolar concentration induced a substantial increase in the surface pressure of monolayers prepared from the phospholipids and their mixtures mentioned above, possibly by penetrating them. This effect is stronger in the case of monolayers prepared from anionic phospholipids alone and weaker when monolayers were prepared from mixtures with PC. The weakest effect was observed in the case of phosphatidylinositol-4,5-bisphosphate monolayers. An interaction of brain spectrin with monolayers prepared from anionic phospholipids (PI/PC 7:3 and PA/PC 7:3) was inhibited (PI/PC much stronger than PA/PC) by purified erythrocyte ankyrin, which indicates that the binding site for those lipids is located in the β-subunit, possibly in, or in close proximity of, the ankyrin-binding site. In contrast, erythrocyte spectrin injected into the subphase induced a change in the surface pressure of monolayers prepared from anionic phospholipids, which was equal or smaller than the value of surface pressure change induced by protein without a monolayer. This effect was different from what had been observed previously for monolayers prepared from aminophospholipids and their mixtures with PC, and from the data for nonerythroid spectrin presented here.

Keywords: Brain spectrin (fodrin); Red blood cell (erythrocyte) spectrin; Anionic phospholipid; Monolayer

1. Introduction

It is known that erythrocyte spectrin, one of the major proteins of the mammalian red blood cell skeleton, interacts directly with the lipid domain in natural membranes [1,2] or in model systems [3–6]. This kind of interaction of red blood cell spectrin with membrane lipid bilayers (or monolayers) has been known for 20 years, but its physiological significance is still a matter of dispute. One of the possible roles of this interaction would be serving as an alternative binding site when there is not enough functional ankyrin to accommodate all spectrin tetramers in the membrane, or its affinity to bind spectrin is reduced, for example, upon phosphorylation of ankyrin [7,8].

In our previous studies, we have observed interaction of brain spectrin with membrane phospholipids studied on natural (synaptic plasma) membranes [9] and on artificial lipid mixtures formed from phospholipid liposomes and monolayers [10,11]. We have found that brain spectrin binds directly and rather strongly (K_D in submicromolar range) to liposomes containing aminophospholipids PE and PS and phosphatidylcholine (PC) [10]. A monolayer study indicated that the protein penetrates a monolayer, inducing an increase in monolayer surface pressure [11]. The β-subunit of brain

Abbreviations: DTT, dithiothreitol; EGTA, ethylene glycol bis-(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; PI, phosphatidylinositol; PIP2, phosphatidylinositol-4,5-bisphosphate; PA, phosphatidic acid; PG, phosphatidyl glycerol; DPG (CL), bisphosphatidylglycerol (cardiolipin)

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spectrin binds preferentially to the PE/PC phospholipid monolayer mixtures. Inhibition of this binding by bovine erythrocyte ankyrin indicates that the 15th repeat of the β-subunit could take part in the interaction with lipid domains of membranes [11].

The carboxyl-terminal segment of the β-subunit of brain spectrin contains a pleckstrin homology (PH) domain, approximately 100 amino acids long, which has also been described as a membrane binding site [12]. The expressed, purified PH domain binds to inositol-1,4,5-triphosphate (IP3) vesicles as well as to vesicles containing phosphatidylinositol-4,5-bisphosphate (PIP2) [13].

To examine whether and how brain spectrin and its erythrocyte analogue interacts with anionic phospholipids, such as phosphatidylinositol (PI), PIP2, phosphatidic acid (PA), phosphatidyl glycerol (PG) and bisphosphatidylglycerol (DPG), we tested this interaction in monolayer experiments. It was found that brain spectrin bound anionic phospholipid monolayers by penetrating them (inducing an increase in their surface pressure), while red blood cell spectrin induced a change in the surface pressure of these monolayers which was smaller from the value induced by the protein without a lipid monolayer. Moreover, the effect of brain spectrin on monolayers composed of PIP2 and its mixtures with PC was very weak, indicating lack of engagement of the PH domain. The inhibition of brain spectrin binding to anionic phospholipid monolayers by ankyrin suggests involvement of the ankyrin-binding domain in this interaction.

2. Materials and methods

2.1. Materials

Sephacryl S-500-HR, Sephacryl S-200, DEAE-Sephacel, Sepharose 4-B, dithiothreitol (DTT), ethylene glycol bis-(β-aminoethyl ether) *N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), PI, PIP2, DPG were from Sigma (St. Louis, MO). PA and PG were from Lipids Products (Nutfield, UK).

2.2. Methods

Brain spectrin was isolated from bovine brains according to Bennett et al. [14]. The final preparation, containing only spectrin bands of apparent molecular weights of 280 and 267 kDa, with traces of a proteolytic 160-kDa fragment of the α-spectrin subunit, was tested by SDS-polyacrylamide gel electrophoresis in the Laemmli system [15].

Bovine erythrocyte spectrin was purified by the extraction of red blood cell ghosts with low ionic strength buffer as described previously [16,17] and tested—as above. Isolated proteins were dialysed overnight against an appropriate buffer.

Pig erythrocyte ankyrin was isolated by the extraction of red blood cell ghosts with low and high ionic strength buffer according to Hall and Bennett [18], except that chromatography was carried out on a FPLC ion exchanger chromatography UNO-Q1 (Bio-Rad) column, using a linear gradient (0–0.8 M) of NaCl in the presence of 2 M urea. To remove traces of spectrin, ankyrin was purified by gel filtration on a Sephacryl S-200 column (1×35 cm), equilibrated with 0.6 M KI in 7.5 mM Na_2HPO_4, 1 mM EDTA, 1 mM DTT, 1 mM NaN_3, pH 8.0.

Monolayer measurements were carried out by using a trough of surface area of 24 cm^2 and a Nima tensiometer (Nima Technology, England) at room temperature (20 °C). Subphase buffer (25 ml) containing 5 mM Tris–HCl, 0.5 mM EDTA, 150 mM NaCl, 0.5 mM DTT and 1 mM NaN_3, pH 7.5, was stirred with a small stirrer bar (5×2 mm). Monolayers were formed by the injection of a chloroform solution of a phospholipid mixture with the use of a Hamilton syringe. Aliquots of brain and red blood cell spectrin dialysed against the same buffer were injected into the subphase, and surface pressure (π) measurements were taken.

For the experiments on the inhibition of brain spectrin–phospholipid interaction by erythrocyte ankyrin, indicated concentrations of ankyrin were first incubated with brain spectrin for 30 min at room temperature, and then injected into the subphase.

The data points represent average values of several independent experiments with an average variation not larger than 10%.

3. Results

In our study, we were concerned with brain spectrin interaction with anionic phospholipids tested by the monolayer technique. The data on the interaction of brain and red blood cell spectrin with anionic phospholipids had not been available in the literature, therefore, the effect of both spectrins on anionic phospholipid monolayers pressure was tested.

In Fig. 1, we show an example of the original set of measurements of changes in surface pressure of the PI/PC (5:5) monolayer after the addition of brain spectrin (filled symbols) and of erythrocyte spectrin (empty symbols) to the subphase, for indicated initial surface pressure (π_i) values. Other data (Figs. 2–6) are presented as the dependence of $\Delta\pi$ versus π_i for chosen spectrin concentrations (as indicated in Figs. 2–6). It should be noted that the data represent $\Delta\pi$ from which the value of the protein adsorption without a lipid monolayer for each concentration was subtracted.

The addition of bovine brain spectrin to the subphase buffer induced an increase in the surface pressure ($\Delta\pi$) of the PI monolayer at pH 7.5, which was a function of initial surface pressure (π_i) (Fig. 2A, filled symbols). The change in surface pressure was increased for larger brain spectrin concentrations and at a smaller initial surface pressure of the PI monolayer. The brain spectrin concentration range evok-

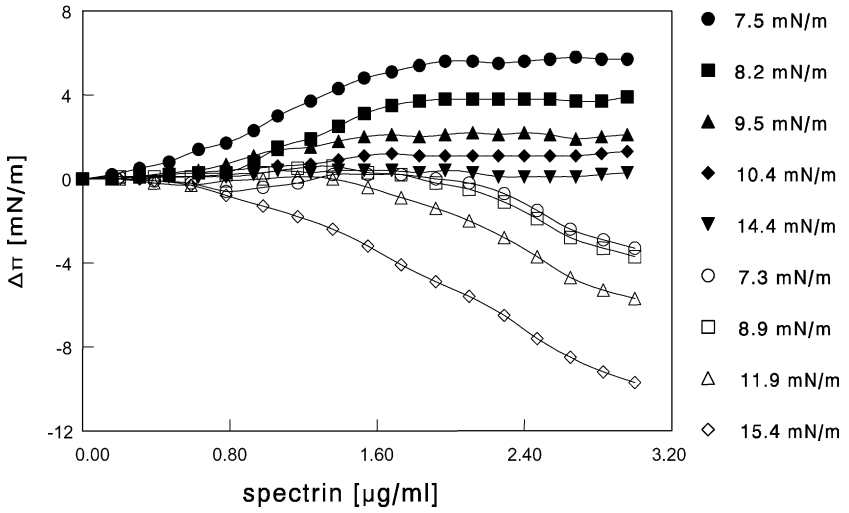

Fig. 1. Change in surface pressure ($\Delta\pi$) of the PI/PC 5:5 monolayer after the addition of brain spectrin (filled symbols) and of red blood cell spectrin (empty symbols) to the subphase, at indicated initial surface pressure (π_i) values. Values of the surface pressure change induced by the protein without a lipid monolayer were subtracted from $\Delta\pi$.

ing this effect was relatively low, that is, below 3 nM, which might indicate a specific interaction. The same effect was observed using a PI/PC monolayer prepared from mixtures of PI/PC ratios 7:3 and 5:5 (Fig. 2B and C).

Fig. 2A–C (empty symbols) shows a change in the surface pressure of the PI, PI/PC (7:3) and PI/PC (5:5) monolayers upon addition of erythrocyte spectrin to the subphase buffer as a function of initial surface pressure (π_i). As a result of these experiments, we observed negative values of surface pressure ($\Delta\pi$) changes, when subtracted from the value of protein adsorption to the surface without a lipid monolayer. Moreover, we observed a larger decrease in the change in surface pressure ($\Delta\pi$) for larger spectrin concentrations and for larger initial surface pressure values of the monolayer. In the case of erythroid spectrin, the changes took place also at nanomolar concentrations.

We tested the effect of bovine brain spectrin on phospholipid monolayer surface pressure also by using other anionic phospholipids, such as PA and its mixtures PA/PC (7:3) and PA/PC (5:5) (Fig. 3A–C), PG and its mixtures PG/PC (7:3) and PG/PC (5:5) (Fig. 4A–C), and DPG and its mixtures DPG/PC (7:3) and DPG/PC (5:5) (Fig. 5A–C). In each case, we observed the same kind of changes in surface pressure ($\Delta\pi$) of the phospholipid monolayer as a function of initial surface pressure (π_i): an increase in the change in surface pressure ($\Delta\pi$) for larger spectrin concentrations and for smaller initial surface pressure of the tested monolayer. In addition, injection of erythrocyte spectrin to the subphase buffer induced the same linear decrease in surface pressure of the monolayers described above, as a function of initial surface pressure (π_i), as in the case of PI and its monolayer mixtures (Figs. 3–5, empty symbols).

In summary, it should be stated that brain spectrin in nanomolar concentrations induced an increase in $\Delta\pi$ which was inversely proportional to the initial film pressure values (π_i). The largest $\Delta\pi$ changes were observed in the case of the PA/PC (7:3) and the smallest in the case of the DPG/PC (5:5) monolayer. Erythrocyte spectrin, however, in nanomolar concentrations induced $\Delta\pi$ changes which were equal or smaller than the value of the protein adsorption without a lipid monolayer. These changes were larger at higher initial surface pressure of the monolayer. These changes are comparable with the data of others (e.g. Shiffer et al. [19]) for monolayers prepared from other lipid mixtures, as well as for other proteins such as erythroid protein 4.1 and spectrin, serum albumin, lysozyme, α-casein, and apolipoprotein. The parameters of an "uncorrected" $\Delta\pi$ value for erythrocyte and brain spectrin at concentrations 5 and 2.5 nM, respectively, resulting from a linear fit of $\Delta\pi = f(\pi_i)$, were similar to those of proteins of rather moderate hydrophobicity. For the brain spectrin, π_i intercept values were from 15 to over 33 mN/m, $\Delta\pi$ intercept 6–25 mN/m and slope between 0.24 and 1.59. For the erythroid spectrin, these values were: 13–26 mN/m (π_i intercept), 12–24.0 mN/m ($\Delta\pi$ intercept) and slope 0.45–1.82 (data not shown).

The data previously obtained by others [12] indicate an interaction of nonerythroid spectrin's PH domain with PIP2-containing vesicles. In this experimental system, this interaction was not well pronounced. In Fig. 6, we show the results of a change in surface pressure of the PIP2, PIP2/PC (7:3) and the PIP2/PC (5:5) monolayers at pH 7.5 upon the addition of bovine brain spectrin to the subphase buffer as a function of initial surface pressure (π_i). The observed effect was much weaker than the increase in surface pressure observed upon the addition of brain spectrin to the subphase of PI, PA, PG, DPG and their monolayer mixtures. It seems that the effect is proportional to the PC concentration in the monolayer. These results indicate a rather low capacity of the PIP2-containing monolayers for nonerythroid spectrin binding.

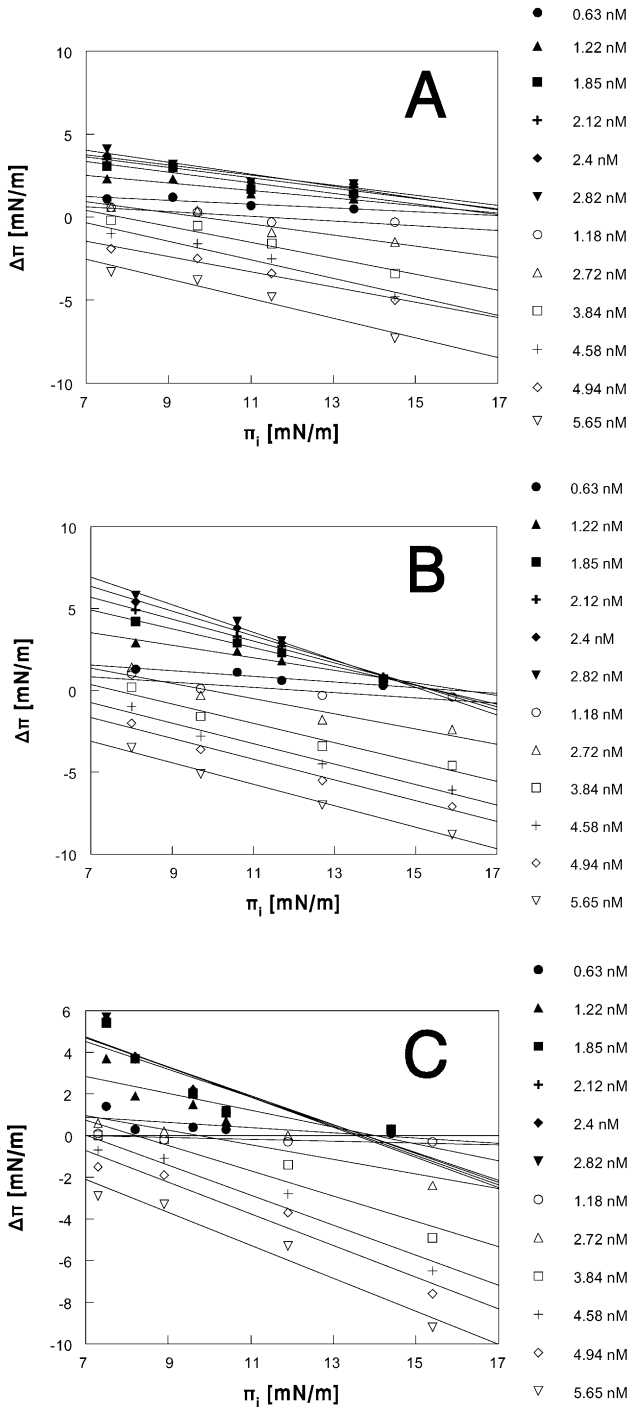

Fig. 2. Change in surface pressure ($\Delta\pi$) of PI and its monolayer mixtures as a function of initial surface pressure (π_i) after the addition of indicated concentrations of bovine brain and erythrocyte spectrin to the subphase. Monolayers formed from: (A) PI, (B) PI/PC 7:3, (C) PI/PC 5:5 (original data presented in Fig. 1). Other details as in Fig. 1.

The measured changes in surface pressure of anionic phospholipid monolayers as a function of π_i after addition of brain spectrin to the subphase and the other, earlier obtained data [11], indicate the penetration of the monolayer film by brain spectrin. One of possible approaches to the identification of anionic phospholipid-binding sites in brain spectrin was to test whether the interaction of brain spectrin was sensitive to inhibition by purified erythrocyte ankyrin. We measured the effect of ankyrin on the binding by brain spectrin to PI/PC 7:3 and PA/PC 7:3 monolayer mixtures.

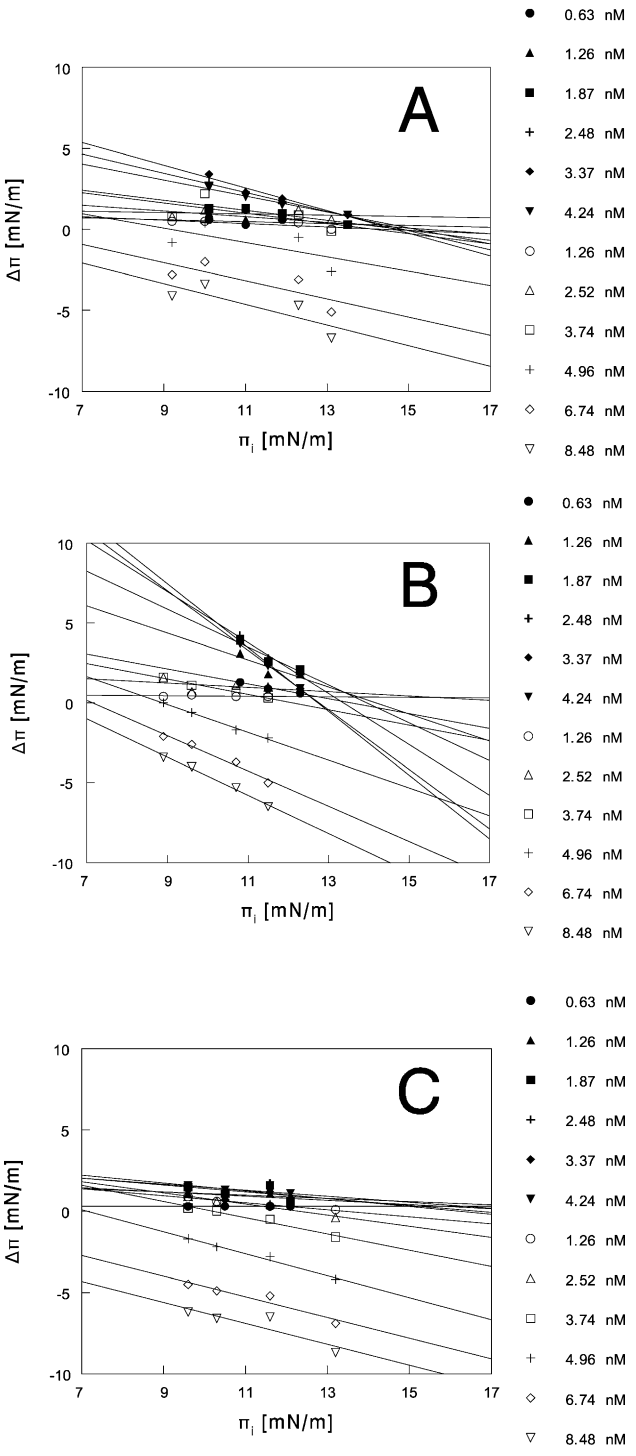

Fig. 3. Change in surface pressure ($\Delta\pi$) of PA and its monolayer mixtures as a function of initial surface pressure (π_i) after the addition of indicated concentrations of bovine brain and erythrocyte spectrin to the subphase. Monolayers formed from: (A) PA, (B) PA/PC 7:3, (C) PA/PC 5:5. Other details as in Fig. 1.

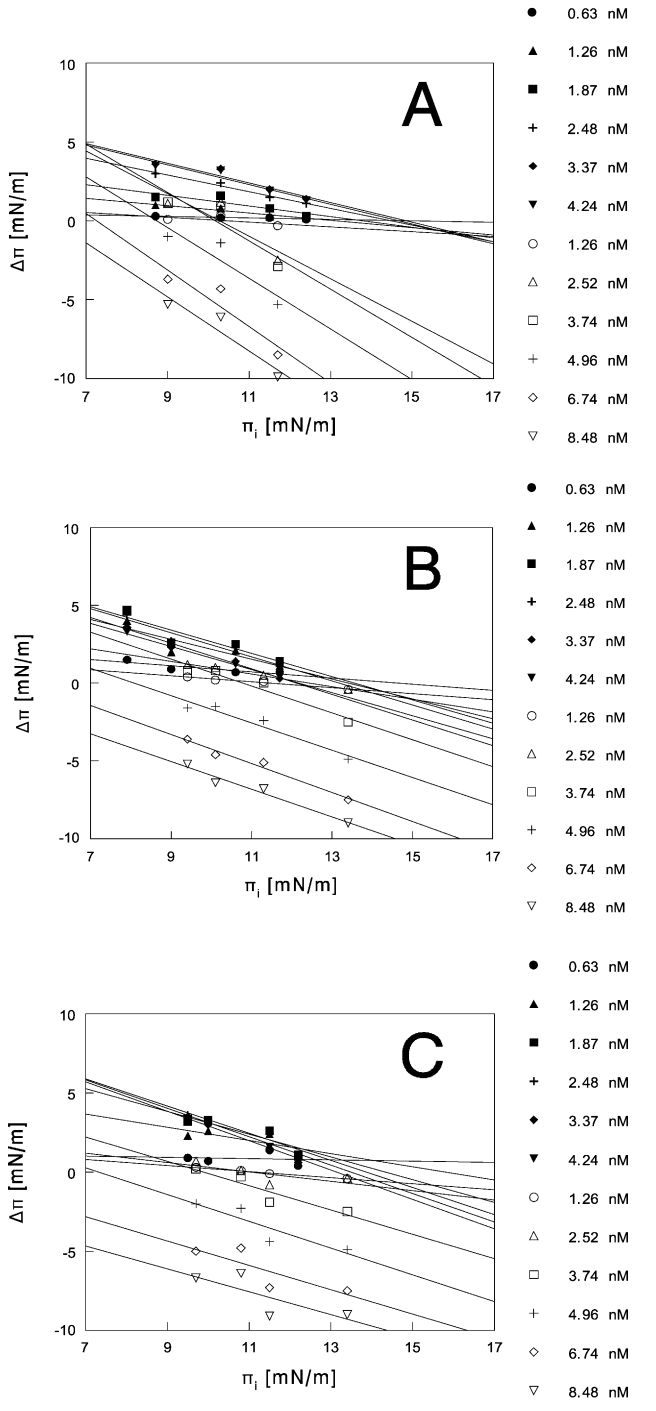

Fig. 4. Change in surface pressure ($\Delta\pi$) of PG and its monolayer mixtures as a function of initial surface pressure (π_i) after the addition of indicated concentrations of bovine brain and erythrocyte spectrin to the subphase. Monolayers formed from: (A) PG, (B) PG/PC 7:3, (C) PG/PC 5:5. Other details as in Fig. 1.

The molar ratio of spectrin to ankyrin varied from 1:0.25 to 1:1. In control experiments, only brain spectrin at the same concentration was added. Stronger inhibition of interaction of brain spectrin with the PI/PC 7:3 monolayer by ankyrin (approx. 50%) was observed in experiments at a 1:1 molar ratio of brain spectrin to ankyrin. A weaker effect (approx. 30%) of inhibition of the interaction of brain spectrin with monolayers by ankyrin was observed in experiments using the PA/PC 7:3 monolayer. Dixon [20] type analysis (Fig. 7A and B) indicates a competitive mechanism with two values of $K_I = 0.603 \pm 0.134$ (S.D.) and 1.181 ± 0.23 (S.D.) nM for

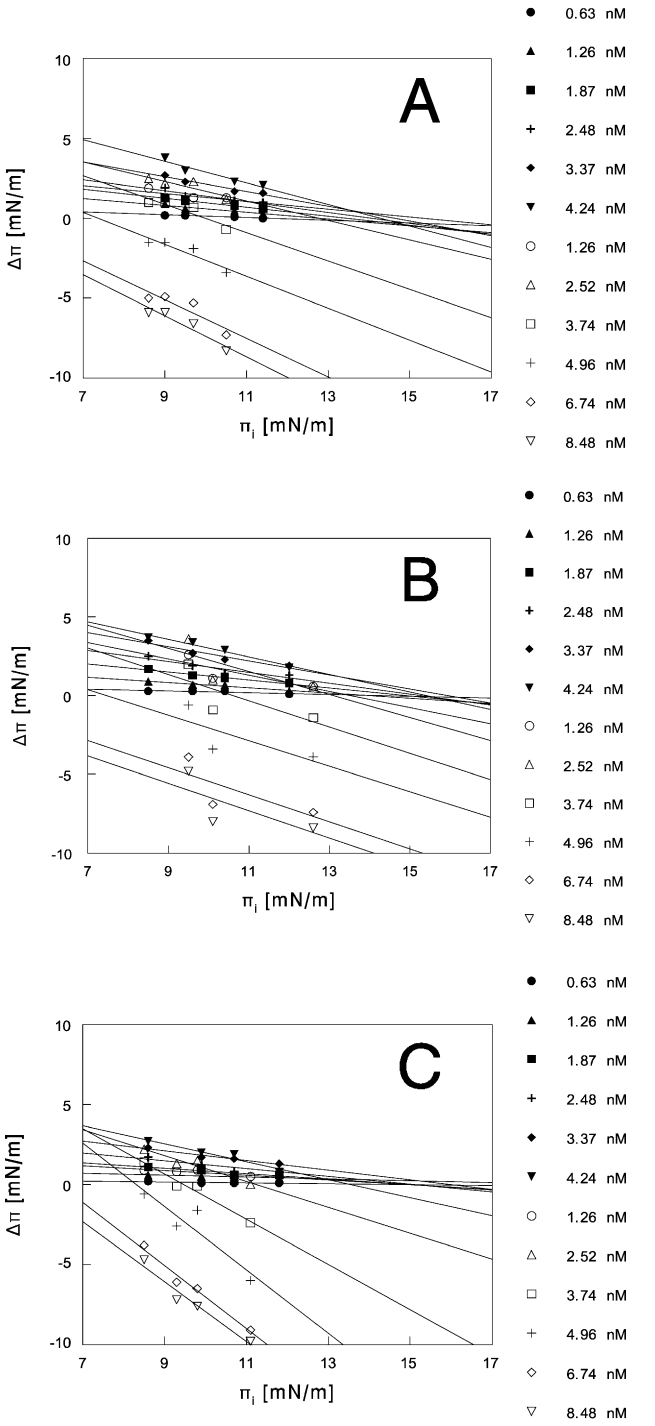

Fig. 5. Change in surface pressure ($\Delta\pi$) of DPG and its monolayer mixtures as a function of initial surface pressure (π_i) after the addition of indicated concentrations of bovine brain and erythrocyte spectrin to the subphase. Monolayers formed from: (A) DPG, (B) DPG/PC 7:3, (C) DPG/PC 5:5. Other details as in Fig. 1.

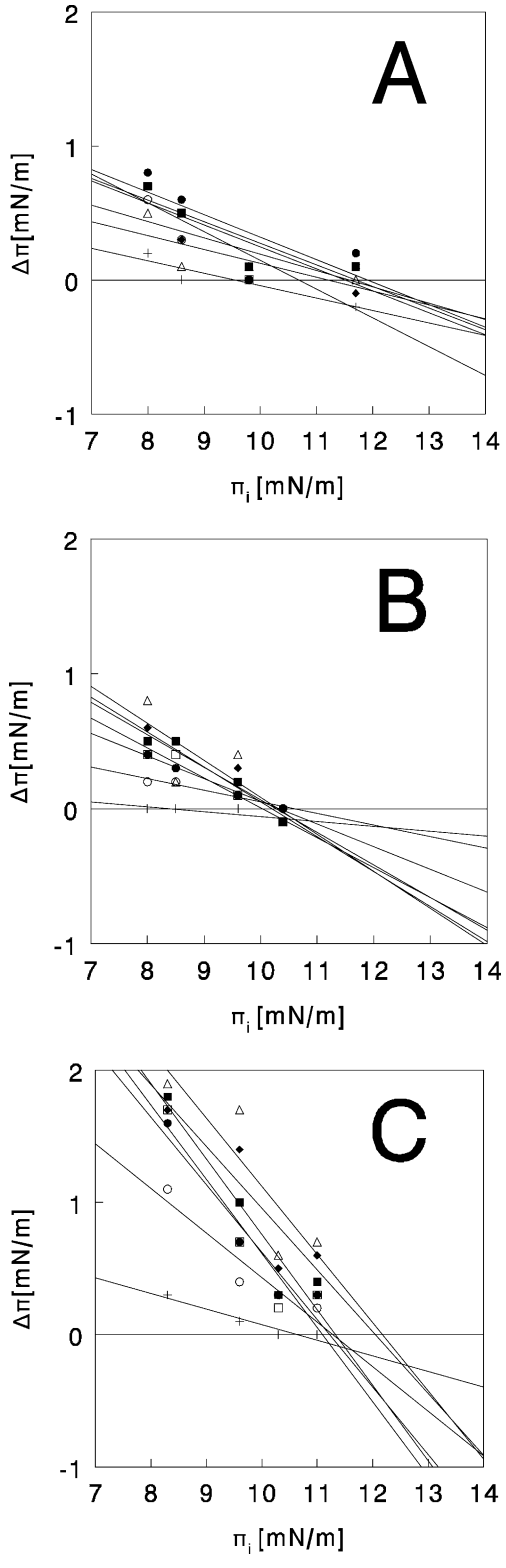

Fig. 6. Change in surface pressure ($\Delta\pi$) of PIP_2 and its monolayer mixtures as a function of initial surface pressure (π_i) after the addition of bovine brain spectrin to the subphase. Monolayers from: (A) PIP2, (B) PIP2/PC 7:3, (C) PIP2/PC 5:5. Brain spectrin concentrations in the subphase: (+) 0.59; (○) 1.17; (●) 1.73; (□) 2.1; (■) 2.47; (♦) 2.83 and (△) 3.35 nM. Other details as in Fig. 1.

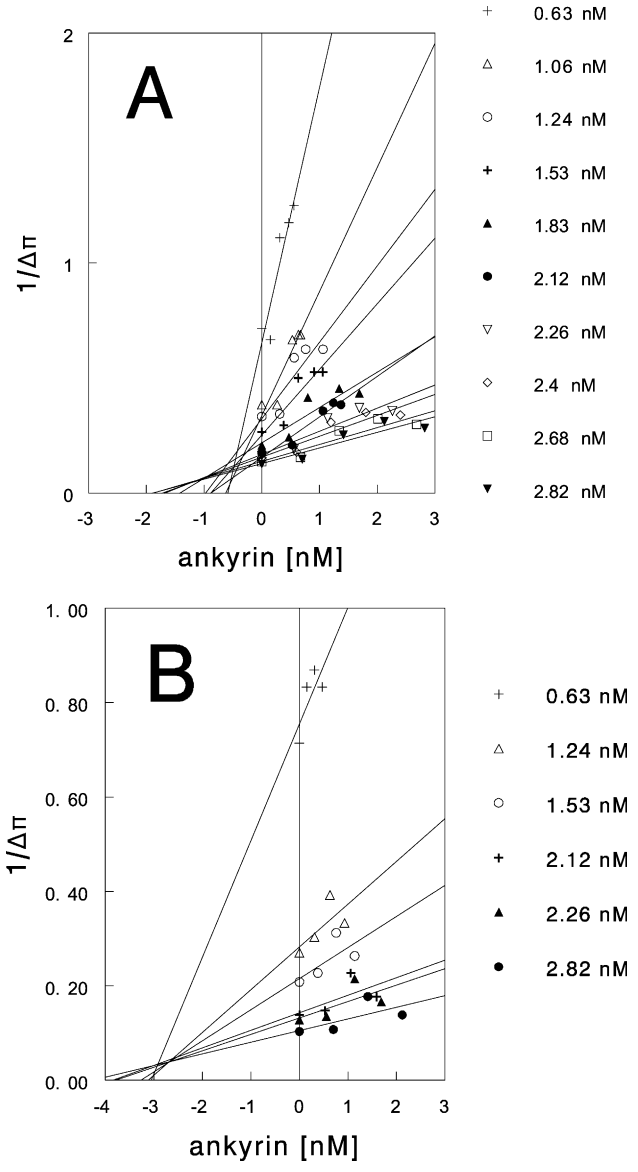

Fig. 7. Inhibition of the binding of bovine brain spectrin to the PI/PC 7:3 monolayers (A), and the PA/PC 7:3 monolayers (B) by erythrocyte ankyrin. (A) Dixon [20] plot of the results obtained for 10 indicated concentrations of brain spectrin. Two different K_I values can be obtained: 0.603 ± 0.134 nM (mean±S.D.) for brain spectrin concentrations in the range of 0.6–1.5 and 1.181 ± 0.23 nM at brain spectrin concentrations over 1.5 nM. (B) Dixon [20] plot of the results obtained for 6 indicated concentrations of brain spectrin. A K_I value can be obtained: 2.788 ± 0.245 nM (mean±S.D.) for brain spectrin concentrations in the range of 0.6–2.8 nM.

the PI/PC 7:3 monolayer mixture, and one value of $K_I = 2.788 \pm 0.245$ (S.D.) nM for the PA/PC 7:3 monolayer mixture, respectively.

4. Discussion

Our previously obtained data demonstrate that brain spectrin binds membrane phospholipids [10,11]. In the experiments presented above, it is shown that this interac-

tion could be demonstrated also by monolayer experiments, although it is difficult to extract quantitative parameters of this interaction (K_D and B_{max}). Instead, applied methods give an insight whether this interaction affects the properties of the lipid layer. The results obtained by using nanomolar concentrations of brain spectrin indicate that this protein could penetrate phospholipid monolayers built from anionic phospholipids, such as PI, PA, phosphatidylglycerol, DPG and their mixtures with PC at the ratio of 7:3 and 5:5. The observed effect was much stronger than in the case of PC alone (see Ref. [11]). Moreover, the effect was independent of the content of PC in the phospholipid–PC mixture (compare Fig. 1 with Fig. 6).

An increase in the surface pressure of a phospholipid monolayer would suggest its penetration by certain segments of brain spectrin molecules. Inhibition of this effect by erythrocyte ankyrin (Fig. 7) suggests that the phospholipid binding site could be located in the β-subunit, approximately at the region of ankyrin binding [21]. These results are similar to those obtained for the inhibition of erythrocyte spectrin interaction with PE/PC vesicles and monolayers by ankyrin [22,23], as well as to those observed for the

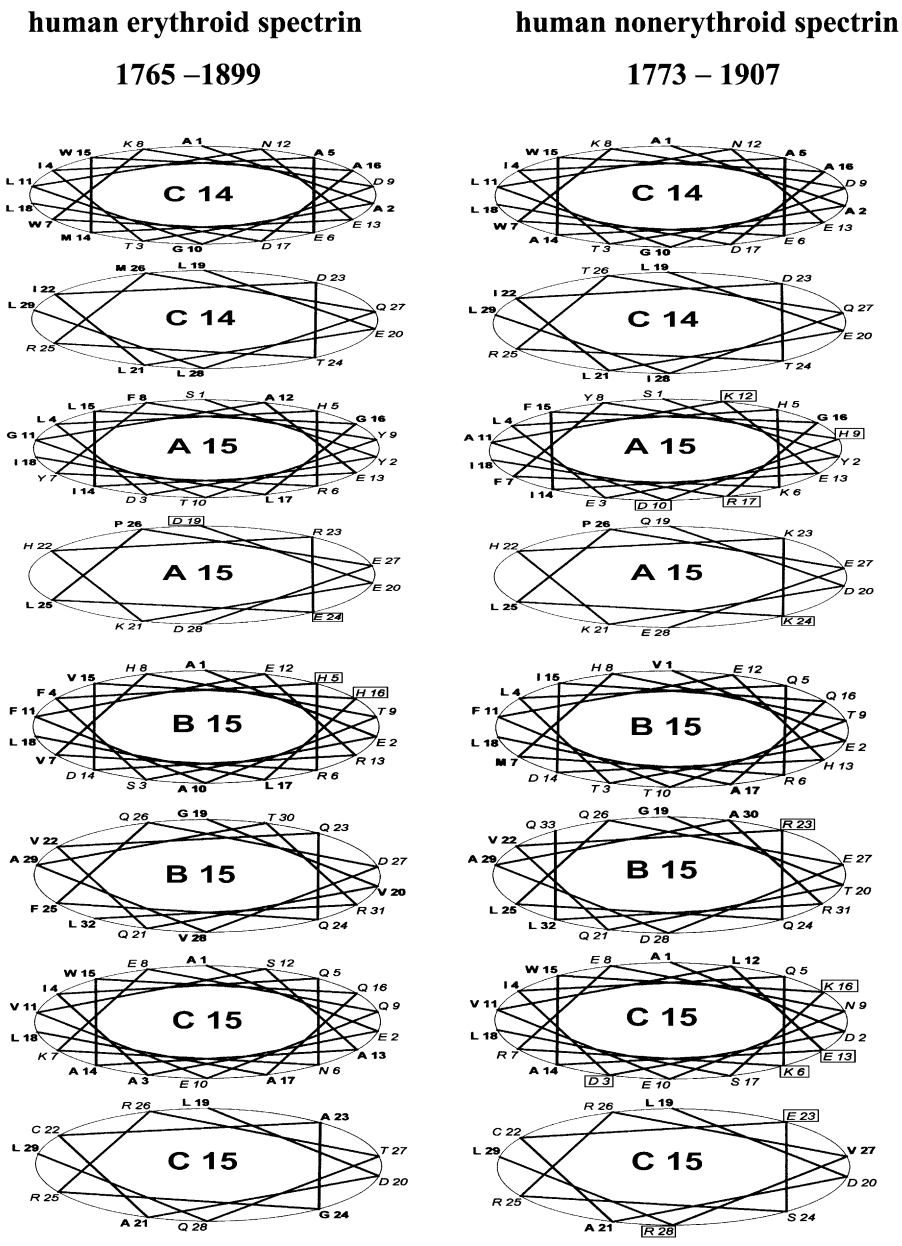

Fig. 8. Comparison of the ankyrin-binding domains of the β-subunit between erythroid spectrin, accession number P11277 (left panel) and nonerythroid spectrin, accession number Q01082 (right panel), see Ref. [21]. Helical wheel presentation. Alignment of helices was taken from Ref. [26]. Charged residues that are different in both sequences are boxed. Linking regions between helices A15 and B15 (erythroid spectrin residues 1822–1830, nonerythroid spectrin residues 1830–1838) which differ by one positive charge (R1834) present in nonerythroid spectrin and B15 and C15 (erythroid spectrin residues 1863–1870, nonerythroid spectrin residues 1871–1878) which are characterised by identical charge distribution are omitted.

inhibition of interaction of brain spectrin with PE/PC monolayers by ankyrin [11]. The reason for the lower sensitivity of PA-containing monolayers to inhibition by ankyrin is not known, but it could be suggested that a smaller head group could account for this effect.

The data given by Wang and Shaw [12] indicate that the PH domain may be involved in lipid binding, and that this binding coincides with the inositol-1,4,5-trisphosphate binding site. These results, indicating an occurrence of other, ankyrin-insensitive sites of phospholipid binding in the spectrin molecule may suggest also functional connection and spatial relationship between the ankyrin-binding site and the PH domain.

In our experiments to determine the binding of brain spectrin with the phospholipid monolayer formed from PIP2 and its mixtures with PC at the ratio of 7:3 and 5:5, we observed a much smaller increase in surface pressure than in that observed for the PI, PA, PG and DPG monolayers. The results presented in Fig. 6 indicate much weaker penetration of the PIP2 monolayer by brain spectrin than in the case of the other anionic phospholipid mixtures. The lack of penetration of PIP2-containing monolayers by brain spectrin is probably due to head-group specificity of β-spectrin's PH domain [24]. It is also possible that in purified brain spectrin, this domain is not exposed to the solution, therefore it does not bind this phospholipid.

Inhibition of the interaction of brain spectrin with anionic phospholipid monolayers indicate an involvement of the β-spectrin region close or identical to the ankyrin-binding domain in this interaction. The obtained K_I-values for inhibition of brain spectrin interaction with PI-containing monolayers are close to the values obtained for the PE/PC monolayer binding by brain spectrin in the presence of ankyrin [22], while higher values, calculated for PA-containing monolayers, might reflect lower affinity of spectrin to this monolayer, which might accompany the lower capacity mentioned above.

All the monolayer experiments were performed using relatively the same, low initial surface pressure values. The results that we obtained (Figs. 1–5, empty symbols) indicate changes in surface pressure upon addition of red blood cell spectrin which were smaller from the surface pressure values evoked by protein adsorption to the surface without an anionic phospholipid monolayer. The effect is more distinct for larger erythrocyte spectrin concentrations and π_i values of the monolayer. The observed surface pressure values could indicate smaller penetration or even a compression of the monolayer. This is somewhat surprising, since the results observed previously indicated a great similarity of the effects of both spectrins in monolayers prepared from aminophospholipid and PC or their mixtures [11,22].

A comparison of the charge of the ankyrin-binding domain (residues 1768–1898) of red blood cell and brain β-spectrin (1776–1906) [21] suggests that the domain of erythrocyte protein contains six more negatively charged amino acid residues than positively charged ones, while brain spectrin contains only two net negative charges. When this comparison is limited to the region of helix A15 and fragment linking helix B15 erythrocyte spectrin contains three net negative charges, while brain spectrin contains three more positive residue than negative residues clustered at the same region of the helix (Fig. 8). This possibly could explain the difference in the effect of both proteins on monolayers composed of anionic phospholipids and their mixtures with PC.

Our data imply a strong, direct interaction between brain spectrin and anionic membrane phospholipids. Its physiological significance, however, is not completely clear, since there are many kinds of proteinaceous binding sites in nonerythroid (particularly neuronal) cell membranes. One possibility is that lipidic binding sites are of particular importance, while ankyrin is either absent or its spectrin-binding activity has been reduced by, for example, phosphorylation [6,7]. Recent data [25] indicate a functional role of aminophospholipid asymmetry in stabilisation of membrane–membrane skeleton interactions. One of the sites of those interactions could be formed by aminophospholipid-rich domains or in particular, in the case of nonerythroid spectrin by anionic phospholipid-rich domains.

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