Brain spectrin (fodrin) interacts with phospholipids as revealed by intrinsic fluorescence quenching and monolayer experiments

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We demonstrate that phospholipid vesicles affect the intrinsic fluorescence of isolated brain spectrin. In the present studies we tested the effects of vesicles prepared from phosphatidylcholine (PtdCho) alone, in addition to vesicles containing PtdCho mixed with other phospholipids [phosphatidylethanolamine (PtdEtn) and phosphatidylserine] as well as from total lipid mixture extracted from brain membrane. The largest effect was observed with PtdEtn/PtdCho (3:2 molar ratio) vesicles; the effect was markedly smaller when vesicles were prepared from egg yolk PtdCho alone. Brain spectrin injected into a subphase induced a substantial increase in the surface pressure of monolayers prepared from phospholipids. Results obtained with this technique indicated that the largest effect is again observed with monolayers prepared from a PtdEtn/PtdCho mixture. The greatest effect was observed when the monolayer contained 50–60 % PtdEtn in a PtdEtn/PtdCho mixture. This interaction occurred at salt and pH optima close to physiological conditions (0.15 M NaCl, pH 7.5). Experiments with isolated spectrin subunits indicated that the effect of the β subunit on the monolayer surface pressure resembled that measured with the whole molecule. Similarly to erythrocyte spectrin–membrane interactions, brain spectrin interactions with PtdEtn/PtdCho monolayer were competitively inhibited by isolated erythrocyte ankyrin. This also suggests that the major phospholipid-binding site is located in the β subunit and indicates the possible physiological significance of this interaction.

Key words: cytoskeletal proteins, phospholipid vesicles, surface pressure.

INTRODUCTION

Spectrin and actin have previously been identified as major proteins of the mammalian erythrocyte skeleton, a proteinaceous network that is formed together with other membrane proteins such as ankyrin, protein 4.1, protein 4.2, adducin and dematin, as well as tropomyosin and tropomodulin (reviewed in [1,2]). Spectrins and spectrin-like proteins are abundant in most animal [3,4] and plant cells [5,6]. One of the best known non-erythroid spectrins is brain spectrin, also known as a fodrin [7,8]. Brain spectrin has the same type of structure as its erythrocyte analogue, with two kinds of polypeptide chain (α and β). Fodrin differs from erythrocyte spectrin in that it has a greater rigidity and inherent stability of the tetramer structure and binds calmodulin strongly.

It is known that erythrocyte spectrin interacts with the lipid domain in natural membranes [9,10] or in a model system ([11,12]; reviewed in [13]). The ability of spectrin to bind hydrophobic ligands such as brominated stearic acid [14,15] fatty acids, or anionic, cationic and zwitterionic detergents [16] lends support to the view that spectrin contains a number of hydrophobic sites. In particular, the βII domain of molecular mass 65 kDa (containing the ankyrin-binding site) [17] was suggested to be rich in such regions [18]. Although an interaction of erythrocyte spectrin with a membrane lipid bilayer (or monolayers) was first reported more than 20 years ago, its physiological significance is still a matter of dispute. Spectrin binding by monolayers and bilayers prepared from phosphatidylethanolamine (PtdEtn)/phosphatidylcholine (PtdCho) was inhibited by purified erythrocyte ankyrin [18a], which indicates that PtdEtn-rich domains might serve as alternative binding sites when there is not enough functional ankyrin to accommodate all spectrin tetramers in the membrane, or that its affinity to bind spectrin is decreased, e.g. after the phosphorylation of ankyrin [19,20].

Using a pelleting assay, we have observed the binding of purified brain spectrin to frozen and thawed liposomes (150–2500 nm in diameter) prepared from PtdEtn/PtdCho (3:2 molar ratio), PtdEtn/phosphatidylserine (PtdSer) (3:2), PtdSer/PtdCho (3:2) and PtdCho phospholipid mixtures [21]. Similar binding curves were obtained for vesicles prepared from the lipids extracted from bovine brain synaptic plasma membranes [21]. K_d (dissociation constant) values obtained in these studies (in the nanomolar range) are similar to those obtained previously (see, for example, [22]; reviewed in [1,23]) for erythrocyte spectrin. In the present studies we applied monolayer and fluorescence quenching techniques to explore this interaction further. We show that sonicated vesicles induce the quenching of intrinsic fluorescence observed with isolated brain spectrin in solution. Results of monolayer experiments indicate that brain spectrin causes a substantial increase in monolayer surface pressure. Results obtained by this technique indicate the optimum of this interaction under physiological conditions. PtdEtn/PtdCho was the most sensitive monolayer to the presence of brain spectrin in the subphase buffer. Further, our studies with isolated brain spectrin subunits and on the inhibition of brain spectrin–PtdEtn/PtdCho monolayer interactions by ankyrin demonstrate that the phospholipid (PtdEtn-rich)-binding site is within the brain spectrin β subunit, at or close to the ankyrin-binding site.

MATERIALS AND METHODS

Materials

Sephacryl S-500-HR, DEAE-Sephacel, acrylamide, Tris, dithiothreitol, EGTA, PtdEtn, PtdSer, dipalmitoyl phosphatidyl-

Abbreviations used: PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine.

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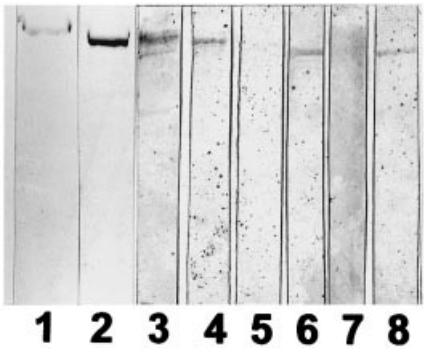

Figure 1 Isolation of brain spectrin subunits

Lanes 1 and 2, Coomassie Blue-stained SDS/PAGE gels of isolated α and β subunits respectively; lanes 3–8, Western blotting analysis with the use of rabbit antisera (1:500 dilution) directed against the separate spectrin α (lanes 3–5) and β (lanes 6–8) subunits. Lanes 3 and 6, brain spectrin; lanes 4 and 7, brain spectrin α subunit; lanes 5 and 8, brain spectrin β subunit. Antigen–antibody complexes were detected with the use of horseradish peroxidase-conjugated goat anti-(rabbit IgG), with 4-chloronaphth-1-ol/H_2O_2 as a substrate.

choline and dipalmitoyl phosphatidylethanolamine were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*,*N*′-Methylenebisacrylamide and EDTA were from Serva Feinbiochemica (Heidelberg, Germany). SDS was from BDH Laboratory Supplies (Poole, Dorset, U.K.).

Brain spectrin and its subunits

Brain spectrin was isolated from bovine brains by the method of Bennett et al. [24]. The final preparation contained only spectrin bands of apparent molecular masses 280 and 267 kDa with traces of a proteolytic 150 kDa fragment of the α-spectrin subunit. Purity was tested by SDS/PAGE in the Laemmli system [25]. Isolated protein was dialysed overnight against an appropriate buffer.

The α and β subunits of brain spectrin were isolated by column chromatography on a DEAE-cellulose (DE23) column in the presence of 7 M urea with a buffer system and a linear gradient as described by Bennett et al. [24]. The efficiency of subunit isolation was tested by 0.1 % SDS/7 % PAGE and Western blotting by the use of anti-(α-spectrin) and anti-(β-spectrin) antibodies (Figure 1). When brain spectrin preparation was stored for 1 week at 4 °C in the presence of 1 μM $CaCl_2$ and then subjected to DEAE-cellulose column chromatography as described above, a 150 kDa fragment (N-terminus) of the α subunit was obtained [26] instead of the intact α subunit. This fragment was the only component of the preparation, as tested by SDS/PAGE.

Lipids and liposomes

Total synaptic plasma-membrane lipids were extracted and analysed by using previously published methods (see [21]). Egg yolk PtdCho was prepared by the method of Van Deenen and de Haas [27].

Liposomes were prepared as follows: lipids or their mixtures were kept at −70 °C. The solvent was evaporated in a rotary evaporator; the lipid sample was then kept under vacuum (oil vacuum pump) for at least 2 h to remove traces of solvent. The dry lipid film was hydrated in 10 mM Tris/HCl/0.15 M NaCl/0.1 mM EGTA/0.1 mM 2-mercaptoethanol (pH 7.5), then shaken vigorously and sonicated (for a total of 20 min) and finally passed several times through first 0.1 μm and then 0.05 μm-pore-size membranes (Nucleopore) in a liposome extruder.

Fluorescence quenching experiments

Fluorescence quenching experiments were performed with a Kontron SFM 25 spectrofluorimeter at a constant temperature of 26 °C. Exciation was at 290 nm and emission was recorded between 320 and 350 nm. Under these conditions, the maximum was found at 337 nm. The control (100 % fluorescence) was a brain spectrin sample to which buffer had been added instead of liposome suspension. This value was corrected for light scattering by subtraction of the value obtained for a sample containing only an appropriate concentration of liposomes in the test buffer: 10 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1 mM EGTA/0.1 mM 2-mercaptoethanol. Fluorescence measurements were taken 15 min after the mixing of brain spectrin with the liposome suspension.

Monolayer experiments

Monolayer measurements were performed with a Wihelmy trough (surface area 24 cm^2) and a Nima tensiometer (Nima Technology, Coventry, U.K.) at room temperature (20 °C). Subphase buffer (25 ml), containing 5 mM Tris/HCl, pH 7.5, 0.5 mM EDTA, 150 mM NaCl, 0.5 mM dithiothreitol and 1 mM NaN_3, was stirred with a small stirrer bar (5 mm × 2 mm). If the salt optimum was to be tested, the test buffer contained the indicated NaCl concentrations. Monolayers were formed by the injection of a chloroform solution of the mixture of phospholipids with a Hamilton syringe. To test the effect of the pH of the phospholipid mixture on brain spectrin, we used monolayers constructed from PtdEtn/PtdCho (3:2) at pH 6.5, 7.0, 7.5 and 8.0. At pH values below 7.5, Mes was substituted for Tris/HCl. Aliquots of brain spectrin, dialysed against the same buffer, were injected into the subphase; the surface pressure (π) measurements were then obtained. For the experiments on the inhibition of brain spectrin–phospholipid interaction by erythrocyte ankyrin, purified [18a,29] ankyrin was first incubated with brain spectrin at the indicated ankyrin concentrations for 30 min at 20 °C and then injected into the subphase.

The data points are averages for several independent experiments with an average variation not larger than 10 %.

RESULTS

It has been shown previously that phospholipid vesicle suspensions added to a solution of erythrocyte spectrin induced quenching of the intrinsic fluorescence of the protein [30], similarly to typical fluorescence quenchers such as brominated fatty acids [14]. We are concerned with the mechanism by which brain spectrin interacts with membrane bilayer phospholipids, and thus we tested whether small vesicles induce the quenching of the intrinsic fluorescence of isolated brain spectrin. Figure 2 shows the results of fluorescence measurements obtained from brain spectrin in the presence of increasing concentrations of small phospholipid vesicles (and vesicles prepared from total lipids of the synaptic plasma membranes). Similarly to erythroid spectrin, lipid vesicles induced a decrease in intrinsic fluorescence of the brain protein. This effect was slightly smaller than that observed for erythrocyte spectrin; for the PtdEtn/PtdCho (3:2) mixture it did not exceed 40 %, whereas for PtdCho alone, small (approx. 10 %) quenching could be observed only in the presence of increased salt concentration (approx. 300 mM) (Figure 2B). A decreased PtdEtn content in the mixture gave a smaller quenching

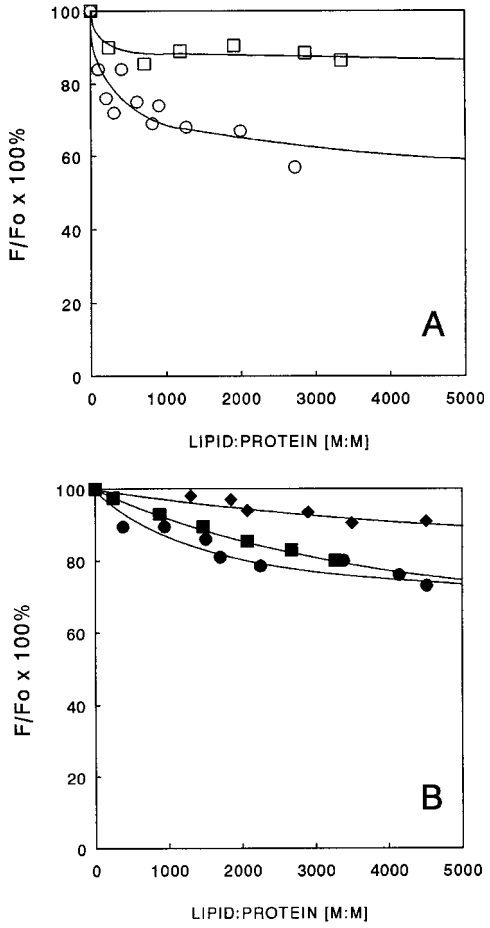

Figure 2 Effect of liposome suspension on the intrinsic fluorescence of isolated brain spectrin

Brain spectrin samples (1.8 ml, approx. 100 μg/ml) were made up in 10 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.1 mM EGTA/0.1 mM 2-mercaptoethanol. Up to 200 μl of liposome suspension was added, and the fluorescence (excitation at 290 nm, emission at 320–350 nm) was recorded 15 min after mixing. (**A**) Liposomes were prepared from PtdEtn/PtdCho (3:2) (○) or 1:9 (□) mixture. (**B**) Liposomes were prepared from: PtdSer/PtdCho (3:2) mixture (■), total lipid extracted from synaptic plasma membrane (●), PtdCho (◆). The effect of PtdCho liposomes was tested in the presence of an additional 150 mM NaCl in the buffer. F_0, fluorescence of brain spectrin; F, fluorescence of brain spectrin in the presence of phospholipid vesicle suspension.

effect (Figure 2A). In addition, liposome suspensions prepared from a total lipid mixture rich in PtdEtn [21] induced substantial quenching of the intrinsic fluorescence of brain spectrin (Figure 2B). The effect of liposome suspension on intrinsic spectrin fluorescence was only weakly sensitive to changes in ionic strength (results not shown). In addition, pH changes induced rather small effects in the fluorescence (within 10%) in the pH range 5.5–8.0. This effect was similar to the effect of pH on erythrocyte spectrin–phospholipid interaction, although no significant minimum at pH 5.5 was observed (results not shown).

The addition of bovine brain spectrin to the subphase buffer induced a change in surface pressure ($\Delta\pi$) of PtdEtn/PtdCho (3:2) monolayers at pH 7.5 as a function of initial surface pressure (π_i) (Figure 3A). The change in surface pressure ($\Delta\pi$) was increased for larger spectrin concentrations and at a smaller initial surface pressure of the monolayer. Control experiments included BSA or haemoglobin solutions added to the subphase formed by the 'test' buffer. At BSA concentrations identical with those of brain spectrin on either a molar basis or a w/v basis, a linear increase in π without saturation could be observed. When haemoglobin solution was injected into the subphase buffer a linear decrease in π was found, also without saturation (results not shown).

To test the effect of phospholipid composition on monolayer surface pressures induced by brain spectrin, we chose some negatively charged and uncharged phospholipids and their mixtures: PtdSer, PtdCho, PtdEtn and PtdEtn/PtdSer (3:2), as well as total lipid mixture extracted from synaptic plasma membrane at pH 7.5. As shown in Figures 3(B)–3(F), an increase in $\Delta\pi$ was observed after the addition of brain spectrin into the subphase, but the values obtained were much smaller. For comparison, the $\Delta\pi$ values obtained at a brain spectrin concentration of 2.3 μg/ml (π_i = 8–9 and 10–11 mN/m) are given in Table 1. These results show that the change in surface pressure under the same conditions was most marked for the monolayer formed from the PtdEtn/PtdCho (3:2) phospholipid mixture.

To determine which PtdEtn/PtdCho ratio was most favourable for interaction with bovine brain spectrin, we changed the composition of the mixtures, using PtdEtn/PtdCho ratios of 1:9, 3:7, 1:1, 3:2 and 4:1, as well as PtdEtn and PtdCho alone at pH 7.5. The results in Table 2 indicate that the largest change in surface pressure induced by brain spectrin in a PtdEtn/PtdCho monolayer was at a PtdEtn content of approx. 50–60%.

When a synthetic phospholipid mixture [dipalmitoyl phosphatidylethanolamine/dipalmitoyl phosphatidylcholine (3:2)] was used to construct a monolayer, the $\Delta\pi$ on the addition of brain spectrin into the subphase was almost exactly the same as with a natural phospholipid (PtdEtn/PtdCho) monolayer (Figure 3G). These two systems differed slightly: in the synthetic lipid monolayer the curves of $\Delta\pi$ against π_i were closer to a straight line.

In experiments to determine the effect of pH on the penetration of the monolayer film with brain spectrin, we measured changes in the surface pressure of PtdEtn/PtdCho (3:2) monolayers as a function of pH at various initial surface pressure values (Figure 4B) as well as at π_i = 9.6 mN/m at various brain spectrin concentrations (Figure 4A). These results indicate an optimum of this interaction at a pH close to 7.5.

Figure 5 shows the dependence of binding of brain spectrin to PtdEtn/PtdCho (3:2) monolayers on NaCl concentration in the range 0–0.5 M at two brain spectrin concentrations, 1.06 and 2.3 nM. The greatest effect was observed at 0.15 M NaCl, which is in good agreement with results obtained previously with an independent method [21].

Further, we addressed the question of whether this interaction (brain spectrin with phospholipid monolayer) is a property of the entire molecule or whether certain regions of the molecule are enriched in phospholipid-binding sites. One method of investigating this was to purify individual brain spectrin subunits (Figure 1) and to test their interaction with PtdEtn/PtdCho (3:2) monolayer at pH 7.5. The results presented in Figure 6 are relative to those from control experiments and measured changes in surface pressure in the subphase buffer after the addition of brain spectrin or its subunits or the α-subunit 150 kDa fragment. We observed an increase in surface pressure with the whole molecule of brain spectrin (Figure 6A) or with the purified β subunit (Figure 6B). The increase observed for the β subunit was larger for smaller π_i than that observed for whole brain spectrin. Results obtained for the α subunit of brain spectrin (Figure 6C) and for its proteolytic 150 kDa fragment (Figure 6D) demonstrate a decrease in surface pressure. Therefore, if we assume that the conformation of isolated spectrin subunits is similar to

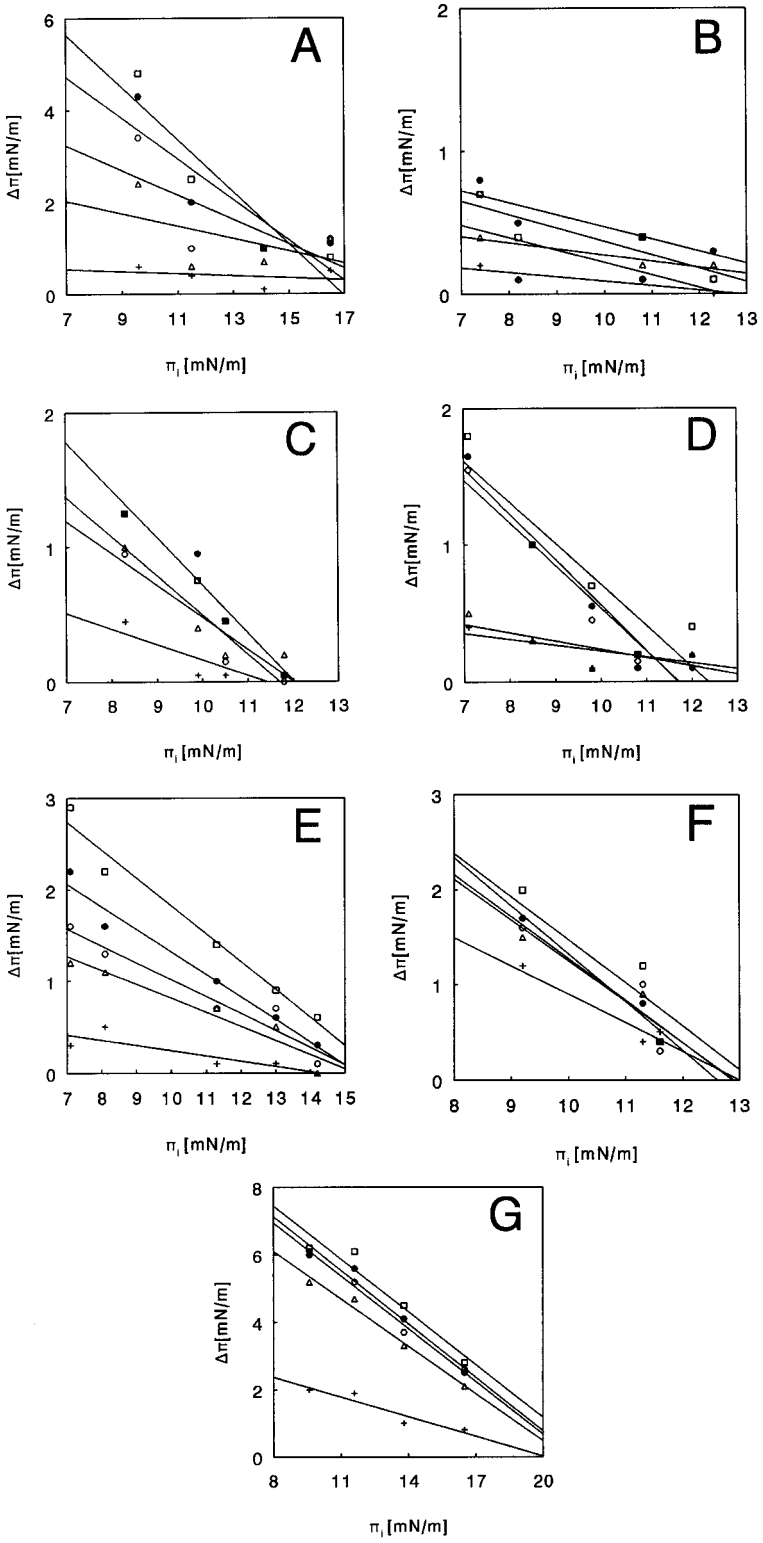

Figure 3 Change in the surface pressure ($\Delta\pi$) of monolayers as a function of the initial surface pressure (π_i) after the addition of bovine brain spectrin to the subphase

Monolayers were formed at pH 7.5 from PtdEtn/PtdCho (3:2) (**A**), PtdCho (**B**), PtdSer (**C**), PtdEtn/PtdSer (3:2) (**D**) or PtdEtn (**E**). Brain spectrin concentrations in the subphase are indicated by the following symbols: +, 0.39; △, 1.17; ○, 1.55; ●, 2.3; □, 2.83 μg/ml. (**F**) A monolayer was formed from the total phospholipid mixture extracted from synaptic plasma membranes; brain spectrin concentrations in the subphase are indicated by the following symbols: +, 1.95; △, 2.42; ○, 2.88; ●, 3.34; □, 3.79 μg/ml. (**G**) Monolayers were formed from dipalmitoyl phosphatidylethanolamine/dipalmitoyl phosphatidylcholine (3:2) at pH 7.5; brain spectrin concentrations in the subphase are indicated by the following symbols: +, 0.31; △, 1.26; ○, 1.56; ●, 2.48; □, 3.07 μg/ml.

Table 1 Change in surface pressure ($\Delta\pi$) of monolayers formed at pH 7.5 from PtdEtn, PtdCho, PtsSer, PtdEtn/PtdCho (3:2), PtdEtn/PtdSer (3:2) and total lipid phospholipid mixtures after the addition of brain spectrin to the subphase

The results are the change in surface pressure of a PtdEtn/PtdCho monolayer after the addition of 2.3 μg/ml brain spectrin to the subphase buffer; π_i is the initial surface pressure.

	$\Delta\pi$ (mN/m)	
Phospholipid	π_i = 8–9 mN/m	π_i = 10–11 mN/m
PtdEtn	0.1	0.1
PtdEtn/PtdCho (3:2)	3.6	1.2
PtdEtn	1.5	0.9
PtdEtn/PtdSer (3:2)	0.4	0.2
PtdSer	0.8	0.35
Total lipid	2.2	0.4

Table 2 Dependence of the change in surface pressure ($\Delta\pi$) of a PtdEtn/PtdCho monolayer on PtdEtn content after the addition of brain spectrin to the subphase

Measurements were taken at pH 7.5. The results are the change in surface pressure of a PtdEtn/PtdCho monolayer after the addition of 2.12 nM brain spectrin to the subphase buffer; π_i is the initial surface pressure.

	$\Delta\pi$ (mN/m)	
PtdEtn content (%)	π_i = 8.0 mN/m	π_i = 10.5 mN/m
0	0.1	0.1
10	0.5	0.2
30	1.3	1.2
50	3.2	1.6
60	4.3	1.2
80	1.0	1.1
100	1.5	0.9

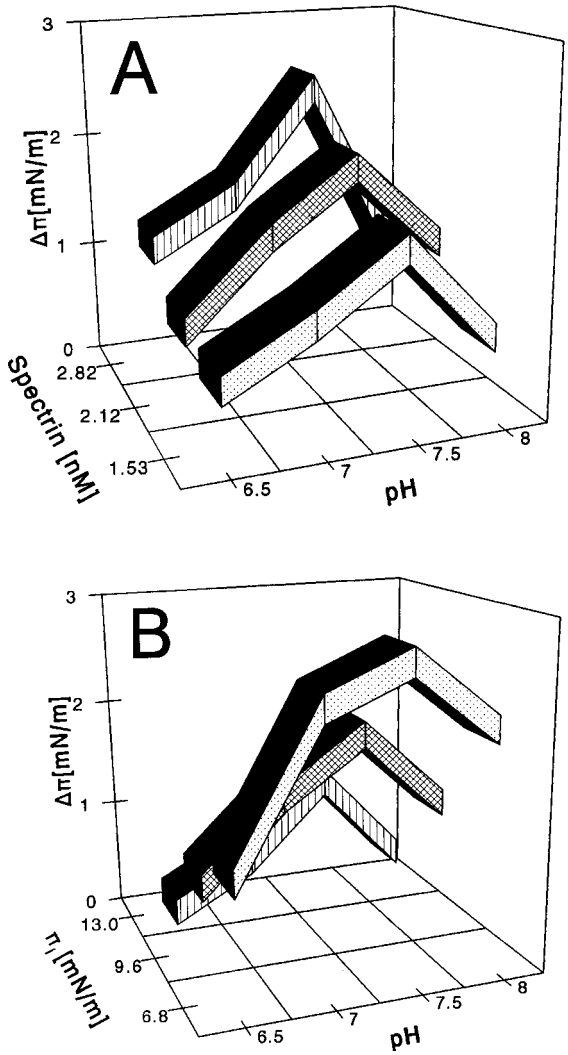

Figure 4 Effect of pH on the binding of brain spectrin to a PtdEtn/PtdCho (3:2) monolayer

(**A**) Results obtained at the indicated brain spectrin concentrations. The initial surface pressure was 9.6 mN/m. (**B**) Results obtained at the indicated initial surface pressure values of the monolayer. The brain spectrin concentration was 2.12 nM.

that of the native spectrin molecule, we conclude that β-spectrin more or less mimics the effect of whole spectrin. The effect of the α subunit or its 150 kDa fragment at comparable concentrations indicates a 'compression' of the monolayer or the extraction of lipid molecules from the monolayer.

Another approach to the identification of phospholipid-binding sites in brain spectrin was to test whether the interaction of brain spectrin was sensitive to inhibition with purified erythrocyte ankyrin. Figure 7 shows the effect of ankyrin on the binding by brain spectrin of a PtdEtn/PtdCho (3:2) mixture monolayer at pH 7.5. Brain spectrin and ankyrin were mixed and incubated at room temperature for 30 min before they were injected into the subphase. The molar ratio of spectrin to ankyrin was changed from 1:0.25 to 1:1.75. In control experiments only brain spectrin at the same concentration as the subphase was added. Figure 7(A) shows three examples of inhibition curve. The largest inhibition of binding of brain spectrin to the PtdEtn/PtdCho monolayer by ankyrin (approx. 40–50 %) was observed in experiments at a 1:1 molar ratio of brain spectrin to ankyrin. These results were similar to those obtained for the inhibition of interaction of erythrocyte spectrin with PtdEtn/PtdCho vesicles by ankyrin [18a], indicating an occurrence of other, 'ankyrin-insensitive', sites of phospholipid binding on the spectrin molecule. Ankyrin alone induced a small (up to 1.5 mN/m at 8 nM ankyrin and 0.5 mN/m at 3 nM ankyrin) linear increase in surface pressure of the PtdEtn/PtdCho monolayer at an initial surface pressure of 9–11 mN/m. When the α subunit of erythrocyte spectrin was used instead of brain spectrin, no difference in $\Delta\pi$ values was observed [31]. A Dixon [32] plot of the results of ten inhibition curves, with the assumption that $\Delta\pi$ corresponded to the amount of 'bound' phospholipid shown in Figure 7(B), indicated that erythrocyte ankyrin competitively inhibited the binding of brain spectrin to the PtdEtn/PtdCho (3:2) monolayer, suggesting that the major phospholipid-binding site (at least in mixtures rich in PtdEtn) is located in the β subunit, at approximately the region of ankyrin binding [17]. In addition, the two values of K_i (inhibition constant, mean $\pm$ S.D.), 0.791 ± 0.047 nM obtained in the brain spectrin concentration range 0.6–1.2 nM, and 1.71 ± 0.42 nM at brain spectrin concentrations above 1.5 nM, suggest a low negative co-operativity in erythrocyte ankyrin–brain spectrin interactions. The results obtained in our studies are lower than the K_d values (2–50 nM, depending on the experimental technique) published by Bennett's

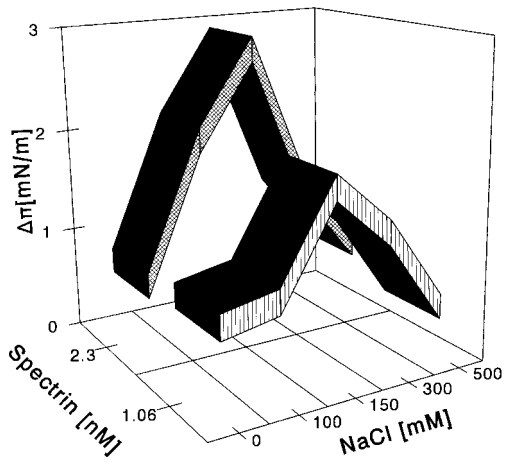

Figure 5 Effect of NaCl concentration in the subphase buffer on the binding of brain spectrin to a PtdEtn/PtdCho (3:2) monolayer at pH 7.5

The brain spectrin concentration was as indicated; π_i was 15.2 mN/m.

group [29,33]. These differences could be due to the different experimental approaches used.

DISCUSSION

The brain spectrin preparation used in this study consisted mostly of 'non-erythroid' type (αIIΣ1/βIIΣ1) spectrin, because this isoform is the most abundant in forebrain tissue. Furthermore, the method of isolation that we used [24] involved the initial isolation of synaptosomes, which are mostly axonal membranes containing exclusively the non-erythroid isoform of spectrin [34].

Our previous results demonstrated that brain spectrin binds phospholipids of large liposomes. From pelleting assays we obtained K_d values in the nanomolar range: from 21 nM for a PtdEtn/PtdCho (3:2) phospholipid mixture at pH 6.0, to 531 nM for PtdCho liposomes at pH 6.0 [21]. It should be noted that the affinity of the interaction between brain spectrin and membrane phospholipids is only an order of magnitude smaller than those between ankyrin and spectrin (K_d in the range 2–50 nM) [29,33]. It is difficult to obtain quantitative parameters of this interaction (K_d and B_{max}) by using the method of quenching of intrinsic fluorescence. However, it yielded information that this interaction affects the properties of the protein. Here we also used surface-pressure measurements of phospholipid monolayers after the addition of brain spectrin (or its subunits or the α subunit fragment) into the subphase buffer as a further demonstration of this interaction. This method, although fast and useful, gives only comparative results, because the measurements were performed at rather low initial surface-pressure values (10–15 mN/m), far from the 30–35 mN/m suggested as being appropriate for comparison with natural membrane bilayers [35]. The results obtained with both methods indicate that for aminophospholipids, and specifically PtdEtn, the effect was much larger than for (egg) PtdCho alone. Moreover the effect was proportional to the content of PtdEtn in the PtdEtn/PtdCho mixture (compare Figure 2A with Figure 3 and Table 2). This result is different from that observed previously in the pelleting assay, in which we did not find substantial differences between

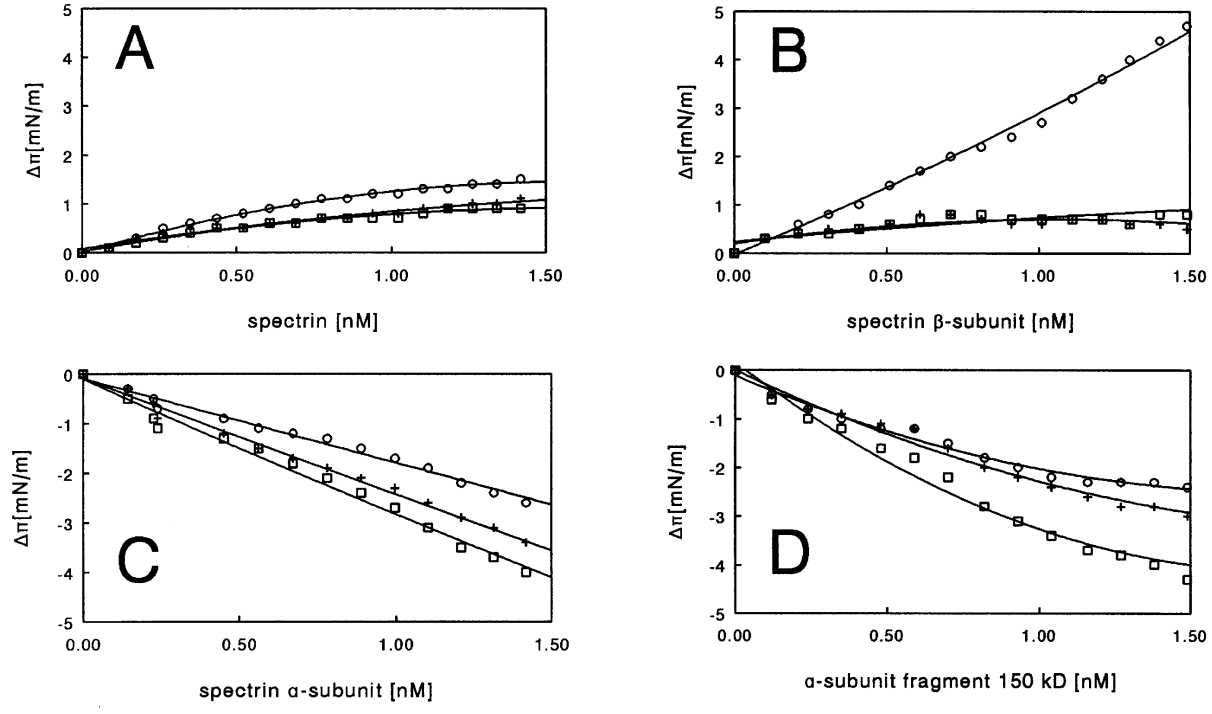

Figure 6 Change in surface pressure of PtdEtn/PtdCho (3:2) monolayer after the addition to the subphase of whole brain spectrin, its separated subunits or the purified 150 kDa fragment of the α-subunit

(**A**) Whole brain spectrin, π_i = 5.5 (○), 8.5 (+) or 12.4 (□) mN/m; (**B**) β subunit of brain spectrin, π_i = 7.7 (○), 10.7 (+) or 12.8 (□) mN/m; (**C**) α subunit of brain spectrin, π_i = 6.4 (○), 8.7 (+) or 13.6 (□) mN/m; (**D**) 150 kDa proteolytic fragment of α subunit of brain spectrin, π_i = 7.0 (○), 8.1 (+) or 12.7 (□) mN/m. Measurements were taken at pH 7.5.

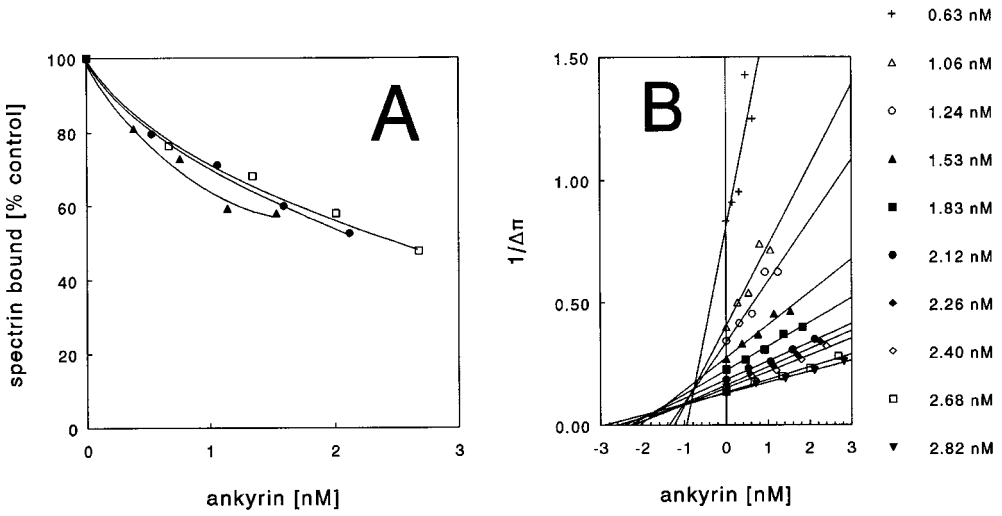

Figure 7 Bovine erythrocyte ankyrin inhibits the binding of bovine brain spectrin to PtdEtn/PtdCho monolayers

(**A**) Inhibition curves obtained at pH 7.5 and π_i values of 9–11 mN/m for brain spectrin concentrations of 1.53 (▲), 2.12 (●) or 2.68 (□) nM. (**B**) Dixon [32] plot of the results obtained for 10 indicated concentrations of brain spectrin. Two different K_i values can be obtained: 0.791 ± 0.047 nM (mean ± S.D.) for brain spectrin concentrations in the range 0.6–1.2 nM, and 1.71 ± 0.42 nM at brain spectrin concentrations over 1.5 nM.

either K_d values or B_{max} values between PtdCho and aminophospholipid vesicles [21]. In particular, the latter parameter corresponds to the maximal fluorescence quenching effect or to the maximal $\Delta\pi$ values. However, it should be noted that these values also depend on many other parameters, such as the initial surface pressure and the salt concentration in the solution. One of the major factors underlying the differences between the results of previous studies, using large liposomes [21], and those presented here could be the packing density of the lipid bilayer or monolayer. In fluorescence experiments in which small vesicles were used, the bilayer experiences an additional stress due to the smaller radius of curvature [36] than in large vesicles. As mentioned above, the monolayer experiments described here were performed at relatively low initial surface-pressure values. Similarly to our present studies of erythrocyte spectrin, we did not observe substantial differences in K_d and B_{max} values for binding large liposomes composed of similar lipid mixtures [18a]. The specificity of erythrocyte spectrin towards PtdEtn (when mixed with PtdCho) is not related to the formation of the HII phase, as illustrated by mixing erythrocyte spectrin with HII-forming dioleoyl phosphatidylethanolamine, resulting in no quenching of intrinsic fluorescence [37]. An experiment on the effect of brain spectrin on the surface pressure of the dipalmitoyl phosphatidylethanolamine/dipalmitoyl phosphatidylcholine (3:2) monolayer indicates that the difference in $\pi \rightarrow A$ isotherm (a compression curve which gives information on the intermolecular repulsions of the lipid components in monolayers formed at the air/water interface) is not a major factor determining the difference in the magnitude of the effect of brain spectrin on the surface pressure of the phospholipid monolayer.

The optimal conditions for the penetration of phospholipid monolayers by brain spectrin are similar to those commonly considered to be physiological conditions: pH optimum approx. 7.5, sodium salt optimum 0.15 M NaCl. These results are in accordance with our previous studies employing a pelleting assay method [21]. However, no such optima were observed with fluorescence measurements, which might indicate that the differences in the net fluorescence quenching effect are not necessarily dependent on the amount of phospholipid bound or on the pH- or salt-induced conformational changes of protein. Because changes in fluorescence are related to conformational changes (the disposition of tryptophan residues), they need not be identical with changes observed in monolayer experiments. It was previously demonstrated that the binding of phospholipid vesicles affected the conformational changes of erythrocyte spectrin occurring during thermal denaturation [30]. As suggested above, it is possible that there are several kinds of phospholipid-binding site [38]; it is known that many tryptophan residues are present in the spectrin molecule. However, the NMR data of Gratzer and colleagues [15] indicate that only a limited number of erythrocyte spectrin tryptophan residues are engaged in hydrophobic interactions.

The β subunit of brain spectrin induces an effect similar to that of the whole molecule of brain spectrin, i.e. an increase in surface pressure that is probably the result of a penetration of the monolayer. We have previously shown that ankyrin inhibits the binding of erythrocyte spectrin to phospholipid vesicles or monolayers. Because the region of the ankyrin-binding site is highly conservative among spectrins, it is possible that this effect might be observed with brain spectrin. Indeed, the results of our experiments show that the binding of the PtdEtn/PtdCho mixture to brain spectrin is sensitive to inhibition by purified erythrocyte ankyrin. This further substantiates the suggestion that the major phospholipid-binding site is located in the β subunit of the spectrin molecule.

On the basis of our results, we suggest that there is a strong, direct interaction between brain spectrin and neuronal plasma-membrane phospholipids. Its physiological significance, however, is not completely clear. There are many kinds of proteinaceous binding site in non-erythroid (particularly neuronal) cell membranes. Results from our laboratory indicate that neuronal membrane contains spectrin-binding sites that are resistant to proteolysis and treatment with NaOH (W. Diakowski and A. F. Sikorski, unpublished work). It should be noted that even in the model system there are at least two types of lipid-binding site in the brain spectrin molecule: 'ankyrin-sensitive' and 'ankyrin-

insensitive'(see Figure 7). Because it has been also shown that the isolated repeat of the dystrophin rod domain binds strongly to phospholipids [38], it is possible that this kind of 'general' interaction is also present in the brain spectrin molecule.

The role of the membrane skeletal network in the erythrocyte membrane is understood much better than the role of its non-erythroid analogue. Available results indicate that the presence of interactions of skeletal membrane proteins with membrane lipids increase the density of 'support' points for the membrane bilayer; these interactions would therefore participate in creating the mechanical properties of the membrane. We believe that an important class of the interactions described above is the 'ankyrin-sensitive' interaction of brain spectrin with PtdEtn-rich lipid mixtures. The importance of this interaction is further indicated by the following.

(1) The spectrin (tetramer)-to-ankyrin ratio in natural membrane is always 1:1, even though the spectrin tetramer contains two identical ankyrin-binding sites [39]. This was also observed on electron micrographs made by Byers and Branton [40], in which some of the spectrin tetramers contained one or two ankyrin molecules but others contained none. The presence of the latter indicates that in the membrane, spectrin filaments should be maintained by interactions between spectrin and phospholipid (PtdEtn-rich domains).

(2) Mutant mice whose erythroblasts fail to synthesize ankyrin are still able to accumulate approx. 50% spectrin in their erythrocyte membranes [41].

(3) It has been shown that the phosphorylation of ankyrin substantially decreases its affinity for spectrin [19,20].

(4) Ankyrin is one of the last membrane skeleton components synthesized by developing cells [42]. Thus lipidic binding sites are of particular importance during the early stages of membrane biogenesis before all membrane 'adaptor proteins' such as ankyrin have been synthesized and recruited to the membrane.

Moreover the studies by Wang and Shaw [43] indicate that the pleckstrin homology domain might be involved in lipid binding, and that this binding coincides with the 1,3,5-trisphosphoinositol-binding site. The spatial relationship between the ankyrin-binding site and the pleckstrin homology domain within the brain spectrin molecule also suggests a functional connection between lipid binding and spectrin function. This possibility needs to be explored further and is currently the subject of examination in our laboratory.

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