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Characteristics of spectrin-induced leakage of extruded, phosphatidylserine vesicles

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At neutral pH spectrin induces modest leakage of trapped calcein from reverse-phase or extruded, but not sonicated, vesicles composed of phosphatidylserine, but not phosphatidylcholine. The extent of leakage from extruded vesicles is not or is only slightly affected by magnesium ions at a physiological concentration or calcium ions at a greater than physiological concentration, respectively. In addition to accounting for several previously discrepant observations on the lytic effects of spectrin, these findings indicate that some proteins like spectrin may destabilize vesicles with low curvature more readily than vesicles of high curvature, in contrast to certain amphiphilic peptides. 60% less leakage is induced from phosphatidylserine vesicles by heat-denatured than by native spectrin. In contrast, both trypsin- and subtilisin-treated spectrins, if sufficiently digested, induce several-fold more leakage than undigested spectrin. Since spectrin prepared either by 1 M Tris dissociation of Triton-extracted cytoskeletons or by low ionic strength extraction of ghosts released the same amounts of calcein from vesicles of various compositions, these effects are unlikely to reflect artifacts of spectrin preparation. Furthermore, spectrin is unlikely to promote leakage in vivo, since vesicles composed of phosphatidylserine, cholesterol and/or phosphatidylethanolamine, which constitute the lipid composition of the inner monolayer of the red cell membrane, did not leak on addition of spectrin, whereas vesicles composed of phosphatidylserine and phosphatidylcholine, did leak in the presence of spectrin.

Introduction

The red cell skeleton, a protein network on the inner surface of the cell membrane [1,2], is responsible for the elasticity and stability of the red cell as it negotiates the microcirculation [3–5]. The protein spectrin is almost certainly the key component of the skeleton with respect to its elasticity, since the spectrin dimer appears to be highly extensible [6] and constitutes the bulk of the cytoskeletal network. We are interested in how spectrin interacts with the membrane, particularly since a recent theoretical treatment of cytoskeletal elasticity [7] indicates that contacts between spectrin and inner monolayer lipid are likely to affect the elasticity of the circulating red cell. It is well-established that spectrin is attached to the red cell membrane by specific interactions with the proteins ankyrin [8,9] and band 4.1 [9]. The former is, in turn, bound to intrinsic membrane protein band 3 [8], and the latter is, in turn, bound to glycophorin, another intrinsic membrane protein [10,11], or to band 3 [12,13]. In addition to band 4.1 and ankyrin, acidic phospholipids of the type found in the inner monolayer of red cells also have an affinity for spectrin [14–16]. The binding constant for the *in vitro* interaction between spectrin and phosphatidylserine (Takeshita and MacDonald, in the press) is about an order of magnitude less than that for spectrin binding to ankyrin and/or band 4.1 [8,9]. Nevertheless, given an estimated concentration of spectrin at the red cell membrane of $5 \cdot 10^{-4}$ M [17], which considerably exceeds

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Abbreviations: DMSO, dimethylsulfoxide; DOPC, dioleoylphosphatidylcholine; Hepes, *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; PC, phosphatidylcholine; PE, phosphatidylethanolamine; POPE, palmitoyloleoylphosphatidylethanolamine; PS, phosphatidylserine; REV, reverse phase evaporation vesicle; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; SUV, sonicated unilamellar vesicle; UV CD, ultraviolet circulation dichroism.

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the PS-spectrin dissociation constant of approx. 10^{-6} M, and provided that the spectrin-PS dissociation constant measured *in vitro* obtains in the cell, PS-spectrin associations should exist *in vivo* most of the time.

With the aim of characterizing PS-spectrin associations and ultimately assessing the contribution of spectrin–lipid interactions to red cell elasticity, we re-examined earlier, conflicting observations that spectrin does [18] or does not [19] cause an increase in the permeability of negatively charged vesicles to small molecules. We have resolved this apparent difference by affirming that at neutral pH spectrin does induce the leakage of unsonicated, negatively charged vesicles, which were used in the former study [18], but not of sonicated, phosphatidylserine vesicles, which were used in the latter study [19]. The characteristics of the modest, spectrin-induced leakage of the extruded phosphatidylserine vesicles described here suggest that in order to promote leakage, spectrin must perturb the headgroups of the phosphatidylserine bilayer, at least initially.

In addition to affirming that pure, native spectrin induces modest leakage of unsonicated, but not sonicated, acidic lipid vesicles under physiological conditions, we report the following new observations. (1) Neither Ca^{2+} at a greater than physiological concentration nor Mg^{2+} at a physiological concentration synergistically enhance spectrin-induced leakage of unsonicated, acidic phospholipid vesicles. (2) Spectrin does not promote aggregation of phosphatidylserine vesicles undergoing leakage. (3) Heat-denaturation reduces, but proteinase treatment enhances the leakage-inducing activity of spectrin. (4) Spectrin dimer and tetramer prepared by the 1 M Tris method induce the same modest leakage as spectrin dimer eluted from ghosts in low salt. (5) Vesicles composed of inner monolayer, red cell lipids (i.e., phosphatidylserine, phosphatidylethanolamine and cholesterol) – but not vesicles composed of phosphatidylserine and phosphatidylcholine – are refractory to spectrin-induced leakage.

Materials and Methods

Preparation of vesicles. Lipids were from Avanti Polar Lipids (Alabaster, AL). Lipids and vesicles were stored under nitrogen or argon. Extruded vesicles were used in all the experiments unless otherwise mentioned. These were prepared by hydrating dried films of bovine brain phosphatidylserine (PS), egg phosphatidylcholine (PC), dioleoylphosphatidylcholine (DOPC) and palmitoyloleoylphosphatidylethanolamine (POPE) or combinations thereof in 60 mM calcein (Hach Chemical Co.; Loveland, CO), pH 7.4, by freeze-thawing 10 times. The resulting multilamellar vesicles were extruded through two 0.1 μm diameter pore polycarbonate filters (Nuclepore Corp.; Pleasanton, CA) 11 times or more in a push-pull, syringe-mounted extrusion apparatus (MacDonald et al., in the press). Free calcein was separated from the dye-containing liposomes by chromatography on a Sephadex G-75 or Bio-Gel A-5m column equilibrated with 5 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA, pH 7.4 (pH 7.4 Tris buffer). Small unilamellar vesicles (SUVs) were prepared by sonicating freeze-thawed vesicles until the preparation was transparent. Reverse phase evaporation vesicles (REVs) were prepared according to a published procedure [20] and extruded once through one or two 0.1 μm diameter pore polycarbonate filters. Lipid concentrations of the preparations were measured by their phosphorus content [21]. The ratios of included volumes of the vesicles prepared by these different methods were ascertained by lysing aliquots of vesicles containing identical amounts of phospholipid with Triton X-100 and determining the ratios of fluorescence intensities of the resulting solutions.

Leakage determinations. Leakage was measured by a previously described method [22]. The Tris-HCl, pH 7.4 buffer described above was used unless otherwise mentioned. Various amounts of spectrin or melittin (Sigma Chemical, St. Louis, MO; 70% pure by HPLC) were added to 0.2 ml aliquots of vesicles in pH 7.4 Tris buffer at a final lipid concentration of 5–22 μM and vortexed. The fluorescence intensity was recorded for 3 h at room temperature at suitable intervals. The excitation wavelength was 490 nm and the emission wavelength was 520 nm, the bandwidth in both cases being 2.5 nm. In some cases 480 nm and 520 nm interference filters were used, the bandwidths of which were 10 nm. 100% leakage was determined by adding Triton X-100 to each sample to a concentration of 1% (v/v). The background leakage from each lipid sample in the absence of spectrin was subtracted from samples with spectrin to obtain the spectrin-induced, percent leakage. Each value represents the average of duplicates or triplicates of the sample described, which did not vary by more than 10%. The % leakage for each lipid/protein ratio – but not the relative spectrin sensitivities of different lipid combinations – varied from 10–15% with different batches of spectrin. Therefore, each experiment was conducted with the same batch of protein, unless otherwise noted.

Spectrin isolation. Spectrin dimers were isolated from human erythrocytes by a procedure to be published (Takeshita et al., submitted). Erythrocyte ghosts were prepared by lysing the washed cells with hypotonic phosphate buffer at pH 7.4 [23]. The ghosts were solubilized in 2% Triton X-100 buffered with 12 mM Hepes (pH 7.0) in the presence of 0.5 mM ATP, 0.25 mM DTT and 0.5 mM EGTA, and the resulting cytoskeletons separated from lipid, intrinsic membrane proteins and detergent by centrifuging through a layer of 30% sucrose containing 1.5 M NaCl [24]. The cyto-

skeletal proteins were dissolved in 1 M Tris, 0.5 mM EGTA, 0.5 mM ATP (pH 7.0) as described by Ohanian and Gratzer [25], and chromatographed on a Sepharose CL-6B column equilibrated with the same buffer to separate spectrin from band 4.1 and actin. The spectrin dimers, tetramers and oligomers were separated by chromatography on a Sepharose CL-4B column [26]. The protein was concentrated after each chromatography step by precipitation in 50% ammonium sulfate. Protein was assayed by the Bradford method [27]. The purified spectrin consisted solely of the characteristic Band 1-Band 2 doublet on SDS-PAGE analysis [28] and was stored at 4°C in 25 mM Tris-HCl (pH 7.6), 100 mM NaCl, 1 mM Na_2EDTA, 0.02% NaN_3. Spectrin was used before the dimer-tetramer association constant and the near-UV CD spectrum became significantly different from the values measured immediately after protein isolation (Takeshita et al., submitted).

Spectrin was also isolated by the more commonly used method of low salt elution from ghosts [29]. After concentration by precipitation in 50% ammonium sulfate, the extracted protein was subjected to CL-Sepharose 4B chromatography, as was 1 M Tris-extracted spectrin, to obtain spectrin dimers.

Spectrin treatments. Spectrin was denatured by heating a stock solution of the native protein at 0.35 mg/ml in a boiling water bath for 10 min. The solution was reassayed for protein to correct for losses due to precipitation onto the glassware. Spectrin was protease-treated by incubating the native protein with trypsin (Sigma, type I) or with subtilisin (Sigma) in pH 7.4 Tris buffer at spectrin/trypsin (w/w) ratio of 100:1 or spectrin/subtilisin (w/w) ratios of 200:1 at room temperature for 2 h. Digestions were terminated by addition of phenylmethylsulfonyl fluoride (PMSF) in dimethylsulfoxide (DMSO).

Turbidity measurements. Light scattering of vesicles containing 300 μM or 75 μM PS or PC was measured in the absence or presence of spectrin at a lipid/protein ratio of about 300:1 or 75:1 after 3 h at room temperature. Light scattering was determined in a fluorometer with the excitation and emission wavelengths set at 500 nm, 5 nm bandwidths and a homemade mask to reduce the exciting light. Background contributed by spectrin alone was subtracted from the light scattering of spectrin-containing samples.

Results

Time course of spectrin-induced leakage from PS vesicles under conditions of physiological ionic strength and pH

Fig. 1 depicts spectrin-induced leakage of calcein from extruded, PS and PC vesicles. The dye is at self-quenched concentrations in the liposomes; dilution into the external aqueous phase leads to relief of self-quenching and the resulting increase in fluorescence

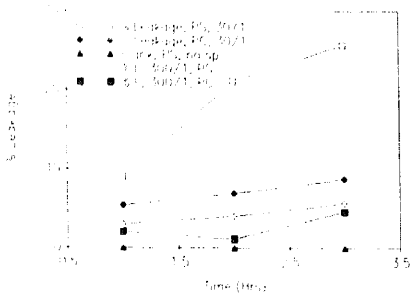

Fig. 1. Percent leakage of calcein induced by spectrin from phospholipid vesicles. PS vesicles at lipid/protein ratio of 30:1 (open squares), PS vesicles at lipid/protein ratio of 300:1 (open diamonds), PS vesicles without spectrin (closed triangles), PC vesicles at lipid/protein of 30:1 (closed diamonds) and PC vesicles at lipid:protein of 300:1 (closed squares). The buffer in each case was 5 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA (pH 7.4). The concentration of PS was held constant while the spectrin concentration varied from 78.7 μg/ml (open squares and closed diamonds) to 7.87 μg/ml (open diamonds and closed squares) to give varying lipid/protein ratios.

intensity is monitored. The fluorescence intensity at a given time is expressed as a percentage of the maximum intensity obtained when all the vesicles are lysed with Triton X-100 and is referred to here as percent leakage. Leakage begins immediately after addition of the protein and continues for at least 3 h. The rate of leakage for the first approximately 10 min is marginally higher than for the subsequent phase and it continues at a relatively constant rate for the duration of the experiment.

The rate of leakage depends significantly on the ratio of spectrin to lipid. In the case of phosphatidylserine vesicles, an increase in the protein/lipid ratio from 1:300 to 1:30 caused an increase in the % leakage from 6% to 26% at the end of the 3 h incubation period. For phosphatidylcholine vesicles, the same increase in the protein/lipid ratio caused an increase in % leakage from 4.6% to 9%.

Spectrin-induced leakage increases with size of vesicle

Sweet and Zull [18] reported about 20% leakage from unsonicated, and presumably multilayered, vesicles composed of dicetyl phosphate and phosphatidylcholine in the presence of spectrin at neutral pH, whereas Juliano et al. [19] reported no leakage from sonicated, PS vesicles at neutral pH. Although sonicated vesicles, the outer monolayers of which are not close-packed and, therefore, under stress, are sometimes more readily perturbed by amphiphilic peptides than unsonicated vesicles (see Ref. 30, for example), it appeared likely that in this case sonicated vesicles might be less responsive to spectrin than unsonicated vesicles. We therefore compared leakage induced by spectrin from SUVs, extruded vesicles and REVs under identical conditions of

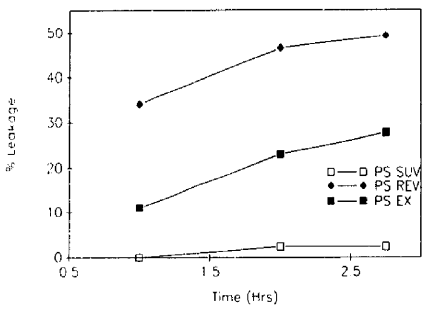

Fig. 2. Percent leakage of calcein induced by spectrin from vesicles of different sizes. PS SUVs (open squares), PS REVs (closed diamonds) and PS extruded vesicles (closed squares). The lipid/protein mole ratio was 22.5 : 1 and lipid concentration was 1 μM for each sample.

concentration and lipid/protein ratio. Spectrin induced most leakage from REVs and least from SUVs (Fig. 2). The trapped volumes of the three liposome samples were determined by lysing aliquots of the liposome preparation with Triton X-100 and measuring the calcein fluorescence of the resulting solutions. By this measurement, the ratio of the included volumes (sizes of the vesicles) was 1 : 2.6 : 5.9 (SUV/extruded/REV). Thus, leakage induced by spectrin was greatest from the largest liposomes and least from the smallest liposomes.

Low concentrations of divalent cations have a negligible effect on spectrin-induced leakage

Since it had been reported that 0.5 mM Ca^{2+} synergized with spectrin at neutral pH to induce leakage of sonicated vesicles [19], it was necessary to determine whether this synergism could be demonstrated with unsonicated vesicles. Given the need for much higher concentrations of Ca^{2+} to induce fusion of LUVs than of SUVs [31], possibly due to higher surface pressure of the outer monolayer of LUVs than SUVs [32], it seemed likely that the previously observed synergism between Ca^{2+} and spectrin reflected primarily an effect of Ca^{2+} on SUVs and not an effect of Ca^{2+} on spectrin. Release of calcein from extruded PS or PC vesicles in the presence of spectrin was measured in the presence and absence of Mg^{2+} or Ca^{2+}. $CaCl_2$ was added to the lipid sample before addition of spectrin to bring the final Ca^{2+} concentration to 0.5 mM, as in [19], which is much higher than the highest concentration measured in normal red cells but close to the Ca^{2+} concentration in sickle cells [33]. 0.5 mM Ca^{2+} caused a slight increase (i.e., from 26 to 33%) in leakage of extruded vesicles also exposed to spectrin (Table I), but not the greater than 400% increase observed by other workers [19] from SUVs also exposed to spectrin. 0.5 mM Ca^{2+} alone did not cause leakage of the vesicles. Addition of $CaCl_2$ after leakage had been induced by spectrin also did not alter the leakage pattern. Addition of $MgCl_2$ at a final concentration of 2 mM, an approximately physiological level [34], did not alter the leakage induced by spectrin.

Spectrin does not cause vesicles to aggregate

Spectrin at a lipid/protein ratio of 288 : 1 did not affect the light scattering of duplicate samples of vesicles containing 300 μM PC or PS, measured after incubation at room temperature for 3 h. PS alone gave a value of 1.65 light intensity units, whereas PS + spectrin gave a value of 1.68 light intensity units. PC alone gave a value of 1.59 light intensity units, whereas PC + spectrin gave a value of 1.72 light intensity units. Spectrin at a lipid/protein ratio of 74 : 1 caused a slight decrease in the light scattering of PS vesicles – i.e., 0.476 light intensity units in the presence of spectrin but 0.589 light intensity units in the absence of spectrin. This lack of increase in light scattering indicates that spectrin does not cause PS vesicles to aggregate.

Heat denaturation decreases the capacity of spectrin to induce leakage of liposomes

The effect of heat denaturation on the capacity of spectrin to induce leakage of liposomes was studied. Heating a 0.35 mg/ml solution of spectrin in 25 mM Tris-HCl, 100 mM NaCl, 0.1 mM EDTA (pH 7.6) in a boiling water bath for 10 min followed by cooling in an ice bath reduced the protein concentration to 0.1 mg/ml, because a considerable amount of the denatured protein was lost on the surface of the glassware. The native protein solution diluted to 0.1 mg/ml induced 11.4% leakage from PS liposomes at a lipid/protein ratio of 70 : 1 while the heat denatured protein at that concentration induced only 4% leakage in 3 h. Denaturing the protein thus considerably reduces the capacity of spectrin to induce leakage of liposomes.

Trypsin or subtilisin treatment increases the vesicle permeabilizing activity of spectrin

Fig. 3 shows that trypsin or subtilisin treatment of spectrin increased its ability to induce leakage from

TABLE I

Leakage of PS and PC vesicles in the presence of spectrin with and without Ca^{2+} or Mg^{2+}

The lipid/protein mole ratio was 79 : 1, and the final lipid concentration was 8 μM. Leakage induced after 3 h of incubation at room temperature by 0.5 mM Ca^{2+} and 2 mM Mg^{2+} in the absence of spectrin was negligible, both from PS and PC (not shown).

Lipid	Divalent cation	Percent leakage
PS	0	26
PS	0.5 mM Ca^{2+}	33
PS	2 mM Mg^{2+}	21
PC	0	9
PC	2 mM Mg^{2+}	7

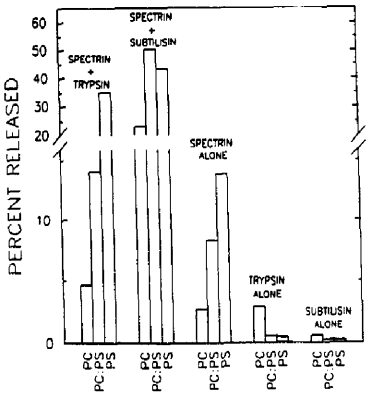

Fig. 3. Percent leakage of calcein induced by proteinase-treated or native spectrin from PS, PS/PC (1:1, mole ratio) and PC vesicles. Spectrin was incubated with trypsin, subtilisin or no proteinase as described in Materials and Methods prior to assay for leakage activity. The final lipid concentration ranged from 19.5 to 23.5 μM and the lipid/protein mole ratio ranged from 176:1 to 146:1, respectively.

PS-containing vesicles. Trypsin treatment had no effect if the digestion was carried out at 4°C (not shown), under which condition spectrin degradation is less extensive. Subtilisin-treated spectrin also was able to induce leakage from PC-containing vesicles, although leakage from PC vesicles was still about 30% less than from PS vesicles. These effects were not due to the proteinases themselves. Thus, proteinase digestion appears to have created lytic sites on spectrin, rather than abolishing them as occurred during heat denaturation.

TABLE II

Spectrin-induced leakage of vesicles containing dioleoylphosphatidylcholine, cholesterol or palmitoyloleoylphosphatidylethanolamine in addition to phosphatidylserine

PS was present at 50 mol% in each combination, the remainder being the other lipid named. The final lipid concentration varied from 4 to 7 μM and the lipid/protein mole ratio varied from about 20:1 to about 100:1 for melittin and about 70:1 to about 350:1 for the various spectrin preparations. Samples were incubated for 3 h at room temperature.

Protein added	Percent leakage from vesicles containing:		
	PS/DOPC [a]	PS/cholesterol	PS/POPE
Dimer from 1 M Tris preparation			
5 μg protein	13.5	1.6	0.65
15 μg protein	21.9	1.2	1.9
Tetramer from 1 M Tris preparation			
5 μg protein	9.5	0.5	0.65
5 μg protein	15.2	0.65	0.4
Dimer from low salt extract			
5 μg protein	8.6	0.15	0.3
15 μg protein	17.7	1.7	0.2
Melittin			
0.1 μg peptide	83.0	92.6	89.5
0.3 μg peptide	96.7	95.3	98.7

Leakage from vesicles composed of PS/DOPC, PS/cholesterol, or PS/POPE induced by different spectrin preparations

To evaluate the physiological significance of spectrin-induced leakage of phosphatidylserine vesicles, vesicles containing inner monolayer, red cell lipids or DOPC – in addition to PS – were tested for leakage on exposure to different preparations (Table II). All vesicles were 50 mol% PS, the remainder being the lipid specified (i.e., DOPC, cholesterol or POPE). Table II shows that spectrin dimer or tetramer from a 1 M Tris preparation or spectrin dimer from a low-salt extract of ghosts induced the release of 8.6 to 21.9% of the calcein trapped in PS/DOPC vesicles but only 0.2 to 1.9% of the calcein trapped in PS/cholesterol or PS/POPE vesicles. Spectrin also induced essentially no release of calcein from vesicles containing PS/cholesterol/egg or dioleoylphosphatidylethanolamine, 1:2:1 (not shown). To establish that PS/cholesterol and PS/POPE vesicles were not merely refractory to calcein release, melittin, a peptide with potent and well-characterized membrane destabilizing activity [35], was demonstrated to cause the release of nearly all calcein (i.e., 83 to 99%) from all three types of vesicles. Thus, the results obtained with spectrin from 1 M Tris preparations cannot be ascribed to the method of spectrin isolation and purification, and it appears unlikely that spectrin induces leakage from red cell membranes which contain cholesterol and phosphatidylethanolamine in addition to phosphatidylserine.

Discussion

Two aspects of spectrin-induced leakage of lipid vesicles indicate that this perturbation results at least initially from the interaction of spectrin with the headgroups of the bilayer: first, spectrin induces leakage at neutral pH from phosphatidylserine, but not phosphatidylcholine, vesicles and second, spectrin induces leakage from vesicles with low curvature (REVs and extruded vesicles) but not from vesicles with high curvature (SUVs). In contrast, a primary interaction of spectrin with the fatty acyl chains of the bilayer would be expected to increase with decreasing vesicle radius, since the bilayer surface becomes more expanded and the fatty acyl chains more exposed, the smaller the vesicle. The amphiphilic peptide, GALA, for example, presumably causes leakage and fusion of SUVs but not LUVs by such a nonpolar interaction [30]. Instead, we found the opposite; REVs and extruded vesicles released significant amounts of trapped dye whereas SUVs released no measurable trapped dye in the presence of spectrin (Fig. 2). An inability of spectrin to distort the bilayer of a limit-sonicated vesicle with maximally

curved walls [36], which were highly resistant to any further distortion, could have contributed to the lack of effect of spectrin on SUVs. Furthermore, the tightness of packing in the inner monolayer, which must be maximal in limit-sonicated SUVs, could have mitigated virtually all permeability-increasing influences *due to hydrophilic interactions* but would present no resistance to penetration by a sufficiently large, hydrophobic moiety which should increase the membrane area and hence relieve the tension due to curvature.

Results of probing the nature of spectrin's interaction with lipid bilayers by other means have been interpreted somewhat differently. Reduction of the enthalpy of the phase transition of sonicated vesicles containing various amounts of dimyristoylphosphatidylserine and at lipid/spectrin mole ratios comparable to those in the present study were taken to reflect a hydrophobic, as well as electrostatic, component of spectrin's interaction with negatively charged lipid bilayers [14]. In another study, the volumes of dimyristoylphosphatidylserine, but not dimyristoylphosphatidylcholine, vesicles, measured by densitometry, increased 60% less when heated through their phase transition temperatures in the presence of spectrin [15]. These data, taken with monolayer area measurements, suggested that spectrin stabilized the gel phase of phosphatidylserine. However, virtually no change occurred in the volumes of vesicles composed of 1:1 and 7:3 mixtures of the same respective lipids heated similarly in the latter study. At any rate, it is not clear that these data are relevant to the interaction of spectrin with lipid bilayers away from a phase transition. It seems possible that the hydrophobic component of the spectrin-lipid interaction indicated in these studies [14,15] may be specific to the interaction of spectrin with negatively charged lipid undergoing a phase transition. A small hydrophobic component of the spectrin-bilayer interaction was also indicated by the quenching of anilinonaphthyl-spectrin by the nitroxide moiety at the C16 position of PS, but not PC, in sonicated vesicles [37]. The extent of quenching was 10% at a lipid/protein ratio of a few hundred, as used here, and 25% at a lipid/protein ratio of a few thousand. The penetration of the bilayer by certain regions – particularly a nonpolar moiety like the anilinonaphthyl group – of a large, complex protein like spectrin is not unexpected. Given that spectrin induces modest leakage of unsonicated, negatively charged vesicles [18] (Fig. 2) but no leakage of sonicated, PS vesicles [19] (Fig. 2), however, the importance of this ostensible, hydrophobic interaction, detected by fluorescence quenching, for the association of spectrin and negatively charged bilayers remains to be assessed.

Our demonstration that spectrin increases the permeability of bilayers in an unstressed, as opposed to stressed, configuration and under physiological conditions also establishes that the previously unexplained difference between a report of modest leakage from large vesicles [18] and a report of no leakage from small vesicles [19] is real and not attributable to differences in spectrin preparation or other unknown variables. That the membrane permeabilizing activity of spectrin can be altered, however, was demonstrated here by heat denaturation of spectrin diminishing and proteinase treatment of spectrin increasing its ability to induce leakage. Nevertheless, it is unlikely that the rest of our results can be attributed to effects of other than native spectrin, since spectrin dimer and tetramer prepared by 1 M Tris dissolution of Triton-extracted cytoskeletons and spectrin dimer eluted from ghosts in low salt all released similar amounts of trapped calcein under the same leakage assay conditions. Further evidence against the creation of lipid binding sites during spectrin preparation is the similar permeabilizing activity of purified spectrin and isolated cytoskeletons produced at an intermediate step in our preparation of spectrin (not shown).

A second, previously unexplained finding is the extensive leakage of sonicated phosphatidylserine vesicles at neutral pH due to synergism between 0.5 mM Ca^{2+} and spectrin [19]. In contrast, we found only a slight increase in calcein leakage from extruded, phosphatidylserine vesicles when Ca^{2+} at 0.5 mM was added to spectrin. Given this lack of a large, synergistic effect of Ca^{2+} and spectrin on extruded vesicles and the more extensive fusion of phosphatidylserine SUVs than LUVs by Ca^{2+} [31], the leakage referred to above [19] may have been due to an effect of Ca^{2+} on phosphatidylserine SUVs, rather than an effect of Ca^{2+} on spectrin. It should also be noted, however, that spectrin has been reported to inhibit, rather than promote, leakage of PS SUV's at 2–10 mM Ca^{2+} by 25% [38]. Competition between Ca^{2+} and spectrin for binding sites on PS at these frankly unphysiological concentrations of Ca^{2+} was invoked to explain this surprising inhibition of vesicle leakage by spectrin. Although Ca^{2+} levels near 0.5 mM have been found in sickled red cells [33] and, therefore, constitute the maximum concentrations relevant to an *in vivo* circumstance, the recently demonstrated encapsulation of red cell Ca^{2+} in endocytic vesicles [39] suggests that even 0.5 mM far exceeds the concentration of Ca^{2+} accessible to spectrin in that pathological circumstance.

Our experiments do not distinguish a small effect of the protein on the entire vesicle population from a larger effect on a subset of the population. Some elementary considerations indicate, however, that the effect on the population is probably uniform. We observe from Fig. 1 that the leakage at 3 h is 26% and the rate is only slowly diminishing. If only a third of the vesicles, for example, were affected, that fraction would have lost 3/4 of its original contents and the leakage rate would have diminished substantially. The affected

population must therefore be much larger than 26% and it appears likely that all vesicles are affected to approximately the same extent. We also considered the possibility that spectrin induced an interaction of the vesicles with one another. Were these interactions strong enough to flatten the surfaces on contact, sufficient stress might be induced in the membranes to cause some leakage of contents. Such a mechanism would also account for the increased leakage induced in larger, as opposed to smaller, vesicles. Leakage due to vesicle aggregation by spectrin can be ruled out, however, since the light-scattering of vesicles undergoing leakage does not increase.

In summary, our re-investigation of the previously controversial, lytic effect of spectrin on acidic phospholipid vesicles [18,19] not only affirms such an effect but also shows that this effect involves polar interactions at least initially and occurs under physiological conditions. We have further established that spectrin is unlikely to have a lytic effect *in vivo*, since vesicles composed of a lipid mixture representative of the inner monolayer of the red cell were refractory to spectrin-induced leakage. In a parallel, as yet unpublished investigation of spectrin binding to vesicles of different lipid compositions, we have found that vesicles composed of such a mixture of lipids nevertheless bind at least as well to spectrin as vesicles composed solely of phosphatidylserine. The demonstration that spectrin in effect discriminates against at least PC in favor of PS shows that spectrin does not recognize the lipid bilayer per se but rather a specific lipid found on the monolayer of the membrane facing the spectrin network. Although clearly secondary in strength to interactions of spectrin with membrane anchors of protein, this interaction of spectrin with lipid may modulate the elastic properties of the membrane [7]. At the very least, lipid–spectrin interactions could restrict configurations of spectrin to only those allowing contact with the inner monolayer of the cell. Some adjustment of the entropy hypothesis of red cell elasticity [3] may be necessary to accommodate such a restriction of spectrin configurations.

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