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SYNERGISTIC EFFECTS OF A MEMBRANE PROTEIN (SPECTRIN) AND Ca^{2+} ON THE Na^+ PERMEABILITY OF PHOSPHOLIPID VESICLES

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SUMMARY

1. Spectrin increases the surface pressure of phosphatidylserine and phosphatidylcholine monolayers when injected below the surface, to a greater extent at acid pH than at neutral pH. This effect is largest for phosphatidylserine monolayers, when increases in surface pressure are seen even at high initial film pressures (30–45 dynes/cm). Albumin shows similar effects.

2. Sonicated phosphatidylserine vesicles show very low $^{22}Na^+$ diffusion rates of 0.05 %/h, equivalent to $9 \cdot 10^{-13}$ cm/sec. The addition of spectrin and albumin increases this rate several orders of magnitude, to 20 and 75 %/h, respectively, but only at acid pH values (3.5–4.5). Spectrin also increases the diffusion rate of phosphatidylcholine vesicles 20-fold at pH 3.5, but has no effect at pH 7.4.

3. Ca^{2+} and protein exhibit a synergistic effect on the permeability of phosphatidylserine and mixed phosphatidylserine/phosphatidylcholine vesicles. In contrast to protein alone, the effect of calcium *plus* protein is seen at both neutral and acid pH. Ca^{2+} does not increase the effect of spectrin on phosphatidylcholine vesicles nor does it alter the penetration of spectrin into phosphatidylserine monolayers. Neither spectrin nor spectrin *plus* calcium have any effect on positively charged stearyl amine/phosphatidylcholine vesicles at pH 7.4.

4. The results are interpreted as indicating that both electrostatic attractions between protein and lipid, and conformational changes in the protein mediate penetration of the protein into the phospholipid bilayer, resulting in the large permeability increases observed. The role of Ca^{2+} in the interactions is discussed. It is suggested that the binding of protein to phospholipid vesicles could produce areas of high negative charge density. Ca^{2+} binding would then be enhanced leading to perturbations of the bilayer structure and to large increases in permeability.

INTRODUCTION

Recent studies have shown that soluble proteins such as lysozyme and cytochrome *c* can interact with phospholipid vesicles, causing a marked increase in their permeability to ions. Studies with lipid monolayers have indicated that the increase

Abbreviation: TES, *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid.

in permeability could be correlated with penetration of protein into the hydrocarbon region of the phospholipid membranes[1,2].

The present communication concerns the interaction of a membrane protein, spectrin, isolated from erythrocyte ghosts[3], with phospholipid vesicles and monolayers. Since Ca^{2+} has been reported to drastically alter the state of aggregation of spectrin[3], and also inhibits the release of spectrin from erythrocyte membranes[4], a study was done on the effects of Ca^{2+} on protein–lipid interactions. A comparison was also made between the effects of spectrin and those of albumin, a soluble protein. The results of these studies indicate that spectrin interacts with phospholipid vesicles only at low pH (3.5). However, Ca^{2+} was found to promote spectrin–phospholipid vesicle interaction at physiological pH, causing profound changes in permeability.

METHODS

Isolation of spectrin

Spectrin was isolated as follows. Erythrocyte ghosts (plasma membranes) were prepared by multiple hypotonic hemolysis of washed human erythrocytes, and loosely bound membrane proteins were extracted by washing the ghosts in isotonic buffer[5]. Spectrin was extracted by incubating the washed membranes for 1 h at room temperature in a solution of 1 mM EDTA at pH 9. The extracted protein fraction was then concentrated by $(NH_4)_2SO_4$ precipitation and purified by gel filtration as described by MARCHESI *et al.*[6]. The purity of the spectrin preparation was assayed by acrylamide gel electrophoresis in the presence of sodium lauryl sulfate[7]. The concentration of spectrin was determined using the published value of the extinction coefficient at 280 nm (ref. 6). Spectrin was not degraded (as shown by electrophoresis) during storage in aqueous buffers at 4° for several days.

Monolayer studies

Monolayer studies were conducted at a constant film area (31.5 cm^2) on a circular polyethylene trough of 100 ml volume; a hanging platinum wire was used to measure surface pressure[8]. Phospholipid dissolved in light petroleum (b.p. 30°–60°) was applied to the surface until the desired initial surface pressure (π_i) was achieved. Protein, and $CaCl_2$ solutions were then injected into the subphase and the change in film pressure ($\Delta\pi$) was recorded continuously[2,8]. The data for $\Delta\pi$ reported represent equilibrium values obtained within 10–30 min after addition of protein.

Vesicle studies

The effect of protein and Ca^{2+} on the Na^+ permeability of sonicated unilamellar phospholipid vesicles was studied as previously described[1,9]. This procedure is a modification of the original method described by BANGHAM *et al.*[10]. Briefly, vesicles were loaded with $^{22}Na^+$ by mechanical dispersion and sonicated under nitrogen in the presence of the isotope, usually at the values of pH and ionic strength used in the subsequent experiment. The loaded vesicles and excess isotope were then separated on a Sephadex G-50 column. Aliquots of the vesicle suspension were placed in dialysis bags and protein added at this time, to a final volume of 1 ml. The amount of phospholipid in each bag was approx. 0.5–1.0 μmoles, measured as inorganic phosphate after $HClO_4$ hydrolysis. The bags were incubated at 25° *versus* 10 vol. of buffer at various

values of pH and in the presence or absence of Ca^{2+}. The amount of $^{22}Na^{+}$ diffusing out of the vesicles was measured by counting aliquots of the dialysis fluid in a liquid scintillation counter. The diffusion rate was expressed as percent of the total incorporated isotope per h. The salt solution used throughout was 10 mM NaCl, 2 mM *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), 2 mM histidine and 0.1 mM EDTA adjusted to the appropriate pH with either HCl or NaOH.

Calculation of the isoionic point of spectrin

Thus far no information is available concerning the titration behavior of spectrin. Thus it was felt necessary to make an estimate of the isoionic point of this protein. We have used the published amino acid composition of spectrin[6] to estimate the isoionic point[11] under the following assumptions: (a) The pK values of the residues in the protein are the same as for the amino acids (Merck index, 1968). (b) The titrable groups are independent and non-interacting. (c) There are no "buried" groups.

This approach is obviously quite crude; however, not enough data on spectrin is available to warrant a more sophisticated approach[11].

Materials

Crystallized human albumin (Pentex) was purchased from Miles Laboratories. L-Histidine·HCl and TES (*N*-tris (hydroxymethyl)methyl-2-aminoethane sulfonic acid) were obtained from Sigma Chemical Co. Sephadex G-50 (coarse) was obtained from Pharmacia. $^{22}Na^{+}$ was obtained from New England Nuclear Corp. as a solution of NaCl (10 mC/mg minimal specific activity). Phosphatidylserine was purified from beef brain and phosphatidylcholine from egg yolks by previously described techniques[12]. To minimize air oxidation the latter stages of purification were carried out under nitrogen, and the final product stored under nitrogen at —50° in individually sealed ampules, as described elsewhere[9]. Both preparations were shown to be pure by thin-layer chromatography in an ammonia-containing solvent[12]. Phospholipid concentrations were determined by estimation of inorganic phosphate after $HClO_4$ hydrolysis. Stearylamine was obtained from K and K Laboratories and was added to phospholipid samples as a solution in chloroform. All other chemicals were analytical reagent grade. Water was twice distilled, the second time in an all-glass apparatus.

RESULTS

Purification and properties of spectrin

Approx. 20 mg of spectrin was obtained from 1 unit of outdated bank blood. The protein was judged by electrophoresis to be free of the other major components of the erythrocyte membrane and of hemoglobin. Several minor high-molecular-weight components and a component with a molecular weight of 70000 were present in small amounts in the spectrin preparation. More than 90% of the protein material in the spectrin preparation was associated with the spectrin band, as determined by gel densitometry (Fig. 1). The isoionic point of spectrin was calculated to be about 4.1 (see METHODS).

Monolayer studies

Spectrin alone at a concentration of 1 μg/ml and at pH 7.4 produces a final surface pressure of 15 dynes/cm when added to the subphase (aqueous solution, 10 mM

NaCl, 2 mM TES, 2 mM histidine, 0.1 mM EDTA) or to the surface; additional amounts of spectrin (2–3 μg/ml) result in only a slight (1 dyne/cm) further increase in surface pressure. Thus the surface activity of spectrin is similar to other soluble proteins[13].

The effect of spectrin on the surface pressure of phospholipid monolayers at different initial film pressures is shown in Fig. 2, where the increase in surface pressure ($\Delta\pi$) due to spectrin is plotted against the initial surface pressure (π_i). Injection of

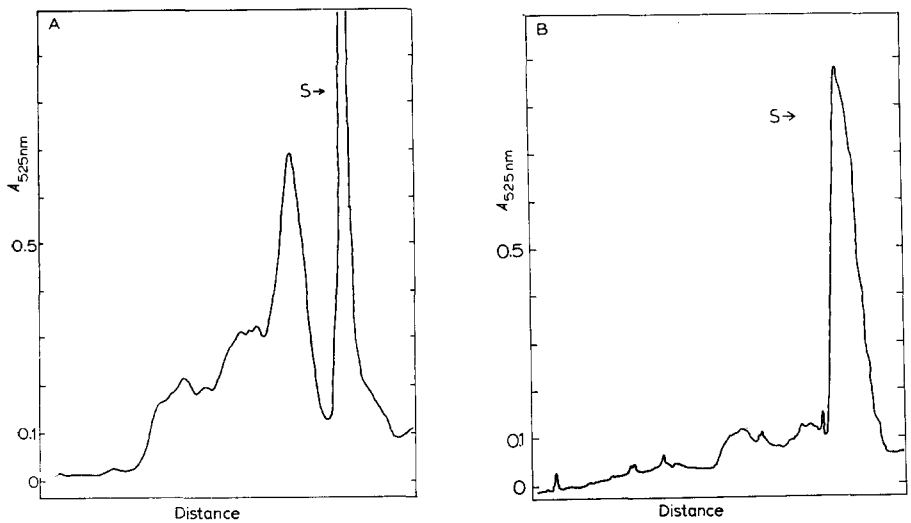

Fig. 1. Gel electrophoresis of spectrin and whole ghost protein. Acrylamide gel electrophoresis in the presence of sodium lauryl sulfate and mercaptoethanol was carried out essentially by the method of DUNKER AND RUECKERT[7]. The buffer used, however, was 100 mM Tris–HCl *plus* 1 mM EDTA (pH 7.6). Coomassie blue stained gels were scanned at 525 nm on a Gilford gel densitometer. The gels shown in this figure were run at 8 mA/tube for 45 min at room temperature. (A) 100 μg of total ghost protein. (B) 25 μg of spectrin. The position of the spectrin peak is indicated by the letter S.

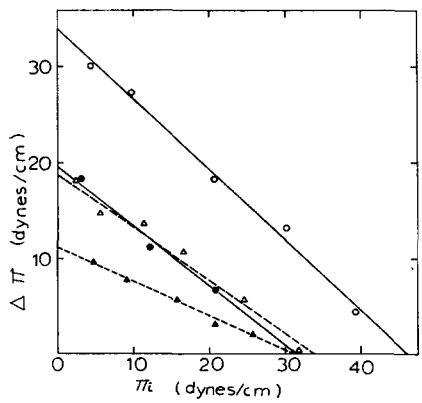

Fig. 2. The effect of spectrin on the surface pressure of phosphatidylserine and phosphatidylcholine films. The initial film pressure π_i and the change in film pressure $\Delta\pi$ were measured as described in the text. Bulk phase was 10 mM NaCl, 2 mM TES, 2 mM histidine and 0.1 mM EDTA adjusted to pH 3.5 or 7.4. ○—○, phosphatidylserine at pH 3.5; ●—●, phosphatidylserine at pH 7.4; △—△, phosphatidylcholine at pH 3.5; ▲—▲, phosphatidylcholine at pH 7.4.

spectrin under phosphatidylserine monolayers at pH 3.5 and at a concentration of 1 μg/ml, causes an increase in the surface pressure of the films up to initial film pressures of 45 dynes/cm. This indicates extensive penetration of the protein into the film[2,13–15]. At pH 7.4 spectrin does not cause an increase in surface pressure above an initial film pressure of 30 dynes/cm. Spectrin also penetrates phosphatidylcholine films at pH 3.5 and 7.4, but only up to 30 dynes/cm initial pressure. As in the case of phosphatidylserine, the $\Delta\pi$ of phosphatidylcholine films is less at pH 7.4 than at 3.5. It should be noted here that albumin has been shown to penetrate into phosphatidylserine[2] and phosphatidylcholine films in a similar fashion.

It can be seen from Fig. 2 that at a film pressure of 25 dynes/cm, where the area per phosphatidylserine or phosphatidylcholine molecule[8] is approximately the same as in phospholipid liquid crystals, 68 Å^2 (ref. 16), spectrin can cause a marked increase in π (15 dynes/cm) at pH 3.5, but only a slight increase (3 dynes/cm) at pH 7.4). In the case of phosphatidylcholine films at an initial pressure of 25 dynes/cm the addition of spectrin causes a $\Delta\pi$ of only 4 dynes/cm at pH 3.5 and an even smaller $\Delta\pi$ of 2 dynes at pH 7.4. These results correlate well with the changes in the Na^+ permeability of phospholipid vesicles discussed below.

Phospholipid vesicle studies

The Na^+ permeability of sonicated phosphatidylserine or phosphatidylcholine vesicles in the absence of protein or Ca^{2+} at 24° is extremely low (0.05 % total diffused per h) at all values of pH from 3.5 to 7.4. The equivalents of $^{22}Na^+$ diffusing per h per mole phospholipid can be calculated on the basis of % total $^{22}Na^+$/h and the μmoles Na^+ captured per μmole phospholipid. For the case of phosphatidylserine vesicles (capture = 0.13 μmole Na^+/μmole lipid) the self-diffusion rate of 0.05 % total/h is 65 pmoles Na^+/μmole lipid per h. On the basis of an area of 68 Å^2 per phospholipid molecule and assuming unilamellar vesicles, the area per μmole of phospholipid is 2000 cm^2 for a bilayer structure. Using this figure, the flux for Na^+ diffusion can thus be calculated as $9 \cdot 10^{-18}$ equiv/cm^2 per sec. For a concentration difference of 10^{-5} moles/cm^3, the permeability coefficient is calculated as $9 \cdot 10^{-13}$ cm/sec. A value of $3.3 \cdot 10^{-13}$ cm/sec was calculated by JOHNSON AND BANGHAM[17] for sonicated phosphatidic acid/phosphatidylcholine vesicles at 37°.

Upon the addition of small amounts of spectrin or of albumin the permeability of phosphatidylserine vesicles increases. A concentration of 100 μg/ml of spectrin results in a diffusion rate of 20 %/h at pH 3.5, and further additions of this protein do not appreciably increase this rate (Fig. 3). In the case of albumin at pH 4.5 the permeability of phosphatidylserine vesicles increases upon addition of albumin up to a concentration of 300 μg/ml when a plateau is achieved at a diffusion rate of 75 %/h. Neither albumin[2] nor spectrin has an appreciable effect on vesicle permeability at pH 7.4 unless much higher concentrations (>1 mg/ml) are used.

The data shown in Fig. 3 represent the diffusion rates for a particular time interval after exposure of the sample to the indicated pH; in the case of spectrin it is the rate for the first h and in the case of albumin for the second hour. Under conditions when the protein does induce an increase in permeability, the percent diffusion rate increases with time until the concentration of isotope within the vesicle declines appreciably. At this time the observed percent diffusion rate begins to decrease presumably because of the marked depletion of isotope.

Effects of protein plus Ca^{2+} on vesicles

As shown in Fig. 4, spectrin at a concentration of 100 μg/ml markedly increases the permeability of phosphatidylserine vesicles to $^{22}Na^+$ when the bulk-phase pH is acidic, but not at pH 7.4. Ca^{2+} alone at 0.5 mM also increases the permeability of phosphatidylserine vesicles. In contrast to the spectrin effect, this increase is not pH dependent in the range pH 3.5 to 7.4 (dotted line in Fig. 4). The combination of spectrin and Ca^{2+}, however, causes a very large increase in the permeability of phosphatidylserine vesicles. This appears to be a synergistic effect since the permeability increase caused by spectrin *plus* Ca^{2+} is 10 or more times the sum of the increases caused by either acting alone. This marked enhancement of permeability also occurs at pH 7.4 where spectrin alone has no effect.

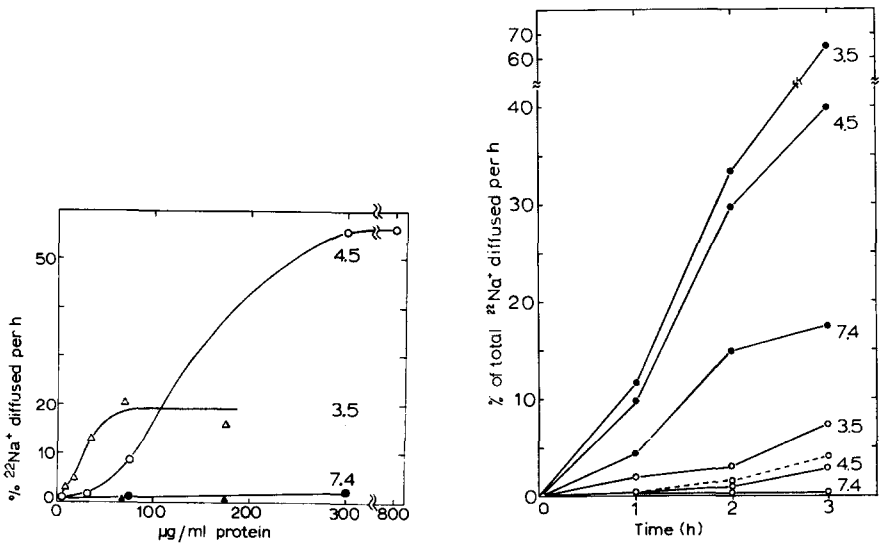

Fig. 3. Concentration dependence of the effect of albumin and spectrin on the permeability of phosphatidylserine vesicles. Note that the permeability of phosphatidylserine vesicles in the absence of protein is quite low (0.05%/h) at either acid or neutral pH. Reaction medium as in Fig. 2. ○—○, albumin at pH 4.5; ●—●, albumin at pH 7.4; △—△, spectrin at pH 3.5; ▲—▲, spectrin at pH 7.4.

Fig. 4. The effect of spectrin and Ca^{2+} on the permeability of phosphatidylserine vesicles. ●—●, spectrin (100 μg/ml) *plus* Ca^{2+} (0.5 mM), at different pH values as indicated on the figure; ○—○, spectrin (100 μg/ml) alone, at different pH values as indicated on the figure; ○---○, Ca^{2+} (0.5 mM) alone; the permeability values were the same for pH 3.5, 4.5 and 7.4. Reaction medium as in Fig. 2.

Fig. 5 shows the effect of spectrin and Ca^{2+} on phosphatidylcholine vesicles. Spectrin causes a small but definite increase in the permeability of phosphatidylcholine vesicles at pH 3.5 but has no effect at pH 7.4. Since phosphatidylcholine vesicles have no net charge in this pH range, the pH dependence of the effect could be ascribed to a change in protein conformation rather than to changes in the charge on the vesicles. The $\Delta\pi$ caused by spectrin in phosphatidylcholine monolayers at pH 3.5 is also substantial (Fig. 2). Ca^{2+} alone exerts no effect on phosphatidylcholine films nor does Ca^{2+} modify the interaction of phosphatidylcholine vesicles and spectrin (Fig. 5).

Spectrin interacts strongly with mixed vesicles of 30 % phosphatidylserine in phosphatidylcholine. Specifically, spectrin (100 μg/ml) can produce a diffusion rate of as much as 20 %/h from vesicles of this type at pH 4.5. This contrasts with a maximal diffusion rate of only 3 %/h produced by spectrin in pure phosphatidylserine vesicles under similar conditions. Spectrin alone has no effect on the mixed vesicles at pH 7.4.

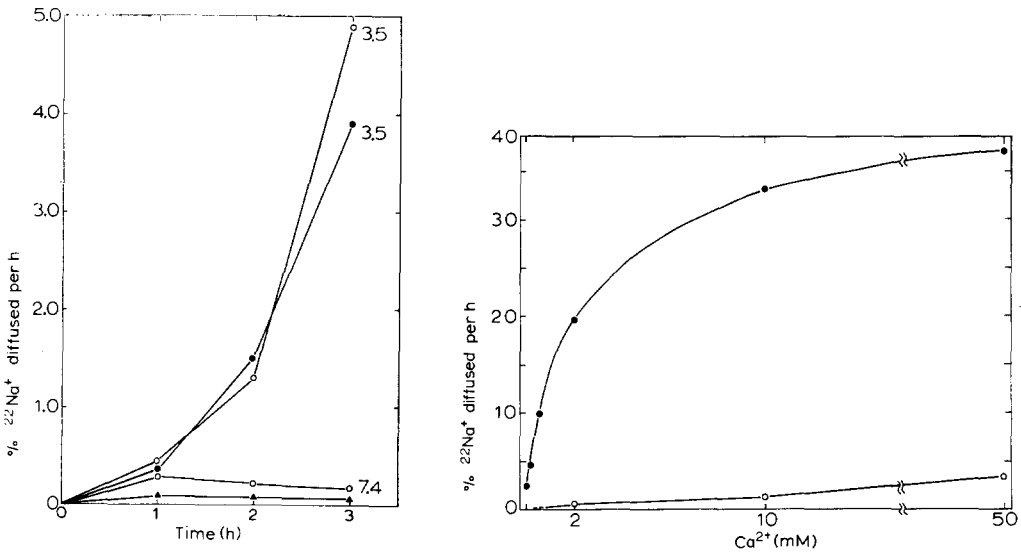

Fig. 5. The effect of spectrin and Ca^{2+} on the permeability of phosphatidylcholine vesicles. ○—○, spectrin (100 μg/ml) alone, at different pH values as indicated on the figure; ●—●, spectrin (100 μg/ml) *plus* Ca^{2+} (0.5 mM), at pH 3.5; ▲—▲, basal permeability rate (no protein or Ca^{2+} added to the vesicles) at pH 7.4, and 3.5. Reaction medium as in Fig. 2.

Fig. 6. The effect of spectrin *plus* Ca^{2+} on the permeability of phosphatidylserine/phosphatidylcholine vesicles. The diffusion rates shown represent the first hour after exposure to Ca^{2+}. The vesicles are 30% phosphatidylserine in phosphatidylcholine on a molar basis, and the ambient pH is 4.5. Reaction medium as in Fig. 2. Note the permeability in the absence of any additions is 0.05–0.1 %/h. ●—●, spectrin (100 μg/ml) *plus* Ca^{2+}; ○—○, Ca^{2+} alone.

Ca^{2+} alone also enhances the permeability of phosphatidylserine/phosphatidylcholine vesicles but its effect is less marked than in the case of pure phosphatidylserine vesicles. Thus the maximum observed diffusion rate from phosphatidylserine/phosphatidylcholine vesicles in the presence of 0.5 mM Ca^{2+} was approximately 0.3 %/h as compared to 4 %/h for pure phosphatidylserine vesicles *plus* Ca^{2+}. The increase in $^{22}Na^+$ permeability induced by Ca^{2+} *plus* spectrin occurs in phosphatidylserine/phosphatidylcholine vesicles at all pH values (3.5, 4.5, 7.4) tested, in a manner similar to the case of pure phosphatidylserine vesicles. The effect of various concentrations of Ca^{2+} with and without the addition of spectrin (100 μg/ml) on the permeability of phosphatidylserine/phosphatidylcholine vesicles at pH 4.5 is illustrated in Fig. 6. The concentration of Ca^{2+} necessary for 50 % of maximal synergistic effect seems to be approx. 1–2 mM.

The Ca^{2+} *plus* spectrin synergistic effect on phosphatidylserine vesicles is only partially antagonized by high ionic strength (100 mM NaCl). Thus the effect can occur

under essentially physiological conditions (Fig. 7). The effect is fully blocked by an excess of EDTA present initially, but only slowly reversed by EDTA after initial exposure of the vesicles *plus* protein to Ca^{2+} (Fig. 7).

Vesicles composed of 10 % stearylamine in phosphatidylcholine have a positive charge at pH 7.4 (ref. 18). Spectrin, Ca^{2+}, or spectrin *plus* Ca^{2+} all fail to significantly affect the permeability of these vesicles at pH 7.4, a pH value at which the protein and vesicles have opposite charges. For example in one experiment the self-diffusion rate during the second hour for stearylamine/phosphatidylcholine vesicles alone was 0.4 %/h while the rate for these vesicles *plus* 100 μg/ml spectrin was 0.7 %/h. The addition of Ca^{2+} decreased this latter rate to 0.3 %/h.

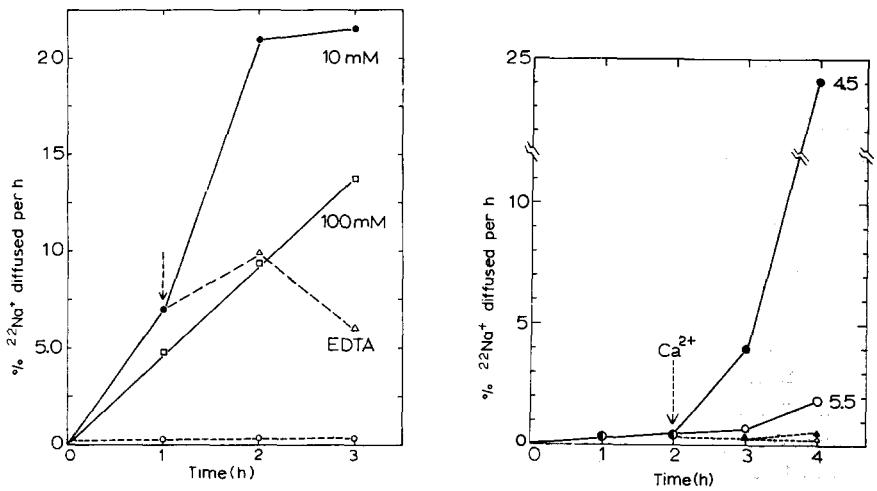

Fig. 7. Effect of ionic strength and chelating agents on the synergistic effect of spectrin and Ca^{2+}. This experiment was conducted at pH 7.4. The amount of spectrin used in each case was 100 μg per bag (1 ml total volume), Ca^{2+} was 0.5 mM and EDTA was 1 mM. At the point indicated by the arrow one of the samples was transferred from a Ca^{2+} containing dialysis medium to an EDTA-containing medium. ●—●, spectrin *plus* Ca^{2+}; □—□, spectrin *plus* Ca^{2+} at high ionic strength (100 mM NaCl); ○----○, spectrin *plus* Ca^{2+} *plus* EDTA from start of experiment; △---△, spectrin *plus* Ca^{2+} transferred to EDTA as indicated by the arrow.

Fig. 8. The effect of albumin on the permeability of phosphatidylserine vesicles. In this experiment the phosphatidylserine vesicles were either exposed to 5 μg/ml albumin, or kept protein free. After 2 h of dialysis against Ca^{2+} free medium the samples were transferred to medium containing 0.5 mM calcium, as indicated by the arrow. ●—●, vesicles *plus* albumin at pH 4.5; ○—○, vesicles *plus* albumin at pH 5.5; ▲---▲, vesicles alone (no protein added) at pH 4.5; △---△, vesicles alone at pH 5.5.

The synergistic effect of Ca^{2+} and protein on the permeability of negatively charged vesicles is not confined to spectrin. Ca^{2+} also markedly enhances the effect of albumin on the permeability of phosphatidylserine vesicles. As can be seen in Fig. 8 a low concentration (5 μg/ml) of albumin has only a minimal effect on the permeability of phosphatidylserine vesicles at pH 4.5 or 5.5. However, upon the addition of Ca^{2+} (0.5 mM) a 50-fold increase in permeability is observed at pH 4.5 and a 10-fold increase at pH 5.5.

Effects of Ca^{2+} plus protein on monolayers

The addition of Ca^{2+} (to 0.5 mM) to the subphase of a phosphatidylserine mono-

layer causes a decrease[8] in film pressure of approx. 10 dynes/cm. If spectrin is added to a phosphatidylserine monolayer at pH 3.5, the film pressure increases (Fig. 2). Subsequent addition of Ca^{2+} causes no further increase in surface pressure but frequently causes a decrease. Similarly, the addition of spectrin to a phosphatidylserine monolayer system previously equilibrated with Ca^{2+} (at pH 3.5) results in an increase in film pressure no greater than that caused by spectrin in a phosphatidylserine monolayer system free of Ca^{2+}. Thus the presence of Ca^{2+} does not seem to augment the penetration of spectrin into phosphatidylserine films.

DISCUSSION AND CONCLUSIONS

Although spectrin is a membrane bound protein, its interactions with phospholipid vesicles qualitatively resemble those of soluble proteins such as albumin[2]. Both have marked permeability effects on negatively charged vesicles at pH values below or near their isoelectric points. At pH 7.4 when these proteins have a net negative charge they fail to interact significantly with negatively charged vesicles. Their penetration into phospholipid monolayers also follows a similar pattern.

Other factors, such as protein conformation as well as charge seem to be important, however, since spectrin fails to interact with positively charged vesicles (stearylamine/phosphatidylcholine) at pH 7.4 when the protein has a net negative charge. Apparently the conformation adopted by spectrin at pH 3.5 is suitable for penetration of phospholipid bilayers, while the conformation at pH 7.4 is less favorable for such interaction. This is supported by the observation that spectrin manifests a greater $\Delta\pi$ effect on phosphatidylcholine monolayers at pH 3.5 than at pH 7.4 (Fig. 2). Moreover, spectrin increases the permeability of phosphatidylcholine vesicles at pH 3.5 but not at pH 7.4 (Fig. 5). Other workers have stressed the importance of protein conformation in the process of vesicle–protein interaction. For example SWEET AND ZULL[19] found that albumin would enhance the glucose diffusion rate of negatively charged lecithin–cholesterol–dicetyl phosphate liquid-crystalline particles (liposomes) at acidic pH, but that the protein failed to affect positively charged lecithin–cholesterol–stearylamine liposomes at neutral pH. In contrast, SWEET AND ZULL[20] have also reported that spectrin can enhance glucose efflux from both negatively and positively charged liposomes at neutral pH. The quantities of spectrin used, however, were more than 10 times those used in the present study, and the magnitude of the enhancement with positively charged liposomes (about 2-fold) would be considered only marginally significant here. It is significant to note, however, that spectrin was found to bind to both positive and negative liposomes, the relative amount bound being higher in the case of positive liposomes[20].

Charge attractions between vesicles and proteins may enhance protein penetration and thus increase permeability, as in the case of spectrin and phosphatidylserine at pH 3.5. However, charge interaction of this type is not an absolute requisite, since at pH 3.5 spectrin enhances the Na^+ efflux from uncharged phosphatidylcholine vesicles (approx. 20 fold), although the effect is less than that with phosphatidylserine vesicles (approx. 100 fold).

As shown previously[2] and also in Fig. 2, protein penetration into phospholipid monolayers, indicated by a $\Delta\pi$ effect, at initial film pressures more than 20 dynes/cm is generally correlated with the effects of the same protein on the permeability of

phospholipid vesicles. The $\Delta\pi$ effect has been interpreted as due to penetration of the protein into the hydrocarbon region of the monolayer, involving hydrophobic associations[2,13–15]. The importance of both ionic and hydrophobic associations has also been discussed by GREEN AND FLEISCHER[27] for mitochondrial membrane components, by ZWAAL AND VAN DEENEN[21] in the recombination of red cell lipids and apoproteins, and by SWEET and ZULL[19] in the interaction of albumin and lipid vesicles. SESSA *et al.*[22] have emphasized the importance of hydrophobic interactions in the lytic effects of the polypeptide mellitin on cells and vesicles.

The observation of a concerted effect of Ca^{2+} and protein on the permeability of phospholipid vesicles is exciting in itself and may be of importance in understanding the function of biological membranes. The precise nature of the interplay between protein and Ca^{2+} in this phenomenon remains unclear. We have entertained two hypothetical explanations or models for the effect. In the first model calcium is considered to enhance the penetration of protein into the hydrocarbon region of the vesicle. Ca^{2+} may bind to protein anionic sites as well as to negative sites on the vesicles, thus decreasing the repulsion between these sites and allowing penetration to take place. Ca^{2+} may also cause a conformational change in the protein which favors penetration. The latter possibility is suggested by the observation that spectrin aggregates in the presence of Ca^{2+} (ref. 3).

The second model is based on the observation that calcium itself can markedly increase the permeability of pure phosphatidylserine vesicles when it is present on only one side of the membrane[8,23]. The magnitude of the increase is a function of Ca^{2+} concentration and of surface charge density. A full explanation of this phenomenon has been presented elsewhere[24], where it was shown that with mixed phosphatidylserine/phosphatidylcholine vesicles, Ca^{2+} is much less effective than in pure phosphatidylserine vesicles. Apparently a high density of anionic sites is necessary for the Ca^{2+} effect to manifest itself. It is conceivable that the presence of protein, Ca^{2+} and negatively charged vesicles may result in the formation of tridentate complexes in which anionic sites on the protein and on the vesicles are the ligands of Ca^{2+}. These complexes may have a higher negative charge density than the unmodified vesicle surfaces, and exhibit a higher affinity for Ca^{2+}. The local perturbations produced by such complexes at the interphase would result in drastic increases in the permeability of phosphatidylserine vesicles, similar to those produced by Ca^{2+} alone at higher concentrations[8]. In this case, the action of the protein could be defined as potentiating the Ca^{2+} effect.

In the case of mixed phosphatidylserine/phosphatidylcholine vesicles, where Ca^{2+} *plus* protein synergism is also seen, the regions of high anionic site density may result from a segregation of phosphatidylserine and phosphatidylcholine molecules with the phosphatidylserine clustering around regions where protein is bound. If such a mechanism does exist it would result in the formation of specialized regions within the plane of the membrane.

At present both models are speculative. However, some available evidence casts doubt on the validity of the first model. First, the synergistic effect is only observed in vesicles which exhibit effects due to Ca^{2+} acting alone (*i.e.* phosphatidylserine and phosphatidylserine/phosphatidylcholine but not phosphatidylcholine); this would rule out the phenomenon due solely to a change in protein conformation. Second, the magnitude of the effect seems greater than can be explained in terms of neutralization of negative charges on the protein by Ca^{2+}. For example 5 μg/ml of

albumin has a similar effect on phosphatidylserine vesicles at pH 5.5 or at 4.5, even though the positive charge on the protein increases by 20 units/mole over this pH range[25]. However, the addition of Ca^{2+} (0.5 mM) to phosphatidylserine vesicles in the presence of albumin (5 μg/ml) results in a diffusion rate increase of 50-fold at pH 4.5 and 10-fold at pH 5.5 compared to albumin alone (Fig. 8). Finally although Ca^{2+} markedly enhances the effect of proteins on vesicle permeability, it has no effect on the penetration of protein into monolayers.

The present study, in conjunction with earlier work[1,2,26] delineates some concepts which may be of importance in understanding the organization of biological membranes. First, it seems clear that exogenous protein can alter the structure of phospholipid bilayers, by means of ionic as well as non-ionic interactions; the alteration is often reflected by a change in the permeability of the bilayer vesicles[1,2,19,20,22]. Second, Ca^{2+}, when distributed asymmetrically across a membrane of anionic phospholipids, have been shown to disrupt the membrane permeability barrier by presumably causing local perturbation of membrane structure[26]. The present work suggest that proteins may amplify the Ca^{2+} effect, especially in bilayers composed of a mixture of neutral and anionic phospholipids, possibly by promoting segregation of the anionic phospholipids. Thus one may speculate that one possible role for Ca^{2+} (and possibly Mg^{2+}) in biological membranes is to bind together negatively charged proteins and anionic lipids under physiological conditions, resulting in the formation of differentiated regions within the plane of the membrane.

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