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THE SPECIFIC INTERACTION OF MYELIN BASIC PROTEIN WITH LIPIDS AT THE AIR–WATER INTERFACE

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SUMMARY

The interaction of A_1 myelin basic proteins and P_1, P_2 peripheral nerve basic proteins with different lipids has been studied at the air–water interface.

Measurement of the change in surface pressure showed a highly specific interaction of A_1 basic protein with negatively charged lipids of myelin such as cerebroside sulphate. Neutral lipids such as lecithin, cholesterol and cerebrosides show markedly less affinity for the A_1 basic protein. The P_1 and P_2 basic proteins did not show specificity to this extent.

Measurement of the surface radioactivity, using ^{131}I-labelled A_1 basic protein, showed that the amount of A_1 basic protein bound to cerebroside sulphate monolayers parallels the pressure increase. The observed specificity, the effects of different solutes, differences in fatty acid chain length of the lipids and the increase in area at constant pressure demonstrate that ionic and hydrophobic forces are involved in the interaction of A_1 basic protein and lipids.

INTRODUCTION

Myelin is not a prototype of a biological membrane but rather a membrane that has a unique function and composition. Central nervous system myelin contains only approximately 20% of protein[1], 30% of which is the A_1 basic protein[2,3]. This protein has been isolated[4] in homogeneous form and the amino acid sequence elucidated[5]. The lipid composition of the myelin is characterized by a high content of charged lipids such as cerebroside sulphate, phosphatidylserine and triphosphoinositides. Other lipids abundantly present are cholesterol, phosphatidylethanolamine and lecithin[6]. Proteolipids, although especially abundant in nervous tissue, are also found in a wide variety of animal and plant tissue[7]. The basic protein is a major structural protein of myelin and is not found in other cellular membranes. Probably it plays a major role in the biogenesis of the myelin structure[8,9] and in the maintenance of the structural integrity of the myelin membrane, as appears from studies on the demyelinating disease multiple sclerosis[10,11]. The biosynthesis of the long hydrocarbon chain cerebrosides was related with the appearance of the basic protein and the formation of compact myelin[11–13].

The possibility of a specific interaction of A_1 basic protein, purified from bovine spinal cord, as well as P_1 and P_2 basic protein from peripheral nerve and myelin lipids was investigated at the air–water interface. The use of ^{131}I-labelled A_1 basic protein and ^{131}I-labelled P_2 basic protein enables one to follow directly the adsorption and desorption from the interface. The interactions of proteins with lipid monolayers provides the investigator with a model[14,15] for the study of the molecular organization of lipids and proteins in oriented interfaces like cell membranes and in the present study, the myelin membrane.

MATERIALS AND METHODS

^{131}I, carrier free, and ^{125}I were purchased from New England Nuclear, U.S.A. Lactoperoxidase, grade B, was purchased from B.D.H. England.

All reagents and solvents used were of analytical grade.

Preparation of proteins

The A_1 basic protein (mol. wt 18235) was purified from bovine spinal cord as already described[16]. The purification of P_1 (mol. wt 14200) and P_2 (mol. wt 12200) basic proteins of the peripheral nerve has been described[17].

Preparation of lipids

The different lipid fractions and the pure lipids were prepared as described[16,18]. Cerebrosides and sphingomyelin were prepared from the ether-insoluble fraction of ox spinal cord lipids[19]. The lipids were dissolved in chloroform–methanol (98:2, v/v), and loaded on a column of aluminium oxide[20]. The choline-containing lipids which contaminate these lipids were hydrolysed and extracted[21]. Sphingomyelin was crystallized from hot ethyl acetate containing 5% (v/v) methanol. Phosphatidylethanolamine was purified from ox brain by using silicic acid column chromatography. Phosphatidylcholine was either synthetic[22] or isolated from egg yolk[23]. Cholesterol was purified by triple recrystallization from ethanol.

All lipid preparations were shown to be more than 99% pure[24]. The determination of the lipid composition of the lipid mixtures has been described[16]. The lipid composition of the total central nervous system lipids of the acid fraction is given in Table I. Cerebroside and cerebroside sulphate were determined according to the method of Neskovec *et al.*[25]. Protein was determined according to Lowry *et al.*[26] or by using the amino acid analysis of protein hydrolysates.

The iodination of the basic proteins

The iodination of the basic proteins was performed as described by Morrison and Bayse[27] at pH 7.4 and at room temperature. $1.4 \cdot 10^{-7}$ mole protein were iodinated using two additions of KI, H_2O_2 and lactoperoxidase. The reaction was stopped by the addition of 0.5 ml of 0.5 M β-mercaptoethanol. Excess of iodine was removed by dialysis against non-radioactive iodine and water containing β-mercaptoethanol. The incorporation of iodine was found to be 2 to 5 iodine atoms per molecule of A_1 basic protein, depending on the iodination time. P_2 basic protein could incorporate 2 iodine atoms per molecule. The A_1 protein used for the monolayer experiments contained 2 iodine atoms per molecule with a specific activity of 10^5 to 10^7 cpm/mg protein (0.1 to 10 μCi/mg protein).

TABLE I

LIPID COMPOSITION OF CENTRAL NERVOUS TISSUE FRACTIONS

Lipid species	*Weight % of total lipid*	
	Total central nervous system lipids[9]	*Central nervous system acidic lipids*[9]
Cholesterol	29.3	
Phospholipid	42.2	
Cerebrosides	28.5	
Cerebroside sulphate		36.5
Serine phosphoglycerides	18.0	37.0
Ethanolamine phosphoglycerides	37.0	—
Inositol phosphoglycerides	1.7	3.0
Choline phosphoglycerides	21.0	—
Sphingomyelin	19.2	—
Phosphatidic acid	1.2	12.4
Polyphosphoinositides	0.7	5.0
Cardiolipin	—	3.9
Unknown	1.0	2.2

Determination of surface characteristics

Pressure increase measurements of lipid monolayers after injection of basic proteins were performed at the air–water interface in a teflon trough, 8 cm in diameter and 3 cm deep. The trough was filled with 10^{-2} M Tris–acetate-buffered water of the desired pH. The water used had been distilled from alkaline permanganate and then redistilled. In order to free concentrated solutions of salt and urea from surface-active material, they were passed through active carbon. Proteins were injected underneath the monolayer from a solution of 1 mg/ml. The subphase was stirred with a magnetic bar. The surface pressure was determined with a recording R.I.I.C. LM 500 electrobalance. The surface radioactivity was measured with a gas flow detector (Nuclear Chicago 8731). The gas used was helium 3% butane, the window Micramil 150 μg/cm^2, 4.2 cm $\times$ 1.3 cm.

Collection of lipid–protein films was performed on a trough (65 cm $\times$ 14 cm) which was divided into two sections by a bar with a 5-mm slit in the middle. The interaction of the lipid monolayer and A_1 basic protein was allowed to take place in one compartment of the trough. The lipid–protein film was then transferred to the other compartment with protein-free subphase without loss of pressure and subsequently collected and lyophilized. For this purpose the buffer used was 0.1 M triethylamine–carbonate, pH 8.0. The complex was hydrolysed in 6 M HCl in evacuated tubes for 24 h at 110 °C. The quantity of lysine and arginine was determined from the amino acid analysis and the amount of protein was calculated.

The γ-radiation was measured with a Tridar auto γ-spectrometer, Packard Model 3002.

RESULTS

The surface activity of the pure proteins was tested by injecting A_1 basic protein, P_1 and P_2 basic protein in the subphase in concentrations up to 2 μg/ml. No change in surface tension was observed even after 1 h.

Fig. 1A shows the effect of the initial surface pressure of the monomolecular film on the ability of A_1 basic protein to interact with lipids at the interface. The most striking pressure increase is observed for cerebroside sulphate. At an initial pressure of 5–12 dynes/cm the pressure increase reaches 20 dynes/cm. The pressure increase is less marked at higher initial pressures. However, at an initial pressure of 27 dynes/cm, the pressure increase is still 10 dynes/cm. This means a total film pressure of 37 dynes/cm. For a film of total central nervous system lipids the pressure increase is less pronounced and varies from 12 dynes/cm at an initial pressure of 3 to 6.5 dynes/cm at an initial pressure of 28 dynes/cm. Changes in the film pressure of monomolecular films of egg lecithin are only observed at initial pressures below 20dynes/cm. At high initial pressures practically no pressure increase is found.

Fig. 1B gives an example of some actual recordings of the pressure change of total central nervous system lipid films after the injection of basic protein at different initial pressures. The interaction is very rapid and was completed within 5 min. To eliminate the possibility of errors due to the differences in wetting of the Wilhelmy

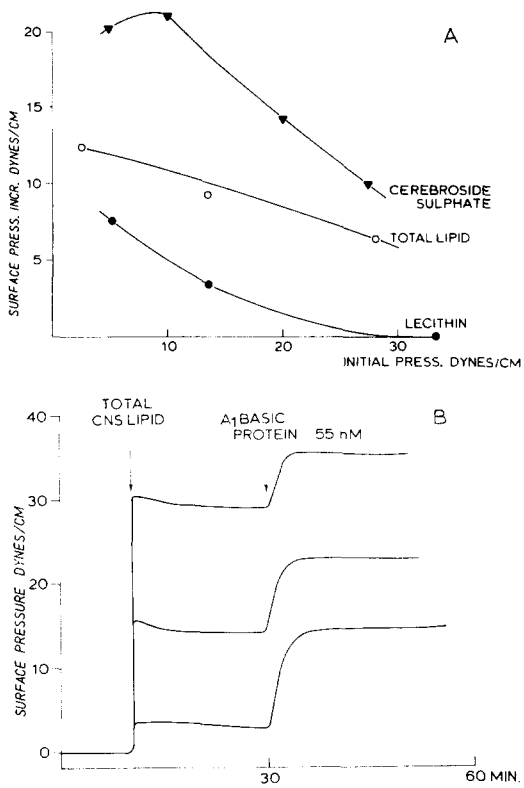

Fig. 1. (A) Surface pressure increase after the injection of A_1 basic protein underneath a monolayer of cerebroside sulphate (▼), total central nervous system lipid (○) and egg lecithin (●) at different initial pressures. The protein concentration was 110 nM in a subphase of 10^{-2} M Tris-acetate buffer, pH 5.0. (B) Automatically recorded pressure increases after the injection of A_1 basic protein underneath a monolayer of total central nervous system (CNS) lipid at different initial pressures. The protein concentration was 55 nM in a subphase of 10^{-2} M Tris-acetate buffer, pH 5.0.

plate the interaction was also measured with the Langmuir trough, which gave the same result. Since the proteins used in this study are positively charged and most of the lipids negatively charged, the effect of pH on the pressure increase was tested.

Fig. 2 shows that high pressure increases are observed for the acidic lipids and for the cerebroside sulphate. A variation of subphase pH from pH 8 varies the pressure increase of cerebroside sulphate from 18 to 24 dynes/cm (at an initial pressure of 10 dynes/cm). The lipid is however fully ionised above pH 3.5[29]. Also for the total lipids a similar pH dependence can be observed. The changes can be brought about by changes in the charge of the lipids or the protein conformation. Monomolecular films of pure cholesterol and lecithin are not influenced by the pH in the range studied. However, significant changes in pressure increase are observed in the presence of basic protein at different pH values. In this case these differences have to be due to differences in charge and structure of the protein.

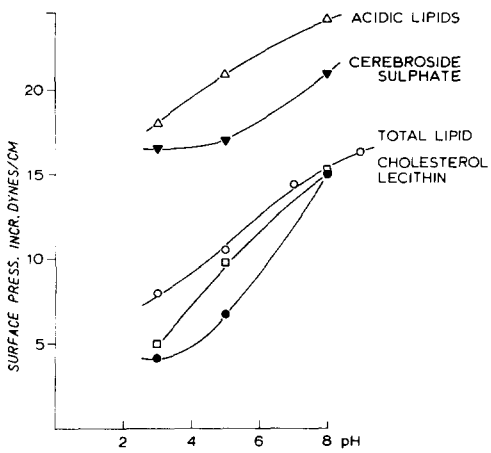

Fig. 2. Surface pressure increase after the injection of A_1 basic protein underneath a monolayer of the acidic lipid fraction from central nervous system (△), cerebroside sulphate (▼), the total lipid fraction from central nervous system (○), cholesterol (□) and lecithin (●) at different pH values. The protein concentration was 110 nM in a subphase of 10^{-2} M Tris–acetate buffer, pH 5.0. The initial film pressure was 10 dynes/cm.

To investigate the affinity of myelin proteins for specific myelin lipids the penetration of basic protein into different lipid monolayers was studied at pH 5.0 and an initial pressure of 10 dynes/cm. It is clearly shown in Fig. 3A that high-pressure increases of 20 dynes/cm are found for negatively charged lipids such as cardiolipin, the acidic lipid fraction from myelin and cerebroside sulphate. For cardiolipin the highest protein concentrations are required to saturated the lipid layer. The less negatively charged lipids such as the total lipid fraction from myelin and phosphatidylserine show an intermediate pressure increase of 10 dynes/cm. Lipids with no net charge such as sphingomyelin, lecithin and cerebrosides show a pressure increase of no more than 5 dynes/cm. Fig. 3B shows the actual recordings of pressure changes of acidic lipid caused by basic protein. Final protein concentrations of 7 and 22 nM show a rather slow penetration of the lipid monolayer. Higher protein concentrations of 55 or 110 nM show a rapid penetration and saturation of the monolayer.

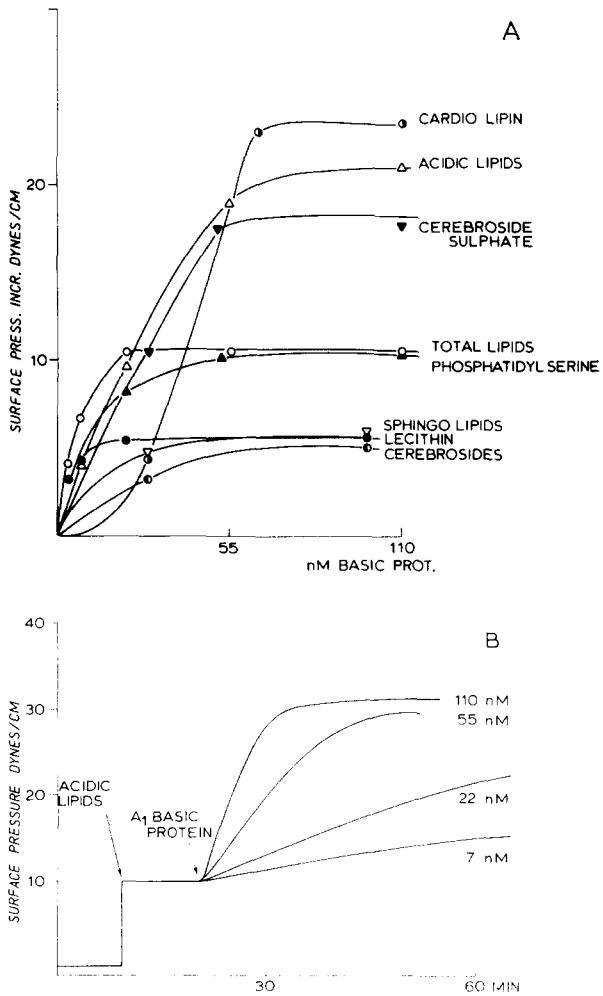

Fig. 3. (A) Surface pressure increase after the injection of different amounts of A_1 basic protein underneath a monolayer of cardiolipin (◑), the acidic lipid fraction (△), cerebroside sulphate (▼), the total lipid fraction from central nervous system (○), phosphatidylserine (▲), sphingomyelin (▽), lecithin (●) and cerebrosides (◐). The initial film pressure was 10 dynes/cm. The subphase was a 10^{-2} M Tris–acetate buffer, pH 5.0. (B) Automatically recorded pressure increases after the injection of different amounts of A_1 basic protein underneath a monolayer of the acidic lipid fraction of central nervous system. The initial film pressure was 10 dynes/cm. The subphase was a 10^{-2} M Tris–acetate buffer, pH 5.0.

Although the ultimate effect with the neutral lipids is less than with the acidic lipids the pressure increase is more rapid and is completed within 15 min. Figs 4A and 4B show the effect of the peripheral basic proteins P_1 and P_2 on different lipid monolayers. Although the highest pressure increases are found for the negatively charged lipids such as acidic lipids and cerebroside sulphate, and a somewhat smaller pressure increase for lecithin, it is clear that the P_1 and P_2 basic proteins do not show specificity to the extent found for A_1 basic protein.

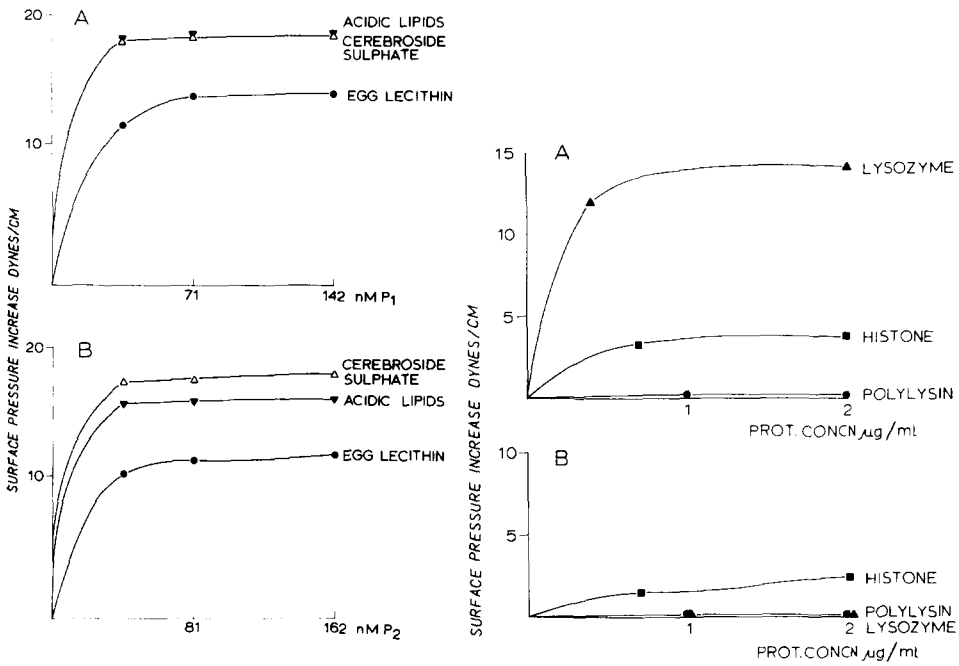

Fig. 4. Surface pressure increase after the injection of different amounts of P_1 and P_2 basic proteins respectively underneath a monolayer of cerebroside sulphate (▼), the acidic lipid fraction from central nervous system (△) and egg lecithin (●). The initial film pressure was 10 dynes/cm. The subphase was a 10^{-2} M Tris–acetate buffer, pH 5.0.

Fig. 5. (A) Surface pressure increase after the injection of different amounts of lysozyme, histone and polylysine underneath the acidic lipid fraction of central nervous system. The initial film was 10 dynes/cm. The subphase was a 10^{-2} M Tris–acetate, pH 5.0. (B) The same proteins are injected underneath an egg lecithin monolayer.

For comparison also some other basic proteins from other sources were investigated for their ability to affect the pressure of lipid monolayers (Fig. 5A, B). Only lysozyme shows a strong pressure increase with the acidic lipid fraction. Arginine-rich histone and especially polylysine have nearly no effect. None of these proteins increased the pressure of lecithin monolayers (Fig. 5B). It can be concluded that lysozyme is able to penetrate especially negatively charged monolayers. The strongly positively charged polylysine on the other hand, is not able to penetrate a monolayer of cerebroside sulphate or phosphatidylserine, but only adsorbs to the charged interface. After the injection of polylysine initially even a reduction in pressure is observed followed by a slow increase to the initial film pressure. To determine whether hydrophobic forces are also involved in the formation of the A_1 basic protein–lipid complex in addition to ionic forces, the effect of fatty acid chain length was studied. Fig. 6 shows the interaction of A_1 basic protein with a series of disaturated lecithins. The interaction is significantly decreased with decreasing chain length. The pressure increase for dilauroyl lecithin is twice the increase denoted for dioctanoyl lecithin. It has to be noted that the chain length and not the liquidity of the lipid monolayer is the determining factor since the same pressure increase is found

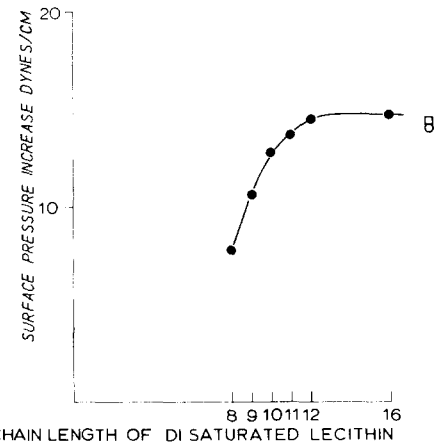

Fig. 6. The effect of lecithin fatty acid chain length on the surface pressure increase after the injection of A_1 basic protein. ●, dioctanoyl lecithin (di $C_{8:0}$ PC), dinonanoyl lecithin (di $C_{9:0}$ PC), didecanoyl lecithin (di $C_{10:0}$ PC), diundecyloyl lecithin (di $C_{11:0}$ PC), dilauroyl lecithin (di $C_{12:0}$ PC) and dipalmitoyl lecithin (di $C_{16:0}$ PC), respectively; □, dioleoyl lecithin (di $C_{18:1}$ PC); ○, dilinoleoyl lecithin (di $C_{18:2}$ PC).

for dipalmitoyl lecithin, dioleoyl lecithin and dilinoleoyl lecithin (Fig. 6). Besides the increase in pressure at constant area also the increase in area at constant pressure can be measured. For a cerebroside sulphate monolayer (initial pressure 10 dynes/cm) an area increase of 200% was found after the interaction with A_1 basic protein.

To study what percentage of the protein molecules is penetrating the lipid monolayer ^{131}I-labelled basic protein was used. Measurement of the surface radioactivity makes it possible to check if the differences in pressure increase observed with different lipids correlate with different amounts of protein penetrating the monolayer.

The pressure increase caused by the ^{131}I-labelled protein is practically identical to that of the unlabelled protein (Fig. 7A) so that the properties of the protein are not affected by the ^{131}I label. Also the peptide mapping of this labelled protein was found to be identical. Absolutely no change in surface pressure and surface radioactivity are measured when protein is injected in the absence of a monomolecular lipid layer. This means that the radioactivity is measurable only when the protein has interacted with a suitable film at the air–water interface.

In Figs 7A and 7B the simultaneous recordings of the pressure increase and radioactivity increase are presented. Fig. 7C shows that there is a linear dependence at concentrations smaller than 28 nM. The increase in pressure corresponds with the increase in surface radioactivity. At higher protein concentrations the monolayer becomes saturated and relatively more protein is adsorbed to the interface. Perhaps at higher protein concentrations this protein is bound to the surface by protein–protein interactions. The amount of protein (concentration 55 nM) bound to a cerebroside sulphate monolayer (initial pressure 10 dynes/cm) can be calculated when the specific radioactivity is known. The lipid:protein ratio can also be determined by moving the lipid–protein complex to a protein-free subphase as described in Methods. No loss in surface pressure was observed so that no desorption of protein

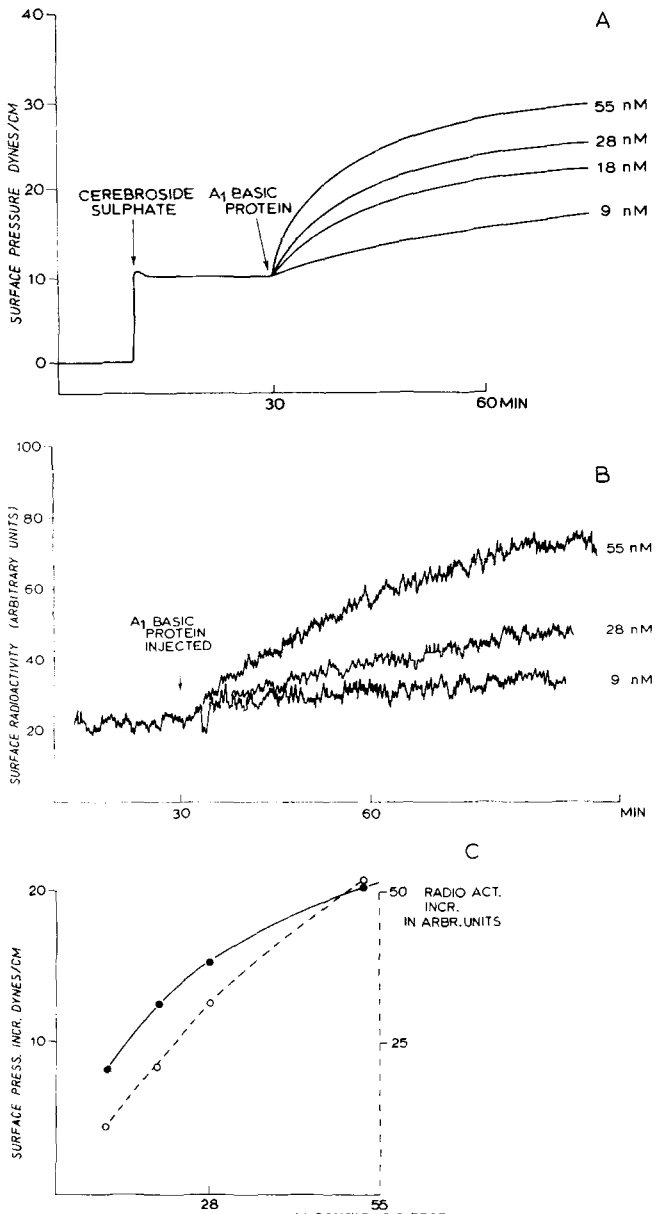

Fig. 7. (A) Automatically recorded pressure increases after the injection of different amounts of ^{131}I-labelled A_1 basic protein underneath a monolayer of cerebroside sulphate. The initial film pressure was 10 dynes/cm. The subphase was a 10^{-2} M Tris–acetate buffer, pH 8.0. (B) Automatically recorded surface radioactivity increases after the injection of different amounts of ^{131}I-labelled A_1 basic protein underneath a monolayer of cerebroside sulphate. The initial film pressure was 10 dynes/cm. The subphase was a 10^{-2} M Tris–acetate buffer, pH 8.0. (C) Surface pressure and surface radioactivity increase after the injection of ^{131}I-labelled A_1 basic protein. Data taken from A and B. The solid line represents the increase in surface pressure. The dotted line represents the increase in surface radioactivity.

was apparent even after 30 min. The monolayer was collected and analysed for cerebroside sulphate and protein. The lipid:protein molar ratio determined by both methods was approximately 30:1. In the case of lecithin monolayers only half of this amount of protein is bound to the lipid layer.

Since we demonstrated that the basic protein shows a high affinity for negatively charged lipids, the effect of charge inhibition by Ca^{2+} was studied. It is known that Ca^{2+} can bind strongly to negatively charged lipids[28,29]. Injection of $^{45}Ca^{2+}$ in the aqueous phase in the absence of a monolayer gives rise to an increase in the radioactivity measured (Fig. 8). This means that a high percentage of the subphase radioactivity is measured, which is in agreement with the observations of Quinn and Dawson[28]. Spreading of a cerebroside sulphate monolayer caused a rapid adsorption of Ca^{2+} to the interface and an increase in radioactivity. No desorption of Ca^{2+} was detected even after a longer period of time (*e.g.* 1 h). Since only labelled $^{45}Ca^{2+}$ was used, these trace amounts did not affect the surface pressure of the lipid film. Injection of basic protein underneath the monolayer caused a rapid increase in film pressure due to the penetration of the protein into the lipid phase. At the same time the $^{45}Ca^{2+}$ surface radioactivity decreased, indicating a complete expelling of Ca^{2+} from the lipid interface. A loss of 90–100% of $^{45}Ca^{2+}$ from the lipid phase is also found for the acidic lipid fraction and triphosphoinositides monolayers containing 20% egg lecithin.

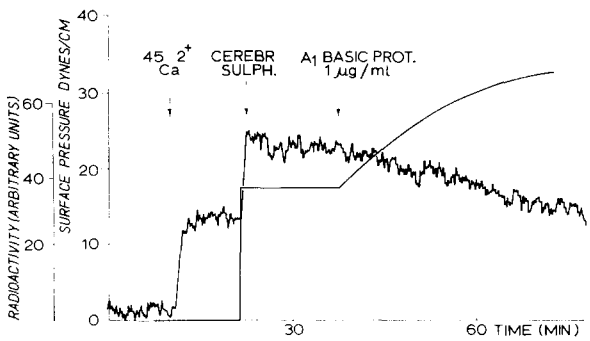

Fig. 8. The effect of Ca^{2+} on the interaction of A_1 basic protein with a monolayer of cerebroside sulphate. The penetration of the protein was measured by the change in surface pressure. The desorption of $^{45}Ca^{2+}$ was measured by the change in surface radioactivity.

When the initial pressure of the cerebroside sulphate film is raised from 10 to 21 dynes/cm, more $^{45}Ca^{2+}$ was found to be bound. Even at this pressure 85% of the $^{45}Ca^{2+}$ can be expelled by A_1 basic protein. No binding of $^{45}Ca^{2+}$ can be detected when the A_1 basic protein is injected and bound to the lipid layer. Polylysine, a basic protein which is found to interact only electrostatically with lipid monolayers, is also able to reject $^{45}Ca^{2+}$ from the lipid monolayer. Also the effect of other solutes on the lipid–protein interaction has been tested. The interaction of A_1 basic protein was not significantly affected by the presence of 2 or 4 M urea. Only the rate of interaction was reduced in the presence of urea. Spreading of the protein on a 4 M urea solution did not increase the surface activity. The P_1 and P_2 basic proteins behaved differently. The rate of interaction with lipids was not reduced on a 4 M urea subphase. However,

the surface activity of the P_1 and P_2 basic protein itself was increased on 4 M urea. 1 M NaCl reduced the pressure increase of a cerebroside sulphate monolayer with 8.0 dynes/cm at pH 8.0. Such a reduction was also found in the presence of 0.25 M KCNS. The effect of solutes could indicate the presence of ionic and hydrophobic interactions between cerebroside sulphate and protein. The effect on phosphatidylserine monolayers was less pronounced both at pH 8.0 and pH 5.0. When the quantity of Arg-(dimethyl) of the A_1 basic protein[5] adsorbed to cerebroside sulphate monolayers was determined, compared with the amount of this amino acid in the lipid-free protein, an increase of 25 to 35% of the Arg-(dimethyl) was found in the protein complexed with the lipid monolayer.

DISCUSSION

For many lipid-protein recombinations poorly defined proteins have been used, and the lipids used were often different from those found in the natural environment of the protein. It is dangerous to extrapolate or generalise the results obtained from such experiments. The actual lipid-protein interaction can depend on the lipid polar head groups, the arrangement of these moieties and the charge distribution. Also the fatty acyl distribution in the lipids will play an important role especially when hydrophobic interactions are involved. The spatial structure of the protein as well as the presence or absence of ions can essentially influence a physiologically significant interaction. It is very likely that most lipid-protein interactions are very specific for the compounds involved. The myelin basic protein is one of the few proteins of which the structure has been elucidated to a high extent and is known to be a membrane protein. The interfacial measurements demonstrate that A_1 basic protein from myelin can interact preferentially with highly negatively charged lipids, and these lipids occur to a particularly high extent in the myelin membrane. The pressure increase of the lipid monolayer due to the penetration of A_1 basic protein is most significant with cardiolipin, cerebroside sulphate and acidic lipid fraction derived from central nervous system lipids and contains cerebroside sulphate. All these lipids form liquid-expanded monolayers[29]. The pressure increase parallels the protein adsorption to the lipid monolayer as measured by surface radioactivity. The A_1 basic protein must have a high affinity for cerebroside sulphate since the complex can be transferred to a protein-free subphase without loss of radioactivity. The interaction with weaker negatively charged lipids such as phosphatidylserine and phosphatidylethanolamine as well as neutral lipids such as cerebroside, lecithin and cholesterol is much smaller. The small pressure increase is not attributed to the state of the film. Condensed films of cerebroside, cholesterol and saturated lecithin and expanded films of unsaturated lecithin gave an identical result when tested. The basic proteins P_1 and P_2 isolated from the peripheral nerve show a different behaviour and a less pronounced specificity for negatively charged monolayers. Basic proteins from other sources, such as lysozyme, histone and polylysine, show little pressure change in either neutral or negatively charged monolayers with the exception of lysozyme. The effect of pH on the interaction of the A_1 basic protein with lecithin monolayers can be attributed to neutralization of the 10 histidine residues in the protein molecule, which probably is accompanied by a conformational change and more effective penetration of the protein into the lipid film. The same protein quantity was shown to interact with

the lipid monolayer at pH 5.0 and pH 8.0 while the pressure increase at pH 8.0 was twice as high. The P_1 and P_2 basic protein which contains only two histidine residues did not show this pH effect. The experiments with 2 and 4 M urea show that the ultimate pressure increase of A_1 basic protein is not affected, but only the rate is decreased. This decrease in rate of interaction could be explained by the fact that urea dissociates intramolecular hydrogen bonds which are important for the microstructure of the protein and, in our case, determined the rate of interaction with the negatively charged lipid monolayer. The basic proteins from peripheral nerve show a different behaviour. The surface activity of these proteins is increased by urea, which could indicate a higher degree of structure as compared to the A_1 basic protein. This illustrates once more the specificity of the lipid–protein interactions so that the interaction of myelin basic proteins cannot be generalised. A_1 basic protein is found to displace Ca^{2+} completely from a cerebroside sulphate monolayer. This indicates that for the association of lipid and A_1 basic protein ionic forces are very effective. This makes it very unlikely that a cerebroside sulphate–A_1 basic protein–Ca^{2+} complex can be formed. The present experiments demonstrate already that charge interactions (high affinity for negatively charged lipids, influence of high salt concentration, displacement of Ca^{2+}) as well as hydrophobic interactions (influence of fatty acid chain length, high increase in area at constant pressure) play an important part in the basic protein sulpholipid interaction. In a subsequent paper, describing the effect of proteolytic enzymes on the interfacial complex of lipid–basic protein, the specificity of the complex as well as the parts of the protein which are involved will be further elucidated.

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