Hypothesis

Band 3 protein–cholesterol interactions in erythrocyte membranes

Possible role in anion transport and dependency on membrane phospholipid

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Band 3 protein of the human erythrocyte membrane, the anion transport protein, possesses a high affinity steroid binding site. In mixed phospholipid–cholesterol monolayers, the state of occupancy of this site is positively correlated with their cholesterol and sphingomyelin content and negatively with their glycerophospholipid content. We suggest that, in the erythrocyte membrane, the binding site is an inhibitory site of anion transport and that the modulation of its state of occupancy by the membrane lipid is responsible for the negative correlation of anion transport with the membrane's content of cholesterol and sphingomyelin and the positive correlation with the phosphatidylcholine content

Erythrocyte membrane *Band 3 protein* *Cholesterol* *Phospholipid* *Anion transport* *Lipid monolayer*

1. INTRODUCTION

Anion transport across the erythrocyte membrane, which is mediated by band 3, the membrane's main integral protein (review [1]), is strongly influenced by the lipid composition of the membrane:

(1) It decreases after enrichment and increases after depletion of membrane cholesterol, as shown with human erythrocyte membranes [2];
(2) When different mammalian species are compared, it is found to be negatively correlated with the sphingomyelin content and positively with the phosphatidylcholine content of the membranes [3,4].

The reason for this dependency of anion transport on membrane lipid is not clear; however, an attempt has been made to correlate the influence of phospholipids with their fatty acid composition [3,4].

We have described the incorporation of isolated band 3 protein (solubilized and purified in acetic acid and afterwards transferred into neutral aqueous solutions) into monolayers of cholesterol and cholesterol analogues [5,6]. The strong and specific interaction between band 3 and cholesterol found in these studies has led us to suggest that band 3 possesses a sterol binding site, the shape of which closely follows the shape of the cholesterol molecule [6]. The existence of this site has been confirmed by binding studies using equilibrium dialysis (Passing, R. and Schubert, D., submitted). Experiments on the incorporation of band 3 into mixed phospholipid–sterol monolayers described in [7] and extended here, have now revealed that the band 3–cholesterol interaction is strongly in-

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fluenced by the relative amount and composition of the phospholipid part of the monolayers. When our results are applied to the erythrocyte membrane, the seemingly unrelated findings on the lipid dependency of anion transport could be explained on the basis of the assumption that the sterol binding site of band 3 is an inhibitory site of anion transport.

2. MONOLAYER EXPERIMENTS

2.1. *Monolayers composed of cholesterol and mixtures of glycerophospholipids*

Solubilized band 3 protein from human erythrocyte membranes induces large changes $\Delta\pi$ in the surface pressure of monolayers of cholesterol at the air–water interface but, at pH values of 7–10, much smaller changes in phospholipid monolayers [5,6]. However, with mixed monolayers composed of cholesterol and a glycerophospholipid with varying mole fraction X of sterol in the mixture, $\Delta\pi$ (X) only increases towards the high values that are characteristic of cholesterol monolayers if $X > 0.67$; $\Delta\pi$ (X) at lower cholesterol content is virtually identical to the value obtained with the pure phosphoglyceride [7]. Analogous results are obtained when, in those experiments, a mixture of glycerophospholipids is used instead of a single glycerophospholipid (fig. 1). Thus, the cholesterol molecules are unavailable for interaction with band 3 protein unless the relative molar cholesterol content in the mixed monolayers exceeds ~0.67, regardless of the number of different glycerophospholipid classes present (for interpretation see [7]).

2.2. *Monolayers composed of cholesterol and phospholipid mixtures simulating the outer or inner half of the human erythrocyte membrane*

The behaviour of mixed monolayers of cholesterol and sphingomyelin towards band 3 protein

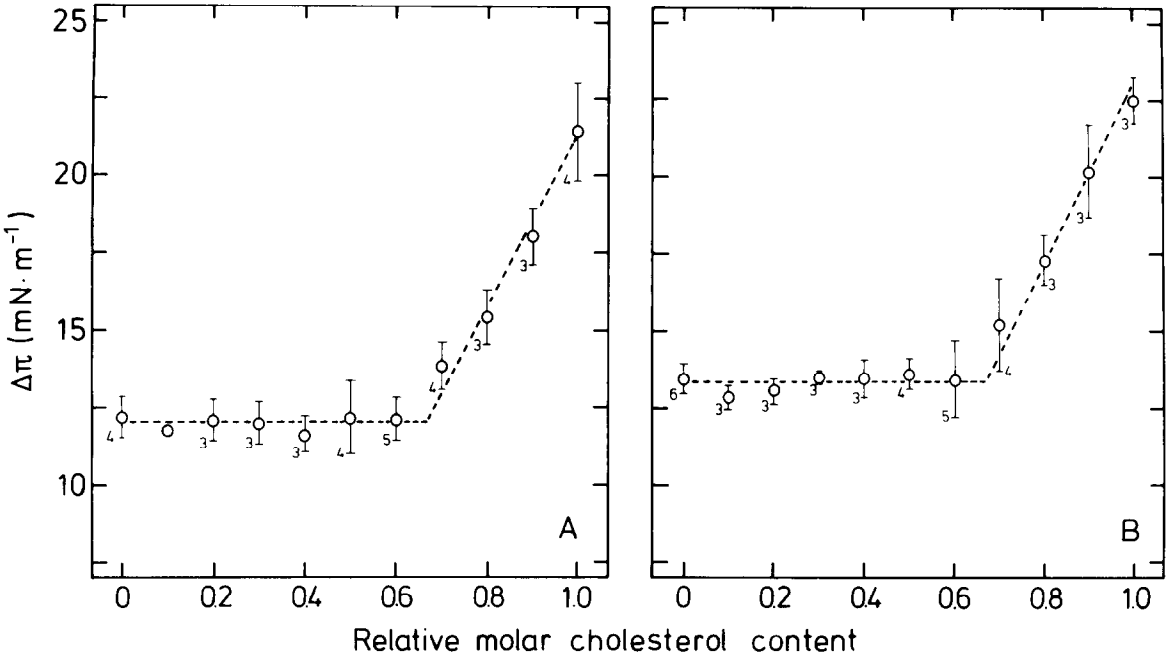

Fig. 1. Dependency of $\Delta\pi$ on relative molar cholesterol content for monolayers composed of cholesterol plus mixtures of glycerophospholipids. The compositions of the latter were (molar ratios) phosphatidylcholine/phosphatidylethanolamine, 44:12 (A) and phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine, 15:47:28 (B) and correspond to that present in the outer and inner half of the human erythrocyte membrane [8]. Initial lipid pressure: $\pi_i = 10$ $mN.m^{-1}$. The bars and figures at the data points represent standard deviation and number of measurements, respectively. The dotted lines were drawn as in [7]. For techniques and materials, see [6,9].

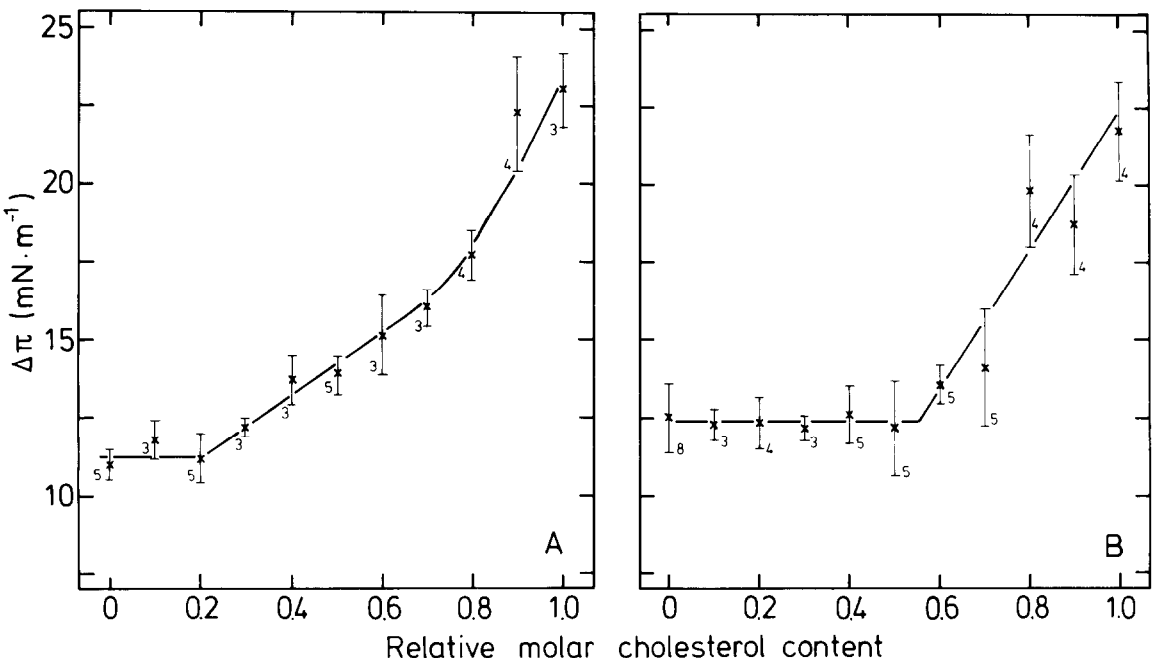

Fig. 2. Dependency of $\Delta\pi$ on relative molar cholesterol content for monolayers of cholesterol plus a phospholipid mixture simulating the outer half (A) phosphatidylcholine/phosphatidylethanolamine/sphingomyelin (44:12:44) and the inner half (B) phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine/sphingomyelin (15:47:28:10) of the human erythrocyte membrane [8]. The sphingomyelin used was from bovine spinal cord. Virtually identical results were obtained when sphingomyelin from bovine erythrocytes was used. For further details see legend to fig. 1.

differs greatly from that of cholesterol–glycerophospholipid monolayers: with these mixtures there is a linear increase of $\Delta\pi(X)$ with increasing cholesterol content, X, starting from $X = 0$ [7]. As a consequence, the interactions of mixed cholesterol–glycerophospholipid–sphingomyelin monolayers with band 3 are strongly influenced by the sphingomyelin content of the mixtures. This is demonstrated in fig. 2 for the phospholipid mixtures that simulate the outer or inner half of the human erythrocyte membrane, the molar ratio of sphingomyelin and total phospholipid in these mixtures being 0.44 and 0.10, respectively [8]. As can be seen from the figure, for the mixture simulating the outer membrane leaflet $\Delta\pi(X)$ is constant for $0 \leqslant X \leqslant 0.2$, but increases distinctly and linearly with increasing X for $0.2 < X \leqslant 0.7$, until the slope of $\Delta\pi(X)$ increases further at still higher values of X. On the other hand, with the phospholipids of the inner leaflet, $\Delta\pi(X)$ increases with increasing X only if X is $\leqslant 0.55$. Cholesterol thus becomes available for interactions with band 3 protein at molar ratios (sterol:total lipid) exceeding 0.2 in lipid monolayers when the phospholipid composition corresponds to that of the outer half of the erythrocyte membrane (due to the high sphingomyelin content of the mixture), but only at molar ratios exceeding ~0.55 in monolayers simulating the membrane's inner half.

3. IMPLICATIONS FOR BAND 3-CHOLESTEROL INTERACTIONS IN THE ERYTHROCYTE MEMBRANE

The molar ratio cholesterol/cholesterol plus phospholipid in the human erythrocyte membrane is ~0.42 [2,10], cholesterol thus being the membrane's most abundant lipid species. The distribution of the sterol between the two membrane leaflets is not clear. Two studies arrived at the conclusion that ~⅔rds of the cholesterol are located in the outer membrane half [11,12], whereas a nearly symmetrical distribution was deduced from another study [13]. Thus the molar ratio chol-

esterol/cholesterol plus phospholipid will probably be somewhere between 0.42 and 0.56 in the outer monolayer of the erythrocyte membrane, but between 0.28 and 0.42 in the inner monolayer. As mentioned above, 44% of the phospholipid of the outer monolayer is sphingomyelin, another 44% phosphatidylcholine [8,14]. In the inner monolayer, sphingomyelin amounts to only 10% of the phospholipid [8,14] (it is completely absent in rat erythrocyte membranes [15,16]) and phosphatidylcholine 15% [8,14].

If we assume that the results of our monolayer experiments are applicable to the situation in the human erythrocyte membrane, the following conclusions can be drawn:

(1) Band 3-cholesterol interactions can occur in the outer half of the membrane, due to its high cholesterol and sphingomyelin content;
(2) Removal of cholesterol from the outer membrane half would reduce the number of band 3 molecules interacting with the sterol, whereas enrichment of cholesterol would increase it;
(3) Similarly, exchange of sphingomyelin against phosphatidylcholine would reduce band 3-cholesterol interactions, whereas exchange of phosphatidylcholine against sphingomyelin would increase them;
(4) Under normal conditions, band 3-cholesterol interactions cannot occur in the inner monolayer of the erythrocyte membrane*.

In drawing these conclusions, we have disregarded the possibility that the band 3-cholesterol interaction described for the monolayer system could be a preparation artifact. We think that this possibility is very unlikely, because of the high specificity and high affinity of the interaction observed both in the monolayer experiments [6] and by equilibrium dialysis (Passing, R. and Schubert, D. submitted).

* The structural basis of the unavailability of cholesterol for band 3 in mixed cholesterol–glycerophospholipid monolayers at relative molar sterol contents below 0.67 has been assumed to be the formation of cholesterol–glycerophospholipid complexes of stoichiometry 2:1 [7]. Band 3–cholesterol interactions could possibly occur also in the inner monolayer of the erythrocyte membrane if, in the membrane, the stoichiometries of the complexes were different from those in the monolayers [7]. However, this would not invalidate the other conclusions drawn

4. POSSIBLE FUNCTIONAL ROLE OF BAND 3-CHOLESTEROL INTERACTIONS AND ITS MODULATION BY PHOSPHOLIPIDS

In the preceding paragraph, correlations have been deduced from monolayer experiments between the occurrence of band 3–cholesterol interactions in the human erythrocyte membrane and the membrane's lipid composition, especially its content of cholesterol, sphingomyelin (both positive) and phosphatidylcholine (negative)**. As described in the introduction, anion transport across the erythrocyte membrane, which is mediated by band 3 protein, is also correlated with the membrane's content of the three lipid species mentioned: negatively with the cholesterol and sphingomyelin content, and positively with the content of phosphatidylcholine [2–4]. We now suggest that the two groups of correlations have a common basis, namely that the sterol binding site of band 3 is an inhibitory site of anion transport and that its state of occupancy is modulated by the membrane phospholipid just as described in the preceding paragraph. Such a behaviour would directly lead to the dependency of anion transport on membrane lipid described above (especially if it is assumed that the sterol binding site of band 3 is located in the outer monolayers of the erythrocyte membrane). It would also be consistent with the recent finding that sulphate transport by reconstituted band 3 protein in liposomes containing, besides ~25 mol% cholesterol, varying amounts of phosphatidylcholine and sphingomyelin, decreases strongly at high sphingomyelin content [17,18]. Evidence of whether our suggestion is valid or not may come from reconstitution experiments.

In summary, we suggest that a cholesterol binding site on band 3 protein could be an inhibitory site of anion transport. This site, probably located in the outer leaflet of the erythrocyte membrane, would be only partly occupied in normal erythrocyte membranes, due to an interference from cholesterol–phospholipid interactions. The glycerophospholipids (mainly phosphatidylcholine) would contribute by shielding cholesterol

** The applicability of the results of our monolayer experiments to the situation in the erythrocyte membrane at present cannot be proven, but has to be regarded as part of our hypothesis

from protein–sterol interaction, and thus preventing the sterol from inhibiting anion transport, whereas sphingomyelin would contribute by making cholesterol available for interaction with band 3, thus indirectly inhibiting transport.

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REFERENCES

[1] Cabantchik, Z.J., Knauf, P.A. and Rothstein, A. (1978) Biochim. Biophys. Acta 515, 239–302.
[2] Grunze, M., Forst, B. and Deuticke, B. (1980) Biochim. Biophys. Acta 600, 860–869.
[3] Gruber, W. and Deuticke, B. (1973) J. Membr. Biol. 13, 19–36.
[4] Lu, Y.-B. and Chow, E.I. (1982) Biochim. Biophys. Acta 689, 485–489.
[5] Klappauf, E. and Schubert, D. (1977) FEBS Lett. 80, 423–425.
[6] Klappauf, E. and Schubert, D. (1979) Hoppe-Seyler's Z. Physiol. Chem. 360, 1225–1235.
[7] Schubert, D. and Marie, H. (1982) Biochim. Biophys. Acta 684, 75–82.
[8] Zwaal, R.F.A., Comfurius, P. and Van Deenen, L.L.M. (1977) Nature 268, 358–360.
[9] Schubert, D. and Klappauf, E. (1980) Hoppe Seyler's Z. Physiol. Chem. 361, 1171–1177.
[10] Johnson, S.M. (1979) Anal. Biochem. 95, 344–350.
[11] Fisher, K.A. (1976) Proc. Natl. Acad. Sci. USA 73, 173–177.
[12] Schroeder, F. (1981) FEBS Lett. 135, 127–130.
[13] Blau, L. and Bittman, R. (1978) J. Biol. Chem. 253, 8366–8368.
[14] Zwaal, R.F.A., Roelofsen, B. and Colley, C.M. (1973) Biochim. Biophys. Acta 300, 159–182.
[15] Renooj, W., Van Golde, L.M.G., Zwaal, R.F.A. and Van Deenen, L.L.M. (1976) Eur. J. Biochem. 61, 53–58.
[16] Crain, R.C. and Zilversmit, D.B. (1980) Biochemistry 19, 1440–1447.
[17] Köhne, W. and Deuticke, B. (1982) Pflüger's Arch. 392 (suppl.), R 16.
[18] Köhne, W. (1982) PhD Thesis, RWTH Aachen.