Effects of Vitamin A in the Presence of Vitamins D_3, E, K_1 on Red Cell Membrane Structure

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Z. Naturforsch. **39 c**, 749–752 (1984); received January 3, 1984

Lipophilic Vitamins, Erythrocyte Membrane, Cell Fusion, Fluorescence Anisotropy

The incubation of erythrocyte ghosts with mixtures of vitamins A+D_3, A+E, A+K_1 produces decrease or increase of fluorescence anisotropy r of the DPH probe, depending on the vitamin/vitamin ratio. We found a correlation between the order parameter S and cell fusion phenomena observed by phase contrast microscopy.

1. Introduction

In a previous work [1] we observed that high doses of lipid soluble vitamins A, E, K_1 on the erythrocyte membranes induce the formation of lipid phases different from the bilayer, increase membrane fluidity and produce cell fusion. Vitamin D_3, on the contrary, stabilizes the bilayer phase, increases the membrane microviscosity and induces erythrocyte aggregation without fusion. These results suggest that the presence of external agents such as lipid soluble vitamins under condition of low enthalpy, may produce molecular movements and rearrangements that result in remarkable modifications of the bilayer organization.

Preliminary experiments [2] indicated that the lytic effect of retinol on membranes is significantly reduced by the presence of vitamin D_3 or K_1. This phenomenon would be in agreement with the stabilizing effect of the D_3 vitamin, but it is less predictable taking into account the fusogenic activity of K_1 vitamin. In the present work we study the effect of vitamin A on erythrocyte membranes in the presence of high concentrations of vitamin D_3, E and K_1 respectively, by spectrofluorimetry and *in vitro* cell fusion, with the aim of getting more insight on the possible protection of these vitamins towards the toxic effect of vitamin A.

2. Materials and Methods

Vitamin A (all-trans retinol), Vitamin E (α-tocoferol) and DPH (1,6-diphenyl-1,3,5-hexatriene) were purchased from Sigma (Saint Louis MO); Vitamin D_3 (cholecalciferol), Vitamin K_1 (3-phythylmenadione) and D_2O were purchased from Merck AG (Darmstadt). Vitamins A, E, D_3 and K_1 were quantitated and used as described in a previous paper [1].

For fluorescence measurements we used the following vitamin/vitamin ratios:

1. Vitamins A + E; vitamin E concentration was kept constant at 200 μM (vitamin/lipid ratio = 4/1) and vitamin A concentration varied between 0.05 μM and 200 μM.

2. Vitamins A + K_1; vitamin K_1 concentration was kept constant at 200 μM (vitamin/lipid ratio = 4/1) and that of vitamin A varied as before.

3. Vitamins A + D_3; we prepared two different sets of mixtures of these two vitamins in order to assay the effects of two different vitamin D_3 concentrations (8 μM and 200 μM); the vitamin A concentration was varied as before.

The same vitamin/lipid ratio was used for erythrocyte incubation *in vitro* and for the experiments on erythrocyte ghosts. The lipid concentration was assayed following Zilversmit [3]. Cholesterol determinations were performed by the method of Roschlan [4]. Experiments on *in vitro* cell fusion were carried out on human (A, Rh+) and chicken erythrocytes as indicated in ref. [1]. Incubation of the red cells with the proper vitamin mixtures was performed at 37 °C for periods of 5, 10, 15, 20, 30 and 40 min. The cells were then immediately observed under a phase contrast microscope (Zeiss).

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Cell fusion was quantitated by counting at least 25 microscopic fields at magnification of 250 ×.

Resealed erythrocyte ghosts were prepared according to ref. [1]. All ghosts preparations were diluted with buffer (0.06 M KCl, 0.12 M NaCl, 0.001 M Tris, pH 7.45) to a final lipid concentration of 5 μM. Incorporation of the DPH probe was obtained according to the procedure of Lenz [5]. 3 μl of 2×10^{-3} M DPH in tetrahydrofuran was added to 3 ml of a ghost suspension. The samples were kept for 90 min at room temperature to allow a complete binding of the DPH probe to the membrane. Fluorescence anisotropy was measured using a MPF 44 Perkin Elmer spectrofluorimeter equipped with accessories for polarization as in Shinitzky *et al.* [6].

3. Results

1. Vitamins A + E

Fig. 1 A shows that a contemporary presence of vitamins A and E at concentrations ranging from 0.05 μM to 20 μM of vitamin A does not induce significant variations in the fluorescence anisotropy *r* of the DPH probe. At concentrations exceeding 20 μM a small decrease of the fluorescence anisotropy is observed.

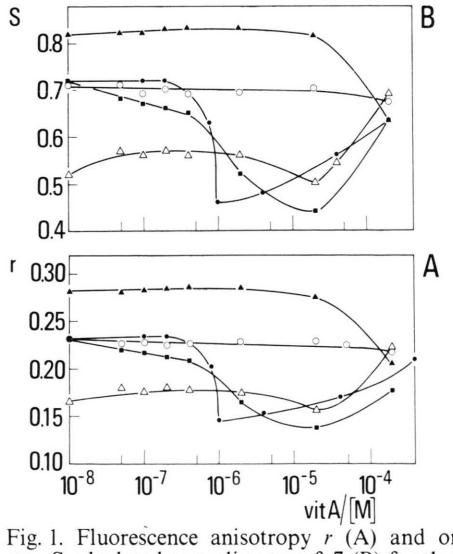


Fig. 1. Fluorescence anisotropy *r* (A) and order parameter *S* calculated according to ref. 7 (B) for the DPH probe in erythrocyte membrane as a function of vitamin A concentration, in the presence of ▲ 200 μM vitamin D_3; ■ 8 μM vitamin D_3; △ 200 μM vitamin K_1; ○ 200 μM vitamin E. Effect of vitamin A alone ● is reported for a comparison.

Incubation of chicken erythrocytes with the proper mixtures of the two vitamins shows no occurrence of cell fusion up to vitamin A concentration of 50 μM; above this concentration few fusion events occur (20%) at 10 min of incubation. When vitamin A concentration reaches the 200 μM value (vitamin A/vitamin E molar ratio = 1/1) a great number of fusions are observed at 10 min of incubation (60%) (Fig. 2). For all the vitamin/vitamin molar ratios, incubation produces cell lysis starting after 20 min of incubation and completed after 40 min.

2. Vitamins A + K_1

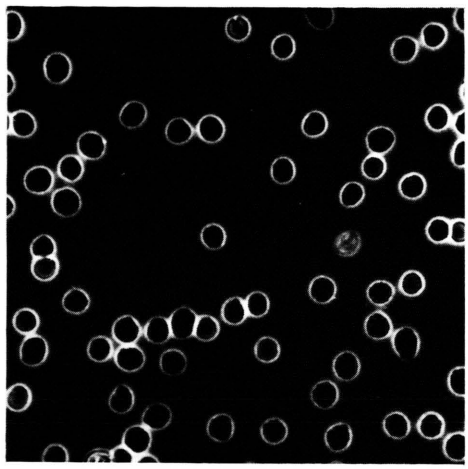
Fig. 1 A compares also the effects of vitamin A alone with the effects of vitamins A + K_1. It can be seen that in both cases fluorescence anisotropy has similar trends.

In the fusion experiments carried on both in the presence of vitamin A alone and vitamins A and K_1, at the same lipid/vitamin ratios used for fluorescence measurements, a great number of fusions (60%) is observed (Fig. 2). This effect is maximum at vitamin A or vitamins A + K_1 concentrations close to those that produce the minimum value in *r* curves.

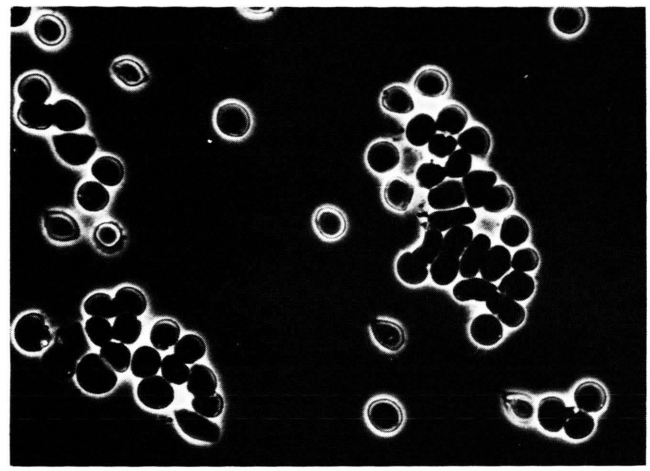
When the two vitamins were present at high doses and at equimolar ratios (200 μM vitamin A + 200 μM vitamin K_1, or 400 μM vitamin A + 400 μM vitamin K_1) no cell fusion was detactable. Other *in vitro* experiments were also performed with different vitamin concentrations and it was observed that for vitamin K_1 to vitamin A ratio 2:1 with 874 μM vitamin K_1, few fusion events occurred less than 5%; the same behavior was observed in the presence of vitamin K_1 to vitamin A ratio 1:2 with 218 μM vitamin K_1. On the other hand, at high vitamin K_1 to vitamin A ratios (1:4) with 109 μM vitamin K_1 extensive fusion phenomena were produced (80%).

3. Vitamins A + D_3

In Fig. 1 A the effects of vitamins A + D_3 mixtures are also compared to the effects of vitamin A alone. The fluorescence anisotropy data show that, at low doses of vitamin D_3, the effects induced by vitamin A are dominating. On the contrary, in the presene of high doses of vitamin D_3 and low vitamin A concentrations, very high values of fluorescence anisotropy, even higher than those



a)



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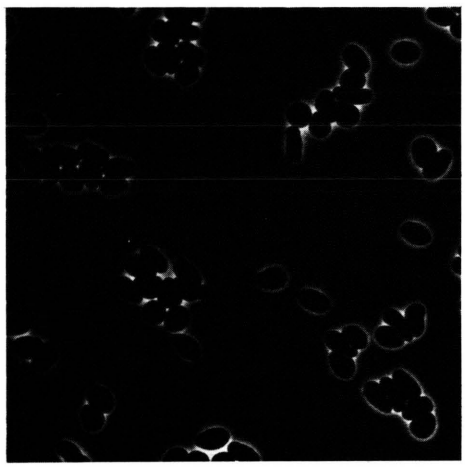

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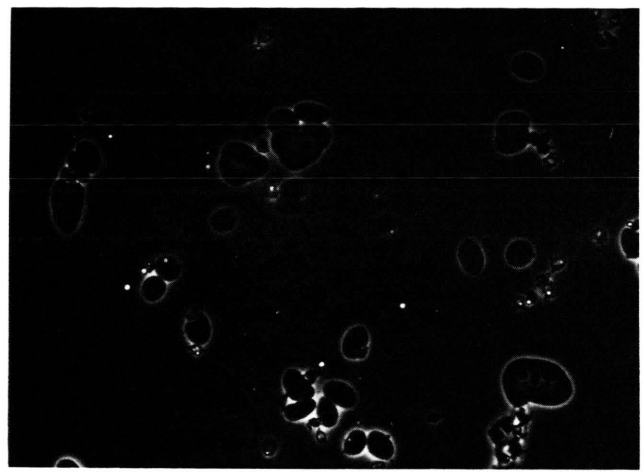

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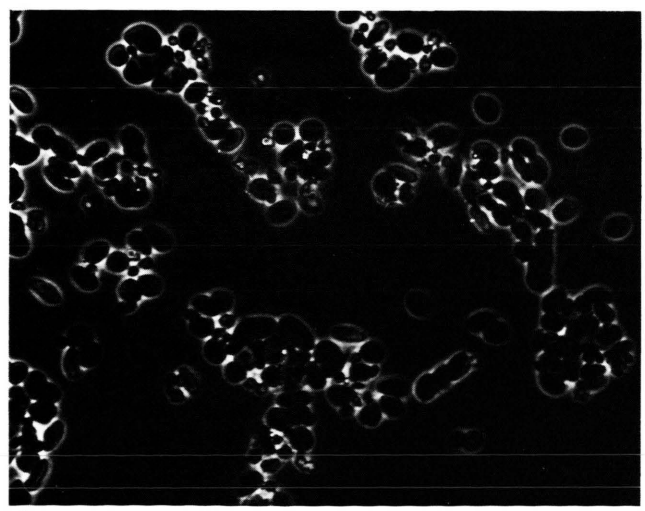

e)

Fig. 2. Phase contrast micrographs of erythrocytes after incubation with vitamins for 10 min at 37 °C; a) human erythrocytes, control sample; b) human erythrocytes in the presence of 200 μM vitamin A + 200 μM vitamin E; c) chicken erythrocytes, control sample; d) chicken erythrocytes in the presence of 100 μM vitamin A + 200 μM vitamin D_3; e) chicken erythrocytes in the presence of 50 μM of vitamin A + 200 μM vitamin K_1.

observed for vitamin D_3 alone, are recorded. When vitamin A concentration reaches 1×10^{-4} M, in the presence of 2×10^{-4} M vitamin D_3, a sharp decrease in the fluorescence anisotropy r of the DPH probe occurs.

The *in vitro* cell fusion experiments show that, at low vitamin D_3 to vitamin A ratios, the pattern of fusion observed is similar to what observed in the presence of vitamin A alone. On the other hand, high doses of vitamin D_3 (200 μM), fail to induce a significant number of fusion events at least until vitamin A concentration is 100 μM (Fig. 2).

In fact, at these doses, an approximative number of 20% fusions are observed in 2–4 cell syncytia. At higher concentrations of both vitamins, extensive fusions are induced (more than 60%), for 5–10 min of incubation. For longer incubation times, cells show extensive lysis.

4. Discussion

The present results indicate that incubation of erythrocyte ghosts with mixtures of the assayed vitamins produces increase or decrease of fluorescence anisotropy r. Following a recent suggestion by Pottel *et al.* [7] we have quantified the observed changes in r by calculating the orientational order parameter S (Fig. 1 B). In fact, for high values of r_s/r_0, calculation of S by a semi-empirical method gives reliable values. In our cases S follows the same trend of the fluorescence anisotropy r.

A general correlation between S, reflecting the packing of the lipids in the membrane, and the occurrence of fusion events holds at least for the tested vitamin concentrations. Moreover for a r_0 we have used the value $r_0 = 0.395$.

The effects produced by vitamin A could be at least partially decreased by the presence of a second lipid soluble vitamin. Due to their lipophilic structure these vitamins can be incorporated in the lipid portion of the cell membrane, therefore producing new molecular equilibria depending on the interactions which can be established either between vitamins and membrane components or/and between the vitamins themselves.

Considering the data concerning vitamins A + K_1 and vitamin A, we may suggest that above a critical concentration, namely 20 μM, these vitamins may exist in the membrane as microdomains leading to an increase of the packing of the membrane and therefore preventing cell fusion phenomena. On the contrary, lower concentrations exert a perturbing effect on the membrane by decreasing lipid packing and causing cell fusion.

Membrane behavior from both fluorescence and fusion experiments under the combined effects of vitamins A and D_3 would be consistent with this hypothesis. In fact keeping in mind that vitamin D_3 alone increases fluorescence anisotropy [1], when the concentration of vitamin D_3 is higher than that of vitamin A its stabilizing effects on the membrane are dominating: we do not observe fusion events and S remains very high. On the contrary, when vitamin A is higher or comparable to vitamin D_3 concentrations, S is strongly reduced and parallely cell fusion phenomena are observed.

Considering that vitamin D_3, similarly to cholesterol [8], increases the order parameter, it is very probable that vitamin D_3 at high concentrations forms microdomains. High doses of vitamin A may perturbe this situation.

A similar explanation holds also for interpretation of the effects of vitamins A + E at the assayed concentrations. In this case the stabilizing action of vitamin E could be dependent on the saturated nature of its hydrocarbon chain.

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