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MEMBRANE LIPID FLUIDITY AS RATE LIMITING IN THE CONCANAVALIN A-MEDIATED AGGLUTINATION OF pyBHK CELLS

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Summary

The initial rate of concanavalin A-mediated agglutination of polyoma transformed Baby Hamster Kidney (pyBHK) cells follows Arrhenius kinetics. There is a smooth decrease in the agglutination rate from 37°C to 22°C with an activation energy of 11.8 ± 0.2 kcal/mol in this region. There is a sharp decrease in agglutination rate below 22°C. The addition of 0.1 mM 1,3-di-*tert*-2-hydroxyl-5-methylbenzene, a lipid perturber, increases the agglutination rate by a factor of two and increases the membrane lipid fluidity as determined by the spin label method. The rotational correlation time of the spin label 2N14 (2,2-dimethyl-5-dodecyl-5-methyloxazolidine-*N*-oxide) was measured. The sum of the enthalpy of activation of rotational diffusion and the enthalpy of activation of translational diffusion is very nearly equal to the enthalpy of activation of agglutination. This is consistent with the rate limiting step of agglutination being receptor diffusion, which is probably limited in pyBHK cells by membrane lipid fluidity.

Introduction

Plant lectins agglutinate cells by binding to the plasma membrane and forming cross-bridges between cells. Transformed variants are usually agglutinated at a lower lectin concentration than normal cells [1]. The state of the membrane lipids has been shown to affect the agglutination process [2,3]. The agglutination behaviour of cells can be modified by changing the fatty acid composition of the membrane using lipid-depleted serum and exogenous fatty

Abbreviation: BHT, 1,3-di-*tert*-butyl-2-hydroxyl-5-methylbenzene.

acids. The addition of more saturated fatty acids tends to increase the minimum temperature at which concanavalin A and wheat germ agglutinin-mediated agglutination occur [2]. The critical temperatures for the binding of concanavalin A to mouse LM cells and the concanavalin A-mediated hemadsorption of rabbit erythrocytes to mouse LM cells are also increased by more saturated fatty acids [3]. More unsaturated fatty acids tend to decrease the critical temperatures for these processes, possibly indicating the requirement for a certain degree of membrane fluidity. The fatty acid composition has also been shown to shift the optimum temperature for mitogenic stimulation of lymphocytes by concanavalin A. Presumably, diffusion of concanavalin A receptors is necessary for agglutination and the state of the membrane lipids may affect receptor diffusion.

As reported in this study, we have altered membrane lipid fluidity, meameasured by the ESR spin label method, by varing the temperature and by addition of the lipid perturbing compound BHT. Increasing the fluidity by either method increases the initial rate of concanavalin A-mediated agglutination of pyBHK cells. A thermodynamic analysis for this system is consistent with the rotational diffusion and the translational diffusion being the rate limiting steps for agglutination.

Materials and Methods

Cell culture. BHK (baby hamster kidney) cells and pyBHK (polyoma transformed BHK) cells were grown in Eagle's minimal essential medium supplemented with L-glutamine and 15% fetal calf serum with 100 units/ml penicillin and 100 μg/ml streptomycin. Cells to be used for experimental purposes were grown in roller bottles, harvested in mid-log phase with 0.05% EDTA and maintained in suspension at 37°C in a spinner flask containing calcium- and magnesium-free Eagle's basal medium supplemented with 15% fetal calf serum for the duration of the experiment (usually 4 h).

Agglutination assay. Cells were removed from the suspension culture as required, centrifuged at $200 \times g$ for 5 min and resuspended to 10^6 cells/ml in phosphate buffered saline containing calcium and magnesium. The agglutination assay was performed by adding 0.5 ml of this cell suspension to 0.5 ml of a concanavalin A (Sigma Chemical Co., grade IV) or a phosphate-buffered saline solution in a 5 ml polystyrene tube. The final concanavalin A concentration was 1 mg/ml for the pyBHK cells and 2 mg/ml for the BHK cells. The tubes were placed in a temperature controlled shaker bath (Eberback Corp.) set at a shaking rate of 170 cycles/min. The agglutination was quantitated by counting the number of single cells remaining as a function of time. This was accomplished with a Celloscope cell sizer (Particle Data Corp.) interfaced with a digital computer (Hewlett Packard, 2116C) for data analysis.

BHT treatment. The BHT, purchased from Aldrich Chemical Co., was introduced into the cells in the following manner: A stock solution of BHT was made by dissolving the BHT in ethanol. An aliquot of this solution was added to phosphate-buffered saline and vortexed vigorously to disperse the BHT. This was added to an equal volume of cells in phosphate-buffered saline resulting in a final cell concentration of 10^6 cells/ml and a BHT concentration of 0.1 or

0.2 mM. The ethanol concentration was maintained below 0.5%.

Electron spin resonance. The rotational correlation time of the nitroxide spin label 2N14 (2,2-dimethyl-5-dodecyl-5-methyloxazolidine-*N*-oxide) was used as a measure of lipid fluidity. Cell homogenates of untreated and BHT-treated cells were prepared using a Dounce homogenizer. The homogenates were centrifuged in a Beckman Airfuge for 10 min at 140 000 × *g* and 25 μl of pellet added to a small test tube which had 8.4 nmol of 2N14 dried from an ethanol solution onto the side of the tube. The test tube was vortexed for 30 s to dissolve the 2N14 into the membranes. $K_3Fe(CN)_6$ (5 mM) was added to stop nitroxide reduction of the spin label by cellular reducing agents. ESR spectra were taken immediately after sample preparation using a Varian E-12 spectrometer.

The spin label 2N14 consists of an alkane chain 14 carbons long and a nitroxide radical containing oxazolidine ring attached to the second carbon [5]. In membranes it undergoes isotropic rotation and a parameter describing the rate of rotation, the rotational correlation time (τ_c) can be calculated from the relation

$$\tau_c(s) = 6.9 \cdot 10^{-10} w_1 \left[\left(\frac{h_1}{h_{-1}}\right)^{1/2} - 1\right]$$

where w_1 is the low field line width and h_1 and h_{-1} are the low and high field line heights, respectively, from the first derivative spectrum. This is a modification of the formula given in Ref. 6 which is rearrangement of an expression first given in Ref. 7. The constant value of $6.9 \cdot 10^{-10}$ was determined from the parameters given in Ref. 8. The low and high field lines were used to calculate τ_c because occasionally spin exchange can increase the area of the mid field line, which can lead to large errors in τ_c. The rotational correlation time can be related to the viscosity by the Stoke's relation

$$\tau_c = 4\pi R^3 \eta / 3kT$$

as previously described [9]. All values of τ_c calculated from spin label spectra are only relative and may differ from the true values by a factor as great as two or three, due primarily to rotational anisotropy [6]. Thus absolute values of τ_c are unimportant, but comparisons between values are quite meaningful. Since viscosity and fluidity are inversely related, we define fluidity as being proportional to $1/\tau_c$.

Results

Kinetics of agglutination

The rate of agglutination was determined from the decrease in the number of single cells as a function of time after addition of concanavalin A. Shown in Fig. 1 are representative curves obtained for concanavalin A (1 mg/ml) mediated agglutination with and without concanavalin A for pyBHK cells at 22°C. The number of single cells remaining as a function of time was fitted to an empirical quadratic equation

$$\ln[S(t)] = At^2 + Bt + C$$

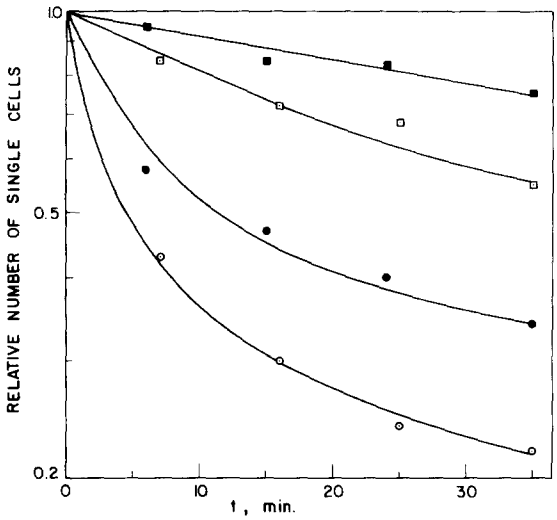

Fig. 1. The number of single cells as a function of time after addition of concanavalin A for pyBHK cells at 22°C. The curves are the best parabolic fit through the experimentally determined points. Single cells: ■, without concanavalin A; ●, with 1 mg/ml concanavalin A; □, with 0.1 mM BHT and no concanavalin A; ○, with BHT and concanavalin A.

where $S(t)$ is the number of single cells and A, B and C are constants. The $\ln[S(t)]$ was plotted since this gves a straight line (the constant A is nearly zero) at low rates of agglutination. The initial rate of agglutination, the absolute value of the slope at $t = 0$, is given by

$$\left|\frac{dS(t)}{dt}\right| t = 0 \Big| = |B|$$

for $S(0)$ normalized to one. The initial rate of concanavalin A-mediated agglutination was determined by subtracting the rate of nonspecific agglutination in the absence of concanavalin A from that in the presence of concanavalin A. All values of concanavalin A-mediated agglutination were corrected for the nonspecific agglutination. The addition of 0.1 mM BHT increased both the concanavalin A-mediated agglutination and the nonspecific agglutination.

Sugar specificity

The concanavalin A-mediated agglutination is specifically inhibited by the sugar D-(+)-mannose. D-(+)-Mannose was added to the concanavalin A in phosphate-buffered saline and incubated for 5 min before addition of the cells to give a final concentration of 4 mM D-(+)-mannose. This inhibited the concanavalin A-mediated agglutination to 6% of the control value for cells without BHT and to 10% of the control value for cells treated with 0.1 mM BHT.

Effect of temperature

The effect of temperature upon the initial rate of agglutination for pyBHK cells is shown in Fig. 2. Between 22 and 37°C each point is the average of three

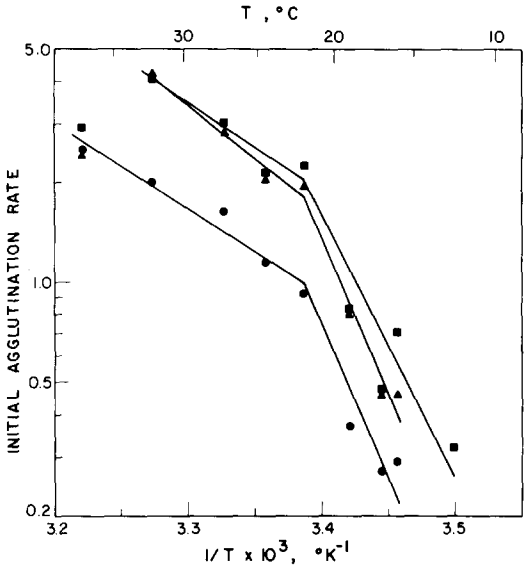

Fig. 2. The ln of the initial rate of concanavalin A-mediated agglutination ($|dS(t)/dt|t = 0$) as a function of $1/T$ for pyBHK cells. ●, without BHT; ▲, with 0.1 mM BHT; ■, with 0.2 mM BHT.

or more separate experiments and below 22°C each point is the average of two experiments. The initial rate of agglutination was normalized to one at 22°C. The Arrhenius plot gives a straight line from 22 to 37°C, indicating a temperature dependence of the form $e^{-E_A/kT}$ where E_A is the activation energy of agglutination and k is the Boltzman constant. The linear Arrhenius plot between 22 and 37°C in Fig. 1 indicates only one rate-limiting step in the agglutination process in this temperature range [10]. The rate of agglutination decreases with decreasing temperature to 22°C. Below this point there is a sharp decrease in agglutination, which becomes undetectable at 12.5°C. The break at 22°C may be due to concanavalin A-binding since agglutination is a multistep process [3]. 3T3 and SV101-3T3 cells also have a critical temperature below which the agglutination is nearly zero [2]. This is about 15°C for both cell types.

Effect of BHT

Addition of BHT increases the initial rate of agglutination. Between 22 and 33°C there is a 2-fold increase after addition of 0.1 or 0.2 mM BHT. BHT also slightly lowers the temperature at which a detectable level of agglutination takes place. At 37°C BHT did not increase the rate of agglutination, and in some experiments lowered it. BHT sensitizes cells to lysis by mechanical stress at higher temperatures (Campbell, S. and Lepock, J.R., unpublished results) which may contribute to the lower than expected agglutination at 37°C.

The activation energies (E_A) were determined from the slope of the three curves above 22°C. They are 11.8 ± 0.2 kcal/mol for the control cells, 14.4 ± 1.6 kcal/mol for the cells with 0.1 mM BHT, and 11.9 ± 2.0 kcal/mol for the cells with 0.2 mM BHT. Thus BHT has little effect upon the activation energy of agglutination.

Agglutination of BHK cells

BHT also increases the initial rate of concanavalin A-mediated agglutination of BHK cells. At 22°C the agglutination rate was 2.3 ± 0.4 (mean ± S.E.; 10 determinations) times greater with 0.1 mM BHT. The agglutination rate was considerably lower for the BHK cells compared to the pyBHK cells, but both seemed to be affected to a similar degree by BHT.

Membrane fluidity

BHT is a membrane lipid perturber and increases membrane fluidity as shown in Fig. 3. The correlation time of rotation is proportional to the viscosity of the environment of the spin label probe and the fluidity is therefore proportional to $1/\tau_c$. The values of τ_c in Fig. 3 for each curve are from three separate cell preparations and the lines are a least-squares fit of the experimental points. Treatment with BHT decreases the measured τ_c values and hence increases membrane fluidity. There is little if any difference between the 0.1 and 0.2 mM BHT treated cells. BHT dissolves into membrane lipids which are probably saturated at 0.1 mM BHT or lower. The decrease in τ_c at 37°C is 28% compared to the controls. The activation energy for each curve is approximately the same and the value for the control cells is 2.85 ± 0.1 kcal/mol. This is the activation energy of rotation since τ_c is dependent upon the rotation of the spin label.

The membrane fluidity of intact cells was also measured and BHT decreased the value of τ_c by an amount comparable to that shown in Fig. 3. The values obtained for the cell homogenates are probably more reliable since cells reduce nitroxide spin labels to a non-paramagnetic state. The $Fe(CN)_6^{3-}$ reoxidizes the nitroxide group at the cell surface. Thus, in intact cells the outer membranes of the cell may be preferentially probed [11]. If BHT should alter the rate of reduction, a different fraction of membranes might be probed which could produce a difference in measured membrane fluidity. In homogenized cells all membranes are exposed to ferricyanide and differences in τ_c must be due to differences in membrane fluidity.

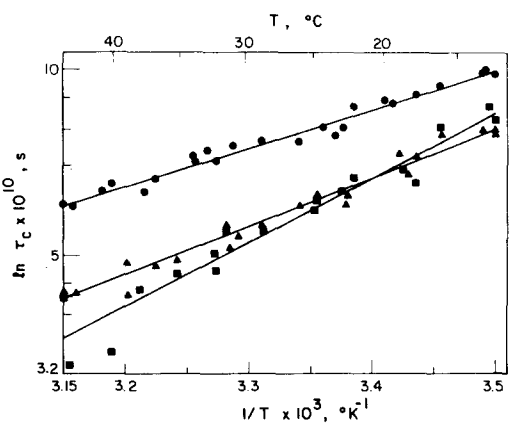

Fig. 3. The ln of the correlation time of rotation (τ_c) of the spin label 2N14 in pyBHK membranes as a function of 1/T. ●, without BHT; ▲, with 0.1 mM BHT; ■, with 0.2 mM BHT. (The line with the smallest τ_c values above 22°C is the best fit for the 0.2 mM BHT treated cells.)

The initial rate of agglutination of the pyBHK cells at 37°C is 2.62 from Fig. 2. The cells treated with 0.1 mM BHT agglutinate at this same rate at 26°C. Thus treatment with 0.1 mM BHT is equivalent to a 11°C temperature change. From Fig. 3, equivalent τ_c values are obtained at 37°C for the control cells and at 21°C for cells treated with 0.1 mM BHT, a 16°C temperature change. Therefore the change in agglutination and the change in membrane lipid fluidity due to BHT agree to within 30%.

Discussion

The rate of agglutination can be represented by the Eyring equation if there is only a single rate limiting step [12]. That is

$$S = kT/h \; e^{-\Delta G^{\neq}/RT}$$

where k is the Boltzman constant, h is the Planck constant, R is the gas constant and $\Delta G^{\neq}$ the free energy of activation of agglutination. Thus the rate of agglutination is determined by the free energy of activation, which is composed of two parts, the enthalpy ($\Delta H^{\neq}$) and entropy ($\Delta S^{\neq}$) of activation. The relationship between these terms is $\Delta G^{\neq} = \Delta H^{\neq} - T\Delta S^{\neq}$. $\Delta H^{\neq}$ can be determined from the activation energy using the relation $\Delta H^{\neq} = E_A - RT$. The value of $\Delta H^{\neq}$ is 11.2 ± 0.2 kcal/mol from the slope of the curve in Fig. 2 for the cells not treated with BHT.

If diffusion of the membrane receptor for concanavalin A is the rate limiting step for the agglutination process, the enthalpy of activation of diffusion should be equal to $\Delta H^{\neq}$ determined above for agglutination. (For a general discussion of rate limiting steps and enthalpy see Ref. 13.) Diffusion of receptors can be separated into rotational and translational diffusion. The above data for τ_c gives a value for $\Delta H^{\neq}$ of rotation of 2.3 ± 0.1 kcal/mol. The $\Delta H^{\neq}$ of translation for the concanavalin A receptor can be determined from the diffusion measurements made by Schlessinger et al. [14]. Replotting the mobility data from their Fig. 3 in an Arrhenius fashion gives a straight line with all points, except the 37°C point, falling on the line. The average $\Delta H^{\neq}$ of translation determined from these curves is 9.7 ± 2.1 kcal/mol. This value was determined for L-6 rat embryo myoblasts and may not be the same for the pyBHK cells. The sum of $\Delta H^{\neq}$ of rotation and $\Delta H^{\neq}$ of translation is 11.8 ± 2.1 kcal/mol, quite close to 11.2 ± 0.2 kcal/mol, the $\Delta H^{\neq}$ of agglutination. The above is consistent with the diffusion (translational and rotational) of the concanavalin A receptors being the rate limiting step of agglutination.

BHT increases the rate of agglutination, but does not affect $\Delta H^{\neq}$. An increase in the rate of agglutination implies a decrease in $\Delta G^{\neq}$. BHT must therefore increase $\Delta S^{\neq}$, the entropy of activation of agglutination. This is reasonable since BHT acts as perturber and increases membrane lipid fluidity. Since nearly all of the increase in agglutination caused by BHT can be accounted for by an increase in membrane fluidity and the rate of agglutination appears to be limited by receptor diffusion, the diffusion of membrane receptors on the cell surface of pyBHK cells may be limited by membrane lipid fluidity.

Breaks in the rate of agglutination as a function of temperature have been suggested to be due to membrane lipid phase transitions [2,3]. In Fig. 3 no

break is seen in the correlation time of the spin label 2N14, but this does not rule out any transitions in pyBHK cells since in a heterogeneous mixture of lipids 2N14 appears to partition into the more fluid phases and may not detect some liquid-crystalline to gel transitions [15]. If the break in agglutination at 20°C is due to a membrane phase transition, it is not clear why the BHT does not lower it, but if the break is non-membrane related then the BHT would not be expected to have any effect.

After treatment with lectins, receptors can redistribute in the plane of the membrane to form patches and caps [1]. There is a correlation between ease of cap formation and lectin-mediated agglutination in some cell types [16,17]. Treatment of murine 3T3 cells with proteases increases their concanavalin A-mediated agglutination and also the rate of cap formation [18,19]. If receptor redistribution into caps is necessary for agglutination, then any constraints on laterl receptor mobility, or diffusion, should affect agglutination. Membrane lipid fluidity is one such constraint. The cellular cytoskeletal system has also been strongly implicated in the control of receptor mobility [1]. If an increase in membrane lipid fluidity increases receptor diffusion and therefore the rate of cap formation, then it should also increase agglutination.

There is also evidence that caps may not be necessary for agglutination. An inverse correlation has been reported between the formation of caps and concanavalin A-mediated agglutination of normal lymphocytes and several malignant lymphocytes [20], and for normal and neoplastic rat glial cells [21]. The curves in Fig. 1 tend to support the hypothesis that the formation of caps is not necessary as the curve shapes are consistent with the highest rate of agglutination occurring immediately after addition of concanavalin A. Even if cap formation is not necessary for agglutination, receptor mobility is necessary since the receptors on different cells, being crosslinked by the lectin, must still be able to move so that they are properly aligned. Also agglutination does not occur below a critical temperature, presumably the point at which membrane lipids begin to solidify [2,3]. The binding of the lectin RCA_2 to liposomes containing glycolipids is influenced by the state of the fatty acyl chains [22]. Fluidity is increased and binding is much more rapid at higher temperatures and when the liposomes contain shorter chains.

Therefore the physical state of the membrane will affect the rate of agglutination independent of the distribution of the membrane receptors required for agglutination. However, membrane lipid fluidity is obviously not the only factor affecting agglutination. Cytochalasin B alters the concanavalin A-mediated agglutination of sarcoma 180 mouse ascites tumor cells but appears to have no effect on plasma membrane fluidity [23]. There are large differences in the agglutination of normal and transformed cells, but no reason to believe that their membranes differ in fluidity [24]. Still, fluidity can influence agglutination and may play an important role in cell types in which the cytoskeletal system does not appear to be involved in lectin-mediated agglutination [25].

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