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Different Arachidonate and Palmitate Binding Capacities of the Human Red Cell Membrane

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Abstract. Human red cell membrane bindings of arachidonate and palmitate at pH 7.3 are investigated at temperatures between 0 and 38°C by equilibrating ghosts with the long-chain fatty acids bound to bovine serum albumin in molar ratios (ν) within the physiological range (<1.7). Linearized relations of ghost uptakes and fatty acid monomer concentrations in buffer provide estimates of the binding capacities and corresponding equilibrium dissociation constants (K_{dm}). The temperature-independent arachidonate binding capacity, 5.5 ± 0.5 nmol g^{-1} packed ghosts, is approximately fivefold smaller than that of palmitate, 26.6 ± 2.0 nmol g^{-1}. While K_{dm} of arachidonate binding 5.1 ± 0.5 nM is temperature independent, K_{dm} of palmitate increases with temperature from 3.7 nM at 0°C to 12.7 nM at 38°C.

The large difference in binding capacities suggests the presence of at least two different fatty acid binding domains in human red cell membranes.

Key words: Arachidonate — Palmitate — Human red cell membrane — Equilibrium dissociation constant

Introduction

Goodman [10] discovered a considerable palmitate (PA) binding capacity of the human red cell membrane characterized by high affinity comparable to that of albumin. The just discovered fast turnover of albumin bound long-chain fatty acids could be understood, if plasma membranes of other cells including liver cells had a similar binding property and the binder mediated the membrane transport. A similar binding was reported later for laurate [15], but the suggested involvement in PA transport was only recently substantiated by the finding that asymmetrically distributed high affinity sites in fact mediate the membrane transport [3, 4]. Goodman [10] suggested that the binder was a protein, a receptor, because the lipid bilayer accounted for a negligible fraction of the binding, if the solubility in membrane lipids was comparable with that in heptane. The validity of this argument was rejected much later based on investigations with artificial bilayers [13]. However, the discussion of this vexed question is postponed, because it is elucidated by the present data.

The present work deals with the parameters of equilibrium binding to the red cell membrane of two very different long-chain fatty acids, PA and AR, when the water-phase concentrations are within the physiological range defined by complexes with bovine serum albumin (BSA). The basis is the improved information on albumin bindings, provided by the recently designed method with red cell ghosts [5, 6], suggesting similar BSA affinities of PA and AR. The results of major interest are (i) that the capacity of the membrane to bind AR, not known before, is approximately only some 20% of the determined PA binding capacity, and (ii) that the affinities to the ghost membrane at 38°C of both fatty acids are higher than to BSA.

Materials and Methods

[(5,6,8,9,11,12,14,15)-^{3}H] Arachidonic acid, sp. act. 209 Ci/mmol and [9,10)-^{3}H] palmitic acid, sp. act. 54 Ci/mmol were obtained from Amersham International, Amersham, England. Unlabeled fatty acids (FA) were obtained from Sigma. Labeled FA were purified monthly by column chromatography [3, 4], ascertaining the elution pattern of a single component. The scintillation fluid Ultima Gold was purchased from Packard Instrument (Downers Grove, IL). Bovine serum albumin (BSA) (Behring Institute, Germany) was defatted according to the method of Chen [7].

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Preparation of Ghosts

Preparation of BSA-free ghosts from human red cells was carried out as described previously [3]. After resealing of ghosts at 38°C for 45 min, the pink ghosts were washed three times with 165 mM KCl, 2 mM phosphate buffer, pH 7.3, containing 0.02 mM EDTA/EGTA (1:1) and once with the same buffer containing 0.01% BSA. Hereafter, the ghosts were stored in the 0.01% BSA buffer at 4°C and used within two days.

Preparation of Charge-Buffers

The amounts of [^{3}H]FA and of unlabeled FA to give 1–5 μCi ml^{-1} and final molar ratios of FA to BSA (ν) from 0.01 to 1.7 were dissolved in 50 μl benzene, just enough to moisten 200 mg glassbeads (diameter 0.1 mm). The benzene was sublimated at a pressure of about 16 mm Hg. Up to 10 μg FA (area per molecule assumed to be 20×10^{-14} mm^2 [14]) can be deposited as a monomolecular layer, as the surface area of the beads is about 4,500 mm^2. Charge-buffers were prepared by shaking such FA-loaded beads with a solution of 0.01% BSA in buffer for 15 min at room temperature.

Fatty Acid Uptake by Ghosts

Ghosts were packed by centrifugation 7 min at 36,470 × g at the appropriate temperature. This resulted in trapping of 20% extracellular buffer [3]. Equilibration was conducted in a series of tubes containing one volume of packed ghost (called V_g ml) to one-and-a-half volume of charge-buffer. After equilibration for 50 min at 0°C, 40 min at 5°C, 30 min at 15°C or 15 min at 23°C and 38°C, the tubes were centrifuged again. Counting rates were determined in the charge-buffer before (C_b dpm/ml) equilibration to ascertain the specific activity of fatty acids. The uptake of fatty acids by ghosts (membrane bound M, nmol/g ghosts) and final ν values are calculated on the basis of counting rates in the charge-buffers after equilibration (C_a dpm/ml) according to Eqs. (1) and (2), taking in consideration that the extracellular volume trapped in packed ghosts is 20%.

$$M = (C_b\ 1.5\ V_g - C_a\ (1.5\ V_g + 0.2\ V_g))/ (0.8\ V_g\ S) \text{ nmol/g} \quad (1)$$

$$\nu = C_a/(S\ 1.5),\ 1.5 \text{ nmol/ml equals } 0.01\% \text{ BSA}, \quad (2)$$

where S is the specific activity of the acids in dpm/nmol.

K_d Determination

The equilibrium constants, K_d's, for the dissociation of BSA:FA complexes are defined for N equivalent binding sites on BSA as

$$K_d = ([\text{FA}]\ (N - \nu))/\nu \quad (3)$$

The equilibrium constants, K_{dm}'s, for the dissociation of FA from a single type of binding site on the ghost membranes are

$$K_{dm} = ([\text{FA}]\ (C - M))/M \quad (4)$$

where C is the binding capacity and M the binding in nmol/g ghosts. At equilibrium [FA] is a common factor in Eqs. (3) and (4). Eliminating [FA] from the two equations gives the Wilkinson linearization [16]:

$$(\nu/(N - \nu))/M = (1/C)(\nu/(N - \nu)) + (K_{dm}/K_d)\ (1/C) \quad (5)$$

In a plot of $(\nu/(n - \nu))/M$ *vs.* $\nu/(N - \nu)$ the slope defines the capacity of binding and the intercept the ratio of the two equilibrium dissociation constants. With known equilibrium constants, K_d's, for the PA and AR dissociation from albumin [5, 6], we get the equilibrium constants, K_{dm}'s, for the dissociation from ghost membranes. The occupancies ($O_c = M/C$) are calculated by Eq. (5):

Statistics and Data Analyses

We have used the methods described by Armitage [1] to calculate the statistics, weighted means and probabilities of significance of differences. The linear regression procedure given by STATGRAPHIC version 5 is used to determine the best fit of the data to Eq. (5).

Results and Discussion

Unfortunately, direct determinations of fatty acid water-phase concentrations along with uptake are impossible in the case of AR, because of the very low ghost binding capacity of this acid. The ghosts are partially depleted, not only by the washing required to remove the charge buffer, but also in the dilution series of ghost suspensions required to measure the water-phase concentrations [6]. This is not so in the case of PA [5], but in general we believe it is appropriate to use Eq. (3) to calculate [FA] because the precision of measured [FA] is inferior to the precision of K_d, estimated on the basis of a large number of single [FA] values.

The Table shows the measured binding capacities and equilibrium dissociation constants of membrane binding at different temperatures. The PA binding capacity is smaller than that measured by Goodman [10]. When studying the binding parameters of the specific membrane binding it is important to realize that some unspecific adsorption may occur of FA, if the water-phase concentration is high, i.e., at high ν values. In such cases, unspecific binding to BSA occurs [6]. This may have been the case in the studies of Goodman [10]. We have confined our studies to the range of ν for which binding sites are less than half saturated, as shown in the Table. The disadvantage is, on the other hand, that the precision of the estimated parameter values is in some cases not impressive, although all the data fit the linearized equilibrium Eq. (5) with highly significant correlation coefficients. Two examples are given in Fig. 1 of the seven plots used to estimate the parameters presented in the Table, one for AR and one for PA. The symmetrical distribution of data points along the regression line fits with only one equilibrium constant and shows that a simple lipid/water phase partition is absent. Such a partition appears as a regression with an insignificant correlation coefficient and a deviation from linear regression at high ν values if combined with a limited number of binding sites. The data are interpreted in terms of Eq. (5), which implies that the entire uptake (M) is bound reversibly. That this is the case for our ghost prepara-

Table 1. Binding capacities (C, nmol/g ghost (equal to 9×10^9 ghosts)) of red cell ghosts for palmitate and arachidonate, the dissociation constants (K_{dm}) at different temperatures as defined by Eq. (5), using the previously determined equilibrium constants K_d's for dissociation of fatty acid-BSA complexes[a] and the occupancies (O_c)[b]

	C (nmol/g)	K_{dm}/K_d	K_{dm} nM	O_c	v	r
Palmitate ($N = 2$)						
0°C ($n = 26$)	32.0 ± 6.0[c]	1.45 ± 0.30	3.65	≤0.40	≤1.0	0.73
5°C ($n = 15$)	23.0 ± 2.0	1.33 ± 0.12	5.20	≤0.44	≤1.0	0.96
15°C ($n = 13$)	26.5 ± 2.6	1.36 ± 0.14	9.50	≤0.45	≤1.0	0.92
38°C ($n = 21$)	25.0 ± 3.8	0.58 ± 0.09	12.70	≤0.38	≤0.52	0.83
	26.6 ± 2.0					
Arachidonate						
0°C ($n = 17$)	6.49 ± 1.06[d]	1.03 ± 0.17	4.45	≤0.52	≤1.68	0.84
23°C ($n = 10$)	4.81 ± 0.41	0.36 ± 0.03	5.98	≤0.64	≤1.18	0.97
38°C ($n = 20$)	5.30 ± 1.60	0.17 ± 0.05	4.85	≤0.56	≤0.26	0.63*
	5.50 ± 0.50		5.09			

n: number of determinations. *r:* correlation coefficient, $P << 0.001$ except for (*) where $P < 0.002$. *v:* molar ratio of fatty acid to albumin and *N:* number of equivalent binding sites of the fatty acids on bovine serum albumin. [a][5, 6]. [b](O_c defined as M/C, Eq. 5). [c]No significant difference from 5°C experiments ($t = 1.4$, $P = 0.1$). [d]No significant difference from 23°C experiments ($t = 1.3$, $P = 0.2$).

tions is known from numerous studies of PA efflux to large volumes of buffer with BSA [3, 4]. The initial ghost tracer bindings are quantitatively recovered in the medium at equilibrium. The binding parameter values of PA at 0°C are different from those suggested previously by us [3]. Erroneously high values of water-phase concentrations suggested a K_{dm} of 13.5 nM [3], much higher than the true value presented in the Table.

It is remarkable that the affinity of the membrane is higher than that of BSA, particularly in the case of AR at 38°C. Provided there is similar FA binding to BSA and to human serum albumin, then the mentioned prediction of Goodman [10] is fulfilled.

The binding parameter values are estimated by assuming two and three equivalent binding sites on BSA for PA and AR, respectively (Table). The studies of PA binding to BSA [5], however, did not preclude entirely three equivalent binding sites. If three sites are present, then the estimated membrane capacity of PA binding is considerably greater, some 50%, and thus approximately eight times greater than that of AR binding.

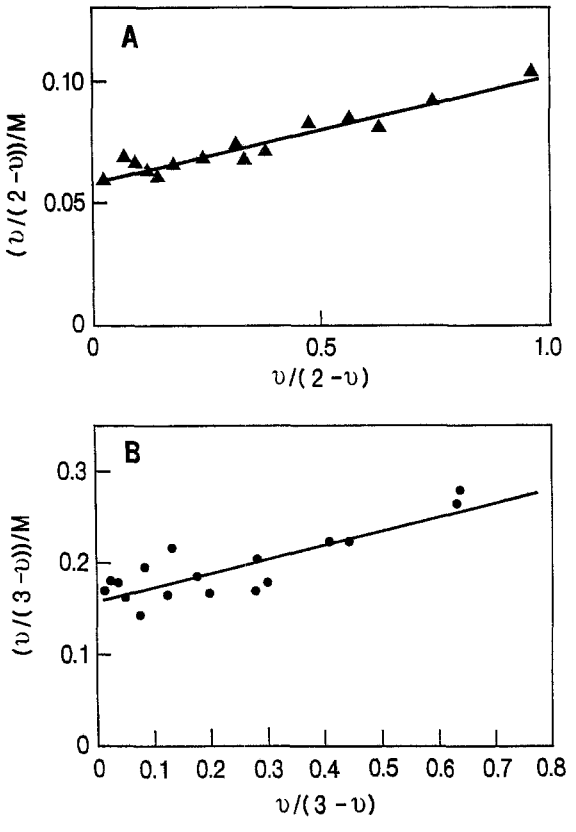

Fig. 1. Regression analyses of fatty acid binding data according to Eq. (5). (*A*) Analysis of palmitate binding data and corresponding v values below 1, at 5°C in terms of two equivalent binding sites on bovine serum albumin. The regression line is $Y = 0.0434$ (±0.0036) $X + 0.0584$ (±0.0016) $r = 0.96$, $t = 12$ and $n = 15$. (*B*) analysis of arachidonate binding data and corresponding v values ≤1.68 at 0°C in terms of three equivalent binding sites on bovine serum albumin. The regression line is $Y = 0.154$ (±0.025) $X + 0.158$ (±0.008) $r = 0.84$, $t = 6.1$ and $n = 17$.

The Nature of Membrane Binding of FA

The interaction of FA with biological membranes is a very disputed subject. Goodman [10] suggested a protein as a binder based on the heptane-water partition coefficient [11]. However, the lipid bilayer accounts for a very large fraction if it is comparable with phospholipid vesicles [13]. Not only was the reported uptake in vesicles several times greater than the phase partition between water and an organic phase, but the uptake was apparently also limited. In contrast, Conrad and Singer [8, 9] have demonstrated that phospholipid vesicles are

not valid models for biological membranes with regard to binding of amphipathic molecules such as chlorpromazin and 1-decanol. The amphipaths were less soluble in biological membranes, including the red cell membrane, than in water, whereas the solubilities in vesicles were some 10^3-fold greater than in water. The phenomenon was explained by a large internal pressure in biological membranes and the permeability across membranes of the amphipaths was suggested to be confined to the boundaries of integral proteins, since fatty acid analogues were found to interact with hydrophobic domains of red cell ghost membrane proteins [2].

The present observations can be understood from this picture, since lipid boundaries with different integral protein domains may fit PA and AR differently. By the same token, above saturation of the binding sites characterized in the present study, i.e., at ν values above 2–3, additional bindings may be attributed to other protein-lipid interphases.

Specificity of the Binding Property

Analysis in terms of a single class of binding sites for AR and PA gives 3.6×10^5 and 17.7×10^5 AR and PA binding sites per cell, respectively, as 1 g ghosts contains 9×10^9 cells. These values are similar to that of the glucose transporter in red cells (3×10^5 sites per cell) [12]. The AR and PA binding affinities are very different. Not only are K_{dm} of AR-binding temperature independent in contrast to K_{dm} of PA, but the affinity at 38°C of AR is approximately 2.5 times higher than that of PA. In addition, PA and AR bindings are asymmetrically distributed in the ghost membrane, with PA mainly bound to the inner leaflet [3] and AR mainly to the outer leaflet (I.N. Bojesen and E. Bojesen, submitted). To us these results are incompatible with fatty acid binding in the lipid bilayer. Much more likely is that hydrophobic domains of integral proteins are involved in the binding of the two long-chain FA. Approximately five times more domains are involved in PA than in AR binding and therefore also in the permeations, as discussed before. This implies the possibility that different cell types may differentiate between various FA differently with regard to permeation. This is of course out of the question, if one assumes that the lipid bilayer of the cell membranes alone mediates the permeation.

One may speculate on the significance of the high FA transport capacity of the red cell membrane as the erythrocytes make use of FA only in remodeling the FA composition of membrane phospholipids. Thus, we suggest that proteins involved in FA transport may have other functions as well.

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