ADENOSINE 3',5' MONOPHOSPHATE BINDS ONLY TO THE INNER SURFACE OF HUMAN ERYTHROCYTE MEMBRANES[+]

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Summary: The binding of adenosine 3',5' monophosphate (cyclic AMP) to each surface of the isolated human erythrocyte membrane was measured. Unsealed ghosts, in which both membrane faces are accessible, and sealed inside-out vesicles, which expose only the cytoplasmic side of the membrane, both bound approximately 6,000 cyclic AMP molecules per cell membrane equivalent with a dissociation constant, $K \simeq 2.5 \times 10^{-9}$. The binding of this nucleotide by preparations rich in sealed ghosts and right-side-out vesicles, which sequester the inner surface, was limited and could be correlated precisely with small amounts of exposed cytoplasmic surface. We conclude that these binding sites for cyclic AMP are confined to the cytoplasmic side of the erythrocyte membrane.

The presence of a cyclic AMP[1] binding activity on isolated human erythrocyte membrane ghosts has recently been demonstrated (1,2), but its function is unknown. It is our impression that all of the proteins in the human red cell membrane thus far examined are asymmetrically disposed with respect to the two membrane surfaces, presumably reflecting their function (cf. 3,4).[2] We undertook the determination of the orientation of the cyclic AMP binding activity to help elucidate its role in the intact erythrocyte and to extend our understanding of membrane "sidedness".

MATERIALS AND METHODS

Cyclic AMP was purchased from Sigma Chemical Company. We are grateful

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1 The abbreviation used is cyclic AMP for adenosine 3',5' monophosphate.

2 J. A. Kant and T. L. Steck, unpublished observations.

Table I

The Accessibility of the Outer and Inner Membrane Surfaces

in Erythrocyte Membrane Preparations

Membrane Preparations (predominant species)	% of total activity accessible[1]		% of total cyclic AMP binding sites accessible[4]
	Acetyl-cholinesterase[2]	Glyceraldehyde 3-P Dehydrogenase[3]	
Sealed Ghosts	102	14	15
Sealed Right-side-out Vesicles	79	26	30
Sealed Inside-out Vesicles	8	92	90
Unsealed Ghosts	109	81	82

Membranes were prepared as described in "Materials and Methods" and assayed for exposed and total marker activity as indicated.

[1] $\text{\% of total activity accessible} = \frac{\text{Activity in untreated membranes}}{\text{Activity in membranes treated with 0.1\% Triton X-100}} \times 100$

[2] Assayed according to Steck and Kant (4). Final reagent concentrations in 0.8 ml: acetylthiocholine chloride, 0.78 mM; 5,5'-dithiobis(2-nitrobenzoic acid), 0.625 mM; Na phosphate buffer (pH 7.5), 85 mM (cf. 8).

[3] Assayed according to Steck and Kant (4). Final reagent concentrations in 1.0 ml: glyceraldehyde 3-P, 1.5 mM; NAD^+, 1.0 mM; Na arsenate, 12.0 mM; Na pyrophosphate buffer (pH 8.4), 21.6-24.6 mM (cf. 11).

[4] Calculated from data points in Fig. 1, assuming for the equilibrium binding of cyclic AMP $K = 2.5 \times 10^{-9}$ M and a total cyclic AMP capacity of 1.0×10^{-20} moles per membrane equivalent. See text for details.

to Dr. Lucia Rothman for cyclic [8-^{3}H] AMP (Schwarz Mann, 28 Ci per mmole), which was demonstrated to be greater than 99% chromatographically pure.

Membranes were prepared as follows: a) unsealed ghosts by hemolysis of washed human erythrocytes in 5 mM Na phosphate, pH 8.0 (4-6); b) sealed ghosts by hemolysis of erythrocytes in 5 mM Na phosphate, pH 8.0, which contained 1 mM $MgSO_4$ (3,4); and c) sealed inside-out and right-side-out vesicles

by a 30 minute incubation of unsealed ghosts in 40 volumes of ice-cold 0.5 mM Na phosphate, pH 8.0-8.5, followed by the addition of 0.1 mM $MgSO_4$ to the right-side-out vesicle preparation (4,6,7). Following homogenization, sealed vesicles were purified from unsealed species by centrifugation on continuous Dextran T-110 (Pharmacia) gradients (density = 1.005-1.050 g/ml) (4,6,7).

The cyclic AMP binding assay was adapted from that of Rubin et al. (1), as described in the legend to Fig. 1. Less than 5% breakdown of sealed membranes occurred during the binding assay.

RESULTS AND DISCUSSION

We tested four types of membrane preparations for their ability to bind cyclic AMP. Each was enriched in membranes which exposed one surface or the other (or both) to the bathing medium. We gauged the exposure of each surface by the accessibility of an enzyme localized at that surface to its substrates (Table I, two left-hand columns). The increment in enzyme activity evoked by the addition of detergent represented that fraction sequestered by the interposition of a sealed membrane. Glyceraldehyde 3-P dehydrogenase activity, a marker for the cytoplasmic surface of the membrane (3,4), was found to be mostly latent in preparations of sealed ghosts and right-side-out vesicles, while acetylcholinesterase, an outer surface marker (cf. 3,4,7), was readily available. The preparation rich in inside-out vesicles showed the reciprocal pattern of accessibility for these markers. Only the unsealed ghosts possessed great accessibility of both enzyme markers to their substrates.

Fig. 1 demonstrates that unsealed ghosts and sealed inside-out vesicles bound cyclic AMP in a nearly identical fashion. Sealed ghosts and right-side-out vesicles took up significantly less nucleotide. It is clear that the cytoplasmic membrane surface took up cyclic AMP, since burying 92% of the external surface in the inside-out vesicle preparation did not diminish the uptake of the nucleotide, while sequestration of the cytoplasmic surface in sealed ghosts and right-side-out vesicles reduced their binding capacity.[3]

[3] Drs. C. S. Rubin and O. M. Rosen have made similar observations (personal communication).

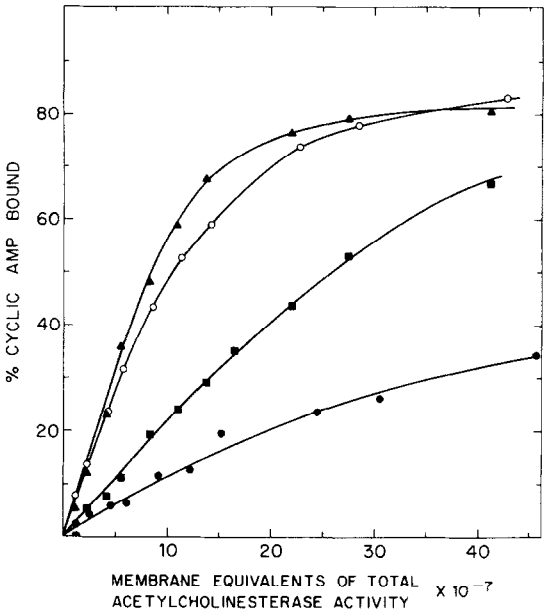

Fig. 1. The binding of cyclic AMP to four erythrocyte membrane preparations. Varying amounts of the sealed ghost (●—●), sealed right-side-out vesicle (■—■), sealed inside-out vesicle (▲—▲), and unsealed ghost (O—O) preparations described in Table I were incubated on ice for 90 minutes with 2 nM cyclic [8-^{3}H] AMP (5,000 cpm) and 10 mM $MgSO_4$ in 50 mM Na phosphate (pH 7.0) (cf. 1). Total incubation volume was 0.25 ml. Samples were then centrifuged in a Spinco type 40 rotor at 2-4° (15 minutes at 15,000 rpm for ghosts; 30 minutes at 30,000 rpm for vesicles) and 0.1 ml aliquots of the supernatant fluid taken for determination of radioactivity by liquid scintillation spectrometry. Bound cyclic AMP was calculated from supernatant and input radioactivity.

Ghosts were counted using a model A Coulter counter. Vesicles were normalized to ghost membranes by their total acetylcholinesterase activity (4,8) which, unlike protein (9), is completely retained by the vesicles.[2] Total acetylcholinesterase content is that measured after the breakdown of permeability barriers by 0.1% Triton X-100. One membrane equivalent is equal to the total acetylcholinesterase activity of one ghost.

It could be concluded that cyclic AMP was bound only to the cytoplasmic surface if it were demonstrated that the binding observed in right-side-out membrane preparations is solely attributable to contaminants which expose the inner surface to cyclic AMP. A direct test of this hypothesis is to compare the accessibility pattern of cyclic AMP binding sites to that of the cytoplasmic surface marker, glyceraldehyde 3-P dehydrogenase. The fraction of binding sites accessible in any membrane preparation is given by the number of available sites divided by the number of total sites. Available binding sites can be estimated

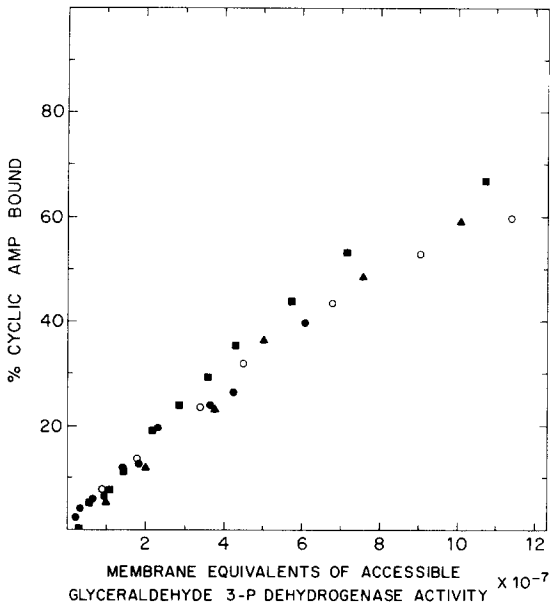

Fig. 2. The relationship of cyclic AMP binding to the cytoplasmic surface accessible in four erythrocyte membrane preparations. Data from Fig. 1 (below 70% cyclic AMP bound) have been plotted as a function of the glyceraldehyde 3-P dehydrogenase accessible in each incubation mixture. Membrane equivalents of accessible glyceraldehyde 3-P dehydrogenase = membrane equivalents of total acetylcholinesterase activity (from Fig. 1) x % of total glyceraldehyde 3-P dehydrogenase activity accessible/100 (from Table I). Symbols as in Fig. 1.

from the data shown in Fig. 1 and the equilibrium relationship, $K = \frac{(cAMP)(S)}{(cAMP\text{-}S)}$, where K is the dissociation constant and the bracketed symbols represent the molar concentrations of free cyclic AMP and nucleotide binding sites and their 1:1 complex. The total number of sites per membrane must be measured in completely unsealed ghosts, and is assumed to be invariant among the various membrane preparations. The dissociation constant and binding capacity of the erythrocyte membrane for cyclic AMP have been reported by other workers (1,2); however, the full availability of both membrane surfaces to the nucleotide was not assured in those studies. We therefore determined these values for unsealed ghosts approaching total accessibility of both surfaces. We obtained a linear double reciprocal plot (10) with $K = 2.5 \times 10^{-9}$ M and a capacity of 1.0×10^{-20} moles (6,000 molecules) of cyclic AMP per membrane equivalent [i.e. 17.5 pmoles per mg protein, assuming 5.7×10^{-10} mg protein per ghost

(5)]. These values agree reasonably well with the previous reports (1,2).

Cyclic AMP accessibility was estimated for each data point in Fig. 1 and averaged for each membrane preparation. The values (Table I, right-hand column) demonstrate that the fraction of total cyclic AMP binding activity accessible in each membrane preparation is not significantly different from that of glyceraldehyde 3-P dehydrogenase. On this basis, all of the cyclic AMP binding activity can be ascribed to the cytoplasmic membrane surface.

Independent verification of this conclusion was obtained without the assumptions or calculations discussed above by replotting the binding data in Fig. 1 against the accessible glyceraldehyde 3-P dehydrogenase activity added to each incubation mixture. It is clear from this plot (Fig. 2) that the binding of cyclic AMP in each preparation is an expression of the availability of the cytoplasmic side of the membrane, as represented by glyceraldehyde 3-P dehydrogenase.

This study affirms a general direct approach to "sidedness" studies on membrane constituents. Through correlation with membrane markers of known orientation, the distribution of additional components can be analyzed unambiguously, even in preparations where both surfaces are accessible to a variable extent. This treatment avoids the difficult task of purifying to homogeneity sealed membranes of a single orientation. The unilateral localization of the cyclic AMP-binding site is reminiscent of the strict asymmetry found thus far for other red cell membrane functions (cf. 3,4). That this cyclic AMP binding activity can be attributed solely to specific sites on the inner membrane surface should help in understanding its role in the intact erythrocyte.

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