STUDIES ON PROTEIN KINASE ACTIVITY AND THE BINDING OF ADENOSINE 3'5-MONOPHOSPHATE BY MEMBRANES OF HEREDITARY SPHEROCYTOSIS ERYTHROCYTES

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Summary: Evidence has been presented recently of a deficiency of an endogenous membrane-associated protein kinase in erythrocytes of patients with hereditary spherocytosis (HS). We have measured endogenous protein kinase activity in erythrocyte membranes of 4 HS subjects using different membrane isolation and reaction conditions and find that the phosphorylation of the spectrin component (mean ± S.E. 17.1 ± 1.2 pmoles/10 mins per mg protein) is not significantly different to that of 4 normal controls (mean ± S.E. 20.7 ± 1.1 pmoles/10 mins per mg protein). Phosphorylation of exogenous proteins such as casein and protamine is also not deficient in HS erythrocyte membranes. Adenosine 3'5-monophosphate (cyclic AMP) binding to normal and HS erythrocyte membranes was also studied using a Millipore filtration assay. The affinity of cyclic AMP for erythrocyte membranes as determined by Hill plots of binding data from 4 HS subjects (K_D mean ± S.E. = 2.2 ± 0.2 nM) was not significantly different to 4 normal controls (K_D mean ± S.E. = 2.8 ± 0.6 nM). The rate of dissociation of bound cyclic AMP from HS membranes was also similar to control membranes. We thus cannot confirm the prediction by others that an abnormality of cyclic AMP interaction with the erythrocyte membrane underlies HS.

Introduction: Human erythrocyte ghosts contain an endogenous membrane-associated adenosine 3'5-monophosphate* (cyclic AMP)-dependent protein kinase which catalyses the phosphorylation of endogenous membrane proteins as well as exogenous proteins (1,2,3,4). One of the major endogenous proteins phosphorylated is spectrin, a 200,000 M.W. filamentous protein localized to the inner surface of red cell membranes. Yawata et al. (5) have recently shown that microtubular denaturants such as vinblastine, lead to selective depletion of spectrin, generating cells with all the properties of HS from normal cells. These effects were completely inhibited by cyclic AMP or cyclic GMP. They predicted that an abnormal cyclic-nucleotide-microtubule interaction might under lie the hemolytic anemia of HS. This suggestion was supported by the recent

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* Abbreviations used: Cyclic AMP, adenosine 3'5-monophosphate; HS, hereditary spherocytosis

report of Greenquist and Shohet (6) of a deficiency in cyclic AMP-catalyzed phosphorylation of endogenous membrane proteins including spectrin in HS. Since it has been suggested by Kuo and Greengard (7) that the effects of cyclic AMP are mediated through the phosphorylation of specific proteins by protein kinases and since binding of cyclic AMP to a specific receptor is the initial step in protein kinase activation (8,9), we have directly tested the possibility that an alteration of binding of cyclic AMP to the receptor protein in HS erythrocytes might underlie some of the abnormalities in these cells. We have also measured the phosphorylation of endogenous and exogenous proteins by membrane protein kinase of HS erythrocytes using different membrane isolation and reaction conditions and cannot confirm the findings of Greenquist and Shohet (6) of decreased cyclic AMP-dependent protein kinase activity in these cells.

Materials and Methods: Freshly drawn heparinized blood was obtained from normal controls and splenectomized HS subjects with normal reticulocyte counts. Erythrocyte ghosts were prepared by the method of Dodge et al (10) after hypotonic lysis in 20 mosM sodium phosphate buffer pH 7.4. After the final wash the ghosts were re-equilibrated in 20 mosM sodium phosphate pH 7.0, and stored overnight at -20°. Protein kinase activity was assayed in a reaction mixture of 0.2 ml containing 0.05 M sodium phosphate buffer (pH 7.0), 0.02 M $MgSO_4$, 50 uM [γ-^{32}p] ATP (2 x 10^5cpm per nmole), 0.01 M dithiothreitol, 50 ug membrane protein and where indicated 2.0 uM cyclic AMP, 0.2 mg protamine or 0.2 mg casein. Incubations were carried out for 5 minutes at 30° unless otherwise noted. The reaction was terminated as described by Guthrow et al.(2), the final pellet was dissolved in 0.5 ml Soluene 350 and 10 ml Dimilume scintillation mixture added and counted in a Packard Tri-Carb liquid scintillation counter. The phosphorylation of exogenous proteins was determined after correction for endogenous membrane phosphorylation determined in parallel assays. A further correction was made for background radioactivity in parellel assays containing boiled membranes. In experiments in which phosphorylated ghosts were fractionated on polyacrylamide gels, the incubation volume was 1 ml with the same concentrations of reagents as above, except that exogenous proteins were omitted. In these experiments, the reaction was stopped after 10 minutes by adding 15 ml ice-cold 20 mosM sodium phosphate buffer (pH 7.0) and the membranes immediately centrifuged and washed twice at 20,000 x g, for 15 minutes. The membrane polypeptides were separated and stained in 5.6% polyacrylamide gels containing 1% sodium dodecylsulphate as described by Fairbanks et al. (11). Unstained gels were sliced into 1.5 mm segments, 0.5 ml Soluene 350 added and counted as above.

Cyclic AMP binding assays were carried out in a 0.2 ml reaction mixture which contained 0.05 M sodium phosphate buffer pH 7.0, 10 mM Mg^{2+}, 50 ug of membrane protein, and cyclic [^{3}H] AMP (27.5 Ci/mmole) in concentrations varying from 0.1 nM to 91 nM. After a 24 hour incubation at 0°C, the binding of cyclic [^{3}H] AMP to the membrane protein was determined by Millipore filtration and subsequent counting in a Packard Tri-Carb liquid scintillation spectrometer as described by Rubin et al. (1). Control assays were carried

out with boiled membranes. Rates of dissociation of membrane-bound cyclic [^{3}H] AMP were determined by allowing binding of 91 nM cyclic [^{3}H] AMP to proceed for 3 hours at 0° in a 1 ml reaction mixture containing 0.05 M sodium Phosphate buffer pH 7.0, 10 mM Mg^{2+}, and 250 ug of membrane protein. At zero time a 0.1 ml aliquot was removed for measurement of binding, and 0.1 ml of 10 mM unlabelled cyclic AMP in water was then added. Bound cyclic [^{3}H] AMP was then measured at various times in 0.1 ml aliquots by the same procedure as for the standard binding assay. Protein was measured by the method of Lowry et al. (12) using bovine serum albumin as standard.

Results and Discussion: ^{32}P-labelling of the membrane polypeptides of normal erythrocyte membranes in the absence of cyclic AMP is shown in Fig. 1. The numbering system is that of Fairbanks et al. (11). Component II is the major polypeptide phosphorylated with only minor labelling of component III and a polypeptide (labelled IVc by Rubin and Rosen (3)) in a position between bands IV and V. These findings are similar to those of other workers (2,3), but are different to those of Greenquist and Shohet (6) and Roses and Appel (4) who found that component III (M.W. approximately 90,000) was also a major substrate for phosphorylation in the absence of cyclic AMP. The ^{32}P-labelling pattern of erythrocyte membranes obtained from 4 splenectomized HS subjects was

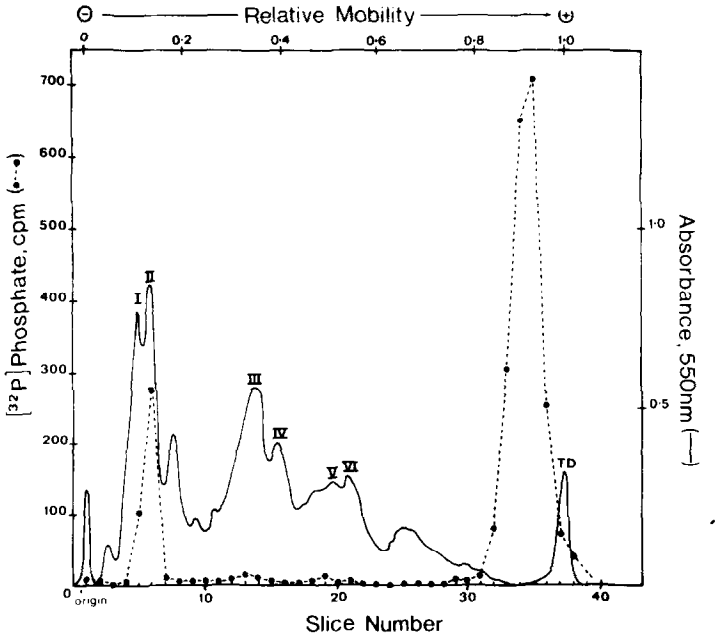

Fig. 1: Sodium dodecylsulphate-polyacrylamide gel electrophoresis of control erythrocyte ghosts phosphorylated in the absence of cyclic AMP. 100 ug of membrane protein was applied. Incubation conditions were as described under methods. T.D. denotes the position of the tracking dye.

qualitatively identical to that of the control membranes, while the quantitative labelling of component II (mean ± S.E. 17.1 ± 1.2 pmoles/10 mins per mg protein) was not significantly different to that of 4 control preparations (mean ± S.E. 20.7 ± 1.1 pmoles/10 mins per mg protein). The individual results are shown in Table I. Addition of cyclic AMP to either control or HS membranes resulted in variable stimulation of phosphorylation of component II (Table I), the mean values showing no significant difference between the two subject groups We were unable to show a consistent stimulating effect of cyclic AMP on phosphorylation of either component III or Ivc. We are thus unable to confirm the findings of Greenquist and Shohet (6) of decreased phosphorylation of component II in HS erythrocyte membranes. A possible explanation for these

Table I

Phosphorylation of component II of normal and HS erythrocyte membranes in the presence and absence of cyclic AMP. (Incubation conditions were as described under methods).

Subject		[^{32}P] Phosphate incorporated	
		No cyclic AMP	Cyclic AMP
		pmoles/10 minutes per mg protein	
Control	1	19.5	22.5
	2	19.2	21.6
	3	20.0	25.7
	4	23.9	20.3
mean ± S.E.		20.7 ± 1.1	22.5 ± 1.2
HS	1	16.0	18.0
	2	14.3	21.5
	3	18.9	17.6
	4	19.3	22.4
mean ± S.E.		17.1 ± 1.2	19.9 ± 1.1

discrepant findings might be in the different hemolysing and washing buffer (0.01 M Tris-HCl pH 7.4) used by these workers in the membrane isolation procedure, and in the different reaction conditions, either of which might lead to selective loss or inhibition of endogenous membrane protein kinase in HS erythrocytes.

Further support for our finding of normal protein kinase activity in HS membranes is the finding of normal phosphorylation of the exogenous proteins protamine and casein by erythrocyte membranes from 3 subjects with HS, both in the presence and absence of cyclic AMP. These data are shown in Table II. Cyclic AMP had a variable effect on phosphorylation of protamine, stimulating phosphorylation in 2 of the 3 HS subjects, with no effect in any of the control preparations. Cyclic AMP had little effect on casein phosphorylation in one HS and one control subject and inhibited phosphorylation slightly in 2 HS and 2 control subjects.

Table II

Phosphorylation of casein and protamine by normal and HS erythrocyte membranes in the presence and absence of cyclic AMP. (Incubation conditions were as described under methods).

Subject		[^{32}P] Phosphate incorporated			
		Protamine		Casein	
		No cyclic AMP	Cyclic AMP	No cyclic AMP	Cyclic AMP
		pmoles/minute per mg protein			
Control	1	166	166	1046	1123
	2	232	235	1005	720
	3	93	93	688	576
HS	1	198	214	1139	950
	2	291	531	931	732
	3	225	307	785	790

We have furthermore assessed the binding of cyclic AMP to HS erythrocyte membranes in view of the hypothesis that an abnormal cyclic nucleotide-membrane interaction underlies HS. The binding of cyclic [^{3}H] AMP to erythrocyte membranes in the standard binding assay with a saturating amount of cyclic [^{3}H] AMP (91 nM) in 5 normal controls (mean ± S.E. 9.6 ± 2.3 pmoles bound per mg protein) was not significantly different to that found in 5 HS subjects (mean ± S.E. 8.0 ± 1.6 pmoles bound per mg protein). The apparent binding affinities of normal and HS membranes for cyclic AMP were determined by measuring binding as a function of cyclic AMP concentration over a wide range of cyclic AMP concentrations. Using double reciprocal plots and concentrations of cyclic AMP of 0.1-91 nM, (Figs. 2 and 3) we could not obtain linear plots as has been claimed by others (1,2), and have been unable by this method to estimate the apparent dissociation constant for the "cyclic

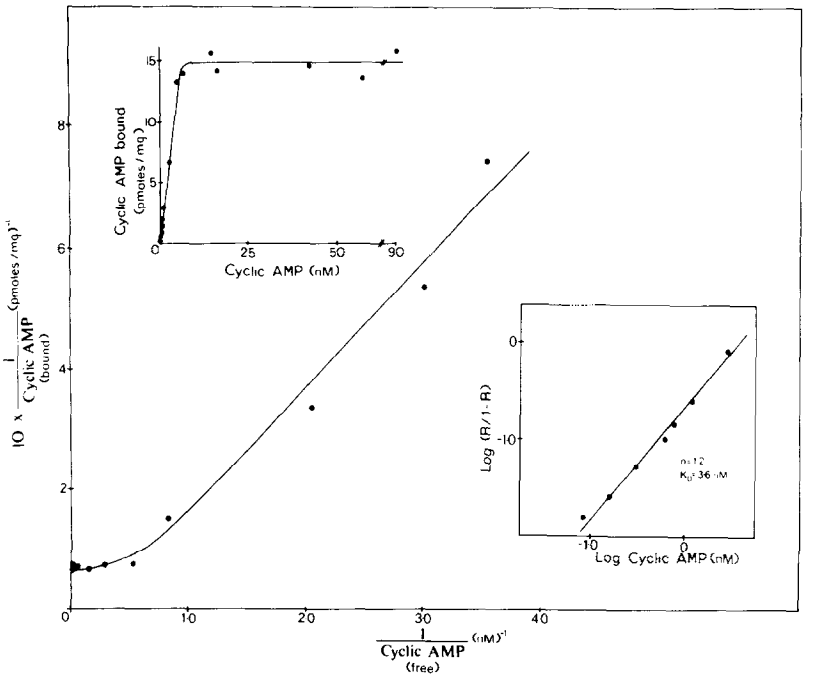

Fig. 2: The binding of cyclic AMP to normal erythrocyte membranes. Each point represents the mean of duplicate estimations. The concentration of cyclic [^{3}H] AMP was varied over the range 0.1 to 91 nM and the data of the binding curves (top inset) were used to construct the double reciprocal plot and the Hill plot (bottom inset).

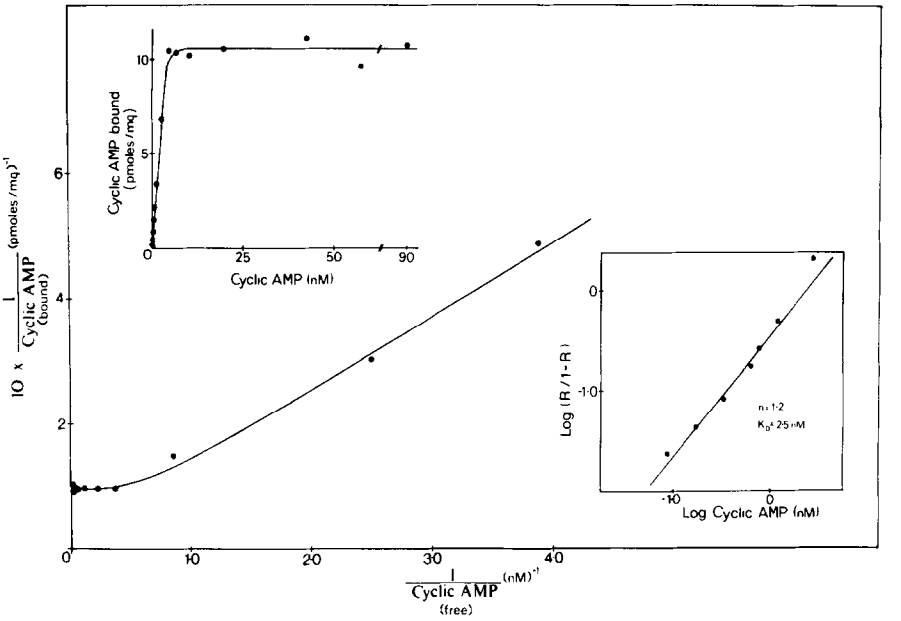

Fig. 3: The binding of cyclic AMP to HS erythrocyte membranes. Each point represents the mean of duplicate estimations. The concentration of cyclic [^{3}H] AMP was varied over the range 0.1 to 91 nM and the data of the binding curves (top inset) were used to construct the double reciprocal plot and the Hill plot (bottom inset).

AMP-membrane complex". We have resorted to Hill plots of the binding data in four normal subjects and obtain an apparent dissociation constant mean ± S.E. of 2.8 ± 0.6 nM which is not significantly different to the mean ± S.E. of 2.2 ± 0.2 nM found in 4 subjects with HS. These values agree closely with those reported by others (1,2,13) for normal erythrocyte membranes. The slope of the Hill plot (n), represents the interaction co-efficient and is equivalent to the minimum number of binding sites per binding molecule. The interaction co-efficient (n) was 1.2 in all eight preparations tested indicating a slight co-operative effect in the binding of cyclic AMP to red cell membranes. Representative double-reciprocal and Hill plots of the binding data of a control and HS membrane preparation are shown in Figs. 2 and 3 respectively. In addition, the rate of dissociation of bound cyclic [^{3}H] AMP from control and HS membranes over a period of 52 hours is very similar (fig. 4).

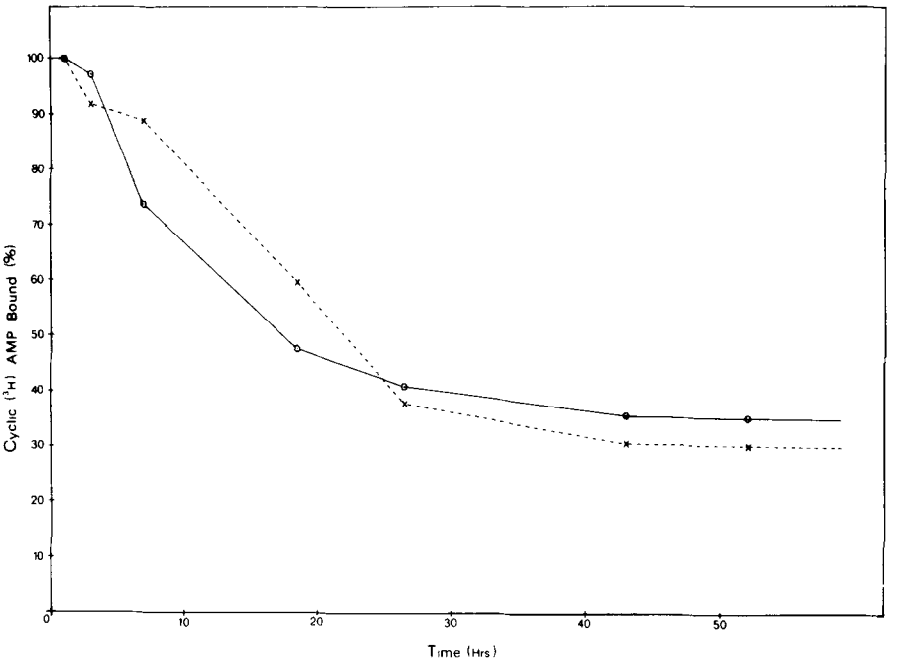

Fig. 4: Rates of dissociation of bound cyclic AMP from normal (x....x) and HS (o——o) erythrocyte membranes. Each point represents the mean of estimations of three normal and three HS membrane preparations. Binding of 91 nM cyclic [^{3}H] AMP proceeded initially for 3 hours at 0° (see methods). Results are expressed as a percentage of the radioactivity bound at zero.

We conclude that the binding affinity of cyclic AMP for HS membranes does not differ from that of normal cells. These studies thus do not support the contention of others (5) that an abnormal cyclic-nucleotide-membrane interaction underlies HS. It should be noted that the evidence for this prediction was based on studies in which cyclic AMP was shown to prevent the development of spherocytic changes in normal cells treated with vinblastine or colchichine (5). Direct studies of cyclic AMP interaction with HS cells have until now not been reported. Taken together, our findings thus indicate there is no abnormality of either the catalytic or binding site of the endogenous membrane protein kinase of HS erythrocytes.

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