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THE EFFECT OF MEMBRANE PREPARATION AND CELLULAR MATURATION ON HUMAN ERYTHROCYTE ADENYLATE CYCLASE

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We found that adenylate cyclase activity of human erythrocytes is potentially labile during isolation of their plasmalemma. Addition of 1 mM EGTA to solution used to remove hemoglobin from lysed cells protected activity. Human erythrocyte adenylate cyclase is minimally activated by catecholamines, in the absence or presence of exogenous guanyl nucleotide, but substantially by 5′-guanylyl imidodiphosphate or sodium fluoride and concentration-dependently by Mg^{2+} or Mn^{2+}. Basal catalytic activity is an age-dependent component of the human erythrocyte; 5′-guanylyl imidodiphosphate- or fluoride-activated activities decline with cellular maturation proportionally to the decrease in basal activity.

Adenylate cyclase is considered to be fundamentally different in mature, nonnucleated, human erythrocytes and mature, nucleated erythrocytes from other species. Catalytic activity, readily measured in preparations of erythrocyte plasmalemma from rat [1–3], frog [4] and turkey [4–6], until recent studies [7,8] was considered to be virtually indetectable in human red cell ghosts [1,3,9–13]. Adenylate cyclase of other red cells was found to be highly responsive to stimulation by beta-adrenergic receptor agonists, but human red cells usually were found to be devoid of this response; until recent studies [14–16], the number of beta-adrenergic receptors was found too low for measurement [17,18].

We found that enzyme lability during isolation of human red cell ghosts accounts for the low apparent activity of adenylate cyclase. We separated cells by density gradient centrifugation and, with enzyme lability inhibited, found that activity and responses to fluoride or guanyl ncleotide declined with cell age. The fundamental properties of adenylate cyclase may be similar in human and other mammalian erythrocytes; the human enzyme appears to be unusually vulnerable during membrane isolation.

Experimental procedures

Materials. [α-^{32}P]ATP (1 mCi/ml) was purchased from Amersham, [^{3}H]cAMP from New England Nuclear. Periodically, the [^{3}H]cAMP was purified on Dowex AG 50W-X4. This resin and Dowex AG 50W-X8 were obtained from BioRad. Sigma supplied neutral alumina (WN-3), enzymes, Ficoll, metrizoic acid, 5′-guanylyl imidodiphosphate (p[NH]ppG), and cyclic AMP. Fisher supplied other reagent-grade chemicals.

Healthy volunteers from the laboratory donated blood samples.

Preparation of red cell membranes. Venous blood was drawn into a syringe containing 3.8% sodium citrate (9 parts blood to 1 part citrate) and immediately applied to 2 cm^3 columns of α-

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Abbreviations: EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N′*-tetraacetic acid; p[NH]ppG, 5′-guanylyl imidodiphosphate.

cellulose : cellulose (1 : 1), suspended in 0.9% NaCl. Erythrocytes eluting from these columns were collected at 4°C in polycarbonate centrifuge tubes. This method of Beutler [19] was confirmed by us with Wright staining of blood smears and by automated counting to remove all white blood cells and platelets.

All subsequent steps were performed at 4°C. The red cells were diluted with 6 vol. of 150 mM NaCl/5 mM Tris-HCl, pH 7.5 and centrifuged at $1000 \times g$ for 10 min, unless they were to be separated by density gradient centrifugation. Cells to be applied to gradients were spun for 5 min at $500 \times g$. Tubes containing unfractionated cells or cells which had been separated on density gradients were immersed in liquid nitrogen. The frozen red cell pellets were gradually thawed into 30 ml of 129 mM NaCl/5 mM EDTA/2 mM dithiothreitol/20 mM Tris-HCl, pH 7.20 by vigorous vortexing. The suspension was centrifuged at $30000 \times g$ for 10 min. The supernatant was carefully aspirated; pelleted membranes were swirled gently and transferred to clean tubes, leaving behind a small pellet of unlysed cells. Routinely, the lysed cells were resuspended in 1 mM EGTA/75 mM Tris-HCl, pH 7.65 and centrifuged at $30000 \times g$ for 10 min. The membranes were resuspended twice in this buffer and recollected by centrifugation. Membranes, pale pink in color, usually were assayed without subsequent dilution. The minimal interval from thawing frozen cells to assaying ghosts was about one hour. This method of preparing ghosts differed importantly from that of Hoffman et al. [20] only in the addition of EGTA to the buffer used to wash lysed cells; consequences of this modification are discussed below. Intentional prolongation of any step of this method by 30 min had no effect on the activity of adenylate cyclase, implying that activity was not substantially lost during membrane isolation. As described below, membranes were also prepared without EGTA in these washing steps or in 5 mM phosphate buffer, pH 8, with or without EGTA. Whole cells, at the stage of their freezing in liquid nitrogen, or isolated membranes could be stored for at least two weeks in liquid N_2 without change in enzyme activity or responsiveness.

Density gradient centrifugation. Discontinuous Ficoll-metrizamide gradients were prepared as described by Galbraith and Watts [21]. Cells were diluted 1 : 3 with 150 mM NaCl/5 mM Tris-HCl, pH 7.5 before application to the gradient. Calculated densities of Ficoll-metrizamide layers were: 1.110, 1.118, 1.127, 1.132, and 1.150. Cells banding at interfaces were removed to polycarbonate centrifuge tubes by Pasteur pipet. Methylene blue staining revealed reticulocytes to be confined to the upper two layers of the gradient, most concentrated in the relatively sparse upper layer, as previously described [21]. Cells were washed once by dilution in 20 vol. of 150 mM NaCl/5 mM Tris-HCl, pH 7.5, and centrifuged at $10000 \times g$ for 10 min. Membranes were then prepared as described above. For some experiments, it was necessary to pool cells from two or more preparations.

Adenylate cyclase assay. Adenylate cyclase was measured as previously described [22], essentially by the method of White and Karr [23]. Routinely, total ATP concentration was 1 mM, total Mg concentration 10 mM. For kinetic studies, free Mg concentration was maintained at 2 mM; ATP concentrations were 0.103, 0.204, 0.411, and 0.617 mM. Consequently, ionized Mg was held almost constant, while calculated MgATP was, respectively, 0.1, 0.2, 0.4, and 0.6 mM [24]. Erythrocyte basal and stimulated activities were linear for at least one hour at 37°C; routinely, 30 min was used. Activities were linear with protein between 2.0 and 8.0 mg. Protein was determined on membranes solubilized in 0.25 N NaOH, 2% deoxycholate by the method of Lowry et al. [25], with bovine serum albumin as standard.

Results and Discussion

With continued washing at 4°C in 75 mM Tris-HCl, pH 7.65, or 5 mM sodium phosphate, pH 8.0, basal and fluoride-stimulated adenylate cyclase activities were progressively lost. Activity in membranes washed three times in Tris buffer without EGTA was almost absent, whether the enzyme was assayed with 2 mM Mn^{2+} or 10 mM Mg^{2+}. In membranes prepared in phosphate buffer without EGTA, activity was reduced by 65%. Addition of 1 mM EGTA, especially to the Tris buffer, inhibited loss of activity (Table I). Supernatants from membranes prepared without or with

TABLE I

COMPARISON OF ACTIVITIES AFTER SUCCESSIVE WASHES OF ERYTHROCYTE MEMBRANES

Successive washes in 5 mM sodium phosphate buffer, pH 8.0, or 75 mM Tris-HCl buffer, pH 7.65, with (+) or without (−) 1 mM EGTA. Activity after one EGTA-containing wash is represented as 100% and other values as % of this activity. Means ± S.D. derive from three experiments.

Buffer	No. of washes	Relative activity			
		Basal		10^{-2} M F	
		−	+	−	+
Tris-HCl	1	74.2 ± 6.33	100	89.5 ±12.5	100
	2	35.7 ±28.6	105 ± 12.3	42.4 ±26.4	113 ±16.2
	3	3.20 ± 2.81	108 ±11.6	5.15 ± 3.42	97.6 ± 10.6
Phosphate	1	77.8 ± 6.84	100	90.8 ± 5.12	100
	2	68.1 ± 7.71	87.3 ± 7.57	70.0 ± 4.12	86.1 ± 12.0
	3	34.7 ± 9.26	74.5 ± 19.4	36.5 ± 5.20	80.2 ± 9.97

EGTA, concentrated by lyophilization, had no adenylate cyclase activity, assayed with Mg^{2+} or Mn^{2+} as divalent cation. For membranes prepared with EGTA in Tris buffer, V_m, was 0.0969 ± 0.0245 pmol/mg per min with a free Mg^{2+} concentration of 2 mM; K_m for MgATP was 0.134 ± 0.0412 (mean ± S.D., three preparations).

Fluoride- and p[NH]ppG-activated activities declined proportionally to basal activity with cell age (Table II). In the two upper layers of every preparation, isoproterenol elicited a slight (< 10%) increase of activity in the presence or absence (data not shown) of p[NH]ppG. The increment from 10 μM isoproterenol was eliminated by 10 μM L-propranolol.

Mawatari et al. [26] reported values similar to

TABLE II

EFFECT OF ERYTHROCYTE AGING ON ADENYLATE CYCLASE ACTIVITY

Red cells distribute according to age on density gradients, with youngest cells in the uppermost layer (layer 1). Adenylate cyclase activities followed this distribution, with activity declining as a function of cell age. Numbers in parentheses are the probabilities by Student's *t*-test (two-tailed) that activity for a layer is different from that in the layer below. Means of basal activities represent values from five different gradients; means of stimulated activities represent values from three different gradients. Concentrations of sodium fluoride (F), 5'-guanylyl imidodiphosphate (p[NH]ppG), and isoproterenol (ISO) were as shown; Mg^{2+} concentration for these assays was 10 mM.

Gradient layer	Adenylate cyclase activity (pmol/mg per min)			
	Basal	F(10^{-2} M)	p[NH]ppG (10^{-5} M)	p[NH]ppG (10^{-5} M) + ISO (10^{-4} M)
1	0.828 ± 0.470 (0.0083)	56.2 ±28.2 (0.033)	19.2 ±8.10 (0.0173)	20.7 ±7.86 (0.0134)
2	0.186 ± 0.0717 (0.0149)	15.1 ± 3.75 (0.049)	4.23 ± 1.35 (0.121)	4.97 ± 1.22 (0.0667)
3	0.0931 ± 0.0318 (0.192)	8.43 ± 3.88 (0.027)	2.64 ± 1.49 (0.40)	2.91 ± 1.46 (0.337)
4	0.0760 ± 0.0266	6.28 ± 3.95	2.33 ± 1.28	2.42 ± 1.17
Unfractionated	0.215 ± 0.0296	15.3 ± 2.40	5.31 ± 0.719	5.77 ± 0.807

ours on unfractionated cells; however, we could not rely on the method of Mellman et al. [27] to isolate erythrocytes. The method of Dodge et al. [28], was used in most other studies of human erythrocyte adenylate cyclase [10–13,19]. The low results previously reported with this method can be explained by the loss of adenylate cyclase activity which occurs with successive washes of erythrocyte membranes in phosphate buffer lacking a chelator of divalent cations (Table II). Fischer and collaborators [7,8], who modified Dodge's method to include 1 mM EDTA in initial washes of lysed cells, had results similar to ours.

Stabilization of human red cell adenylate cyclase activity during membrane isolation permitted us to examine the maturational effect on adenylate cyclase of human cells, previously studied in detail with rodent erythrocytes [29–33]. In previous studies of human cells, proportion of reticulocytes and activity of adenylate cyclase correlated [8] and fluoride-stimulated activity was shown to decline with cell age [29]. Our results are consistent with these studies and with maturational loss of catalytic subunits with preservation of N-protein, which is known to be normal in function and concentration in mature human erythrocytes.

We found that human erythrocyte adenylate cyclase has a minimal but reproducible response to catecholamines. This observation supplements that of Sager [16] who found that the cyclic AMP content of human erythrocytes, preincubated with theophylline, could be dose-dependently incremented by incubation with isoproterenol. If beta receptors in human red cells are normal in number and function [16] and if concentration of N-protein is normal [34], the anemic response of human red cell adenylate cyclase to catecholamines [1,3] is unexplained. The use of human erythrocytes in complementation assays also requires further study: the possibility exists that inactivation of catalytic subunits during membrane isolation is reversible during a complementation assay.

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