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Reduction of diferric transferrin by SV40 transformed pineal cells stimulates Na^+/H^+ antiport activity

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Transplasmalemma electron transport by HeLa and pineal cells to reduce external ferricyanide is associated with proton release from the cells. Diferric transferrin also acts as an electron acceptor for the transmembrane oxidoreductase. We now show that reduction of external diferric transferrin by RPNA-209-1 SV40 transformed pineal cells is accompanied by proton release from the cells. The stoichiometry of proton release to electron transfer is much greater than would be expected from aniostropic electron flow across the membrane through protonated carriers. The proton release is not stimulated by apotransferrin and the diferric transferrin-stimulated activity is inhibited by apotransferrin. Apotransferrin also inhibits reduction of diferric transferrin by these cells. The proton release is dependent on external sodium ions and is inhibited by amiloride, which indicates that the proton release is mediated by the Na^+/H^+ antiport and that this antiport is activated by electron transport through the transmembrane dehydrogenase. Growth stimulation by diferric transferrin or other external oxidants can be based in part on activation of the Na^+/H^+ antiport.

Introduction

Transplasmalemma electron transport has been observed in many types of cells [1–5]. Evidence for this activity has been developed by several types of observation. Reduction of impermeable electron acceptors such as ferricyanide is found with intact cells and perfused tissue [6,7]. This reduction of external oxidants is associated with oxidation of cytosolic NADH [8]. NADH dehydrogenase activity has been associated with the plasma membrane by histochemical studies on intact cells and isolated membranes [9–11]. With isolated erythrocyte membranes, a part of the NADH : ferricyanide reductase activity has been shown to function only with open membrane vesicles and not with closed vesicles [12], indicating that exposure of both side of the membrane is necessary for activity.

The reduction of external electron acceptors, such as ferricyanide, by transplasmalemma electron transport, has been shown to be accompanied by proton release from the cells into the external media. Dormandy and Zarday [1] showed that reduction of ferricyanide was accompanied by

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proton release from erythrocytes. Ferricyanide reduction by HeLa cells has also been shown to be accompanied by proton release by Sun et al. [2,3], but the proton release was saturated at a much lower ferricyanide concentration (0.4 mM) than was electron transport (1.2 mM). Inhibition of HeLa cells electron transport by low concentrations of adriamycin and related antitumor drugs was accompanied by inhibition of proton release induced by ferricyanide [13].

Electron transport across a membrane through a protonated electron carrier can be a basis for proton movement through the membrane, as proposed by Mitchell [14]. This proton release would be expected to be equal to electron flow and show an H^+/e^- ratio of one. On the other hand, oxidation-reduction change in a membrane protein may cause conformational changes in the protein which could activate a proton channel in the redox protein or in an adjacent protein. This action could allow release of multiple protons per electron.

With oxidant-stimulated proton release by animal cells the observed ratio of protons released to electrons transferred is higher than 1. For HeLa cells in 10 mM NaCl plus salts and sucrose the H^+/e^- ratio is greater than 1.5 [2,3]. For pineal cells (SV40 transformed) the H^+/e^- is 3.2 when assayed in 250 mM sucrose [15]. For Ehrlich ascites cells the ratio is 3.3 when both ferricyanide reduction and proton release are measured in 250 mM sucrose [16]. SV40 transformed fetal rat liver cells assayed in 10 mM NaCl with other salts and 100 mM sucrose have an H^+/e^- ratio of 15 [17]. It is apparent that the stoichiometry of proton and electron release under the conditions used is much greater than 1 and therefore not based on a simple movement of protons by oxidation reduction of a protonated transmembrane electron carrier.

We have recently shown that the transplasmalemma electron transport enzymes can reduce the iron in diferric transferrin outside the cell and suggest that diferric transferrin is the natural electron acceptor for the transmembrane enzyme [18]. In this report we will show that the reduction of diferric transferrin by RPNA 209-1 SV40 transformed rat pineal cells is accompanied by proton release from the cells and that this proton release is greater than would be expected from direct coupling to electron flow.

We find that proton release from transformed pineal cells stimulated by ferricyanide or diferric transferrin reduction is dependent on sodium ions and is amiloride sensitive which is consistent with proton release through the Na^+/H^+ antiport.

Methods

Rat pineal cells (RPNA 209-1) infected with a temperature-sensitive strain (tsA209) of SV40 [15] were obtained from Dr. J.Y. Chou. These cells show the transformed phenotype when cultured at 33° C but revert to the nontransformed phenotype at 40° C. Cells were cultured in modified minimal essential media with 4% fetal bovine serum at 33° C [19]. Doubling time is 24 h. Cells were harvested and washed in buffer A (140 mM NaCl/25 mM KCl/0.6 mM Na_2HPO_4/25 mM Trizma base/0.05 mM EDTA (pH 7.4)). Final suspension was at 0.1 g wet weight per ml.

Reduction of iron in diferric transferrin was assayed by following the formation of ferrous bathophenanthroline sulfonate according to Avron and Shavit [20]. Absorbance change at 535 nm was subtracted from absorbance at 600 nm with the dual beam on the DW2a Aminco spectrophotometer. Extinction coefficient difference is 17.1 $mM^{-1} \cdot cm^{-1}$. The assay mixture in 2.5 ml buffer A contained 3.4 μM diferric transferrin, 3.3 μM bathophenanthroline sulfonate and 10–50 mg wet weight cells. Diferric transferrin (absorbance ratio 465 : 280 over 0.04) was prepared according to Karin and Mintz [21] or obtained from Miles Laboratories. Apotransferrin was from Sigma. Assay temperature was 37° C except where stoichiometric comparison is made to proton release where 24° C is used. For comparison of electron transport and proton release, assays were done in sucrose or salt solutions with low buffer capacity (1.5 mM Trischloride) and at various pH values.

Ferricyanide reduction was measured in 2.5 ml buffer A with 0.1 mM potassium ferricyanide and 10–20 mg wet weight cells at 37° C. Change in absorbance at 500 nm was subtracted from absorbance at 420 nm on the dual beam of the Amino DW2a. Extinction coefficient is 1 $mM^{-1} \cdot cm^{-1}$. For comparison with proton release sucrose or salt solutions with 1.5 mM Tris (pH 7.4) were

used instead of buffer A at 24° C.

Oxidant-stimulated proton release was measured by following changes in pH of low buffer capacity medium outside of cells. A Corning combination electrode was immersed in a thermostated chamber with 3.0 ml assay mixture at 24° C. The reaction mixture was continuously bubbled with air to remove excess CO_2. The same results are obtained by bubbling with CO_2-free air passed through NaOH solution. Cells, 10–20 mg wet weight, were equilibrated in the buffer solution to minimum pH change before addition of diferric transferrin (3.4 μm) or potassium ferricyanide (0.1 mM). Oxidant-stimulated proton release was calculated from the difference in pH change before and after oxidant addition. Proton release was calibrated by adding 10–50 nmol HCl at the end of each assay. The media was 250 mM sucrose which was replaced by sodium chloride or choline chloride at concentrations up to 150 mM. 1.5 mM Tris-choloride was present in all solutions as a buffer at pH 7.4 (starting pH).

Inhibitors such as amiloride, apotransferrin or adriamycin were added with cells during the equilibration 3–5 min before addition of diferric transferrin or ferricyanide. Since ferricyanide shows reduction in the presence of amiloride it was necessary to wait for the rapid reaction with amiloride to end before measuring the rate of reduction. This measurement was corrected by subtracting activity found in the presence of amiloride without cells. Diferric transferrin was not reduced by amiloride.

Results

Addition of potassium ferircyanide to transformed pineal cells caused immediate release of protons. The rate of proton release was greatest in sodium chloride solution and much less in choline chloride. There was also only a slow rate of proton release in 250 mM sucrose (Fig. 1). Potassium ferrocyanide did not induce proton release. Addition of diferric transferrin in 150 mM NaCl gave a faster rate of proton release than ferricyanide (Fig. 2). In 150 mM choline chloride or sucrose the diferric transferrin-induced proton release was much less than in sodium chloride solution (Fig. 3, Table I).

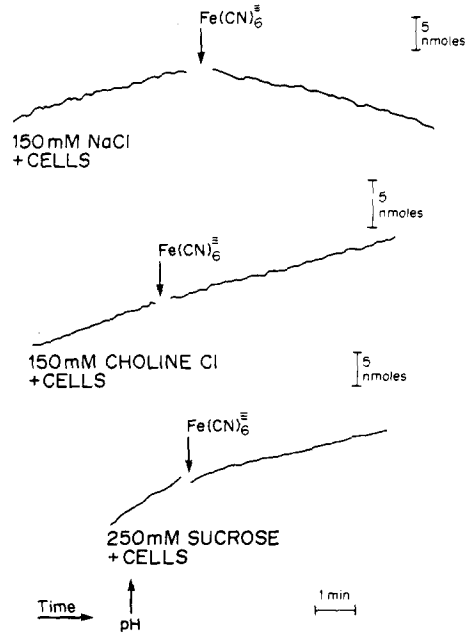

Fig. 1. Induction of proton release from pineal cells by ferricyanide. Cells equilibrated in indicated buffer solution until proton exchange is minimized, then potassium ferircyanide (0.1 mM) is added as indicated. pH change was measured with a combination glass electrode. All solutions contain 1.5 mM Tris chloride to dampen pH change and proton release is calibrated by adding 0.01 M HCl at the end of each experiment. The chamber is constantly bubbled with air to remove CO_2.

In contrast to the slow proton release induced by ferricyanide, the reduction of ferricyanide by the pineal cells was much faster than the rate of

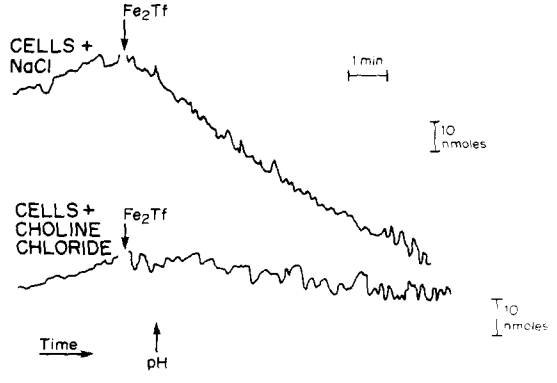

Fig. 2. Induction of proton release from pineal cells with diferric transferrin. Procedure same as for Fig. 1 except that 17 μM diferric transferrin (Miles) was added after equilibration. Salt concentration 150 mM, 10 mg cells.

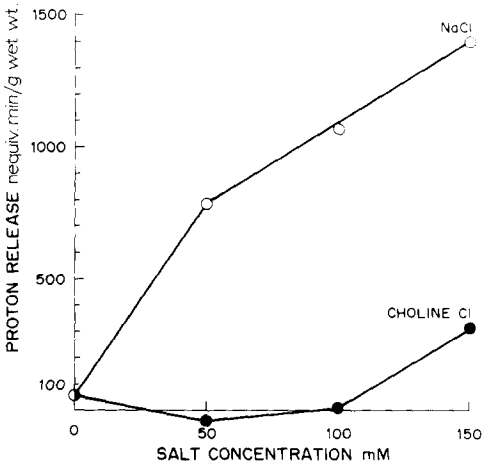

Fig. 3. Effect of sodium chloride and choline chloride on diferric transferrin-induced proton release rate from pineal cells. Assay as shown in Fig. 2.

diferric transferrin reduction. Both ferricyanide reduction and diferric transferrin reduction were faster in sodium chloride than in choline chloride or sucrose (Table II).

Apotransferrin has been shown to inhibit reduction of diferric transferrin by HeLa cells [18] so it would be expected to inhibit proton flow based on the reduction as is seen in Table III. On the other hand, apotransferrin is only a weak inhibitor of ferricyanide reduction and, as seen in Table III, produces low inhibition of ferricyanide-induced proton release.

Since diferric transferrin is strongly bound at the transferrin receptor, it is possible that binding of the transferrin to the receptor is sufficient to activate proton release. As seen in Table III, apotransferrin alone did not activate proton release.

Amiloride acts as an inhibitor of the Na^+/H^+ antiport. As seen in Table III, amiloride-inhibited proton release induced by both ferricyanide and diferric transferrin from the pineal cells. Concentrations of amiloride (0.2 mM) which inhibit the oxidant-induced proton release also inhibited

TABLE I

STIMULATION OF PROTON RELEASE BY RPN A209-1 CELLS WITH FERRICYANIDE AND DIFERRIC TRANSFERRIN

Results are representative of six experiments.

Salts in assay medium	Rate of proton release after addition of oxidant (nequiv. H^+/min per g wet wt.)	
	ferricyanide (0.1 mM)	diferric transferrin (17 μM)
150 mM NaCl	266	1406
150 mM Choline chloride	52	313
250 mM Sucrose	167	50

TABLE II

EFFECT OF SODIUM IONS ON THE REDUCTION RATE OF FERRICYANIDE AND DIFERRIC TRANSFERRIN BY PINEAL CELLS RPN-A209-1

RPN-A209-1 SV40 pineal cells were grown at 33° C permissive temperature for 2 days [12]. Potassium ferricyanide was used as electron acceptor. Results are representative of four experiments.

Acceptor:	Reduction rate (nmol/min per g wet wt.)	
	ferricyanide (slow)	diferric transferrin
250 mM sucrose	18	7
50 mM NaCl	52	11
100 mM NaCl	71	19.7
150 mM NaCl	101	16.5
150 mM choline chloride	34	5.2

TABLE III

CONTROL OF PROTON RELEASE BY INHIBITORS OF ELECTRON TRANSPORT AND AMILORIDE

All assays were done as 150 mM NaCl/1.5 mM Tris-HCl (pH 7.4)/RPN-A209-1 cells 33° C culture. Fe_2Tf, diferric transferrin; ApoTf, apotransferrin.

Oxidant	Inhibitor	Protein release (nmol min per g wet wt.)
Fe_2Tf	—	1406
Fe_2Tf	17 μM apotransferrin	323
ApoTf	—	0
Fe_2Tf	—	1956
Fe_2Tf	0.2 mM amiloride	500
Ferricyanide	—	628
Ferricyanide	apotransferrin	490
Ferricyanide	0.2 mM amiloride	79

TABLE IV

EFFECT OF AMILORIDE ON REDUCTION OF DIFERRIC TRANSFERRIN AND FERRICYANIDE BY PINEAL CELLS

Ferricyanide reduction can only be measured by the slow rate after 2 min or more after a correction for reduction by amiloride directly in absence of cells. Results are representative of five experiments. Fe_2Tf, diferric transferrin.

Concentrations of amiloride added	Reduction rate (nmol/min per g wet wt.)	
	ferricyanide (slow)	Fe_2Tf
None	113	19.2
0.1 mM	75	7.5
0.2 mM	30	4.0
0.4 mM	33	6.0

electron transport to ferricyanide or diferric transferrin (Table IV).

The ratio of protons released to electron flow was not much greater than 5 under optimal salt conditions (see Introduction for activity previously observed in lower salt concentrations) with ferricyanide as oxidant (Table V). However, in 150 mM sodium chloride the H^+/e^- ratio by pineal cells in the presence of diferric transferrin can be 100 or greater (Table V).

TABLE V

STOICHIOMETRY OF PROTON RELEASE BY REDOX AGENTS. PINEAL CELLS SV40

Results are expressed as nequiv./min per g wet wt. Fe_2Tf, diferric transferrin.

	Ferricyanide reduction (slow) per g wet wt.	$Fe(CN)_6$ proton release per g wet wt.	H^+/e^- per g wet wt.
250 mM sucrose	18	81	4.5
150 mM choline	34	0	0
150 mM sodium chloride	101	540	5.3
	Transferrin reduction	Fe_2Tf proton release	
250 mM sucrose	7	88	12.6
150 mM choline chloride	5.6	0	0
150 mM sodium chloride	16.5	1530	93

Discussion

Diferric transferrin is well recognized as necessary for the growth of many mammalian cells [22,23]. Its primary function has been envisioned as a source of iron for enzymes necessary for DNA synthesis and energy conversion in the cell [24–26].

The mechanism of iron uptake is proposed to be based on uptake into endosomes of diferric transferrin bound to the transferrin receptor. The iron is removed in the endosome and the apotransferrin is returned to the cell surface [27–29]. It has also been suggested that the diferric transferrin iron can be reduced to ferrous iron at the cell surface [30–32], and we have recently demonstrated that the transplasma membrane electrocton transport acts as a diferric transferrin reductase [18]. Since other non-ferric electron acceptors for this transmembrane electron transport can replace diferric transferrin in stimulation of cell growth [33,34], we have suggested that electron transport to diferric transferrin is a second role for this protein in growth stimulation [18,33].

The role of the Na^+/H^+ antiport in stimulation of cell growth has been extensively examined [35–37]. Removal of protons from the cell increases cytoplasmic pH [41] and this has been associated with activation of DNA synthesis, protein phosphorylation, and other vital functions [38–40]. Activation of the Na^+/H^+ antiport by mitogens and growth factors has been attributed to increasing the positive allosteric response of the Na^+/H^+ exchanger to protons [37,41] possibly by increasing the affinity of the activator site on the cytoplasmic side of the membrane for protons [41,42].

External oxidants increase proton release by cells [1,2,15]. The proton excretion induced by the external oxidant could be carried out as a consequence of anisotropic arrangement of oxidoreductase enzymes in the membrane [14,43] or by activation of an H^+ exchanger in the membrane. The high ratio of protons released per electron transferred across the membrane to ferricyanide and especially to diferric transferrin mitigates against a simple protonated redox carrier as the mechanism for proton transfer. The alternative

would be activation of a proton exchanger across the membrane to allow either passive or energized proton movement. Garcia Cañero and coworkers have proposed that the reduction of external ferricyanide by HeLa and liver cells is associated with activation of proton movement through the sodium/proton antiport (Ref. 44 and Garcia Cañero, R. and Guerra, M.A., personal communication). They have shown that reduction of external ferricyanide is dependent on a high concentration (greater than 50 mM) of sodium ions, and that the ferricyanide reduction is accompanied by uptake of sodium ions. They also find that amiloride, a specific inhibitor of the Na^+/H^+ antiport inhibits ferricyanide reduction by the HeLa or rat liver cells. The observations in this paper are consistent with their interpretation [44] that the Na^+/H^+ antiport is the exchanger through which protons are released, and that the electron flow through the membrane is coupled to the activation of that antiport. The proton release induced by both ferircyanide and diferric transferrin is dependent on external sodium and is inhibited by amiloride at concentrations appropriate to inhibition of the Na^+/H^+ antiport.

The inhibition of ferricyanide or diferric transferrin reduction by inactivation of the antiport, either through sodium deficiency or by amiloride, is remarkable. The inhibition of the redox system when the antiport is not active implies a close association of the antiport protein and the dehydrogenase. We have previously proposed that the plasma membrane dehydrogenase can act to protonize adjacent sites on the plasma membrane to control activity of associated protins, e.g., adenylate cyclase [3,45,46]. The release of a proton in the surface layer of the membrane from the NADH dehydrogenase could activate the allosteric proton activator site on the sodium/proton antiport [37]. This would allow activation of the antiport without a change in the bulk cytoplasmic pH and would allow it to stay active even if the internal pH increases. It remains to be established how the external sodium site, which is also the amiloride inhibition site [41], can control the electron transport across the membrane. A further point of interest is why reduction of iron in diferric transferrin induces more proton flow through the antiport than does reduction of ferricyanide which supports much greater electron flow.

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