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Mucosal surface ferricyanide reductase activity in mouse duodenum

David J. Pountney, Kishor B. Raja, Matthew J. Bottwood*, John M. Wrigglesworth* & Robert J. Simpson

*Department of Clinical Biochemistry, King's College School of Medicine and Dentistry, London and * Metals in Biology and Medicine Centre, Division of Life Sciences, King's College London, London, UK*

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Mouse duodenum possesses mucosal surface ferricyanide reductase activity. The reducing activity, determined *in vitro* by measuring ferrocyanide production from ferricyanide, was found to be greater in duodenal fragments when compared with ileal fragments. Experiments with right-side out tied-off duodenal sacs show that reduction occurs mainly on the mucosal side and indicates that the reducing activity is associated with the brush border membrane. Experiments using mice with increased levels of iron absorption (hypoxic, iron-deficient) showed corresponding increases in reducing activity. The increase was present in duodenal but not ileal fragments. Inhibitor studies showed no effect of several compounds which inhibit other, more characterized, transplasma membrane reductases. In particular, doxorubicin (10 μM) and quinacrine (1 mM) were without effect on duodenal mucosal transplasma membrane reducing activity. Depolarization of the membrane potential with high medium K^+ inhibited reducing activity. *N*-ethyl malemide (1 mM) was a potent inhibitor, but iodoacetate was found to be less inhibitory. Comparision with inhibitory effects on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) demonstrated that the effect of *N*-ethyl malemide on reducing activity was not secondary to GAPDH. Collectively these results indicate that mouse duodenum possesses mucosal surface transplasma membrane ferricyanide reductase activity and that the activity is correlated with the process of intestinal iron absorption. Furthermore, the reducing activity appears to be distinct from other reported transplasma membrane reductases.

Keywords: brush border membrane, duodenum, ferricyanide, iron absorption, reduction

Introduction

The mechanism of absorption of iron by the intestinal mucosal cell and its regulation is not fully understood. The absorption process includes at least two distinct steps: (1) entry of iron into the mucosal cell (uptake) and (2) transfer to the portal circulation. Previous studies have demonstrated more than one route of entry of inorganic iron into the duodenal mucosa of mice (Simpson *et al.* 1989). The major pathway for uptake is mainly localized to the duodenum, and is dependent on cellular metabolism and on the brush border membrane potential gradient (Raja *et al.* 1989).

An inducible mucosal surface Fe(III) reduction has recently been implicated in duodenal Fe(III) uptake (Raja *et al.* 1992), using the Fe(II) chelator ferrozine to detect Fe(III) reduction. Crane *et al.* (1985) have hypothesized that transplasma membrane redox activity could be involved in mucosal Fe(III) uptake. This group of workers have characterized transplasma membrane ferricyanide reductase in a variety of systems. In particular, it has been shown that the yeast *Saccharomyces cerevisiae* has the ability to reduce externally added ferricyanide (Crane *et al.* 1982). An investigation of iron uptake by this organism implicates a regulated transplasma membrane Fe(III) reductase as a component of the iron uptake system. A gene coding for the reductase has been cloned and shown to have homology with the well-characterized neutrophil respiratory burst transplasma membrane O_2 reductase (Dancis *et al.* 1992). This latter activity is dependent on intracellular NADPH as a source of reducing equivalents (Segal & Abo 1993). NADH-dependent ferricyanide reductases in plasma membrane preparations from other tissues have also been described (Crane *et al.* 1982).

In the present study, investigations have been carried out to characterize a surface reductive process in mouse intestinal fragments and tied-off sacs using the electron acceptor ferricyanide, and given the membrane impermeable nature

Address for correspondence: R. J. Simpson, Department of Clinical Biochemistry, King's College School of Medicine and Dentistry, Bessemer Road, London SE5 9PJ, UK. Fax: (+44) (0)171 737 7434; email: R.Simpson@BAY.CC.KCL.AC.UK.

of ferricyanide, to ascertain if the reductive process is a truly extracellular observation confined to the mucosal surface. Two methods of measuring ferricyanide reduction have been examined: (1) a determination of ferrocyanide produced from ferricyanide and (2) a continuous monitoring of ferricyanide disappearance. Once the complementary nature of the two assays was established, the effect of known inhibitors and modulators of characterized transplasma membrane redox systems of other cell types on the intestinal reductive process was investigated. The results show the presence of a mucosal surface ferricyanide reductase activity in mouse duodenum that is regulated in parallel with the process of intestinal iron absorption and with an inhibitory response suggesting distinction from the transplasma membrane redox systems of other intact cells.

Materials and methods

Reagents

All chemicals and biochemicals were from either BDH Laboratory Supplies, Merck (Poole, UK) or Sigma (Poole, UK).

Animals

Male mice, CD1 strain, 6–8 weeks old (Charles Rivers, Margate, UK), were used throughout. Hypoxia was produced by placing mice in a hypobaric chamber maintained at 53.4 kPa (0.5 atm) for 3 days. Dietary iron deficiency was induced by feeding 3 week old mice with a low iron diet for 3–4 weeks. The diet was prepared according to the method of Pearson *et al.* (1967) and consisted of vitamin free casein (18%), hydrogenated cotton seed oil (5%), sucrose (73%), adequate vitamins and iron-free mineral mixtures. Controls received the same diet except that it was supplemented with iron as $FeCl_3$ (125 mg kg^{-1} diet).

In vitro *ferrocyanide production studies*

Ferricyanide reduction, assayed by a modification of the procedure described by Avron & Shavitt (1963), was followed by monitoring ferrocyanide production using a redox assay based on observing Fe(II) production with the specific ferrous ion chelator ferrozine. Intestinal tissue (rinsed in saline) was incubated at 37°C in incubation buffer (16 mM HEPES, pH 7.4, 3.5 mM KCl, 125 mM NaCl, 10 mM glucose), plus potassium ferricyanide (100 μM). Aliquots of 200 μl were removed at different times and added to 50 μl of 40% TCA. These samples were first centrifuged for 5 min at 10 000 *g* to remove any cells and tissue particles, and then 200 μl of the supernatant was added to an assay medium (560 μl 2 M Na-acetate, pH 4.8, 10 μl 100 mM ferrozine, 100 μl 10 mM $FeCl_3$ in 10 mM HCl).

Ferrocyanide (produced by tissue reduction of ferricyanide), is able to reduce medium ferric iron to the ferrous form, which then binds the specific ferrous ion chelator ferrozine, producing a stable, purple colored complex which can be quantitatively assayed at 562 nm. A blank reference value is first obtained by assaying the HEPES buffer incubation medium in the absence of tissue and ferricyanide. The tissue is then added, and after 5 min the medium sampled and assayed to determine the background reducing rate. Ferricyanide is then added and the continuously-oxygenated medium sampled every 5 min during a 15 min incubation at 37°C.

In vitro *ferricyanide disappearance studies*

Conversion of potassium ferricyanide to ferrocyanide was measured by monitoring absorbance at 410 nm in a Pye Unicam SP8000 spectrophotometer together with absorbance changes at 550 nm at 30 s intervals to correct for background scattering due to slow tissue degradation or secretion of light absorbing materials during the time-course of an incubation. A low dead-volume flow cell was connected to an incubation vessel via an LKB peristaltic pump (model Broma 12000). The incubation vessel was essentially a conical base Sterilin universal container (30 ml capacity), with a connecting tube carrying medium to the flow cell and tube returning medium from the flow cell to the vessel. A small piece of plastic gauze fixed at the base ensured that the tissue sample was not in contact with the opening of the outflow tubing and also prevented O_2 bubbles entering the tubing. The incubation vessel was positioned in a waterbath set at 37°C and the spectrophotometer cuvette was maintained at 37°C with a circulatory waterbath.

Incubation buffer (2.5 ml 16 mM HEPES, pH 7.4, 3.5 mM KCl, 125 mM NaCl, 10 mM glucose) was added and allowed to circulate through the system for 2 min to provide an absorbance baseline. The absorbance of ferricyanide in the absence of tissue was determined by addition of 50 μl 10 mM ferricyanide. For all experiments, the pump speed was set at 2 ml min^{-1} and the gauge of all connecting tubing was 0.5 mm, in order to minimize the time taken for medium to reach the flow cell.

Single fragments of tissue (about 30–60 mg) prepared from the duodenum (first 5 cm from pylorus) or whole tied-off duodenal sacs were rinsed in warm saline, blotted to remove mucosal food particles and then added to the incubation vessel containing 2.5 ml of incubation buffer which was continuously oxygenated. Oxygenation also provided an effective way of producing constant mixing of the medium. Immediately after the addition of the tissue, the absorbance was measured at 410 and 550 nm at 30 s intervals for 5 min prior to the addition of 50 μl 10 mM potassium ferricyanide. The ratio of absorbance (550:410 nm) in the absence of ferricyanide was found to change over time in the presence of tissue and the absorbance values in the presence of ferricyanide had to be corrected for this effect. Several experiments were performed over a longer time period with no ferricyanide present in order to establish the magnitude of background changes in absorbance. After recording the changes in absorbance over 10–20 min as described, the tissue was removed, gently blotted and the wet weight determined.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) assay

Duodenal fragments of approximately equal wet weight were assayed for ferrocyanide production as described above. Of the individual tissue incubations, three contained either 1 mM *N*-ethyl malemide, iodoacetate or *p*-chloromercuriphenyl sulfonate and a fourth, with no addition, served as a control. Upon completion of the assay the fragments were gently blotted, rinsed twice in 150 mM NaCl 10 mM HEPES (pH 7.4) and homogenized in 0.5 ml of homogenization buffer; 2.7 mM EDTA (neutralized), 5.0% v/v β-mercaptoethanol, using a 1 ml Dounce homogenizer. GAPDH was assayed as soon as possible thereafter, essentially as described by Beutler (1975), following the disappearance of NADH at 340 nm. An assay cuvette contained 100 μl 5 mM EDTA, 1 M Tris HCl, pH 8.0, 100 μl 100 mM $MgCl_2$, 100 μl 2 mM NADH, 400 μl 20 mM ATP (neutralized), 10 μl PGK (50 U ml^{-1}), 15 μl homogenate and 185 μl H_2O. Substitution of 3-PGA by distilled water served as a blank. The cuvettes were incubated in the spectrophotometer at 37°C for 10 min prior to initiating the reaction with the addition of 100 μl 100 mM 3-PGA. The decrease in optical density at 340 nm was measured against that of the blank at 37°C for 20 min.

Statistical analysis

Where appropriate, data was analyzed by a series of two-sample Student's *t*-tests. The assumption of normality for the *t*-tests was valid, since Minitabs normal score test gave no significant evidence that the data were not from normal distributions. Where multiple comparisions were performed, the Student–Newman–Keuls modification was used (Glantz & Slinker 1990).

Results and discussion

The reducing activity [1161 ± 80 (SEM, $n=7$) pmol mg gut^{-1} $10 min^{-1}$ incubation with ferricyanide] measured by the ferrous-ferrozine production method was found to be greater in duodenal fragments, when compared with ileal fragments [836 ± 159 (SEM, $n=6$)] ($P<0.05$) (Figure 1a).

Duodenal fragments from hypoxic mice had an increased reducing activity [1614 ± 167 (SEM, $n=6$)] ($p<0.05$), but no response was seen with hypoxic ileal fragments [1055 ± 118 (SEM, $n=6$)]. Hypoxic mouse duodenum has been shown to have elevated levels of iron uptake (Raja *et al.* 1987), and the parallel increase in reducing activity seen with duodenal fragments from hypoxic mice indicates that reduction and uptake are related steps. The results also suggest that any regulation of the reducing activity to adapt to hypoxic conditions is confined to the duodenum.

When ferricyanide was omitted from the medium a basal production of Fe(II)-ferrozine was observed (Figure 1b). This basal activity, however, was independent of intestinal location or hypoxia. However, because of this low basal activity, inhibitor and kinetic studies were performed with tissue from hypoxic mice. Figure 2 shows the dependence of reduction rate on medium ferricyanide concentration. The

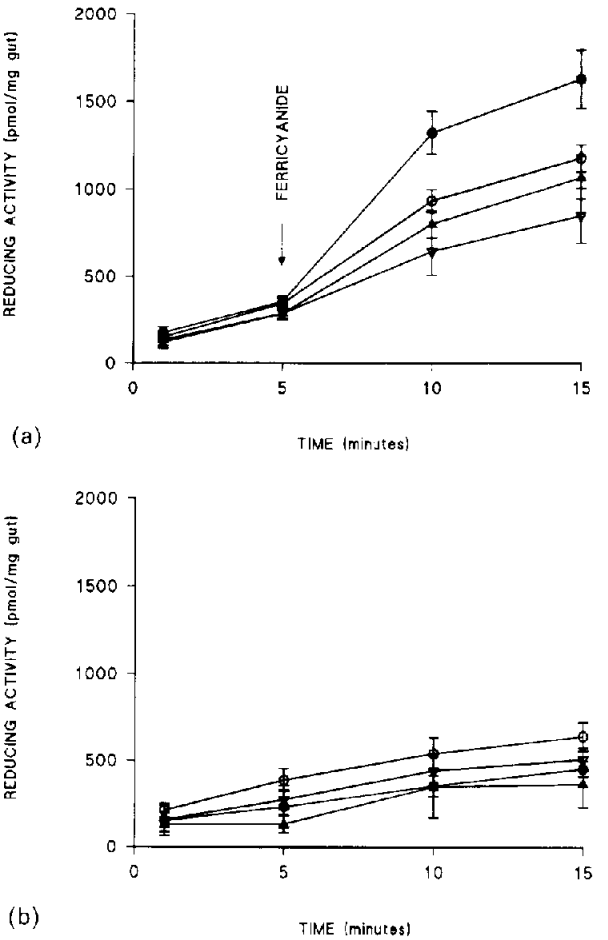

Figure 1. Ferrocyanide production in the presence of mouse gut. (**a**) Reducing activity (pmol mg^{-1} gut) was determined by the method of Avron & Shavitt (1963), in *in vitro* incubations of duodenal (○) and ileal (▽) fragments from normal mice and duodenal (●) and ileal (▲) fragments from hypoxic mice, before and after addition of 100 μM ferricyanide to the medium. (**b**) Background reducing activity in incubations as in (**a**) only without addition of ferricyanide. Further details are given in the text. Values are means ± SEM, $n=6$–7.

data was fitted to a hyperbolic curve with an apparent K_m of 185 μM.

Figure 3 shows representative timecourses for the disappearance (reduction) of ferricyanide measured directly by monitoring medium absorbance at 410 nm of *in vitro* incubations of duodenal tissue. The initial rates of ferricyanide reduction were determined from non-linear curve-fits using a mathematical program (Fig P, Biosoft). Although many curves were non-linear, the apparent K_m determined above would be inconsistent with a single substrate Michaelis–Menten reaction progress curve as product depletion could not generate curvature unless the K_m was much higher than the value observed in Figure 2. Studies of initial rates of ferricyanide disappearance were also consistent with an apparent K_m below 200 μM. It was

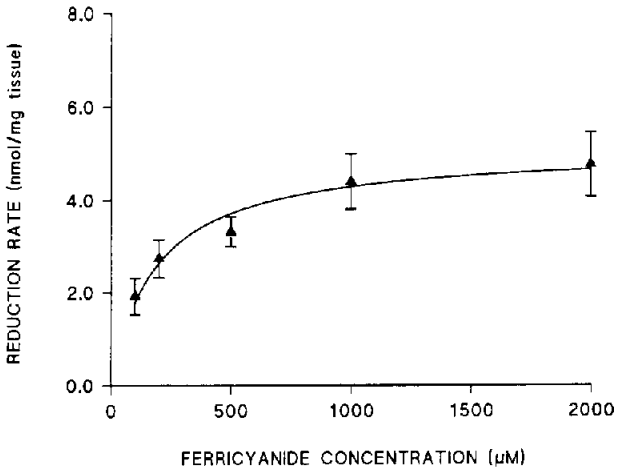

Figure 2. Apparent kinetics for ferricyanide reduction by hypoxic mouse duodenum. Ferricyanide reduction after 5 min incubation of duodenal fragments from hypoxic mice was determined as in Figure 1. Background reducing activity was corrected by subtracting activity determined immediately before ferricyanide addition. The data are fitted by a hyperbolic curve with an apparent K_m of 185 μM and V_{max} of 5.1 nmol min^{-1} mg^{-1} tissue.

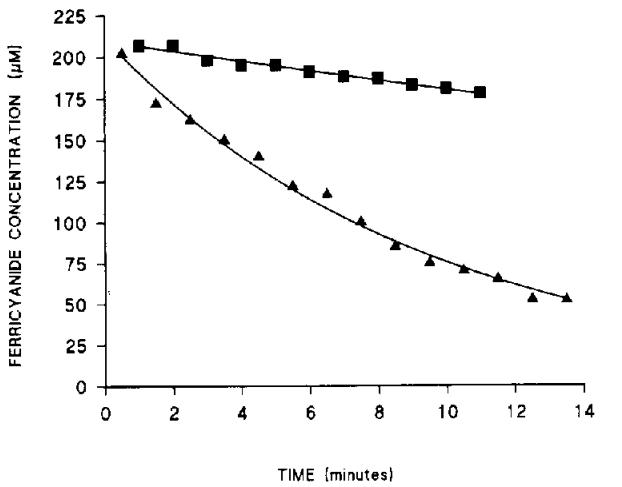

Figure 3. Ferricyanide reduction by mouse duodenal fragments. Disappearance (reduction) of ferricyanide measured directly by monitoring medium absorbance at 410 nm of *in vitro* incubations of duodenal tissue from normal (■) and hypoxic (▲) mice. Changes in background absorbance were corrected by monitoring absorbance at 550 nm. Full details are given in the text.

concluded that curvature resulted from product inhibition or loss of tissue viability.

Ferricyanide reduction by everted sacs of duodenum is responsive to prior hypoxic treatment of mice (Table 1). Serosal side-out sacs have little capacity to reduce ferricyanide, suggesting that the reducing activity is located on the mucosal surface.

Iron-deficient and 3 day hypoxic mice have previously been shown to have increased levels of iron uptake (Raja *et al.* 1987). Table 2 shows the results of determinations of ferricyanide reduction in proximal intestinal tissue from normal, 3 day hypoxic, iron-deficient and iron-replete mice. The induction of ferricyanide reducing activity is similar in both groups with increased iron absorption (hypoxic, iron-deficient). The significant increase in reducing activity observed in the iron-deficient group as compared to the iron-replete group parallels reported elevated levels of iron uptake for iron-deficient mice (Raja *et al.* 1989), and supports the hypothesis that reduction and uptake are related steps. Incubation media that had been pre-incubated with duodenal tissue from hypoxic mice for 5 min had virtually no capacity to reduce ferricyanide after removal of the tissue and subsequent addition of ferricyanide, thus providing further evidence against a significant intestinal release of reducing factors.

Both methods of assay (ferrous-ferrozine production and ferricyanide disappearance) were examined for their comparability by using the same incubations for each assay (200 μM ferricyanide was used). A good quantitative agreement between the two methods was obtained, using either hypoxic or normal duodenum as can be seen in Figure 4. This demonstrates that no significant re-oxidation of ferricyanide occurs in the oxygenated medium.

Table 1. Ferricyanide reduction by duodenal tied-off sacs

Experimental group	Ferricyanide reduction (nmol mg tissue^{-1} min^{-1})
Normal mucosal sacs	0.406 ± 0.051 (6)
Hypoxic mucosal sacs	1.343 ± 0.210 (5)[a]
Hypoxic serosal sacs	0.043 ± 0.014 (5)[b]

Initial rates of ferricyanide reduction by mouse duodenum were determined by observing changes in absorbance at 410 nm in the medium of *in vitro* incubations of everted (mucosal), and non-everted (serosal), tied-off duodenal sacs. Full details are given in the methods section. Data are means ± SEM for (*n*) observations.

[a] $P < 0.01$ as compared to normal mucosal sacs.

[b] $P < 0.005$ as compared to hypoxic mucosal sacs.

Table 2. Ferricyanide reduction by duodenal tissue fragments

Experimental group	Ferricyanide reduction (nmol mg tissue^{-1} min^{-1})
Normal	0.315 ± 0.034 (5)
Hypoxic	1.112 ± 0.126 (5)[a]
Fe-replete	0.488 ± 0.128 (5)
Fe-deficient	1.170 ± 0.220 (7)[b]

Initial rates of ferricyanide reduction by mouse duodenal fragments were determined by observing changes in absorbance at 410 nm in the medium of *in vitro* incubations. Full details are given in the methods section. Data are means ± SEM for (*n*) incubations.

[a] $P < 0.005$.

[b] $P < 0.05$.

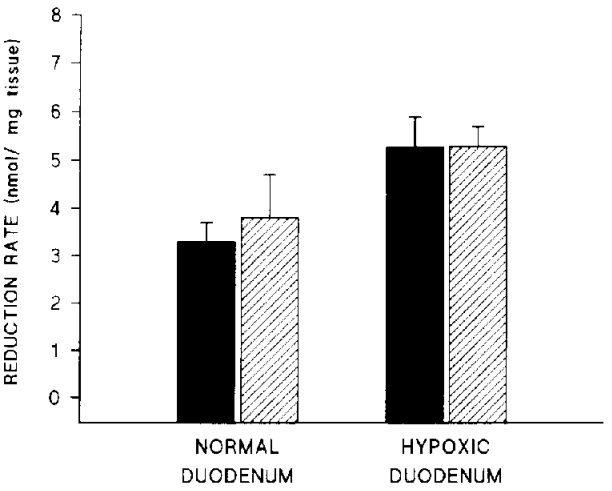

Figure 4. A comparison of the two methods for measuring ferricyanide reduction by mouse duodenum: ■, ferrocyanide production; ▨, ferricyanide disappearance (200 μM ferricyanide was used). Full details of the two assay procedures can be found in the text. Incubation time was 9 min. Values are means ± SEM, $n = 3$.

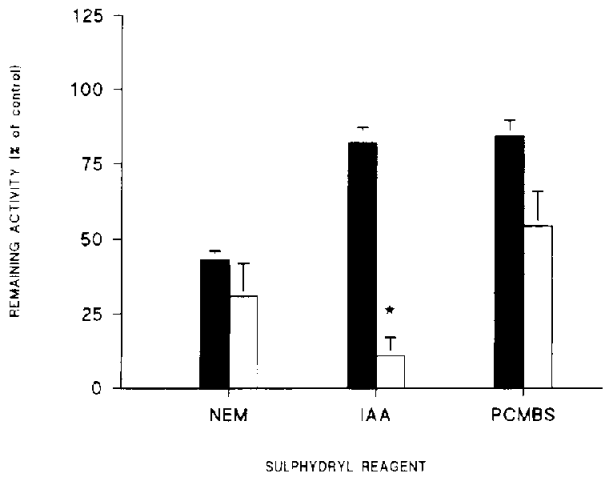

Figure 5. The effect of sulfydryl reagents on duodenal ferricyanide reducing activity (■) and cellular GAPDH (□). Duodenal fragments were incubated with 1 mM *N*-ethyl malemide (NEM), iodoacetate (IAA) or *p*-chloromercuriphenyl sulfonate (PCMBS), and ferricyanide reducing activity determined. The tissue was then rinsed, homogenized and assayed for GAPDH as detailed in the text. In each experiment, enzymic activities were expressed relative to the mean of duplicate control tissue incubations. Data are means ± SEM for 8–13 experiments. * For comparision between GAPDH and ferricyanide reducing activity, only iodoacetate incubations differed significantly, $P < 0.01$.

Figure 5 shows the inhibition of duodenal ferricyanide reducing activity (calculated by determination of ferrocyanide production), and cellular GAPDH by the thiol reagents *N*-ethyl malemide, iodoacetate and *p*-chloromercuriphenyl sulfonate. The activity of the glycolytic enzyme, GAPDH, is known to be inhibited by thiol reagents alkylating essential active site thiols (MacQuarrie & Bernhard 1971). The inhibition of duodenal mucosal surface ferricyanide reducing activity by *N*-ethyl malemide indicates the involvement of essential thiols. However, the observed difference in thiol reagent specificity between GAPDH and the reducing component of the duodenal mucosal surface indicates that inhibition of reducing activity is not a secondary effect resulting from an alteration of the metabolic status of the cell due to a partial inhibition of glycolysis. The differential sensitivity of reducing activity to iodoacetate and *N*-ethyl malemide inhibition may arise from reaction of *N*-ethyl malemide with other susceptible groups, e.g. histidine residues, or the environment of a susceptible thiol may exclude reaction with the negatively charged iodoacetate but not the uncharged *N*-ethyl malemide. The finding that the negatively charged iodoacetamide showed similar inhibition (55 ± 7(13)) to *N*-ethyl malemide (43 ± 3(3)) is consistent with the latter hypothesis [data means ± SEM (*n*)% of control activity remaining in presence of inhibitor].

Table 3 demonstrates the effect on reducing activity of substitution of medium Na^+ with K^+ or Rb^+, resulting in membrane depolarization, or the addition of valinomycin resulting in membrane hyperpolarization. The effects resemble those reported on iron uptake (Raja *et al.* 1989) and suggest a membrane potential dependence for the reducing activity.

The two methods used to monitor iron reducing activity are essentially complementary for the investigation of inhibitor effects. Thus inhibitors with strong color in the 560 nm region cannot be used with the ferrous-ferrozine production method, while absorbance at 410 nm is incompatible with the ferricyanide disappearance method. Compounds which themselves are active in the reducing assay system at pH 4.8 but are not reducing at pH 7.4 are incompatible with the ferrous-ferrozine production method but can be studied with the ferricyanide disappearance method.

Table 3. Effect of membrane potential modulating agents on duodenal ferricyanide reducing activity

Incubation media	Ferricyanide reduction (% of control)
K^+	61.0 ± 10.0[a]
Rb^+	67.6 ± 12.7
Valinomycin	114.3 ± 14.6

Duodenal fragments were incubated for 5 min in the presence of ferricyanide and the production of ferrocyanide determined as described in the text. Incubation medium Na^+ was replaced with either K^+ or Rb^+, or 5 μM valinomycin was added as indicated. Ferrocyanide production was expressed relative to Na^+ medium controls incubated in parallel. Data are means ± SEM for three experiments.

[a] $P < 0.05$ as compared to control activity.

Table 4. The effect of various inhibitors on duodenal ferricyanide reducing activity

Inhibitor	Ferricyanide reducing activity (% of control)
Quinacrine (1 mM)	121 ± 12 (3)
Doxorubicin (10 μM)	106 ± 22 (3)
p-Chloromercuriphenyl sulfonate (1 mM)	84 ± 5 (13)
Chloroquinone (0.5 mM)	95 ± 12 (3)

Hypoxic mice were used for all experiments. Inhibition was measured by performing parallel *in vitro* incubations of 1.2 mM ferricyanide with duodenal fragments in the presence or absence of inhibitor. Full details are given in the methods section. Data are means ± SEM for (*n*) experiments.

Certain compounds have been identified as inhibitors of transplasma membrane reductase systems in intact cells. We have investigated the effect of selected inhibitors on the reduction of ferricyanide by mouse duodenum (Table 4). The flavin analog quinacrine, which inhibits respiratory burst O_2 reduction by neutrophils (Cross 1990), has no effect on mouse duodenal ferricyanide reducing activity. The non-penetrating sulfydryl reagent *p*-chloromercuriphenyl sulfonate, a potent inhibitor of some tissue ferricyanide reductases (Crane *et al.* 1985), was relatively ineffective. Doxorubicin has been shown to be particularly effective at inhibiting ferricyanide reduction by cancer cells (Crane *et al.* 1985), but was without effect in mouse duodenum. Cisplatinum was found to decrease activity but by less than 25% at concentrations up to 400 μM (not shown). At the same concentration the activity in cancer cells is decreased by 70% (Crane *et al.* 1985). Imipramine and Chlomipramine have been suggested as potential anti-leishmanial drugs, and have been reported to be effective inhibitors of transplasma membrane reductase in *Leischmania* (Mayer 1993), but in a series of experiments no inhibition of mouse duodenal reducing activity by these compounds (concentrations in the range 100–450 μM) could be demonstrated.

The existance of plasma membrane ferric reductase activity has been described in both procaryotic and eucaryotic cells. Several cells and tissues have been demonstrated to reduce ferricyanide using a transplasma membrane reductase (Crane *et al.* 1985). The ability of yeast to reduce external ferricyanide was first described by Crane *et al.* (1982). Dancis *et al.* (1990) have studied a reductase-deficient mutant of *S. cerevisiae* and shown that the cell surface reductase activity plays an important role in ferric iron uptake. The externally directed reductase activity is regulated by the concentration of iron in the growth medium and maximal activity is induced by iron starvation. The responsible gene in *S. cerevisiae* has been isolated and sequenced (Dancis *et al.* 1992). The present data demonstrates that mouse duodenum also possesses a transplasma membrane ferricyanide reductase activity which is induced in parallel with increased iron absorption and is localized to the duodenum. Ferricyanide does not enter cells and together with ferrocyanide is biologically inert. This, together with the absence of a significant release of reducing factors into the medium, suggests that the reductive process is occurring at a cellular level on the mucosal surface. Further work is needed to ascertain whether reduction and uptake of iron are the function of separate entities or the combined function of a transplasma reductase–carrier complex in the brush border membrane of the duodenum.

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