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Divalent metal inhibition of non-haem iron uptake across the rat duodenal brush border membrane

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Duodenal Fe^{2+} uptake is essential to body Fe^{2+} homeostasis, but the interaction of metals with the uptake process remains unclear. The present study compared the effects of four essential trace metals (Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+}) with two toxic metals (Pb^{2+} and Cd^{2+}) on Fe^{2+} uptake across the brush border membrane of villus-attached duodenal enterocytes. Everted rat duodenum was exposed to buffer containing 0·2 mM-$^{59}Fe^{2+}$–ascorbate with or without the competing metal (2 mM) and the tissue was then processed for autoradiography allowing Fe^{2+} uptake to be determined at specific crypt–villus regions. The quantification method ensured that uptake by cells, rather than Fe^{2+} binding to the tissue surface, was measured. Fe^{2+} uptake was significantly inhibited by Cd^{2+} in upper villus enterocytes only and Pb^{2+} was without effect on Fe^{2+} uptake. The inhibition by Cd^{2+} was not due to general cell damage as judged by the release of lactate dehydrogenase from tissue into incubation fluid. Essential divalent trace metals reduced uptake significantly along the whole length of the crypt–villus axis. Cd^{2+} uptake, measured separately, took place at all regions of the villus–crypt axis, highest uptake being into crypt enterocytes. The very different uptake profiles for Cd^{2+} and Fe^{2+} suggests that the divalent metal transporter 1 is not the principal transporter of Cd^{2+}. The addition of Fe^{2+} to incubation buffer inhibited Cd^{2+} uptake by both crypt and villus enterocytes. The possibility that the inhibitory actions of Fe^{2+} and Cd^{2+} on the uptakes of Cd^{2+} and Fe^{2+} respectively can be explained by a non-competitive action or the involvement of an additional metal transporter is discussed.

Intestinal iron transport: Duodenum: Cadmium uptake: Brush border membrane

The absence of a mechanism for controlling Fe^{2+} excretion makes the process for uptake of the metal across the intestine crucial to body iron homeostasis. The level of dietary Fe^{2+} influences duodenal uptake of Fe^{2+} (Charlton & Bothwell, 1983; Smith *et al.* 2000) and although the response is thought to alter the expression of the divalent metal transporter (DMT) 1 at the brush border membrane (BBM) (Oates *et al.* 2000; Trinder *et al.* 2000) the mechanisms involved remain poorly understood. Essential trace metals also affect Fe^{2+} uptake in predictable ways (Rucker *et al.* 1994) and studies using *Xenopus* oocytes expressing DMT1 have implied that interactions of divalent trace metals with Fe^{2+} uptake take place through competition for DMT1 (Gunshin *et al.* 1997). This situation is however complicated by the fact that DMT1 may also transport Cd^{2+} and Pb^{2+} (Andrews *et al.* 1999; Tallkvist *et al.* 2001), metals which are highly toxic and chemically distinct from the other essential trace metals (Frausto da Silva & Williams, 1991). We therefore considered it worthwhile to compare and analyse the ability of Cd^{2+} and Pb^{2+}, as well as essential trace metals, to inhibit Fe^{2+} uptake by intact duodenal mucosa. Since uptake of Fe^{2+} across the BBM and expression of DMT1 follow a similar developmental profile along the villus (O'Riordan *et al.* 1995; Trinder *et al.* 2000), autoradiographic methods were used to determine Fe^{2+} uptake across the BBM at specific loci along the villus axis. Care was taken to ensure that our autoradiographic method was a measure of Fe^{2+} uptake across the brush border, rather than binding of the metal to the mucosal surface.

The aims of the present study were to demonstrate the importance of using intact duodenal epithelium as a

Abbreviations: BBM, brush border membrane; DMT1, divalent metal transporter 1; LDH, lactate dehydrogenase.

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useful model for assessing both the manner in which Fe^{2+} crosses the intestine and the way in which other important trace metals might affect Fe^{2+} absorption. A short account of this work has been published previously (Shenoy *et al.* 2001).

Materials and methods

Chemicals

$^{59}FeCl_3$ and $^{109}CdCl_2$ were obtained from NEN Life Science Products (Hounslow, Middlesex, UK). All other chemicals were of Analar Grade from Sigma Limited (Poole, Dorset, UK) or Merck Limited (Poole, Dorset, UK).

Iron and cadmium uptake

All experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986. The method used to measure Fe^{2+} uptake was similar to that described previously (O'Riordan *et al.* 1995; Smith *et al.* 2000). Briefly, it involved removing duodenal tissue from Sprague-Dawley rats (230–250 g), anaesthetized with pentobarbitone sodium (90 mg/kg, intraperitoneal), and everting the tissue before dividing it into two equal lengths. One of these was stored in cooled saline whilst the other was pre-incubated for 5 min in oxygenated buffer, pH 7·1 (mM: Hepes 16, glucose 10, KCl 3·5, $MgSO_4$ 10, $CaCl_2$ 1, NaCl 125) followed by 5 min incubation in buffer containing 0·2 mM-$^{59}Fe^{2+}$ (complexed with 4 mM ascorbic acid; $^{59}Fe^{2+}$ specific activity 0·52 MBq/ml), with or without the competing metal (2 mM). Previous studies using Caco-2 cells have shown that a buffer pH range of 6·5–7·1 is appropriate for Fe^{2+} uptake (Worthington *et al.* 2000), that 0·2 mM is an appropriate concentration for measurement of Fe^{2+} uptake (Cox & Peters, 1980; Raja *et al.* 1987) and that Fe^{2+} entry into duodenal tissue increases linearly with time for up to 10 min (Cox & Peters, 1979; Raja *et al.* 1989). The stored tissue was then taken through the same two incubation procedures. The order in which tissues were incubated with $^{59}Fe^{2+}$ alone or with the competing metal was alternated to avoid possible effects of a 10 min storage time on Fe^{2+} uptake. Subsequent washing of the tissues to displace surface-bound Fe and fixation and processing of the tissues for autoradiography was carried out as described previously (O'Riordan *et al.* 1995; Smith *et al.* 2000). The uptake of 0·2 mM along the villus and the effects of 2 mM-Fe^{2+} in the incubation buffer were measured using an identical uptake method, again utilizing paired tissue sections.

Quantitation of metal uptake

Fe^{2+} uptake was assessed by autoradiography using a Vanox AH-2 microscope (Olympus, London, UK) at a final magnification of ×4000 to detect Ag grains under dark field illumination through a photoelectric cell built into the microscope. Under these conditions Ag grains on autoradiographs were easily identified as separate spots of light. Fainter, non-specific light present in sectioned tissue was largely eliminated by closing the iris and passing emitted light through a light balancing daylight filter before recording the light intensity as an exposure time determined automatically by the apparatus. Measurements of light intensity used a scanning spot of 25 μm diameter placed manually over the cytosolic region of enterocytes at different positions along the crypt–villus axis of sectioned villi. Single readings of light intensity were taken for each region of the villus. These readings were however taken from three villi per animal. Inter-villus variation in exposure time ranged from 7.8 to 14.8 s in the tips of these villi. This variation was substantially reduced (9.5 to 11.5 s) by taking mean values obtained by analysing tissue from six animals.

The validity of this quantitative method was assessed separately by comparing exposure times with counts of Ag grains carried out directly on enlarged photographs of the same areas of villi. Results showed a linear relation between the increase in grain counts and a decrease in exposure time from 19 to 5 s (results not shown). The negative slope of this graph (−7·2 grains/s exposure time) then allowed the conversion of exposure times to Ag grain counts and thus the quantitation of Fe^{2+} uptake. The advantage of this method compared with microdensitometry was that it shortened considerably the time needed to determine uptake in selected areas of the villus. This was particularly important in the present work that involved taking measurements from villi obtained from a large number of rats.

Microdensitometry

The use of microdensitometry to determine the positional location of transporter expression along villi has already been described in detail (Smith *et al.* 2000). Briefly, it involves routine preparation of autoradiographs of sectioned villi that are then scanned manually using an M85 microdensitometer (Vickers, Pudsey, UK). Readings of optical density are taken sequentially from crypt to villus tip. The final information obtained is sufficiently detailed to detect minor changes in the expression of transport function.

Lactate dehydrogenase activity

The pre-incubation and incubation protocols used were similar to those described for measurement of Fe^{2+} uptake. However, only the bottom half of everted U-shaped intestinal segments were immersed in buffer in order to ensure that the cut ends of the segment could not release lactate dehydrogenase (LDH) into the buffer. LDH activity was measured after exposure to buffer, with or without 2 mM-Cd^{2+}, using a commercial assay kit (Sigma).

Statistics

Mean values with their standard errors for Fe^{2+} uptake were calculated by analysis of three villi from each of six rats. Mean values with their standard errors for Cd^{2+} uptake were calculated by analysis of three villi taken from each of eight rats. Differences between means were

evaluated using the paired Student's *t* test. Values of $P > 0{\cdot}05$ were considered to be not significantly different.

Results

Divalent metal inhibition of iron transport

Results showing the ability of five different divalent metals to inhibit Fe^{2+} uptake at four different locations along sectioned duodenal villi are given in Fig. 1. In the absence of competing metal, Fe^{2+} uptake more than doubled as enterocytes migrated from crypts through to the lower villus. This increased uptake then continued until enterocytes reached the mid-villus but no further change in transport occurred during subsequent enterocyte migration. A virtually identical developmental profile for Fe^{2+} transport was recorded in the presence of Cr^{3+}, a finding that supports results obtained from oocytes showing that only divalent metals are transported by DMT1 (Gunshin *et al.* 1997).

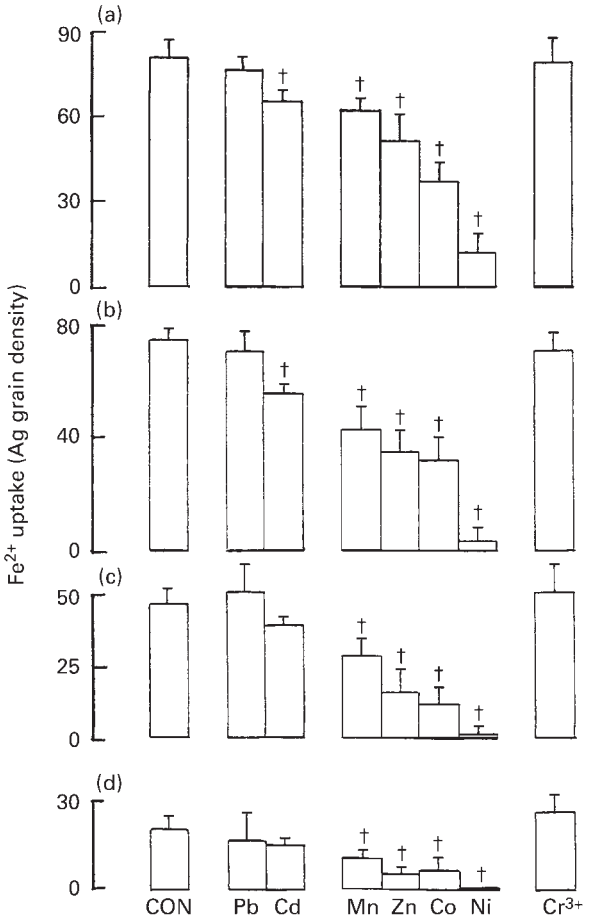

Fig. 1. Divalent metal inhibition of Fe^{2+} uptake by rat duodenum. Everted duodenum was incubated for 5 min with 0·2 mM-$^{59}Fe^{2+}$ with or without 2 mM of the non-radioactive competing metal. Tissues were later processed to produce autoradiographs of sectioned villi and Fe^{2+} uptakes were determined by Ag grain counts of the enterocyte cytosolic region at different positions along the crypt–villus axis. (a), Tip; (b), mid; (c), base; (d), crypt. The villus base, mid-villus and villus tip represent areas 25, 50 and 80 % respectively from the crypt–villus junction. Values are means with standard errors shown by vertical bars. The control value (CON) is the mean (*n* 42) for Fe^{2+} uptake determined in the absence of competing metal. Mean values were significantly different from control values: †$P < 0{\cdot}05$ to $P < 0{\cdot}001$.

Differences were, however, seen between the ability of various divalent metals to inhibit Fe^{2+} uptake. Results for the mid-villus (Fig. 1) show for example that mean inhibitions of Fe^{2+} uptake using tissue from six rats to be as follows (%): Cd^{2+} 18·0, Mn^{2+} 26·0, Zn^{2+} 42·8, Co^{2+} 53·2, Ni^{2+} 87·8. Pb^{2+} was unable to inhibit Fe^{2+} uptake significantly at any of the four villus regions implying that uptake of Pb^{2+} across the BBM, like Cr^{3+}, does not utilize the DMT1 transporter.

The statistical significance of the remaining differences seen between control and test measurements of Fe^{2+} uptake were assessed using the paired *t* test. All divalent metals tested, apart from Pb^{2+}, caused significant inhibition of Fe^{2+} uptake. Further paired *t* tests carried out on uptake data using other regions of the villus and crypts showed Mn^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} to cause significant inhibition of Fe^{2+} uptake. Inhibition by Cd^{2+} was, however, only statistically significant at the upper half of the villus whilst Pb^{2+} remained unable to inhibit Fe^{2+} uptake at any of the crypt and villus locations tested. These results provide strong evidence against the involvement of DMT1 with respect to Pb^{2+} uptake. The case for DMT1-mediated Cd^{2+} uptake, however, required further investigation.

Cadmium–iron interactions along duodenal villi

The profile describing increasing Fe^{2+} uptake during enterocyte migration along villi is already well documented (O'Riordan *et al.* 1995; Smith *et al.* 2000), but no comparable results exist for Cd^{2+} uptake along the crypt–villus axis. Experiments using $^{59}Fe^{2+}$ and $^{109}Cd^{2+}$ were therefore carried out to compare directly the uptake profiles of the two metals. The results obtained are shown in Fig. 2.

Crypt enterocytes displayed the highest uptake of Cd^{2+}, cells at the upper villus having about half the rate of Cd^{2+} uptake compared with those in the crypt. In contrast, the profile for Fe^{2+} uptake showed lowest uptake in the crypt region and highest uptake in the upper villus region (Fig. 2). These results confirm results from measurements taken in four different regions along the crypt–villus axis (control values, Fig. 1).

The shape of the profile for Fe^{2+} uptake corresponds with that shown for DMT1 expression (Trinder *et al.* 2000). However, the very different profile for Cd^{2+} uptake along the crypt–villus axis indicates that the predominant mode of entry of this metal is not DMT1-dependent.

Fe^{2+} inhibition of Cd^{2+} uptake

A final test of whether some Cd^{2+} entry into upper villus enterocytes might still take place through DMT1 involved comparing profiles for Cd^{2+} uptake in the absence and presence of 2 mM-Fe^{2+}. Localized inhibition of Cd^{2+} in upper villus enterocytes in the presence of Fe^{2+} would, in this case, support the view that Cd^{2+} entry was DMT1-mediated.

Fe^{2+} inhibited Cd^{2+} uptake by 40 to 50 % along the entire villus axis (Fig. 3) including those regions of the villus known to be deficient in DMT1. These results therefore

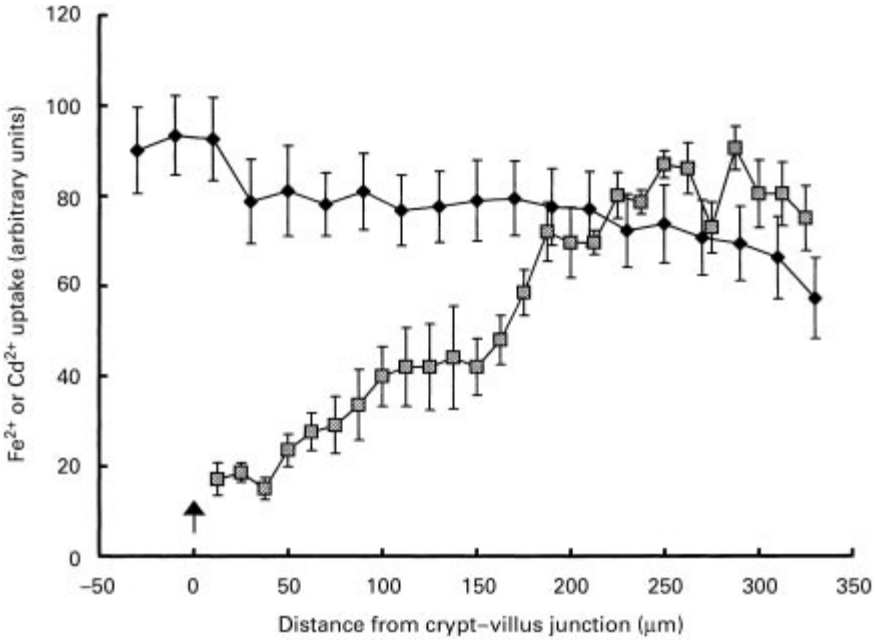

Fig. 2. Positional distribution of Fe^{2+} (▨) and Cd^{2+} (◆) uptake along rat duodenal villi. For details of procedures, see p. 52. Values are means for uptake of the metal determined by the measurement of Ag grain density in the cytosolic region of enterocytes, with standard errors shown by vertical bars. Villus length was 390 μm. ↑, Crypt–villus junction.

provide some evidence for DMT1-independent Cd^{2+} transport taking place across the duodenal BBM.

Lactate dehydrogenase release

Determination of LDH activity in incubation buffer was carried out in order to test whether inhibition of Fe^{2+} uptake by Cd^{2+} was a consequence of non-specific damage to enterocyte function. Incubation of everted duodenum did not affect LDH release ($-Fe^{2+}$ 0·029 (SEM 0·007), $+Fe^{2+}$ 0·023 (SEM 0·006) U/g tissue per min, *n* 5 pairs, $P=0·19$). Surprisingly, Cd^{2+} caused a sig-

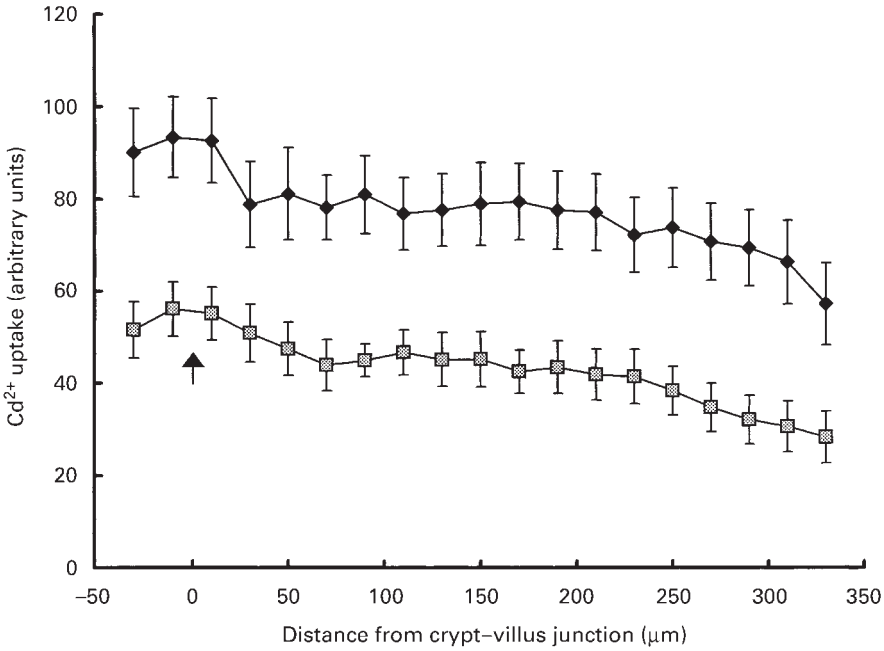

Fig. 3. Inhibition of Cd^{2+} uptake by Fe^{2+}. Results show Cd^{2+} uptake by upper crypt to villus tip enterocytes with or without 2 mM-Fe^{2+} in the incubation buffer. (◆), Fe^{2+} absent; (▨), Fe^{2+} present. For details of procedures, see p. 52. Values are means with standard errors shown by vertical bars. Measurements were taken up to 330 μm of a maximum villus length of 390 μm. ↑, Crypt–villus junction. Mean values for Fe^{2+} present were significantly different from those with Fe^{2+} absent at all points along the crypt–villus axis ($P<0·001$).

nificant decrease in LDH release (64·7 %, $P=0{\cdot}031$, n 5). The possibility that this effect could be caused by a direct inhibitory action of the metal on LDH activity was investigated by measuring enzyme activity, with or without 2 mM Cd^{2+}, in buffer collected after the initial 5 min pre-incubation with duodenum. The metal was found to be without effect on LDH activity ($-Cd^{2+}$ 0·10 (SEM 0·03), $+Cd^{2+}$ 0·10 (SEM 0·03) U/g tissue per min, n 11 pairs, $P=0{\cdot}7$).

Discussion

Fe^{2+} is essential for many cellular processes and the absence of a regulatory process for excretion of the metal means that body Fe^{2+} homeostasis is crucially dependent on the rate of uptake of the metal across the small intestine (Charlton & Bothwell, 1983). On the basis of expression studies using *Xenopus* oocytes, Gunshin *et al.* (1997) proposed that DMT1 is responsible for movement of Fe^{2+} across the duodenal BBM. Levels of DMT1 mRNA within duodenal enterocytes are influenced by dietary Fe^{2+} content (Oates *et al.* 2000; Trinder *et al.* 2000), implying that changes in gene expression of the transporter are involved in control of Fe^{2+} absorption in response to Fe^{2+} status. However, despite the apparent importance of DMT1 in body Fe^{2+} metabolism, there is conflicting information concerning the substrate specificity of this transporter.

Early literature describing interactions between divalent metals on their uptake across the small intestine reflect both the concern of nutritionists to ensure an optimal body balance of essential trace metals and the emphasis by toxicologists of the need to protect animals from the effects of metallic poisons. DMT1 has been reported to transport both essential non-toxic and toxic divalent metals across the membrane of DMT1 mRNA-primed oocytes (Gunshin *et al.* 1997) and to transport Cd^{2+} in Caco-2 cells (Tallkvist *et al.* 2001). Despite these studies, most studies of the role of DMT1 have tended to concentrate on the importance of the transporter in the control of body Fe^{2+} metabolism rather than its ability to absorb toxic heavy metals.

In attempting to broaden this interest in DMT1, it was first thought necessary to test whether DMT1-selectivity for divalent metals, established previously using oocytes, would also apply to villus-attached enterocytes, since the latter preparation is more physiologically relevant to the uptake of divalent metals under normal conditions. In the present study, inhibition of Fe^{2+} uptake by different divalent metals was initially taken to indicate the involvement of DMT1 in transport. Of the six divalent metals tested, only Pb^{2+} failed to inhibit Fe^{2+} transport. Pb^{2+} is nevertheless a toxic metal that is absorbed by the intestine and this has consequences for health (Ferguson, 1990).

The other toxic metal, Cd^{2+}, only inhibited Fe^{2+} transport significantly at the upper villus region. Uptake of Cd^{2+} took place most readily in those regions of the villus containing the least amounts of DMT1 (see Fig. 2 for uptake and Trinder *et al.* (2000) for DMT1 expression). A final surprise was the finding that Fe^{2+} also inhibited Cd^{2+} uptake, this occuring to a similar extent at all levels along the crypt–villus axis, despite the fact that Fe^{2+} transport increases along the villus. All of these effects are not caused by toxic effects of Cd^{2+} on incubated intestine, as assessed by the failure of the metal to cause changes in efflux of LDH from the tissue.

Some of the these inconsistencies could be explained by the presence of additional metal transporters in the intestine. In testing for this, however, it would first be necessary to establish whether metal uptake followed Michaelis–Menten saturation kinetics in order to distinguish between competitive and non-competitive mechanisms of inhibition. This has already been established for Fe^{2+} in oocytes (Gunshin *et al.* 1997) but not for Cd^{2+}, Mn^{2+} and Co^{2+}, each of which we have now shown to be inhibitors of Fe^{2+} transport through DMT1. It is particularly important to make this distinction for Cd^{2+} since this metal is known to inhibit the intestinal transport of Ca^{2+}, threonine and galactose by non-competive means (Hamilton & Smith, 1978; Mesonero *et al.* 1993, 1996). It remains reasonable, however, to assume that inhibition of intestinal Cd^{2+} uptake by Fe^{2+} could be either non-competitive or could involve a different transporter of Fe^{2+}.

The ability of DMT1 to mediate the entry of a wide variety of divalent metals into *Xenopus* oocytes (Gunshin *et al.* 1997) is greater than that found previously for any other type of transporter. This non-selectivity has now been shown to be similar when measuring the ability of divalent cations to inhibit Fe^{2+} uptake by enterocytes (Fig. 1). Caco-2 cells, that express DMT1, also show a wide specificity profile for divalent metals, though many of the inhibitory effects on Fe^{2+} transport proved to be statistically insignificant (Tandy *et al.* 2000).

In conclusion, in an attempt to provide evidence that Pb^{2+} and Cd^{2+} do not enter intact enterocytes via DMT1, the present study has uncovered a new area of uncertainty about the ways in which divalent metals are transported across the duodenal BBM. Relating the uptake of a metal to its ability to act as an inhibitor and to relate both to the state of enterocyte differentiation appears generally to be a powerful way to test existing views of how different metals interact at the mucosal level.

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