

Actions of Cadmium on Basolateral Plasma Membrane Proteins Involved in Calcium Uptake by Fish Intestine

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Summary. The inhibition of Ca^{2+} -ATPase, $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and $\text{Na}^+/\text{Ca}^{2+}$ exchange by Cd^{2+} was studied in fish intestinal basolateral plasma membrane preparations. ATP driven $^{45}\text{Ca}^{2+}$ uptake into inside-out membrane vesicles displayed a K_m for Ca^{2+} of 88 ± 17 nM, and was extremely sensitive to Cd^{2+} with an IC_{50} of 8.2 ± 3.0 μM Cd^{2+} , indicating an inhibition via the Ca^{2+} site. $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity was half-maximally inhibited by micromolar amounts of Cd^{2+} , displaying an IC_{50} of 2.6 ± 0.6 μM Cd^{2+} . Cd^{2+} ions apparently compete for the Mg^{2+} site of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger was inhibited by Cd^{2+} with an IC_{50} of 73 ± 11 nM. Cd^{2+} is a competitive inhibitor of the exchanger via an interaction with the Ca^{2+} site ($K_i = 11$ nM). Bepridil, a Na^+ site specific inhibitor of $\text{Na}^+/\text{Ca}^{2+}$ exchange, induced an additional inhibition, but did not change the K_i of Cd^{2+} . Also, Cd^{2+} is exchanged against Ca^{2+} , albeit to a lesser extent than Ca^{2+} . The exchanger is only partly blocked by the binding of Cd^{2+} . In vivo cadmium that has entered the enterocyte may be shuttled across the basolateral plasma membrane by the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. We conclude that intracellular Cd^{2+} ions will inhibit plasma membrane proteins predominantly via a specific interaction with divalent metal ion sites.

Key Words cadmium · Ca^{2+} -ATPase · $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ · $\text{Na}^+/\text{Ca}^{2+}$ exchange · competitive inhibition · teleost fish

Introduction

Living cells need to extrude calcium continuously: the large inward electrochemical gradient for calcium results in “leakage” of this ion into the cell and, as high Ca^{2+} levels are toxic, it has to be eliminated in order for the cell to function properly. The intracellular Ca^{2+} concentration plays a key role in the regulation of many cellular processes, acting as an intracellular second messenger. Cellular calcium homeostasis is often safeguarded by the ATP-dependent Ca^{2+} pump, a high affinity Ca^{2+} -ATPase [27]. This enzyme belongs to the family of $\text{E}_1\text{-E}_2$ type ion pumps [23], of which the ubiquitous sodium/po-

tassium-dependent ATPase $((\text{Na}^+ + \text{K}^+)\text{-ATPase})$ is the most widely studied member. $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ is considered a pivotal transport ATPase for cellular ion homeostasis. It uses intracellular ATP to extrude Na^+ ions, and to import K^+ ions into the cell. Thereby it maintains the electrochemical gradients for Na^+ and K^+ , which in turn are used by other membrane proteins that are essential for vital processes as cell volume regulation, ion extrusion and nutrient uptake [35]. We have demonstrated both these plasma membrane ion pumps in the intestine of the teleost *Oreochromis mossambicus* (tilapia) [13].

In this freshwater fish intestinal calcium uptake not only depends on the action of the ATP-dependent Ca^{2+} pump, but also on sodium/calcium exchange activity, which is abundantly present in the basolateral membrane. $\text{Na}^+/\text{Ca}^{2+}$ exchange may well be the dominant Ca^{2+} extrusion mechanism, in contrast to higher vertebrates where the ATP-dependent Ca^{2+} pump is most important [21]. Sodium/calcium exchange requires the action of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ for the creation of the electrochemical gradient for Na^+ , which is its main driving force. The apparent maximum exchange velocity is 16 times higher than that reported for a comparable membrane preparation from rat small intestine [13, 17]. The maximum velocity of the fish $\text{Na}^+/\text{Ca}^{2+}$ exchanger exceeded that of the fish ATP-dependent Ca^{2+} pump 34 times. Also, net intestinal calcium uptake was dependent on the existence of a Na^+ gradient across the basolateral cell membrane. These data indicate the involvement of $\text{Na}^+/\text{Ca}^{2+}$ exchange in cell Ca^{2+} homeostasis as well as transcellular Ca^{2+} transport in tilapia intestinal epithelium [13]. With its array of Ca^{2+} transport mechanisms in the basolateral membrane the fish intestine

may provide a suitable model for analyzing toxicological effects of Cd^{2+} on ion transport mechanisms.

In freshwater tilapia the major part of Ca^{2+} uptake takes place via the gills [15]. Uptake of calcium via the gastrointestinal tract may present a significant route of calcium influx, *viz.* in water containing low levels of calcium ("soft water") or when the fish are in need of extra calcium, e.g. during sexual maturation [3, 13]. Branchial Ca^{2+} uptake is readily inhibited by submicromolar amounts of waterborne Cd^{2+} [30, 46, 48]. Cadmium from the water as well as from the food accumulates in the gastrointestinal tract [9, 10]. The latter pathway appears to be most important for uptake of cadmium [7]. In tilapia adapted to soft water dietary cadmium is known to cause hypocalcemia [29]. However, the mechanisms whereby cadmium interferes with calcium uptake in fish intestine have not yet been studied.

For tilapia gills it has been shown that Cd^{2+} is able to pass the apical membrane of the calcium-transporting epithelial cell via the same route as Ca^{2+} [48]. The inhibition of transcellular calcium transport takes place at the basolaterally located Ca^{2+} pumps [46]. In analogy then, our understanding of the interactions of Cd^{2+} with the cytoplasmic substrate and cation sites of proteins in the basolateral plasma membrane will be of primary importance to evaluate the mechanism of inhibition of intestinal Ca^{2+} uptake by Cd^{2+} . The basolateral membrane proteins involved in Ca^{2+} transport are the $\text{Na}^+/\text{Ca}^{2+}$ exchanger, which is dependent on the correct operation of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, and the $\text{Ca}^{2+}\text{-ATPase}$. We report here on the effects of Cd^{2+} on these three mechanisms, which appear to result from an interaction with divalent metal cation sites of the proteins. The $\text{Na}^+/\text{Ca}^{2+}$ exchanger appeared to be able to shuttle Cd^{2+} into the blood.

Materials and Methods

Male tilapia, *Oreochromis mossambicus*, weighing around 250 g were obtained from the electricity plant Bergheim/Niederhausen (Germany). The fish were kept in 100 liter aquaria supplied with running tap water (0.7 mM Ca, 25°C) under a photoperiod of 12 hr of light alternating with 12 hr of darkness, during at least one month before experimentation. Cadmium was not detectable in tap water (detection limit 1 nM). Animals were fed Trouvit® fish pellets (Trouw & Co., Putten, The Netherlands), 4 to 5% body weight per day. Trace amounts of cadmium in the food result in a total cadmium content of mucosal tissue of $6.0 \pm 2.4 \text{ nmol} \cdot \text{g}^{-1}$ dry weight ($n = 6$). The possible consequences of this amount will be discussed below.

Fish were killed by spinal transection. The peritoneal cavity was opened and the intestinal tract removed. The intestine was rapidly flushed with ice-cold saline and processed as described below. Enterocyte basolateral plasma membrane vesicles were prepared as described in detail recently [13].

Briefly, the intestine was cut lengthwise and intestinal mucosa was collected by scraping off the epithelium onto an ice-cooled glass plate. The cells were homogenized in an isotonic sucrose buffer, and nuclei and cellular debris were pelleted by centrifugation at $1,400 \times g_{av}$ for 10 min. The supernatant (containing 75% of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity of the homogenate) was collected and centrifuged for 25 min at $150,000 \times g_{av}$. The resulting pellet consists of two parts: a firm brown part which contains mostly mitochondrial membranes, and a white and fluffy part on top which consists of plasma membranes. The fluffy layer was resuspended in a 250 mmol \cdot liter $^{-1}$ sucrose buffer and subsequently brought to 37% (wt/wt) sucrose by addition of 1.25 volumes of a 60% (wt/wt) sucrose solution. On top of 8 ml of this suspension 4 ml of sucrose buffer was layered, and the assembly was centrifuged isopycnicly for 90 min at $200,000 \times g_{av}$. Using a syringe fitted with a 23-gauge needle the membranes at the interface were collected and mixed with 30 volumes of the final isotonic assay buffer (150 mM KCl, 0.8 mM MgCl_2 and 20 mM HEPES/Tris, pH 7.4). After centrifugation at $180,000 \times g_{av}$ for 35 min, the pellet was rinsed twice with assay buffer and resuspended by 25 passages through a 23-G needle. This final fraction contained 18% of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity present in the homogenate.

The configuration of the tilapia enterocyte plasma membrane vesicle preparation is 29% IOV, 24% ROV and 47% leaky fragments [13]. Protein content of membrane or enzyme preparations was estimated with a commercial reagent kit (Bio-Rad), using bovine serum albumin (BSA) as a reference. Protein recovery with respect to the homogenate was $1.6 \pm 0.5\%$. The membrane preparation was enriched 7.1 times in $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity with respect to the homogenate. Contamination with mitochondrial and apical membrane fragments was minimal, as indicated by the purification factors of 1.1 and 1.2 for succinate dehydrogenase and alkaline phosphatase, respectively.

BUFFERING OF Ca^{2+} , Mg^{2+} AND Cd^{2+} CONCENTRATIONS

All assay media contained (in mM): 0.5 [ethylenebis(oxyethylenenitrilo)]tetraacetic acid (EGTA), 0.5 N-(2-hydroxyethyl)-ethylenediamine- $\text{N},\text{N}',\text{N}'$ -triacetic acid (HEEDTA), and 0.5 nitrilotriacetic acid (NTA). Free calcium, magnesium and cadmium levels were calculated as outlined below. All incubations were performed at 37°C.

We have written a computer program (implemented in Turbo Pascal 5.5® for IBM PC and compatibles; available upon request) that incorporates multiple metal-chelator equilibria to calculate free metal ion concentrations when using metal-chelating substances. The program also corrects for several experimental conditions, known to affect the apparent stability constants used in the calculations. Tsien and Rink [43] noted that textbook stability constants for H^+ are based on $[\text{H}^+]$'s. At nonzero ionic strength $[\text{H}^+]$ is not equal to H^+ activity, of which pH is the negative logarithm. Unfamiliarity with this fact has led to important errors in free metal calculation routines. Several authors have continued to overlook this point. Users of the algorithm of Fabiato and Fabiato [12] should use the correction published by Fabiato [11]. Also, the method of van Heeswijk, Geertsens and van Os [21], which we used previously, does not contain this correction. Furthermore, textbook stability constants are obtained at standard experimental conditions, *viz.* ionic strength and temperature, which are different from those used in most biological in vitro

Table 1. Apparent stability constants of combinations of metals and chelators corrected for use at 37°C and 160 mM total ionic equivalents

Metals	Chelators			
	ATP	EGTA	HEDTA	NTA
H ⁺	K1: 6.40 K2: 3.81	K1: 9.22 K2: 8.65 K3: 2.58	K1: 9.40 K2: 5.16	K1: 9.50 K2: 2.42
Ca ²⁺	K1: 3.70 K2: 1.95	K1: 10.34 K2: 5.10	K1: 8.09 K2: 1.23	K1: 6.33
Mg ²⁺	K1: 4.02 K2: 2.08	K1: 5.10 K2: 3.14	K1: 5.69 K2: 1.27	K1: 5.15
Cd ²⁺	K1: 5.38 K2: 1.67	K1: 15.79 K2: 10.38	K1: 13.09 K2: 2.29	K1: 9.43 K4: -11.92

K1, K2 and K3 represent stability constants for the binding of the metal to the unprotonated, mono- and diprotonated chelator, respectively. K4 is a stability constant for the reaction $MC \cdot H_2O \rightleftharpoons MC \cdot OH + H$ (MC stands for a metal-chelator complex; electrical charges omitted for clarity).

systems. They therefore have to be corrected for these differences.

We converted H⁺ activity to [H⁺] in all our calculations by the use of a semi-empirical form of the Güggenheim-Davies extension of the Debye-Hückel limiting law [20]. This was also used to correct the stability constants *K* for the effects of ionic strength:

$$\log K' = \log K + F \cdot (\log f_j - \log f_j'), \quad (1)$$

where *K'* is the constant after and *K* the constant before correction, *f_j* the activity coefficient of ion *j* at the tabulated ionic strength and *f_j'* that for the desired conditions. *F* is a factor dependent on the electrical charges of the cationic and anionic species relevant to a specific stability constant and is calculated according to different formulae, depending on which stability constant is being corrected for effects of ionic strength. The activity coefficient *f_j* is calculated by:

$$\log f_j = (1.8246 \times 10^6 / (\varepsilon T)^{1.5}) \times (\sqrt{I} / (1 + \sqrt{I}) - 0.25 \times I) \quad (2)$$

where ε is the relative dielectric constant of water (we corrected this constant for the temperature used), *T* the absolute temperature and *I* the ionic strength in ionic equivalents [20].

The effect of temperature is calculated by using Van't Hoff's Isochore [20]:

$$\log K' = \log K - (\Delta H / (\ln(10) \cdot R) \cdot (T'^{-1} - T^{-1})) \quad (3)$$

where *R* is the universal gas constant ($8.314 \times 10^{-3} \text{ kJ} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$) and ΔH is expressed in $\text{kJ} \cdot \text{mol}^{-1}$. Van't Hoff's Isochore was also used to correct *K_w* (water constant; used in some equilibria) for temperature effects.

The contributions of all charged species of metal ions, chelators, complexes and pH buffers to ionic strength were included in the calculation. The p*K_a* of the pH buffers was adjusted for effects of ionic strength. Table 1 lists the corrected stability constants used in our calculations. Calculated Ca²⁺ concentrations

were counterchecked using a Ca²⁺-selective electrode (above $1 \mu\text{M Ca}^{2+}$) or fura-2 fluorescence (below $1 \mu\text{M Ca}^{2+}$) and found to be correct (*see Note Added in Proof*).

Ca²⁺-ATPASE

Ca²⁺-ATPase activity is not necessarily related to a functional Ca²⁺-extrusion mechanism [4, 26]. The vectorial activity of the plasma membrane Ca²⁺ pump is, in our view, more accurately represented by the measurement of ATP-dependent uptake of ⁴⁵Ca into inside-out plasma membrane vesicles. We used this radiochemical measurement of ⁴⁵Ca uptake, since it offers the most sensitive assay for Ca²⁺-ATPase activity functionally related to Ca²⁺ extrusion.

Ideally, to obtain a precise estimate of the *K_i* for Cd²⁺ and given that Cd²⁺ competes with Ca²⁺ for the calcium site of the pump [46, 47, 49], the dose-dependent inhibition of the pump by Cd²⁺ should be established at a [Ca²⁺] near the pump's *K_m* [39]. We used a [Ca²⁺] of almost twice that value (200 nM) to ensure an accurate creation of calculated free Cd²⁺ concentrations: at lower [Ca²⁺]s Cd²⁺ disturbs the calcium-chelating action of the ligand system to such a degree that the buffering action of the ligands for calcium becomes unacceptably small.

The ATP-driven transport of ⁴⁵Ca was assayed using a rapid filtration technique [21]. Uptakes of ⁴⁵Ca into membrane vesicles during 1-min incubations (representing initial velocities of the pump) in the presence of 3 mM ATP and 0.8 mM Mg²⁺ were measured and corrected for uptake in the absence of ATP. The reaction was quenched by a 14-fold dilution in ice-cold isotonic medium containing 0.1 mM LaCl₃, and the suspension was filtered (Schleicher & Schüll ME25, pore size 0.45 μm). Filters were rinsed twice with 2 ml of ice-cold medium and transferred to counting vials. Four ml Aqualuma® was added per vial, filters were allowed to dissolve (30 min at room temperature) and radioactivity was determined in a Pharmacia Wallac 1410 liquid scintillation counter.

(Na⁺ + K⁺)-ATPASE

(Na⁺ + K⁺)-ATPase activity in fish intestinal basolateral plasma membrane vesicles was determined as described earlier [15]. In short, vesicles (10 μg membrane protein) were diluted 50-fold into 500 μl medium, containing 100 mM NaCl, 30 mM imidazole, and either 15 mM KCl or 1 mM ouabain, all adjusted to pH 7.4 with HEPES. In addition, the media contained the cation buffer substances, magnesium, cadmium and Na₂ATP to realize the calculated amounts of free Mg²⁺, Cd²⁺ and ATP. (Na⁺ + K⁺)-ATPase activity was defined as the Na⁺- and K⁺-dependent, ouabain-sensitive phosphatase activity. The assay period was 5 min at 37°C. Inorganic phosphate produced was measured by the colorimetric Fiske-Subbarow technique using a commercially available combined calcium/phosphorus standard (Sigma cat. nr. 360-11) [15].

Saponin was added to maximize the accessibility of the initially resealed membrane vesicles for the reactants. The (Na⁺ + K⁺)-ATPase activity of the membrane preparation was maximally stimulated by 75% by the addition of 0.4 mg saponin per mg membrane protein, at a protein concentration of 0.5–1.0 mg membrane protein per ml.

(Na⁺ + K⁺)-ATPase purified from dog kidney (Sigma cat. nr. A-0142; ouabain-sensitive activity $66 \mu\text{mol P}_i \cdot \text{hr}^{-1} \cdot \text{mg protein}^{-1}$) was suspended in medium containing 250 mM sucrose and

60 mM imidazole, adjusted to pH 7.4 with HEPES. The final protein concentration was 42 mg · ml⁻¹. (Na⁺ + K⁺)-ATPase activity of this preparation was determined using 5 µl of preparation on 500 µl assay medium.

Na⁺/Ca²⁺ EXCHANGE

Na⁺/Ca²⁺ exchange activity in plasma membrane vesicles was assayed as described earlier [13]. Briefly, 5 µl membrane vesicles equilibrated with 150 mM NaCl were added to 120 µl of ⁴⁵Ca-containing media with either 150 mM NaCl (blank) or 150 mM KCl as main salt constituents. The media also contained 0.8 mM free Mg²⁺, mimicking intracellular conditions. Earlier experiments had shown that addition of valinomycin did not further increase exchange velocities in media containing equal amounts of intra- and extravesicular K⁺, indicating that no build-up of membrane potential occurred during the assay period. We therefore did not use valinomycin in the present studies on Na⁺/Ca²⁺ exchange. After 5 sec at 37°C (this represents initial velocities), the reaction was stopped by addition of 1 ml ice-cold isotonic stopping solution containing 1 mM LaCl₃. A 1-ml sample was filtered (Schleicher & Schüll 0.45 µm), and the filter was rinsed three times with 2 ml stopping solution. The difference in ⁴⁵Ca²⁺ accumulated was taken to represent Na⁺-gradient driven Ca²⁺ transport.

Ca²⁺/Ca²⁺ EXCHANGE

Ca²⁺/Ca²⁺ exchange was tested according to Philipson and Nishimoto [28] with minor modifications. Briefly, 5 µl membrane vesicles equilibrated with 150 mM NaCl were added to 70 µl medium with 150 mM KCl and 25 µM ⁴⁵Ca. No calcium-chelating substances were used in these assays. The vesicle Na⁺ gradient will have dissipated 3 min after the primary dilution (*see also* Fig. 2 in ref. 13: Na⁺-gradient driven Ca²⁺ uptake reaches a maximum at *t* = 2 min). Then, the vesicle suspension was diluted 14-fold by addition of 975 µl 150 mM KCl medium containing either no calcium and cadmium, or 25 µM calcium, or 25 µM cadmium. KCl-equilibrated vesicles were used as blanks to correct for ⁴⁵Ca bound to the exterior of vesicles. Efflux of ⁴⁵Ca was stopped by filtration immediately followed by a threefold wash with ice-cold 150 mM KCl medium containing 1 mM LaCl₃.

ISOLATION OF FISH ENTEROCYTES AND LOADING WITH FURA-2

After having obtained the intestine as described above, the intestinal mucosal cells were scraped off the submucosa onto an ice-cooled glass plate using a glass slide. Cells were resuspended in 10 ml basic salt solution (ingredients (in mM): NaCl 140, KCl 5, CaCl₂ 1, MgCl₂ 1, glucose 10, and HEPES 10, adjusted to pH 7.2 with KOH) supplemented with 0.1 mg · ml⁻¹ bovine serum albumin (Sigma, grade V) and 50 µg · ml⁻¹ DNase. This suspension was kept at 0°C for 30 min, filtered through cheese cloth and centrifuged to collect the cells (5 min, 100 × *g*_{av}). The cell pellet was resuspended in 4 ml bicarbonate-Ringer containing NaCl 140 mM, KCl 2.4 mM, CaCl₂ 1.2 mM, MgSO₄ 1.4 mM, KH₂PO₄ 1.3 mM, NaHCO₃ 25 mM, glucose 500 mg/liter, glutamine 290 mg/liter, and Eagle's Minimal Essential Medium amino acids (50×) 20 ml/liter. The cell suspension was distributed in aliquots of 0.4 ml over plastic petri dishes containing one round cover slip (φ 25

mm) each, and put in an incubation chamber at 28°C that was flushed with humidified gas (5% CO₂ and 95% O₂). After 60 min, the cells attached to the cover slips were washed twice with 1 ml medium. Finally, 0.5 ml medium, containing 5 µM fura-2-acetoxymethyl ester (Fura-2/AM; Molecular Probes, Junction City, OR), was added. Cells were incubated for 30 min. The cover slips were subsequently washed twice with 1 ml medium without fura-2/AM and used in the experiments described below.

INTRACELLULAR FREE Ca²⁺ MEASUREMENTS

Fura-2 fluorescence was determined using a Tracor Fluoroplex III system (TN 6500 Rapid Scan Spectrometer; TN 6075 Photon Counter; IBM AT-compatible computer) coupled to a Nikon Diaphot microscope equipped with UV-optics. The excitation wavelength was alternated every 200 msec between 340 and 380 nm. Emitted fluorescence was filtered through a barrier filter (BA520) and measured through the front port of the microscope using a photomultiplier tube (FP 1400 Photon Counting Detector). Intracellular free Ca²⁺ concentrations were calculated according to Grynkiewicz, Poenie and Tsien [18], using a *K*_d of 162 nM [24]. Since the *K*_d for Mg²⁺ is 9.8 mM [18] and intracellular Mg²⁺ levels are unknown (but predicted to be around 1 mM), no correction for competitive binding of Mg²⁺ to fura-2 was applied.

Calibration of fura-2 was performed inside the cells on which an experiment had been performed. Ionomycin was added to the observation chamber (final concentration: 10 µM) to obtain influx of calcium and to assess the maximum fluorescence ratio (*R*_{max}). Next, EGTA (final concentration: 1.25 mM) was added to determine the minimum fluorescence ratio (*R*_{min}). Typical values were 0.5 for *R*_{min}, 0.7 for *R*_{rest} and 9 for *R*_{max}.

CALCULATIONS AND STATISTICS

Kinetic parameters and their standard deviations were derived by nonlinear regression analysis of the data. Statistical significance of differences between mean values was tested using the Mann-Whitney *U*-test.

Results

Ca²⁺-ATPASE

Figure 1 shows the Ca²⁺ dependency of ATP-driven ⁴⁵Ca uptake into inside-out basolateral plasma membrane vesicles. The calculated *K*_m is 88 ± 17 nM Ca²⁺ and the *V*_{max} is 0.81 ± 0.05 nmol Ca²⁺ · min⁻¹ · mg⁻¹ protein.

Figure 2 shows the dose-dependent inhibition of the Ca²⁺-pump by Cd²⁺. Non-linear regression analysis yielded a half-maximal inhibition constant of 8.2 ± 3.0 pM Cd²⁺ ([Ca²⁺] = 200 nM). We were unable to evaluate the kinetics of the inhibition by Cd²⁺, as a result of the low maximal velocity of the pump. Assuming that Cd²⁺ competes with Ca²⁺ for the Ca²⁺ binding site of the pump, as shown for other preparations before [46, 47, 49], the *K*_i (*K*_i =

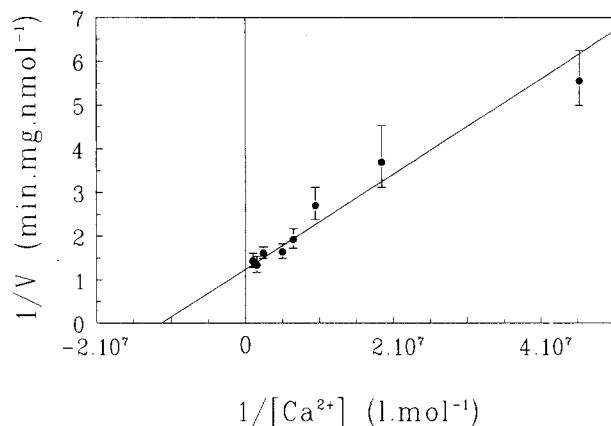


Fig. 1. Double reciprocal plot of the Ca^{2+} dependency of the ATP-dependent Ca-pump in basolateral plasma membrane vesicles from fish intestine. Mean values (\pm SEM) of five experiments are given. Initial rates of ATP-dependent Ca^{2+} uptake were corrected for ATP-independent uptake. The calculated K_m is 88 ± 17 nM, while the computed V_{\max} is 0.81 ± 0.05 nmol \cdot min $^{-1}$ \cdot mg $^{-1}$.

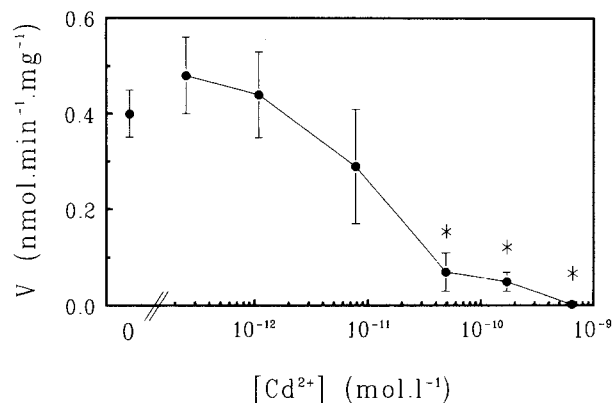


Fig. 2. Cd^{2+} inhibition of the ATP-dependent Ca pump in basolateral plasma membrane vesicles from fish intestine. Mean values (\pm SEM) of nine experiments are given. The free $[\text{Ca}^{2+}]$ was kept constant at 200 nM. Initial rates of ATP-dependent Ca^{2+} uptake were corrected for ATP-independent uptake. Asterisks indicate values significantly different from the control value ($P \leq 0.001$). The calculated IC_{50} is 8.2 ± 3.0 pM Cd^{2+} .

$\text{IC}_{50} \cdot (K_m / (K_m + S))$ for cadmium-inhibition of the ATP-dependent Ca^{2+} transporter in basolateral plasma membranes of fish enterocytes is approximately 3.0 pM.

$(\text{Na}^+ + \text{K}^+)\text{-ATPase}$

Cadmium inhibits the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity in the basolateral plasma membranes isolated from fish intestinal epithelium half-maximally at a concen-

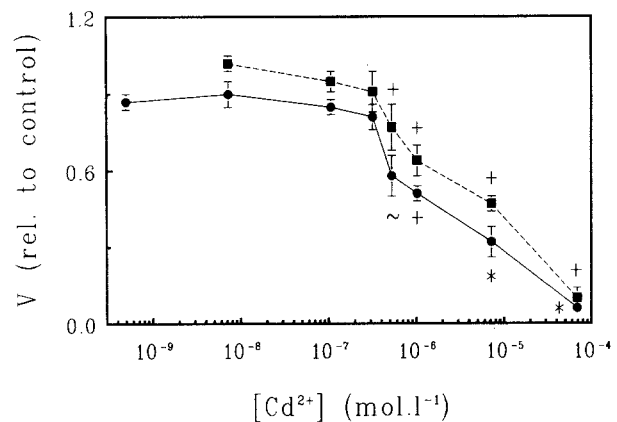


Fig. 3. Cd^{2+} inhibition of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ from fish intestine and dog kidney. Mean values (\pm SEM) are given. Filled circles and solid lines indicate the fish intestinal preparation ($n = 6$), while filled squares and dashed lines represent the dog kidney preparation ($n = 5$). The fish intestinal $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ displayed an activity of 214 ± 27 $\mu\text{mol P}_i \cdot \text{hr}^{-1} \cdot \text{mg protein}^{-1}$, and the dog kidney $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ produced 180 ± 13 $\mu\text{mol P}_i \cdot \text{hr}^{-1} \cdot \text{mg protein}^{-1}$. Free $[\text{Mg}^{2+}]$ was 5 mM. Tildes indicate a $P \leq 0.05$; plus signs a $P \leq 0.01$, and asterisks a $P \leq 0.001$. For fish intestinal $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ the IC_{50} was 2.6 ± 0.6 μM ; for the dog kidney preparation it was 6.3 ± 1.4 μM Cd^{2+} .

tration of 2.6 ± 0.6 μM Cd^{2+} (Fig. 3). In the dog kidney enzyme preparation the IC_{50} for cadmium inhibition was 6.3 ± 1.4 μM ($n = 5$).

To test whether Cd^{2+} specifically interferes with activation of the enzyme by intracellular substrates, we investigated the effects of a fixed concentration of Cd^{2+} on the ATP- and Mg^{2+} -dependent activities of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. ATP exerts both a high-affinity phosphorylation of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and a low-affinity allosteric stimulation. The ATP concentrations used are aimed specifically at discriminating the low-affinity stimulatory action of ATP on the E_2 -form of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, since this action is most important for overall reaction velocity [32]. At 2.5 μM free Cd^{2+} the V_{\max} of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ for activation by ATP decreased to $62 \pm 10\%$ of the control value, while the K_m for ATP had not changed significantly ($P > 0.5$; $n = 3$) from 0.85 ± 0.11 to 0.97 ± 0.16 mM (Fig. 4). Apparently, Cd^{2+} 's inhibition of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ does not take place via the low-affinity site of ATP.

Figure 5 shows the Mg^{2+} dependency of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and the effect of the presence of 2.5 μM Cd^{2+} . Control kinetics were complex, apparently involving more than one action of Mg^{2+} . At low Mg^{2+} concentrations, the high affinity component of the Mg^{2+} dependency of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ displayed a half-maximal activation constant of around 4 μM . With 2.5 μM free Cd^{2+} present,

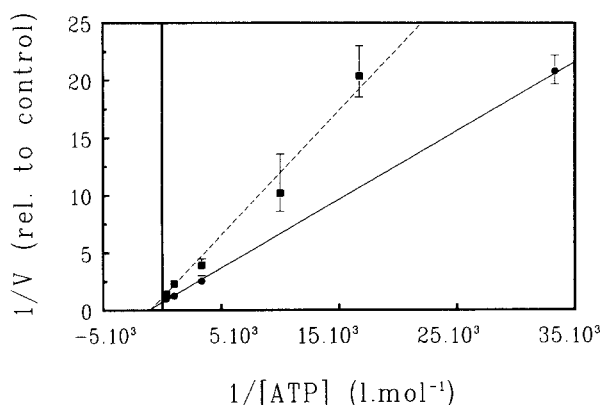


Fig. 4. Double reciprocal plot of the kinetics of the low-affinity ATP site of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ from fish intestine, illustrating the effect of Cd^{2+} on the V_{\max} for ATP of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$. The data are mean values (\pm SEM) for three experiments. The reaction velocity of the control experiment attained at the highest substrate concentration used was taken as 100% (i.e., $137 \pm 26 \mu\text{mol P}_i \cdot \text{hr}^{-1} \cdot \text{mg protein}^{-1}$). Mg^{2+} was kept constant at 5 mM. Control data are represented by filled circles and solid lines, while experimental data are given by filled squares and dashed lines. The presence of $2.5 \mu\text{M Cd}^{2+}$ resulted in a 38% decrease of the calculated V_{\max} , but no significant change in the calculated K_m for ATP.

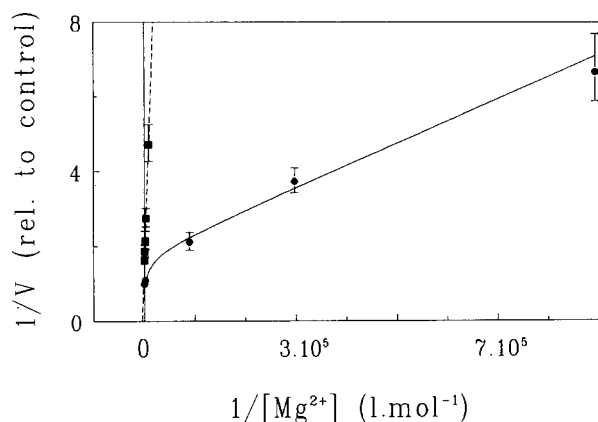


Fig. 5. Double reciprocal plot of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity of fish intestine as a function of Mg^{2+} . Mean values (\pm SEM) for five experiments are given. 100% activity equals $177 \pm 35 \mu\text{mol P}_i \cdot \text{hr}^{-1} \cdot \text{mg protein}^{-1}$ hydrolyzed (attained at the highest $[\text{Mg}^{2+}]$ tested). Control data are indicated by filled circles and solid lines, while experimental data are given by filled squares and dashed lines. The presence of $2.5 \mu\text{M Cd}^{2+}$ yielded a shift in K_m for Mg^{2+} to $243 \pm 13 \mu\text{M}$. The calculated maximal velocity had decreased by 31%.

the activation of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ by Mg^{2+} could be described by a single Michaelis-Menten relationship. The K_m of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ for Mg^{2+} increased to $243 \pm 13 \mu\text{M}$, and the V_{\max} was reduced to $69 \pm 3\%$ of the control value.

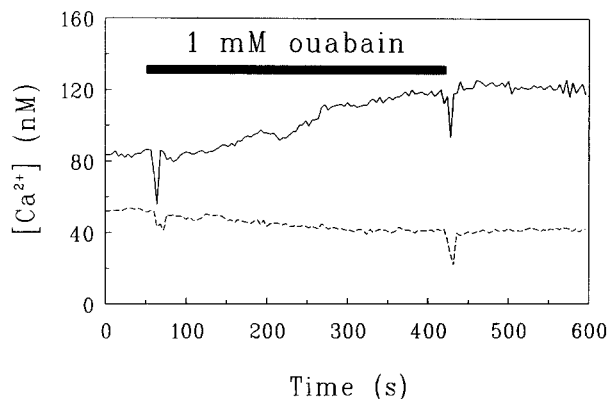


Fig. 6. Two experiments showing the effect of changes in the outside medium on intracellular Ca^{2+} concentration in single freshly isolated tilapia enterocytes. The solid line is a record for a cell kept in an external medium containing 1 mM ouabain. The dashed line is a record of another cell, where the extracellular medium did not contain calcium during the indicated period. The two acute drops in intracellular $[\text{Ca}^{2+}]$ are an artifact due to the solution changes. The rise in intracellular $[\text{Ca}^{2+}]$ was dependent on the presence of extracellular calcium. Similar results were obtained in three other experiments.

$\text{Na}^{2+}/\text{Ca}^{2+}$ EXCHANGE

In isolated fish enterocytes the resting intracellular Ca^{2+} concentration was $85 \pm 21 \text{ nM}$ ($n = 25$). When the outside medium was supplied with 1 mM ouabain (blocking the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ of the cells), the intracellular Ca^{2+} concentration rose. Cells exposed to a ouabain-containing solution without calcium did not exhibit a rise in intracellular Ca^{2+} , indicating that the extracellular Ca^{2+} is necessary for this response to be observed (Fig. 6).

Figure 7 shows the Cd^{2+} concentration dependence of the inhibition by Cd^{2+} of $\text{Na}^+/\text{Ca}^{2+}$ exchange ($n = 5$) at a fixed free Ca^{2+} concentration of $7 \mu\text{M}$. Nonlinear regression analysis yields an IC_{50} of $73 \pm 11 \text{ nM Cd}^{2+}$. Given that Cd^{2+} is a competitive inhibitor of the Ca^{2+} site of the antiporter (see below), we calculated a K_i ($K_i = \text{IC}_{50} \cdot K_m \cdot (K_m + S)^{-1}$) of 11 nM.

The Ca^{2+} kinetics of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger were determined at 0, 5 and 65 nM Cd^{2+} . Substrate dependence of initial velocities obeyed a Michaelis-Menten relationship. In the absence of Cd^{2+} a K_m of $1.21 \pm 0.06 \mu\text{M}$ and a maximal velocity of $18.1 \pm 0.6 \text{ nmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{ protein}$ ($n = 5$) were calculated. Cd^{2+} (5 and 65 nM) had a competitive inhibitory effect on Ca^{2+} kinetics (Fig. 8). The K_m for Ca^{2+} increased significantly to $8.1 \pm 0.5 \mu\text{M}$ at 5 nM Cd^{2+} , and to $16.6 \pm 0.7 \mu\text{M}$ at 65 nM Cd^{2+} ($P < 0.001$). Maximal velocities had not changed significantly ($P > 0.1$).

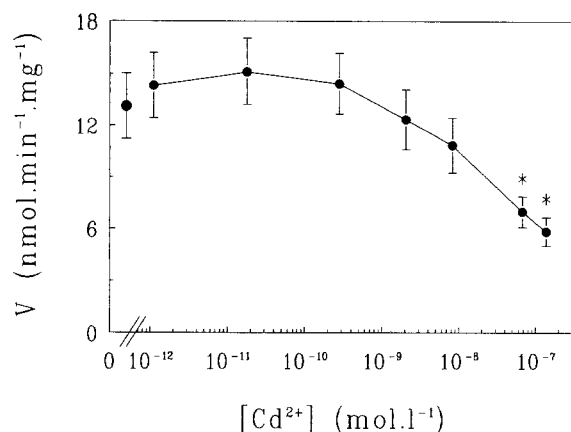


Fig. 7. Inhibition of initial velocities of Na⁺/Ca²⁺ exchange in tilapia basolateral plasma membrane vesicles by Cd²⁺. Free Ca²⁺ concentration was kept constant at 5 μM. An IC₅₀ of 73 ± 11 nM Cd²⁺ was observed. Mean ± SEM of six experiments are shown. Asterisks indicate a significant difference from the control value ($P < 0.01$).

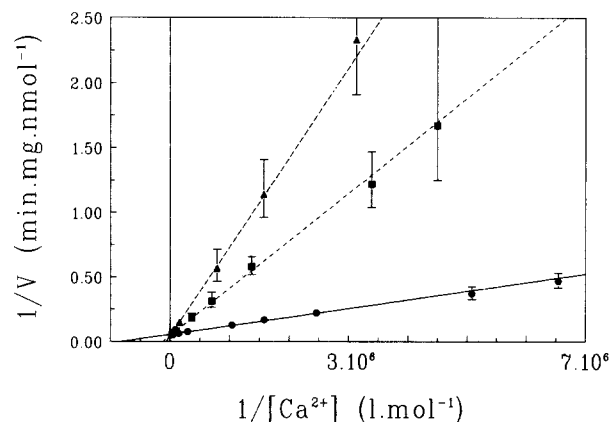


Fig. 8. Lineweaver-Burk plot of the Ca²⁺ dependence of initial velocities of Na⁺/Ca²⁺ exchange. The two fixed Cd²⁺ concentrations (5 and 65 nM Cd²⁺) induce a competitive inhibition for Ca²⁺, increasing Ca²⁺'s K_m but not significantly increasing V_{max} ($P > 0.1$). The K_m for Ca²⁺ activation of the Na⁺/Ca²⁺ exchanger changed from 1.21 ± 0.06 μM to 8.1 ± 0.5 μM in the presence of 5 nM Cd²⁺, and to 16.6 ± 0.7 μM when 65 nM Cd²⁺ was present. Controls ($n = 5$) are shown as filled circles, 5 nM Cd²⁺ values ($n = 3$) as filled squares and 65 nM Cd²⁺ values ($n = 3$) as filled triangles. Means ± SEM are shown. Error bars were not drawn when smaller than the symbols.

Bepridil (80 μM), known to specifically interfere with the exchanger's activation by Na⁺ [16], induced an additional inhibition of the Na⁺/Ca²⁺ exchanger apart from that caused by Cd²⁺ (Fig. 9). Furthermore, the abscissa intercept of the regression line in the Dixon plot (where $[Cd^{2+}] = -K_i + K_i \times K_m^{-1} \cdot [Ca^{2+}]$) remained unchanged, indicating

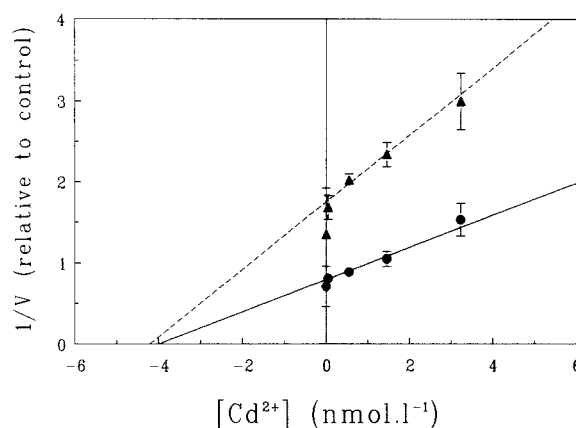


Fig. 9. Dixon plot of Cd²⁺ inhibition of initial velocities of Na⁺/Ca²⁺ exchange and the effect of 80 μM bepridil. Ca²⁺ concentration was kept constant at 2.5 μM. Filled circles indicate data of Cd²⁺ inhibition and filled triangles show data of the inhibition by Cd²⁺ plus bepridil. Means ± SEM ($n = 3$) are shown. Lines were calculated by weighted nonlinear regression analysis. The shared abscissa-intercept indicates that Cd²⁺ and bepridil do not compete for each other's inhibitory site.

Table 2. ⁴⁵Ca efflux from basolateral plasma membrane vesicles

	Fractional loss of ⁴⁵ Ca at $t = 60$ sec
Control	25 ± 15%
25 μM calcium	44 ± 14% ^a
25 μM cadmium	38 ± 14% ^b

^a $P < 0.01$, significantly different from control values; ^b $P < 0.05$. Efflux of ⁴⁵Ca is expressed as the fractional loss of ⁴⁵Ca associated with the vesicles between $t = 0$ and $t = 60$ sec. ⁴⁵Ca was introduced into the vesicles by Na⁺/Ca²⁺ exchange activity during 3 min of preincubation. K⁺-loaded vesicles served as blanks, for which all figures above were corrected. Means ± SD for six experiments are given.

that the K_i of the inhibition by Cd²⁺ remained unchanged. Thus, bepridil does not compete for the site by which Cd²⁺ inhibits Na⁺/Ca²⁺ exchange.

To test whether Cd²⁺ substitutes for Ca²⁺ in the activation of the antiporter we followed an approach originally suggested by Philipson and Nishimoto [28]. Using a differential assay to determine the stimulation of efflux of ⁴⁵Ca accumulated intravesicularly through the action of the exchanger, it was demonstrated that 25 μM extravesicular calcium induces a significant efflux of ⁴⁵Ca (statistically tested as the effect of the treatment on the fractional loss that had occurred after 1 min of efflux; $P < 0.01$). Cd²⁺ also induced a significant ($P < 0.05$) release of ⁴⁵Ca from the vesicles, indicating a functional Ca²⁺/Cd²⁺ exchange (Table 2). Cd²⁺ was less readily exchanged than Ca²⁺ itself.

Discussion

CADMIUM CONTENT OF INTESTINAL TISSUE

Trace amounts of cadmium in the fish food led to a total cadmium content of the mucosal scrapings of $6.0 \text{ nmol} \cdot \text{g}^{-1}$ dry weight. We calculate a total $[\text{Cd}]$ of $1.4 \text{ } \mu\text{M}$ if this amount were to be homogeneously distributed in the epithelial water, i.e. $4.2 \text{ ml} \cdot \text{g}^{-1}$ dry weight [44]. However, sequestration of Cd^{2+} by intracellular calcium stores and binding to calcium binding proteins will minimize the amount of available ionic Cd^{2+} . Ca^{2+} buffering mechanisms (stores and binding proteins) reduce the total $[\text{Ca}]$ of 2.9 mM [45] to a free concentration of 85 nM (this study). Assuming that their affinity for cadmium is (at least) 100-fold higher than for calcium (see e.g. this study and [46, 47]), they will buffer free Cd^{2+} levels to 0.4 pM (or less). We therefore conclude that the trace amounts of cadmium originating from the food do not interfere with the calcium transport mechanisms in tilapia intestinal cells. Moreover, the assays reported on here will not be perturbed by any cadmium possibly associated with the enterocyte plasma membrane vesicles after their isolation, since the use of millimolar amounts of metal chelating substances warrants stable free metal ion concentrations.

Ca^{2+} -ATPASE

The half-maximal activation concentration of the pump for Ca^{2+} ($88 \pm 17 \text{ nM}$) appears to be at variance with the value previously published, i.e. $27 \pm 4 \text{ nM}$ [13]. However, the calculated free calcium concentrations of the latter experiment were not corrected for effects of experimental conditions on the stability constants of chelator-cation complexes, as described above. Recalculation of the previous data yielded a similar K_m value ($65 \pm 8 \text{ nM Ca}^{2+}$).

The K_m found is near the measured $[\text{Ca}^{2+}]_i$ of around 85 nM , and is comparable to those previously published for ATP-dependent ^{45}Ca uptake in plasma membrane vesicles from rat duodenum [49], trout gill [46], rat kidney cortex [21], and rat enterocyte endoplasmic reticulum [5].

The observed sensitivity to inhibition by Cd^{2+} is unlikely to be caused by a competition for the Mg^{2+} site, but rather for the Ca^{2+} site of the enzyme, since ATP-dependent uptake of ^{45}Ca into inside-out membrane vesicles typically requires millimolar amounts of Mg^{2+} for activation, but submicromolar amounts of Ca^{2+} [4]. Moreover, it is well known that Cd^{2+} interacts with the Ca^{2+} site of the enzyme in trout gills [46], rat enterocytes [49] and human erythrocytes [47].

Although the Ca^{2+} affinity of the pump is comparable in the preparations mentioned above, its affinity for Cd^{2+} is remarkably different: the Ca^{2+} pump in trout gill basolateral plasma membranes was half-maximally inhibited at 3 nM Cd^{2+} ($[\text{Ca}^{2+}] = 250 \text{ nM}$) and the rat duodenal Ca^{2+} pump displayed an IC_{50} of 1.6 nM Cd^{2+} at $1 \text{ } \mu\text{M Ca}^{2+}$. Fish intestinal Ca^{2+} pump, however, showed an IC_{50} of $8.2 \pm 3.0 \text{ pM}$ at 200 nM Ca^{2+} . These differences cannot be explained by the different Ca^{2+} concentrations used. We suggest species differences or membrane environment of the pump as possible causes. Whatever the cause of these differences, the very low IC_{50} value indicates an extreme sensitivity to Cd^{2+} for the Ca^{2+} pump of tilapia enterocytes. In vivo, this pump will become impaired once free Cd^{2+} ions are present in intestinal mucosal cells.

$(\text{Na}^+ + \text{K}^+)\text{-ATPASE}$

A purified preparation of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ from dog kidney displayed a somewhat lower affinity for cadmium, i.e. $6.3 \pm 1.4 \text{ } \mu\text{M}$, than $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ in fish intestinal basolateral plasma membrane vesicles ($2.6 \text{ } \mu\text{M Cd}^{2+}$). Other preparations have displayed similar IC_{50} values for Cd^{2+} inhibition of $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ activity: in rat brain synaptosomes half-maximal inhibition occurred at $5.4 \text{ } \mu\text{M}$ [25], in disrupted vascular smooth muscle cells at $10 \text{ } \mu\text{M}$ [40], and in microsomes obtained from frog skin at $30 \text{ } \mu\text{M}$ [37].

The 5 orders of magnitude difference in IC_{50} values observed for Cd^{2+} inhibition of the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ and the $\text{Ca}^{2+}\text{-ATPase}$ leads us to conclude that the affinity for Cd^{2+} is not dictated by the homologous regions of these $\text{E}_1 - \text{E}_2$ class ion pumps. The reduction in V_{max} and the unchanged apparent K_m for ATP caused by $2.5 \text{ } \mu\text{M Cd}^{2+}$ also shows that Cd^{2+} does not exert its inhibitory action on the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ through occupation of the low-affinity ATP site.

$(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ displayed non-Michaelis-Menten behavior with respect to Mg^{2+} . Rossi and Garrahan [33] have taken this to be an indication for the presence of a contaminant amount of Mg^{2+} in the assay media. Analyzing our data along these lines, we calculate a contaminant Mg^{2+} concentration of $1.2 \pm 0.4 \text{ } \mu\text{M}$. The half-maximal activation constant found in this way, i.e. $12.2 \pm 1.7 \text{ } \mu\text{M}$, equals that previously published by Rossi and Garrahan [33], i.e. $11.8 \pm 1.7 \text{ } \mu\text{M}$. However, our use of chelating compounds will prevent errors caused by small contaminations. Therefore, we tend to favor an alternative explanation advanced by Covarrubias and de Weer [6]: the non-Michaelis-Menten behavior ob-

served is the direct consequence of a dual action of Mg²⁺ on a single site of the (Na⁺ + K⁺)-ATPase. The presence of Cd²⁺ changed this behavior drastically: the K_m for Mg²⁺ increased from around 4 μ M to $243 \pm 13 \mu$ M and the kinetics could be adequately described by a normal Michaelis-Menten function. Cd²⁺ apparently competes for the Mg²⁺ site on the (Na⁺ + K⁺)-ATPase.

Na⁺/Ca²⁺ EXCHANGE

The mean intracellular Ca²⁺ concentration of around 85 nM in isolated tilapia enterocytes is in good agreement with literature values of other types of epithelial cells [38]. We used two treatments that greatly decreased net intestinal calcium uptake [13] to test whether the Na⁺/Ca²⁺ exchanger is also involved in intracellular Ca²⁺ homeostasis. Changing the extracellular medium to a medium containing N-methyl-D-glucamine instead of sodium yielded similar results as seen in Fig. 6: the intracellular Ca²⁺ concentration rose rapidly to values slightly above 100 nM (*data not shown*). This is most probably caused by Na⁺/Ca²⁺ exchange working in a "reverse" mode as a result of the change in electrochemical gradient for Na⁺. The relatively rapid onset of the response implies that the exchanger operated near its zero-flux equilibrium, so that a small change in Na⁺ driving force resulted in a reversal of net flux. Indeed, the measured resting free Ca²⁺ concentration is close to the estimated equilibrium value of 67 nM for the Na⁺/Ca²⁺ exchanger (with $[\text{Ca}^{2+}]_o = 1.25 \text{ mM}$ [19], $[\text{Na}^+]_o = 125 \text{ mM}$ [14], $[\text{Na}^+]_i = 9 \text{ mM}$ [38], a membrane potential of -50 mV [2], a stoichiometry of 3 Na⁺ to 1 Ca²⁺, and $[\text{Ca}^{2+}]_i = [\text{Ca}^{2+}]_o \times [\text{Na}^+]_i^3 \times [\text{Na}^+]_o^{-3} \times \exp(V_m F \cdot (RT)^{-1})$).

A potential pitfall in the fura-2 experiments is that intracellular Ca-chelating substances inhibit "reverse-mode" Na⁺/Ca²⁺ exchange in certain cell types [1, 8, 34]. The inhibitory action is not caused by the chelation of intracellular Ca²⁺ ions, since even an excess amount of intracellular Ca²⁺ cannot reverse the inhibition of Ca²⁺ influx [34]. The "reverse-mode" Na⁺/Ca²⁺ exchange activity that we attempted to use in these experiments might be severely inhibited by the presence of intracellular fura-2 [38]. In fact, the modest response of intracellular [Ca²⁺] observed (when viewed against the drastic effects of the same treatments on transcellular Ca²⁺ transport [13]) suggests that such an inhibition did indeed occur: in experiments using fura-2, the role of Na⁺/Ca²⁺ exchange in intracellular Ca²⁺ homeostasis may well be underestimated.

Cd²⁺ was previously shown to inhibit the Na⁺/

Ca²⁺ exchange in mammalian sarcolemmal vesicles with an IC₅₀ of approximately 30 μ M in the presence of 20 μ M calcium (Fig. 2 in ref. 41). In cultured arterial smooth muscle cells the Na⁺/Ca²⁺ exchanger apparently was more sensitive to Cd²⁺ inhibition, since an IC₅₀ of 2.4 μ M was observed at a calcium concentration of 100 μ M (with a K_m for Ca²⁺ of 100 μ M) [36]. No metal chelating substances were used in these studies. Proceeding from these published data and the present study we calculate apparent K_i 's of 11 nM Cd²⁺ for the tilapia Na⁺/Ca²⁺ exchanger, 1.2 μ M for the arterial smooth muscle exchanger and 15 μ M for the antiporter in mammalian sarcolemma. The tilapia Na⁺/Ca²⁺ exchanger is by far the most sensitive of the antiport systems studied, with respect to both Ca²⁺ and Cd²⁺. The Cd²⁺ sensitivity of the exchanger falls midway between the Cd²⁺ sensitivities of the ATP-dependent calcium pump and the (Na⁺ + K⁺)-ATPase in these membranes. The order of sensitivity suggests a correlation between the IC₅₀ for inhibition by Cd²⁺ and the affinity of the divalent metal ion site for which Cd²⁺ competes.

The Ca²⁺ dependence of the Na⁺/Ca²⁺ exchanger obeyed single Michaelis-Menten kinetics (improvements in the procedure to calculate free Ca²⁺, Mg²⁺ and Cd²⁺ concentrations allowed us to reject the previously reported double Michaelis-Menten relationship [13]). The K_m for Ca²⁺ of $1.21 \pm 0.06 \mu$ M is low compared to values published for Na⁺/Ca²⁺ exchange in mammalian sarcolemmal vesicles (around 27 μ M) or in synaptosomal membranes (around 34 μ M) [31]. The higher Ca²⁺ affinity may reflect a species-specific difference. Alternatively, it may arise from the use of calcium chelating substances: Trosper and Philipson [42] state that the apparent K_m of the sarcolemmal Na⁺/Ca²⁺ exchanger for Ca²⁺ was lowered from 21.5 μ M to as low as 1 μ M by the use of 22 μ M EGTA. Moreover, the use of EGTA changed the kinetic behavior such that the data yielded a curved Eadie-Hofstee plot comparable to the one we reported previously [13]. However, our present results show a simple Michaelis-Menten behavior for the Ca²⁺ dependence of tilapia enterocyte Na⁺/Ca²⁺ exchange. Calcium chelators may reduce the Na⁺/Ca²⁺ exchanger's K_m for Ca²⁺, but if calcium binding proteins act in a similar way, this may be important in vivo: in these epithelial cells, where resting Ca²⁺ levels are around 85 nM, a Ca²⁺ extrusion mechanism should respond to changes in submicromolar Ca²⁺ concentrations. The use of calcium chelators is not a prerequisite for the determination of the Ca²⁺ dependence of the Na⁺/Ca²⁺ exchange *per se*, but it is indispensable to accurately establish free Ca²⁺, Mg²⁺ and Cd²⁺ concentrations in the kinetic

assessment of Cd^{2+} inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange. If no metal ion buffering is used, Cd^{2+} ions become bound to membrane structures and reaction vessel walls to a very significant degree. This leads to an overestimation of the IC_{50} of Cd^{2+} . We therefore used calcium chelators in our system to circumvent problems related to this topic.

The kinetic study thus performed showed an exclusive effect of Cd^{2+} on Ca^{2+} affinity, which indicates that Cd^{2+} exerts an inhibition via a competition for the Ca^{2+} site of the exchanger. Pharmacological studies using bepridil supported this hypothesis: a change in the K_i for inhibition by Cd^{2+} was not observed. We conclude that Cd^{2+} inhibits the $\text{Na}^+/\text{Ca}^{2+}$ exchanger solely via a competition for the Ca^{2+} site on the molecule.

It is well known that intracellular Ca^{2+} ions activate $\text{Na}^+/\text{Ca}^{2+}$ exchange (usually measured in its "reversed-mode," i.e. Na^+ efflux/ Ca^{2+} influx) in excitable tissues [1, 8, 34]. The Ca^{2+} affinity of the regulatory site can be much higher than that of the Ca^{2+} transport site, although excised-patch clamp studies on guinea pig sarcolemma showed that binding of Na^+ to the exchanger molecule induces a drop in Ca^{2+} affinity of the regulatory site to around $1\ \mu\text{M}$ [22]. If such a regulatory site exists on the $\text{Na}^+/\text{Ca}^{2+}$ exchanger found in fish intestinal epithelium, binding of Cd^{2+} to it could either mimic Ca^{2+} 's action and stimulate $\text{Na}^+/\text{Ca}^{2+}$ exchange, or exchange could be inhibited by prohibiting Ca^{2+} to bind to the activatory site. The first possibility implies that the observed inhibition by Cd^{2+} stems only from its competition for the Ca^{2+} transport site. The second would result in Cd^{2+} acting on two sites simultaneously, since both sites appear to have similar affinities (for Ca^{2+} , and thus probably for Cd^{2+} , too). The dose-dependent inhibition by Cd^{2+} did not, however, show any signs of cooperativity, which argues against the latter possibility.

The competition of Cd^{2+} for the Ca^{2+} site of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger raises the question whether Cd^{2+} might also be translocated across the cell membrane. We did not observe a $\text{Na}^+/\text{Cd}^{2+}$ exchange activity in this plasma membrane preparation. However, when we tested for $^{45}\text{Ca}^{2+}/\text{Cd}^{2+}$ exchange according to the procedure of Philipson and Nishimoto [28], we found that $25\ \mu\text{M}$ cadmium elicited a significant ^{45}Ca efflux. Trosper and Philipson [41] showed that, in canine cardiac sarcolemmal vesicles, $20\ \mu\text{M}$ cadmium was exchanged against calcium even more effectively than calcium itself. In our experiments calcium was more effective in inducing ^{45}Ca efflux than cadmium.

Does $\text{Ca}^{2+}/\text{Cd}^{2+}$ exchange occur in vivo? The high Cd^{2+} affinity displayed by the exchanger in vitro suggests that intracellular Cd^{2+} ions will com-

pete successfully with Ca^{2+} ions for the Ca^{2+} site of the exchanger. The putative 1 : 1 stoichiometry of an electroneutral $\text{Ca}^{2+}/\text{Cd}^{2+}$ exchange would dictate that the equilibrium ratio for Cd^{2+} ($[\text{Cd}^{2+}]_o/[\text{Cd}^{2+}]_i$) equals that for Ca^{2+} ($[\text{Ca}^{2+}]_o/[\text{Ca}^{2+}]_i$). Hence, an operational $\text{Ca}^{2+}/\text{Cd}^{2+}$ exchange would attempt to create a steady-state $[\text{Cd}^{2+}]$ in the extracellular serosal compartment that exceeds the intracellular $[\text{Cd}^{2+}]$ more than 10,000-fold. Intracellular Cd^{2+} will be exchanged for extracellular Ca^{2+} , leading to a Cd^{2+} efflux through the basolateral membrane of the epithelial cell.

Taken together, our data show that the inhibition by Cd^{2+} of the three membrane proteins studied occurs predominantly through an interaction with divalent metal ion sites. Ca^{2+} extrusion via the calcium pump will become inhibited to a significant degree when intracellular Cd^{2+} concentrations reach the level of $100\ \text{pM}$, whereas the activity of the other $\text{E}_1\text{-E}_2$ type ion pump, the $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$, will only be inhibited significantly by micromolar amounts of Cd^{2+} . $\text{Na}^+/\text{Ca}^{2+}$ exchange activity will decrease when intracellular Cd^{2+} concentrations are in the nanomolar range. Since transcellular calcium uptake in the tilapia intestine mainly depends on $\text{Na}^+/\text{Ca}^{2+}$ exchange activity [13], the inhibition of the ATP-dependent Ca^{2+} pump will result in a minor decrease of intestinal Ca^{2+} uptake. Therefore we conclude that inhibition of the Ca^{2+} - and $(\text{Na}^+ + \text{K}^+)\text{-ATPase}$ is not the main mechanism by which Cd^{2+} suppresses intestinal calcium uptake and disturbs fish calcium homeostasis. Rather, intestinal calcium uptake will become hampered once $\text{Na}^+/\text{Ca}^{2+}$ exchange is inhibited by nanomolar amounts of Cd^{2+} ions. Furthermore, the ability to exchange Ca^{2+} for Cd^{2+} suggests the exchanger as the prime candidate for a mechanism of Cd^{2+} extrusion across the basolateral plasma membrane of the enterocyte. Dietary cadmium will not only accumulate in the tissue, but will be transported into the blood.

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Note Added in Proof

A paper on the calculation of free metal ion concentrations with our computer program (CHELATOR) is currently *in press* in *Biotechniques*. This article and the computer program will become available on *Biotechnet*.