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Characterization of cadmium plasma membrane transport in gills of a mangrove crab *Ucides cordatus*

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ABSTRACT

Membrane pathway for intracellular cadmium (Cd^{2+}) accumulation is not fully elucidated in many organisms and has not been studied in crab gill cells. To characterize membrane Cd^{2+} transport of anterior and posterior gill cells of *Ucides cordatus*, a hypo-hyper-regulating crab, a change in intracellular Cd^{2+} concentration under various experimental conditions was examined by using FluoZin, a fluorescent probe. The membrane Cd^{2+} transport was estimated by the augmentation of FluoZin fluorescence induced by extracellular application of $CdCl_2$ and different inhibitors. Addition of extracellular calcium (Ca^{2+}) to the cells affected little the fluorescence of FluoZin, confirming that Cd^{2+} was the main ion increasing intracellular fluorescence. Ca^{2+} channels blockers (nimodipine and verapamil) decreased Cd^{2+} influx as well as vanadate, a Ca^{2+}-ATPase blocker. Chelating intracellular Ca^{2+} (BAPTA) decreased Cd^{2+} influx in gill cells, while increasing intracellular Ca^{2+} (caffeine) augmented Cd influx. Cd^{2+} and ATP added at different temporal conditions were not effective at increasing intracellular Cd^{2+} accumulation. Ouabain (Na^+/K^+-ATPase inhibitor) increased Cd^{2+} influx probably through a change in intracellular Na and/or a change in cell membrane potential. Routes of Cd^{2+} influx, a non-essential metal, through the gill cell plasma membrane of crabs are suggested.

1. Introduction

Estuarine and coastal areas are regions affected by discharge of heavy metals, mainly cadmium. Cadmium (Cd) plays no role as a trace element and is considered a toxic metal with unknown physiological function for animals. One exception, however, is shown by some diatoms, and in the absence of Zn^{2+}, use Cd^{2+} in the active site of their carbonic anhydrase (Lane and Morel, 2000; Thévenod, 2010). It is known that crabs exposed to Cd up-regulate several antioxidant enzymes, and chronic exposure affect more extensively the metabolic and protein expression profiles than acute exposure to Cd (Silvestre et al., 2006). Cd also causes detrimental effects in respiratory and osmoregulatory functions (see Silvestre et al., 2006).

Membrane pathway for intracellular cadmium transport is not fully elucidated in many organisms (Bridges and Zalups, 2005; Gagnon et al., 2007; Ojo and Wood, 2008; Thévenod, 2010; Verheyen et al., 2012; Kwong and Niyogi, 2012; Li et al., 2012a,b) and has not been studied in crab gill cells, apart from work on perfused gills (Pedersen and Bjerregaard, 1995; Lucu and Obersnel, 1996; Bondgaard and Bjerregaard, 2005). At the cellular level, it is assumed that Cd^{2+} is taken up by cells through transporters for other essential elements as a consequence of low specificity of most membrane transporters (Rainbow and Luoma, 2011). Work with mammals, adopting methodologies for Cd^{2+} transport using radiotracers, or sensitive dyes, has shown unequivocally that Cd^{2+} can enter cells through divalent metal transporters (DMT1), Zn related protein transporters (ZIP) and through mediated receptor endocytosis as a Cd-metallothionein complex, using cell energy (see review by Thévenod, 2010). Other modes of transport such as Ca^{2+} channels are still controversial in mammals (Thévenod, 2010).

For aquatic organisms, studies using whole animals with radioactive tracers show that Cd^{2+} entry in gill epithelium occurs probably via calcium transport routes in fish (Perry and Flik, 1988; Wicklund Glynn et al., 1994), mollusks (Roesijadi and Unger, 1993) and crustaceans (Borowitz and McLaughlin, 1992; Pedersen and Bjerregaard, 1995; Lucu and Obersnel, 1996). Nørum et al. (2005) observed a postmolt increase in cadmium influx in the crab *Carcinus maenas* due to increased transport of cadmium, occurring at least partly, by accidental uptake via calcium transporting proteins. In fish, Cd^{2+} affect Ca^{2+} homeostasis, as both ions compete at the chloride cells of the gills (Lock et al., 1987; Matsuo et al., 2005), and

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apparently the competition occurs with basolateral Ca^{2+}-ATPase and apical Ca^{2+} channels (Lock et al., 1987; Matsuo et al., 2005). For the crab *C. maenas*, Cd^{2+} caused an irreversible inhibition of the osmoregulatory enzyme Na^+/K^+ ATPase (Postel et al., 1998) and a strong inhibition of the respiratory enzyme carbonic anhydrase, affecting the regulation of multiple ions indirectly (Vitale et al., 1999). Additionally, once Cd^{2+} enters a cell, different mechanisms of intracellular detoxification are found. For example, in the bacteria *Staphylococcus aureus* and the yeast *Saccharomyces cerevisiae*, Cd^{2+} is discarded from the cell through a P-type ATPase (Adle et al., 2009; Tommasini et al., 1996; Mielniczki-Pereira et al., 2011). In mammals, however, this mechanism is still debatable (Thévenod, 2010). The binding of Cd^{2+} to metallothionein is an established mechanism of intracellular detoxification in animals and crustaceans (Wallace and Luoma, 2003; Van Kerkhove et al., 2010). In plants, Cd^{2+} is extruded as free ion and as a complexed form, energized by ATP and by an electrochemical gradient (Migocka et al., 2011).

Crustacean's gills are a selective organ that acts between external and internal environment, serving for gas exchange, osmolyte transport, nitrogenous excretion and acid–base balance as well as volume regulation (Lucu and Towle, 2003). In hypo-hyper-regulating crabs, such as *Ucides cordatus*, the posterior gills harbor ionocytes, whereas the anterior gills display a typical gas-exchange epithelium made up of thin cells (see Sá and Zanotto, 2013, for differences in Cu^{2+} transport between both gills). In addition, earlier work has shown that these crabs have functional differences in adjacent gills, enabling strong hypo-hyper-ion regulation (Martinez et al., 1999). Flux-ratio analysis showed active uptake of Na^+ in one gill filament and active extrusion in another, suggesting that hypo-hyper-regulation is performed in different gills (Martinez et al., 1999). It is recognized that gills are immediately exposed to the environment and are the first organs affected by pollutants when ambient water is contaminated. Cadmium exposure, for example, causes a decrease in the functional lamellar area of the gills, impairing energy production (Xuan et al., 2014). In addition, waterborne metals are greatly affected by the water chemistry: amounts of [Ca] and [Mg] (i.e. hardness), pH, alkalinity, dissolved organic matter and other complexing anions (Ojo and Wood, 2008). There is nowadays large evidence of interactions between different metals and *in vitro* cell studies of Cd^{2+} transport, such as this, and the interaction with different metals or ions, can be later validated by *in vivo* experiments. In this case, manipulations of the animal's external environment can be a mechanism that controls the amount of toxic metals that may accumulate in the animals via cellular influx.

Therefore, the objective of this work was to characterize gill cadmium transport (Cd^{2+}) in the presence of calcium, ATP and specific inhibitors of different transporters, in isolated gill cells of the mangrove crab *U. cordatus*. Toxic metal transport and interaction with other ions in cells is an essential part for a better understanding of metal toxicity. In addition, *U. cordatus* is an important source of human food, and could be a possible vehicle of cadmium contamination and transference to human populations. By studying the process of Cd^{2+} transport at the cellular level, unstudied in crab gill cells, the present work answers some questions and open avenues for further research on metal transport interactions in isolated cells of crustaceans.

2. Materials and methods

2.1. Animals

The animals were collected in Itanhaém, São Paulo coast, at the Pescadores Beach, and then, brought to University of São Paulo, where they were acclimatized in the vivarium of the University. The crabs were kept in tanks filled with artificial sea water at 20 ppt, gravel, water filter and pieces of brick for the emersion of the animals. Photoperiod (12:12) was constant as well as the temperature ($22 \pm 3\,^{\circ}C$). Only males in the intermolt period were used. For each set of experiment, $N = 4$ animals were used.

2.2. Cellular dissociation

The gills were separated in anterior and posterior regions. Then, for the gill cells dissociation, the enzymatic method was used, where 10 mL of the extraction solution (NaCl: 22.47 g; KCl: 0.59 g; $NaHCO_3$: 0.22 g; NaH_2PO_4: 0.03 g; Hepes: 0.95 g; glucose: 0.18 g; EDTA: 0.37 g) were mixed with 200 μL of Trypsin (0.05%; Ortega et al., 2011). The gills were immersed in solution and kept in ice, for 15 min. After that, the gills were minced with scissors for 15 min more. The minced gills were filtered in thin mash at 30 μm nylon mesh, put in Falcon tubes (15 mL) and centrifuged for 10 min at $115 \times g$ at 5 °C. After the centrifugation the pellet was resuspended in extraction solution and kept in ice.

2.3. Cellular transport

The gill cells previously dissociated were labeled with FluoZin-3 am (1 μL) during 1 h under shaking at 200 rpm at room temperature. After, the cells were centrifuged at $405 \times g$ for 5 min and then washed in the extraction solution, without EDTA. In the fluorimeter (Biotek), the emission used was 525 nm and the excitation 495 nm. The cellular transport was measured each 90 s in real time. In an Elisa plate, 180 μL of cells were added with $CdCl_2$ concentrations of 0.5, 1.0 and 1.5 μM and at 0.5, 1.0 and 1.5 mM concentrations. The variation in fluorescence was measured for each concentration.

A calibration curve was prepared to transform arbitrary fluorescence units into intracellular Cd^{2+} concentrations, based on Zanotto and Baptista (2011). At the end of the experiment, 180 μL of cells without the exposition of cadmium were added to an Elisa plate with 50 μL of Triton X-100 20% for 15 min. To obtain the minimum fluoresce (F_{min}), it was added 50 μL of HEDTA (1 mM). For the maximum fluorescence (F_{max}), we added 50 μL of Triton X-100 20% for 15 min and 50 μL of $CdCl_2$ (5 mM) in different batch of cells. Intracellular cadmium for every experiment was calculated according to the equation:

$$[Cd^{2+}]i = Kd \times \left(\frac{F - F_{min}}{F_{max} - F}\right)$$

where $[Cd^{2+}]i$ is the intracellular free cadmium concentration (nM); Kd is the dissociation constant of FluoZin (1.88); F is the actual fluorescence measured; F_{min} is the minimum fluorescence and F_{max} is the maximum fluorescence.

The cellular viability was assayed using the Trypan Blue method, where 20 μL of Trypan Blue were added to 200 μL of cells (Tennant, 1964) and put in a Neubauer chamber. The viability was quantified by the visualization of the stained cells (unviable) and translucent cells (viable) in four quadrants and the result was multiplied by 10^4. For all experiments, were used 23×10^4 cells.

2.4. Experimental design

To investigate copper transport, we added the following inhibitors: (a) verapamil (1 μM) and nimodipine (11 mM), which blocks slow calcium channels (Ahearn and Franco, 1993) and L-type voltage-dependent calcium channels (VDCC; Gavazzo et al., 2005) respectively; (b) ATP (1 mM), which inhibits Ca^{2+}-ATPase and Cu^{2+}-ATPase (Wheatly et al., 1999); (c) ouabain (2 mM), a Na^+/K^+-ATPase inhibitor (Zare and Greenaway, 1998); (d) vanadate (100 μM), a

Na^+/K^+ and Ca^{2+}-ATPase inhibitor (Zare and Greenaway, 1998); (e) caffeine (100 μM), which facilitates the release of calcium from the endoplasmic reticulum (Goudeau and Goudeau, 1998); and (f) BAPTA (2 mM) that chelates intracellular Ca^{2+}.

Cells were also incubated with extracellular Ca^{2+} at 1 mM or 20 mM. After, $CdCl_2$ was added at different concentrations and the change in fluorescence was followed.

For experiments with ATP, the cells were exposed to the following treatments: (a) $CdCl_2$ *alone* at 1.0 mM; (b) $CdCl_2$ + ATP at 1 mM *added* together; (c) $CdCl_2$ followed by ATP after 90 s, both at 1 mM; (d) ATP followed by $CdCl_2$ after 90 s, at 1 mM. Then, the fluorescence change was analyzed as intracellular Cd^{2+}.

In the case of experiments with BAPTA, an intracellular Ca^{2+} chelator, cells were incubated with BAPTA at 2 mM together with FluoZin dye during 1 h. Cellular Cd^{2+} transport was performed using $CdCl_2$ in the same concentrations as above.

2.5. Data analysis

Data was collected on 4 animals. One-Way ANOVA was used to compare different inhibitors (treatments) against control. Cadmium transport was also compared between treatments using the kinetic variables, Km and Vmax, when the data was displayed as Cd transport against Cd concentration. Whenever the data was presented as Cd transport against time, all experimentals (different inhibitors or ATP) were compared against control. The statistical package used was Sigma Stat and the data was verified for normality and homogeneity of variance. Data are displayed as means ± SE.

3. Results

Before the start of the experiments with Cd^{2+} transport, we tested FluoZin to see whether the fluorescence of the dye could suffer interference with Ca^{2+} ions. To test that, we added both Cd^{2+} and Ca^{2+} at 1 mM, together or separated, in the presence of isolated cells. The results show that the fluorescence change (arbitrary units) due to Ca^{2+} is at least 3× less than the fluorescence due to Cd^{2+} alone, and the change due to Cd^{2+} and Ca^{2+} together is similar to Cd^{2+} alone (Fig. 1, ANOVA, $P < 0.05$). These results show that the probe/dye used is sensitive to Cd^{2+} only, and reinforce that any change in intracellular Ca^{2+} would not affect our results.

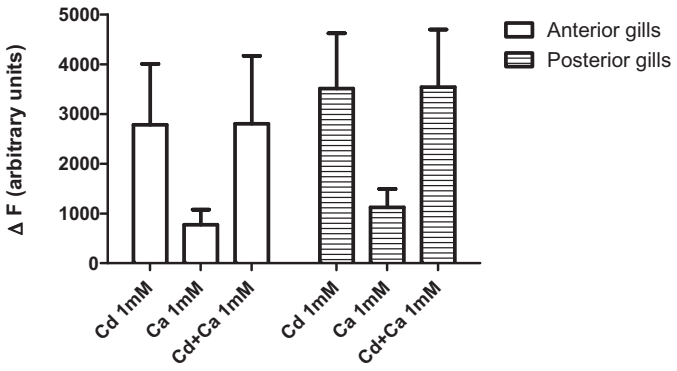


Fig. 1. Fluorescence change (ΔF) as arbitrary units against Cd^{2+} and Ca^{2+} added together or separated, both at 1 mM, in anterior and posterior gill cells.

Next, we tested different concentrations of Cd^{2+} and followed gill cell viability for 2 h (within the experimental transport time) after gill cell exposure to Cd^{2+}. Cd^{2+} added to the cells in μM amounts elicited a change in intracellular Cd^{2+} at levels much lower levels compared to Cd^{2+} added in mM levels (0.15 against 1.0 nM × 23 × 10^4 cells × 90 s^{-1}; Figs. 2A, B and 3). However, the errors associated were much larger for Cd^{2+} in μM amounts. We then tested the cell viability using Trypan Blue at the end of cell exposure to Cd^{2+} (Table 1). There was no difference in cell viability among control and treatments after 2 h. Therefore, we used Cd^{2+} in mM amounts for next experiments due to a lower amount of variation in the data collected.

Next, Cd^{2+} influx at different concentrations was compared between anterior and posterior gill cells (Fig. 4). There was no statistical difference between Km and Vmax for both. Anterior and posterior gill cells transport Cd^{2+} in similar amounts, although there was a slightly lower Km for posterior gill cells, indicating a higher affinity.

Fig. 5A and B shows the effect Cd transport for cells exposed to the Ca^{2+} channel inhibitor nimodipine. Both Km and Vmax for Cd transport were reduced for cells incubated with nimodipine (ANOVA, $P < 0.001$). Vmax was around 3× lower and Km 2× smaller compared to control (Fig. 5A). For posterior gill cells the inhibition occurred only for Vmax (ANOVA, $P < 0.001$, Fig. 5B). Gill cells

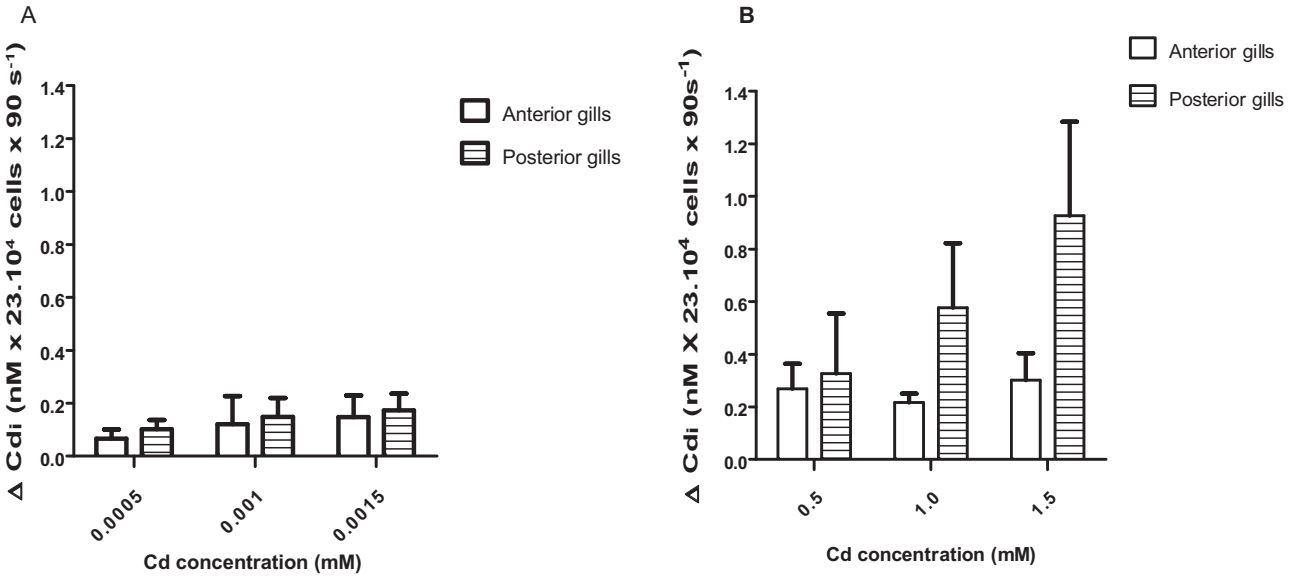


Fig. 2. Variation of intracellular Cd^{2+} concentration (ΔCdi) against extracellular Cd^{2+}, for anterior and posterior gill cells. (A) Cd^{2+} added in lower mM concentration; (B) Cd^{2+} added in higher mM concentration.

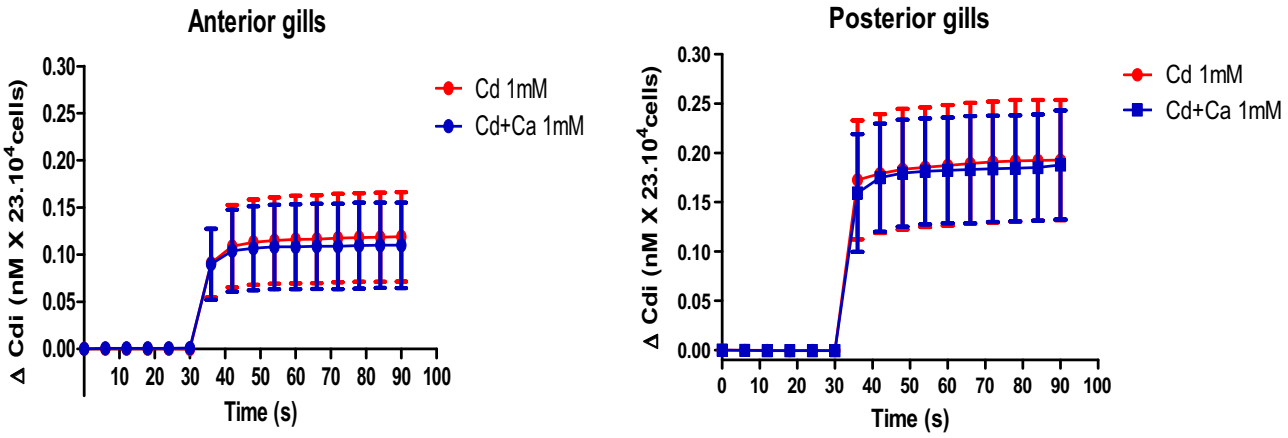


Fig. 3. Relationship between intracellular Cd^{2+} (nM × 23 × 10^4 $cells^{-1}$) and time (s) for anterior and posterior gill cells exposed to Cd^{2+} and Ca^{2+} (1 mM for both). Cd^{2+} was added alone or Cd^{2+} and Ca^{2+} (Cd^{2+} + Ca^{2+}) were added together.

Table 1
Cell viability (%) at the end of Cd^{2+} exposure (2 h) for anterior and posterior gill cells exposed to Cd^{2+} at 0.0015 mM and 1 mM. There was no difference among treatments (ANOVA; $P > 0.05$).

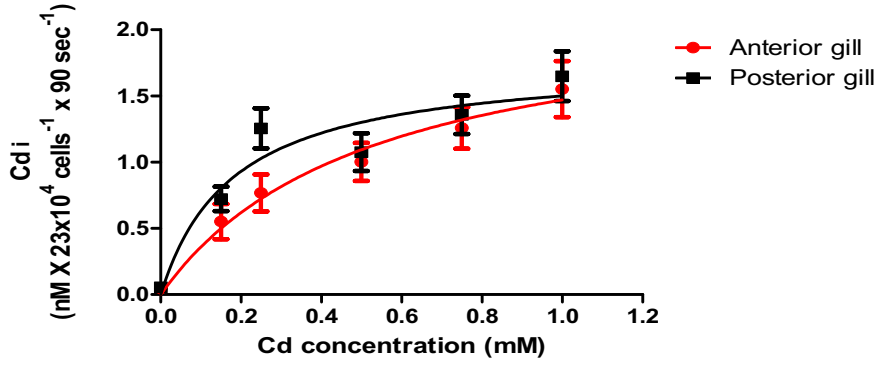
	Control (0 Cd^{2+})	Anterior gills (Cd^{2+} 1 mM)	Anterior gills (Cd^{2+} 0.0015 mM)	Posterior gills (Cd^{2+} 1 mM)	Posterior gills (Cd^{2+} 0.0015 mM)
Cell viability (%)	69.2 ± 5.0%	57.4 ± 7.4%	68.6 ± 4.7%	50.1 ± 5.9%	49.9 ± 5.9%

exposed to verapamil (Fig. 5C and D), caused a decrease in Vmax and Km for anterior gill cells, while for posterior gill cells, Vmax decreased while Km increased 2× (ANOVA, $P < 0.001$, Fig. 5C and D). Vanadate exposure, an inhibitor of Ca-ATPase, caused a decrease in Vmax only for anterior gill cells, while for posterior gill cells there was an increase in Km (a decrease in transport affinity) and decrease in Vmax (ANOVA, $P < 0.001$, Fig. 5E and F).

Anterior and posterior gill cells were incubated with extracellular Ca^{2+} at 1 and 20 mM or caffeine. After 30 s Cd at 1 mM was added alone or to the cells exposed to Ca^{2+} and caffeine (Fig. 6A and B). Results showed an increase for Cd influx for anterior gill cells exposed to caffeine (ANOVA, $P < 0.05$, Fig. 6A) but not for the other treatments. The same was seen for posterior gill cells, although the increase in Cd influx for cells treated with caffeine was not different from cells exposed to Cd and Ca^{2+} at 1 mM (ANOVA, $P < 0.05$, Fig. 6B). The effect of caffeine was, however, higher than cells exposed to the control (Cd alone).

Anterior and posterior gill cells were incubated with vanadate, ouabain and vanadate + ouabain together. After 30 s, Cd at 1 mM was added alone or to the cells exposed to each inhibitor. Fig. 7A shows the effect of Cd^{2+} uptake for anterior gill cells. Intracellular Cd^{2+} was higher in the presence of ouabain compared to Cd alone, but lower when both ouabain and vanadate were added together (ANOVA, $P < 0.05$). The same trend was seen for posterior gill cells, an increase of Cd influx but at higher levels compared to anterior gill cells (Fig. 7B, ANOVA, $P < 0.05$). In addition, Cd transport in the presence of vanadate was statistically lower than control, together with vanadate and ouabain added together (Fig. 7B, ANOVA, $P < 0.05$).

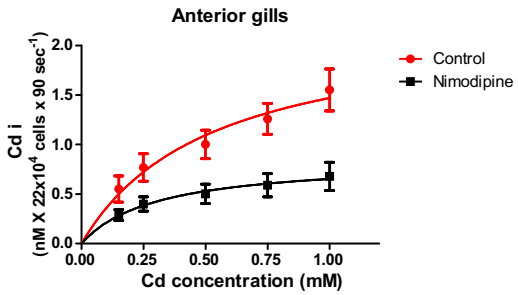
Anterior and posterior gill cells incubated with BAPTA, an intracellular Ca^{2+} chelating agent, resulted in strong inhibition of



Treatment	Km	Vmax
Anterior gill	0.5237	2.241
Posterior gill	0.1809	1.772

Fig. 4. Relationship between intracellular Cd^{2+} (nM × 23 × 10^4 $cells^{-1}$ × 90 s^{-1}) and Cd^{2+} concentration (mM) for both anterior and posterior gill cells. Km and Vmax values are shown and are statistically similar for both gills.

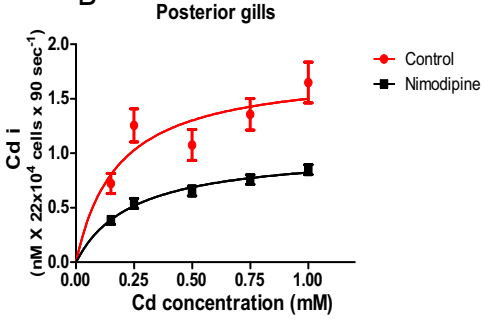
A



Treatment	Km (*)	Vmax(*)
Control	0.5237	2.241
Nimodipine	0.3029	0.8505

(*) P<0.01

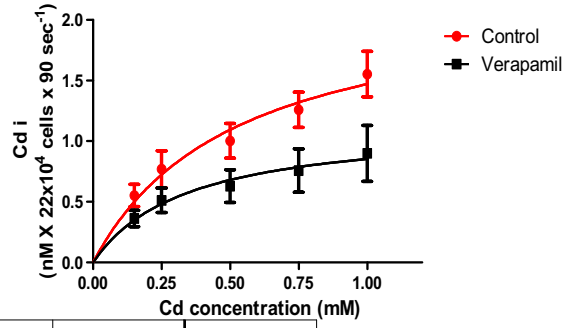
B



Treatment	Km	Vmax(*)
Control	0.1809	1.772
Nimodipine	0.2454	1.026

(*) P<0.01

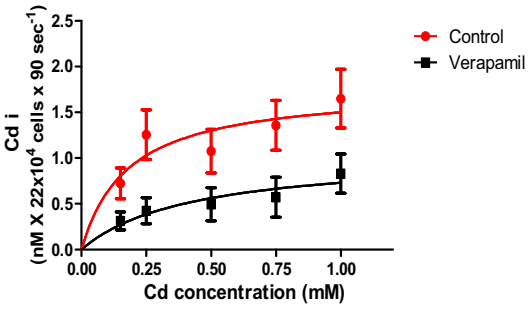
C



Treatment	Km (*)	Vmax (*)
Control	0.5237	2.241
Verapamil	0.3439	1.147

(*) P<0.01

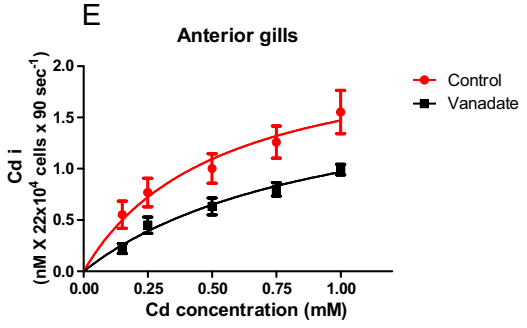
D



Treatment	Km(*)	Vmax(*)
Control	0.1809	1.772
Verapamil	0.4260	1.042

(*) P<0.01

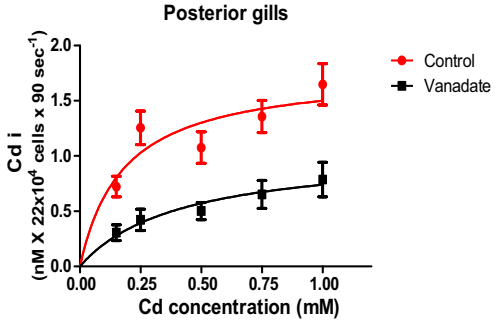
E



Treatment	Km	Vmax(*)
Control	0.5237	2.241
Vanadate	0.9675	1.904

(*) P<0.01

F



Treatment	Km(*)	Vmax(*)
Control	0.1809	1.772
Vanadate	0.4170	1.049

(*) P<0.01

Fig. 5. Relationship between intracellular Cd^{2+} ($nM \times 23 \times 10^4$ cells$^{-1} \times 90\,s^{-1}$) and Cd^{2+} concentration (mM) for both anterior and posterior gill cells and different inhibitors. (A and B) Cells were incubated with nimodipine for 3 min at 11 μM; (C and D) cells were incubated with verapamil for 3 min at 1 μM; (E and F) cells were incubated with vanadate for 3 min at 100 μM. Statistical differences between Km and Vmax are shown as asterisks for each figure (One-Way ANOVA).

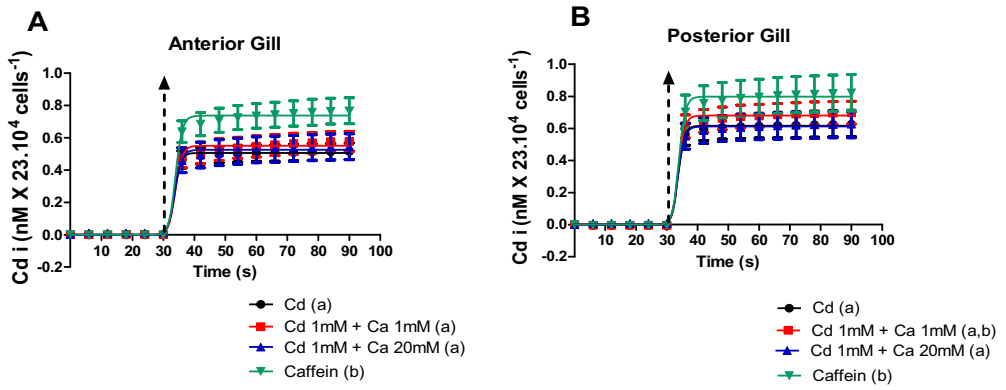


Fig. 6. Relationship between intracellular Cd^{2+} (nM × 23 × 10^4 $cells^{-1}$) and time (s) for anterior and posterior gill cells exposed to Cd^{2+} at 1 mM and different inhibitors or ions. Control at Cd 1 mM; caffeine (100 μM); Cd and Ca^{2+} (1 mM) and Cd at 1 mM and Ca^{2+} at 20 mM. (A) Anterior gill cells; (B) posterior gill cells. The vertical line represents the addition of Cd^{2+} (1 mM) to the gill cells incubated with each different compound for 3 min. Different letters in the legends represent significant statistical differences (ANOVA, $P < 0.05$).

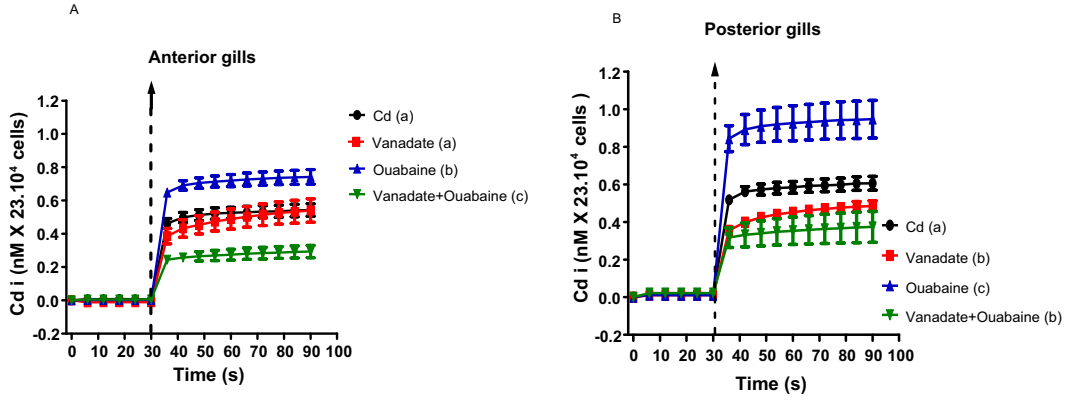
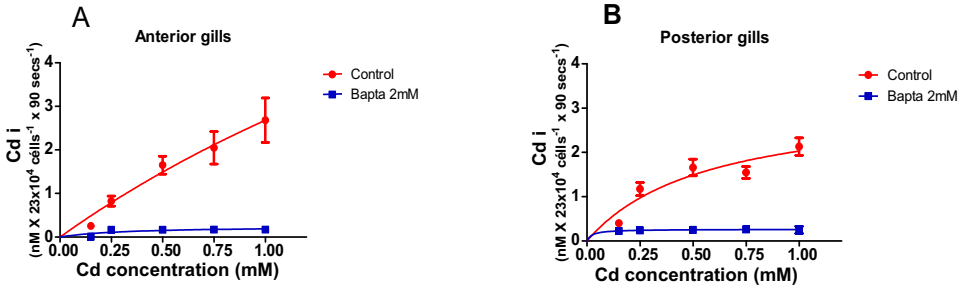


Fig. 7. Relationship between intracellular Cd^{2+} (nM × 23 × 10^4 $cells^{-1}$) and time (s) for anterior and posterior gill cells exposed to Cd^{2+} at 1 mM and different inhibitors. Vanadate (100 μM); ouabain (2 mM) and vanadate + ouabain together. The vertical line represents the addition of Cd^{2+} (1 mM) to the gill cells incubated with each inhibitor for 3 min. Different letters in the legend represent significant statistical differences (ANOVA, $P < 0.05$).

intracellular Cd^{2+} influx for both Km and Vmax (Fig. 8), suggesting a dependence of Cd^{2+} transport and intracellular Ca^{2+} stores.

To understand the mechanism of Cd^{2+} influx and the effect of ATP, anterior and posterior gill cells were exposed to Cd^{2+} alone, Cd^{2+} followed by ATP after 20 s, ATP followed by Cd^{2+} after 20 s and Cd^{2+} + ATP added to the cells at the same time (Fig. 9A and B). The results showed that Cd^{2+} and ATP added in different order did not affect the intracellular levels of the metal (Kruskal–Wallis One Way Analysis of Variance on Ranks, $P > 0.05$). The experiment was performed for both anterior and posterior gill cells, with larger increases in intracellular Cd^{2+} for posterior gill cells compared to anterior gill cells (Fig. 9B).

Fig. 10 shows an integrative view of the suggested mechanisms of Cu transport in crab gill cells according to the results reported here. Overall Cd enter gill cells through: (a) Ca^{2+} channel – nimodipine and verapamil inhibited; (b) Cd^{2+}/Ca^{2+} ($2Na^+$)



Treatment	Km (*)	Vmax(*)
Control	3.910	13.16
Bapta 2mM	0.3248	0.2502

(*) P<0.05

Treatment	Km (*)	Vmax(*)
Control	0.5684	3.185
Bapta 2mM	0.02340	0.2616

(*) P<0.05

Fig. 8. Relationship between intracellular Cd^{2+} (nM × 23 × 10^4 $cells^{-1}$ × 90 s^{-1}) and Cd^{2+} concentration (mM) for both anterior and posterior gill cells incubated with BAPTA, an intracellular Ca^{2+} chelator, for 1 h. Km and Vmax values are shown, and there was a statistically significant difference for Km and Vmax for both gill cells.

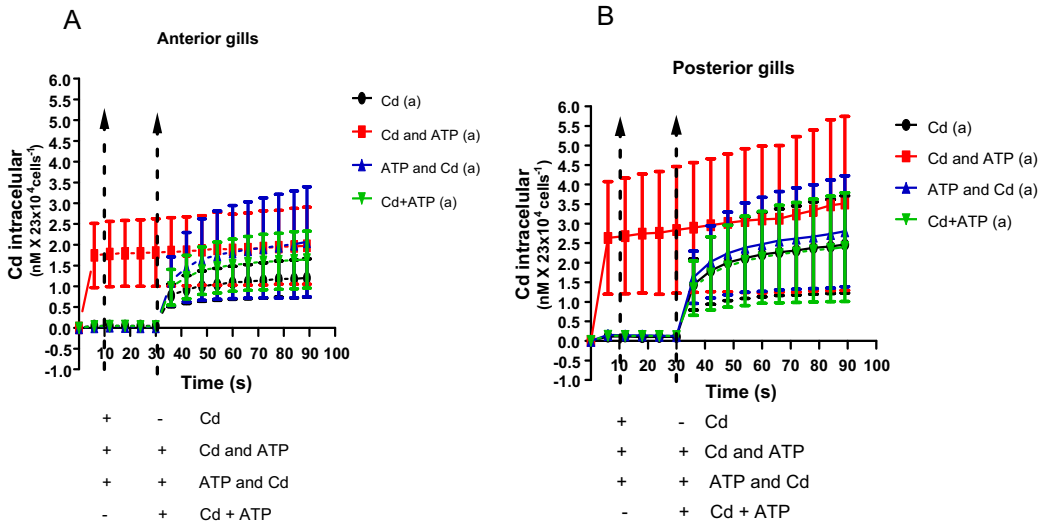


Fig. 9. Relationship between intracellular Cd^{2+} (nM × 23 × 10^4 $cells^{-1}$) and time (s) for anterior and posterior gill cells exposed to Cd^{2+} and ATP (1 mM for both) added at different time points according to the hatched line. Cd^{2+} alone; Cd^{2+} and ATP; ATP and Cd; and Cd^{2+} + ATP added simultaneously. Plus (+) and minus (−) represent the addition or not of the different compounds below the hatched line. There was no statistical difference among the treatments. Similar letters in the legends represent no significant differences.

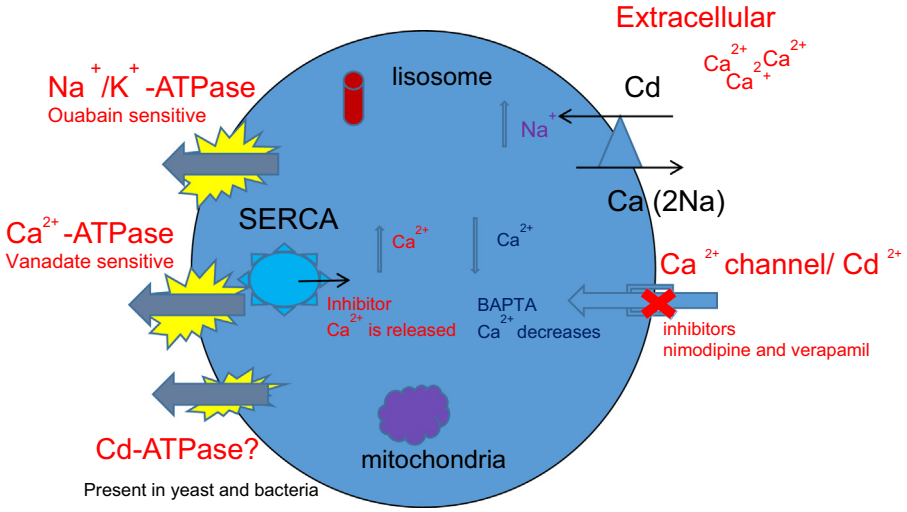


Fig. 10. Working model for Cd^{2+} transport in *Ucides cordatus* gill cells. Cd^{2+} enters gill cells through: (a) Ca^{2+} channel, nimodipine and verapamil inhibited; (b) Cd^{2+}/Ca^{2+} ($2Na^+$) exchanger where intracellular Na^+ increase result in augmented Cd^{2+} influx, increased (caffeine) or decreased intracellular Ca^{2+} (BAPTA), increase or decrease Cd^{2+} influx, respectively. However there was no effect of increased extracellular Ca^{2+}; (c) Na^+/K-ATPase inhibited by ouabain, increased Cd^{2+} influx probably through intracellular Na^+ alteration; (d) Ca^{2+}-ATPase, vanadate sensitive, decreased Cd^{2+} influx, an effect not explained here by an elevation of intracellular Ca^{2+} but through a probable effect in cell membrane potential, resulting in altered Cd^{2+} influx.

exchanger where intracellular Na^+ increase result in augmented Cd^{2+} influx and increased intracellular Ca^{2+} (caffeine) or decreased intracellular Ca^{2+} (BAPTA), increase or decrease Cd^{2+} influx, respectively. However, there was no effect of increased extracellular Ca^{2+}; (c) Na^+/K-ATPase inhibited by ouabain, increased Cd^{2+} influx probably through intracellular Na^+ alteration; (d) Ca^{2+}-ATPase, vanadate sensitive, decreased Cd^{2+} influx, an effect not explained here by an elevation of intracellular Ca^{2+} or a probable effect in cell membrane potential, altering Cd^{2+} influx.

4. Discussion

The use of FluoZin as a fluorescent probe for monitoring changes in intracellular concentration of cadmium was shown here to be effective to monitor this metal influx. The results strongly suggest that Cd^{2+} transport from gill cells of *U. cordatus*, a hypo-hyper-regulating crab occurs through Ca^{2+} transporting proteins due to the transporter sensitivity to Ca^{2+} channel blockers. Additionally the transporter is vanadate sensitive, an ATPase blocker. In the presence of ATP, however, Cd^{2+} influx did not change. Also, the transporter is highly dependent on intracellular Ca^{2+} stores and Na^+ and/or membrane potential, both affecting Cd^{2+} transport. Extracellular Ca^{2+}, on the other hand, did not affect Cd uptake and there were no differences between anterior and posterior gill cells for uptake of Cd^{2+}.

For crustaceans, it has been shown that Cd^{2+} influx is affected by Ca^{2+} concentration using, in these cases, perfused gills cells (Lucu and Obersnel, 1996; Silvestre et al., 2004; Bondgaard and Bjerregaard, 2005). In addition, Cd^{2+} affects Na^+/K^+-ATPase activity when administered in the basolateral side of perfused gills of the crab *C. maenas* (Postel et al., 1998). Another work with perfused gills of *Eriocheir*, an euryhaline crab like the one studied here (Silvestre

et al., 2004), the authors did not see Cd^{2+} influx through posterior gills, only for anterior ones. However, they saw Cd^{2+} accumulation in both gills. Influx rates for *Eriocheir* in that work were around 0.17 nmol Cd^{2+} g^{-1} gill w w h^{-1} (Silvestre et al., 2004). Although it was measured here influx through isolated cells from posterior and anterior gills, intracellular Cd^{2+} accumulation was in the nanomolar range as well. Usually there are 4 different types of cells in crustaceans gills in general (reviewed by Freire et al., 2008), and we expect higher amount of ionocytes from posterior gills compared to anterior gills (see Ortega et al., in press). Interestingly, Pedersen and Bjerregaard (2000) measured influx and efflux across perfused gill cells of *Carcinus* and influx rates were also in the nanomolar range but, surprisingly, efflux rates were very similar to influx rates in their work. In addition, a change in membrane potential difference from negative to positive across perfused gills in that work apparently altered Cd^{2+} influx, caused by the addition of a Na^+-free solution to the perfused gills (Pedersen and Bjerregaard, 2000). It was seen here a Na^+ ion effect, through the inhibition of Na^+/K^+ ATPase and as a result, an augmentation in Cd^{2+} influx. It seems that a change in the membrane potential alters Cd^{2+} cell influx rates. More details on the types of cells found in gills of crustaceans are revised elsewhere (Freire et al., 2008) and were characterized in Ortega et al. (in press).

4.1. Cd^{2+} and Ca^{2+} channels

The addition of Ca^{2+} and Cd^{2+} together to the extracellular medium did not affect Cd^{2+} influx, although Ca^{2+} channel blockers inhibited Cd^{2+} influx. Apparently, the rapid passage of both ions simultaneously through diffusion using channels is not limiting for each ion, however blocking the channel affect Cd^{2+} uptake in gill cells. Epithelial Ca^{2+} channels, in contrast with excitable cells, have been characterized in intestine and kidney of mammals (Nilius et al., 2001) and have a constitutively presence of Ca^{2+} permeability at physiological membrane potentials (Den Dekker et al., 2003) and, in addition, allow passage of other divalent cations. Interestingly, intracellular ATP is necessary to maintain epithelial Ca^{2+} channel activity steady in mammals, although the mechanism of ATP-induced activation is not known yet (Den Dekker et al., 2003). This could be an explanation for results found here on Cd^{2+} influx through epithelial channels in crabs and the fact that Cd^{2+} gill cell transport was affected by vanadate. Epithelial Ca^{2+} channel has not been elucidated for crabs, though. Energy dependence for cell Cd^{2+} uptake has also been reported for oysters gills (Roesijadi and Unger, 1993), and Cd^{2+} transport was also inhibited by calcium channel blockers (see review by Wright, 1995). Moreover, Li et al. (2012a) saw that a freshwater crab exposed to Cd^{2+} had decreased levels of Na^+/K^+-ATPase and Ca^{2+}-ATPase in gills, followed by a lower metallothionein mRNA levels, which is known to chelate intracellular Cd^{2+} in general.

Pancreatic cells possess L-type voltage-dependent calcium channels (VDCC) and represent a pathway of Cd^{2+} influx in these cells (Gavazzo et al., 2005). Nimodipine is a specific inhibitor of these VDCC and was shown to modulate Cd^{2+} influx for insulinoma (pancreatic) cells (Gavazzo et al., 2005). It was seen here a similar effect of the same blocker on Cd^{2+} influx, although the presence of an L-type Ca^{2+} channel has not been characterized in crustacean gill cells. Verapamil, also an L-type Ca^{2+} channel inhibitor, was also effective at inhibiting Cd^{2+} influx seen in our results.

4.2. Cd^{2+} and intracellular Ca^{2+}

A study with rat enterocytes, cells of epithelial origin, showed that extracellular as well as intracellular Ca^{2+} binding sites for Ca^{2+} transporters have affinities for Cd^{2+} two orders of magnitude higher than for Ca^{2+} (Verbost et al., 1987), also observed for fish gills (Verbost et al., 1988). They concluded that Cd^{2+} affect intracellular Ca^{2+} homeostasis due to the extreme sensitivity of the Ca-pumping ATPase in basolateral plasma membrane and other internal ATP-driven Ca^{2+} transport systems. Here, chelating intracellular Ca^{2+} was affecting Cd^{2+} transport negatively, suggesting a dependence of Cd^{2+} for intracellular Ca^{2+} stores. Similarly, the use of caffeine, responsible for releasing Ca^{2+} from sarcoplasmic reticulum (Mandal et al., 2005), affected Cd influx positively. Therefore, a lowering of intracellular Ca^{2+} and an increase of the same ion inside gill cells were affecting intracellular Cd^{2+} influx, strongly suggesting that a Ca^{2+}/Na^+ exchanger in the gill cell plasma membrane could also exchange Cd^{2+}.

4.3. Cd, Na^+/K^+-ATPase and effects of ATP

In addition, the use of extracellular Ca^{2+} was not affecting intracellular Cd^{2+} accumulation as seen for intracellular Ca^{2+}. Here, ouabain affected positively Cd^{2+} influx and again, an increase in intracellular Na^+, through inhibition of the Na^+/K^+-ATPase, could possibly be another factor to reinforce the presence of a $Cd^{2+}/Ca^{2+}(Na^+)$ exchanger in gill cells. Interestingly, the positive effect of ouabain in these cells was lost when vanadate and ouabain were added together, now through a larger decrease in intracellular Cd^{2+} influx. The reason for such effect is not clear at the moment. Probably inhibitors affecting at the same time Na^+/K^+-ATPase plus Ca^{2+}-ATPase generate a large change in major intracellular ions and in the cell electrochemical gradient that affects negatively Cd^{2+} influx and overcomes the effect of ouabain alone.

The presence of a Cu^{2+}-ATPase in crab gill cells was suggested by Sá and Zanotto (2013), as seen by the presence of ATP causing increased influx of intracellular Cu^{2+}. Cd^{2+}, studied here, was not affected by ATP. The essentiality of the metal Cu^{2+} for crustaceans, in contrast with Cd^{2+}, suggest basic differences between transport of both ions. Here some inhibitors had a positive effect on Cd^{2+} influx, not seen for Cu^{2+} (Sá and Zanotto, 2013) and extracellular Ca^{2+} had no effect on Cd^{2+} transport, different from effects seen with intracellular Ca^{2+}. In addition, Zn^{2+} transport in lobster gill cells (Sá et al., 2009), another essential metal, shares many similarities with Cu^{2+} transport in crabs (Sá and Zanotto, 2013), unlike the non-essential metal Cd^{2+} studied here.

5. Conclusions

In conclusion, the present work elucidates the mechanisms of Cd^{2+} influx, a non-essential metal, in crab gill cells. Although the Cd concentrations used here were not similar to environmental Cd^{2+}, it still represents, in a larger scale, how Cd penetrate epithelial cells and how it interacts with other ions. Cd influx appears to occur through Ca^{2+} channels, and further routes of Cd^{2+} influx are evident, that is, through the gill cell plasma membrane Cd^{2+}/Ca^{2+} (Na^+) exchanger due to a dependence of Cd^{2+} on intracellular Ca^{2+} and Na^+ (see Fig. 10 for details). Additionally, alterations in the transporter Na^+/K^+-ATPase through ouabain, augmented Cd influx. The metal influx or efflux is not affected by ATP, and the presence of a Cd^{2+}-ATPase to get rid of excess Cd, as seen in bacterial cells (Thévenod, 2010; Mielniczki-Pereira et al., 2011), appears very unlikely here.

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