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Calcium transport in gill cells of *Ucides cordatus*, a mangrove crab living in variable salinity environments

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ABSTRACT

Crustaceans show discontinuous growth and have been used as a model system for studying cellular mechanisms of calcium transport, which is the main mineral found in their exoskeleton. *Ucides cordatus*, a mangrove crab, is naturally exposed to fluctuations in calcium and salinity. To study calcium transport in this species during isosmotic conditions, dissociated gill cells were marked with fluo-3 and intracellular Ca^{2+} change was followed by adding extracellular Ca^{2+} as $CaCl_2$ (0, 0.1, 0.25, 0.50, 1.0 and 5 mM), together with different inhibitors. For control gill cells, Ca^{2+} transport followed Michaelis–Menten kinetics with $V_{max} = 0.137 \pm 0.001$ $\Delta Ca^{2+}i$ ($\mu M \times 22.10^4$ $cells^{-1} \times 180$ s^{-1}; N = 4; $r^2 = 0.99$); $K_m = 0.989 \pm 0.027$ mM. The use of different inhibitors for gill cells showed that amiloride (Na^+/Ca^{2+} exchange inhibitor) inhibited 80% of Ca^{2+} transport in gill cells (V_{max}). KB-R, an inhibitor of Ca influx in vertebrates, similarly caused a decrease in Ca^{2+} transport and verapamil (Ca^{2+} channel inhibitor) had no effect on Ca^{2+} transport, while nifedipine (another Ca^{2+} channel inhibitor) caused a 20% decrease in Ca^{2+} affinity compared to control values. Ouabain, on the other hand, caused no change in Ca^{2+} transport, while vanadate increased the concentration of intracellular calcium through inhibition of Ca^{2+} efflux probably through the plasma membrane Ca^{2+}-ATPase. Results show that transport kinetics for Ca^{2+} in these crabs under isosmotic conditions is lower compared to a hyper-regulator freshwater crab *Dilocarcinus pagei* studied earlier using fluorescent Ca^{2+} probes. These kinds of studies will help understanding the comparative mechanisms underlying the evolution of Ca transport in crabs living in different environments.

1. Introduction

The diversity of environments where crustaceans are found, whether marine, freshwater or intertidal, suggests the presence of strong regulatory mechanisms either at the organismic level or at the cellular level. Old studies on whole animals have shown that crustaceans display osmoregulatory mechanisms which are well suited to their lifestyle, being marine, freshwater or a combination of the two, such as mangrove habitats or even in more harsh environments such as intertidal habitats (see review by Mantel and Farmer, 1983). Their osmoregulatory capacity can also function for a large variation range of salinity (euryhaline) or for very narrow variations (stenohaline; see Mantel and Farmer 1983, for a comprehensive review). Crustaceans living in mangrove habitats, in particular, are known as strong hypo-hyper-regulators, like *Ucides cordatus* studied here (Martinez et al., 1999). However, no study have looked at cellular Ca^{2+} transport in isolated gill cells of mangrove crabs (see Sá et al., 2009, 2010).

U. cordatus is a typical Brazilian mangrove crab and can osmoregulate at salinities in the range of 2–33 ppt (Santos and Salomão, 1985; Martinez et al., 1999). At these salinities these crabs are able to regulate hemolymph Na tightly, suggesting a strong homeostatic mechanism during exposure to different salinities (Martinez et al., 1998). *U. cordatus* also possess powerful osmoregulatory mechanisms and are able to pump NaCl from external medium in salinities below 20 ppt and extrude NaCl in concentrated media above 26 ppt salinity (Santos and Salomão, 1985). These crabs also show functional differences in gills 5 and 6, enabling strong hypo-hyper-ion regulation (Martinez et al., 1999). Moreover, marked differences in transepithelial potential difference (TEP) and unidirectional sodium fluxes were observed in posterior gills 5 and 6 in *U. cordatus*. Flux-ratio analysis showed active uptake of Na^+ in gill 5 and active extrusion in gill 6, suggesting functional differences in adjacent gills of this crab (Martinez et al., 1999). Similarly, *Pachygrapsus marmoratus*, an intertidal crab, when exposed to 45 ppt salinity, showed increased mRNA expression of the Na/K-ATPase activity but only in a single gill pair (gill 7 out of 9), suggesting that individual gills may be specialized for hypersaline or hyposaline adaptation (Jayasundara et al., 2007). Interestingly, salt secretion during hyperosmotic exposure in crabs apparently involves mechanisms similar to vertebrates, a $Na^+/K^+/2Cl^-$ co-transport basolaterally located that

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extrudes Na^+ and Cl^- (McNamara and Faria, 2012). *Neohelice granulata* (=*Chasmagnathus granulatus*), on the other hand, is a strong hypo-hyper-regulator crab but does not seem to possess individual gill pairs that perform absorption or secretion of salts. Posterior gills transport Na^+ and Cl^- in both directions (Luquet et al., 2002).

Calcium (Ca^{2+}) plasma membrane transporters are very important for crabs because these animals undergo periodic shedding of the exoskeleton due to their molting cycle. Ca concentration varies orders of magnitude from full marine to freshwater habitats (15 mM to 1 mM) and these variations should reflect the nature of Ca^{2+} transporters in the plasma membrane in terms of affinities and maximal transport rates. Transepithelial Ca^{2+} transport in intermolt crustaceans has been studied using purified vesicle preparations of various tissues of seawater and freshwater crustaceans, mainly lobsters and crayfish (Ahearn and Franco, 1993; Flik et al., 1994; Ahearn and Zhuang, 1996; Zhuang and Ahearn, 1996; Wheatly, 1999; Wheatly et al., 2001, 2002). The Ca^{2+} transporters found at the plasma membrane of crustaceans are discussed elsewhere (see review by Ahearn et al., 1999). The technique of fluorescent markers used here, in contrast to vesicle studies, measures changes in intracellular Ca^{2+} associated with net flux of calcium across the plasma membrane of cells and is holistic in nature. This technique discloses cell homeostatic mechanisms and its interaction with intracellular organelles when cells are faced with extracellular Ca variation. This view has been explored using whole gill cells of lobsters (see recent work by Sá et al. (2009)), and using gills and hepatopancreatic cells of a freshwater crab *Dilocarcinus pagei* (Sá et al., 2010; Zanotto and Baptista, 2011, respectively).

Earlier work has shown that Ca^{2+} transport in whole gill cells in *D. pagei*, a full freshwater crab, is concentration dependent and follows a typical Michaelis–Menten kinetics (Sá et al., 2010). The transporters present at the plasma membrane are very similar to Ca^{2+} transporters found in crayfish, crabs and lobsters: hepatopancreatic vesicles of lobsters (Zhuang and Ahearn, 1996, 1998), gill vesicles of a saltwater crab *Carcinus maenas* (Flik et al., 1994; Lucu and Flik, 1999) and crayfish hepatopancreas and antennal glands (Wheatly, 1999; Wheatly et al., 2002). However, the transport rates and affinities vary between studies and should reflect the environment in which these animals live. Knowledge of these factors can help understanding evolutionary capacity for salinity adaptation in crustaceans, particularly for Ca^{2+}.

Therefore, the objective of this work was to study Ca^{2+} transport kinetics in a typical mangrove crab, *U. cordatus*, using dissociated gill cells under isosmotic conditions. A further step will be the study of Ca^{2+} transport separating anterior (respiratory) and posterior gill cells (osmoregulatory) to dissect out how Ca^{2+} transport differs among each cell type. This kind of work will elucidate physiological and biochemical parameters of Ca^{2+} transport for crabs living in diverse environments and help understanding the evolution of these transporters under freshwater and marine habitats.

2. Material and methods

2.1. Animals

For the experiments, the animals (*U. cordatus*) were collected at Itanhaém, São Paulo Coast, and brought to the University where they were acclimatized for a week. 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 ± 3 °C). The salinity at 20 ppt was chosen because at this salinity the animals are at equilibrium with outside environment.

2.2. Cellular dissociation

Four animals were used for each treatment, at intermolt stage and only males, to avoid confounding effects of stage of reproduction. The mean weight was 171.00 ± 15.26 g (n = 4).

Cellular dissociation of whole gills was performed through the enzymatic method, where 15 mL of the extraction solution (NaCl: 395 mM; KCl: 10 mM; $NaHCO_3$: 2.5 mM; NaH_2PO_4: 2.5 mM; Hepes: 3.75 mM; glucose: 1 mM; EDTA: 0.9 mM; p.H. 7.8; ~730 mOsm·Kg^{-1} H_2O) was mixed with 150 μL of trypsin enzyme (0.5%). The gills were soaked in the solution and kept in ice, for 15 min. They were then minced with scissors and taken to the shaker in bath at 30 °C, 1.8 g for 15 min. After that, the minced gills were filtered in thin mesh of 30 μm and centrifuged at 115 *g* and 5 °C for 5 min. The filtered gills were reserved for a second digestion. After the first centrifugation, some of the gill cells that sedimented (pellet) were resuspended in extraction solution and kept in ice. A second digestion was performed using the filtered and reserved gills from the first digestion.

The cellular viability was tested using the Trypan Blue method, mixing 200 μL of cells with 20 μL of Trypan Blue and put in the Neubauer chamber. After that, the viability was quantified by the visualization of the stained cells (non-viable) and translucent cells (viable) in four quadrants, with the result multiplied by 10^4.

2.3. Cellular transport

Ca^{2+} uptake was measured with isolated gill cell suspensions of the semi-terrestrial crab *U. cordatus*. The dye fluo-3, after reconstitution with DMSO, was added to the cells at a final concentration of 4.5 μM dissolved in DMSO according to the Molecular Probes catalog (see details in Sá et al. 2010). Probenecid at a concentration of 0.1 mM was added to the cells to avoid dye release and sequestration (DiVirgilio et al., 1990). The cells were equilibrated with the dye at room temperature in an orbital shaker at 100 rpm for 60 min, after preliminary trials of incubation time and dye incorporation. The preparation was then centrifuged twice at 230 *g* for 10 min to remove the extracellular dye. The same buffer was added, together with probenecid for all cell washes.

2.4. Fluorescence measurements

Spectrofluorimetric measurements with fluo-3 AM were performed by using a FLx 800 Multi-detection Microplate Reader (Bio-Tek) with excitation at 495 nm and emission at 525 nm. In each experiment, an aliquot of 200 μl of cells was placed in the fluorimeter equipped with magnetic stirring at 25 °C. Once the dye signal was stable, known concentrations of Ca^{2+} ($CaCl_2$ — 0, 0.10, 0.25, 0.50, 1.00 and 5.00 mM) were added to the 96 well assay plate (Corning) and the Ca^{2+}-induced changes in fluorescence were measured as an indication of Ca^{2+} uptake across the cell plasma membrane. Different inhibitors were used to assess Ca^{2+} transport, which were added to the assay plates together with the buffer before adding Ca^{2+} at known concentrations.

2.5. Data acquisition and collection

Data acquisition was performed through a KC junior software (Bio-Tek). Curve fitting and the production of the resulting Ca^{2+} influx kinetic constants were performed using SigmaPlot 2001 for Windows. Plots of statistical residuals were graphically inspected to check if the error values of enzyme velocity (fluorescence) against Ca^{2+} concentration were randomly distributed. For the kinetic parameters, the raw fluorescence data was tested to check when the signal was stable after addition of Ca^{2+} at each different concentration. It was found that the signal did not change after 180 s equilibration. Curve fitting of the data was performed, using a two-parameter rectangular hyperbola (Eq. (1)):

$$y = V_{max}.x/(k + x) \quad (1)$$

the variables "x" and "y" represent Ca^{2+} concentration change and fluorescence intensity, respectively. The data were plotted through

Table 1
V_{max} ($\Delta Ca^{2+}i$ μM × 22.10^4 $cells^{-1}$ × 180 s^{-1}) and K_m (mM) values for control and inhibitors (Amiloride 200 μM, N = 4; Verapamil 100 μM, N = 4; Nifedipine 100 μM, N = 4; Ouabain 100 μM, N = 4; K-Br 7943 50 μM, N = 4; Vanadate 10 mM, N = 4) for gill cells of *Ucides cordatus* (one-way ANOVA). Values are means ± s.e.m.

Gill cells *Ucides cordatus*	V_{max} ($\Delta Ca^{2+}{}_i$ μM × 22.10^4 $cells^{-1}$ × 180 s^{-1})	K_m (mM)
Control	0.137 ± 0.001	0.989 ± 0.031
Verapamil	0.139 ± 0.006	1.002 ± 0.122
Amiloride	0.028 ± 0.0211*	2.857 ± 0.024***
Nifedipine	0.014 ± 0.001	0.229 ± 0.041***
Ouabain	0.138 ± 0.012	0.799 ± 0.189
KB-R 7943	0.076 ± 0.006	0.212 ± 0.059***

* $P < 0.05$.
*** $P < 0.001$.

the best fit equation (r^2), which for most of them was around 0.98–0.99.

A calibration curve to transform arbitrary fluorescence units to intracellular Ca^{2+} concentration was performed. For the calibration curve, a method was adapted based on Merrit et al. (1990). At the end of the experiments, cells that were not exposed to extracelular Ca^{2+} were lysed by adding 5 μL of Triton X-100 20%. To obtain minimum fluorescence (Fmin), 5 μL of EGTA 200 mM (Sigma-Aldrich) was added twice. In a different batch of cells, 5 μL of Triton X-100 20% was added, and after 300 s, Ca^{2+} at 25 mM was added to get the maximum fluorescence (Fmax). Intracellular Ca^{2+} for every experiment was calculated according to equation (Eq. (2)):

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

$[Ca^{2+}]i$: intracelular free Ca^{2+} concentration (μM)Kd: dissociation constant of fluo-3 (0.39)F: actual fluorescence measuredFmin: minimum fluorescenceFmax: maximum fluorescence.

2.6. Statistical analysis

The kinetic parameters (V_{max} and K_m) for control and each inhibitor (intracellular Ca^{2+} change μM × 22 × 10^4 cells × 180 s^{-1}) were compared through a one-way ANOVA. Statistical difference was accepted for $P < 0.05$. The errors were random between different substrate concentrations, making it valid to fit a hyperbola to the data (Henderson, 1992).

3. Results

For control gill cells, Ca^{2+} transport followed Michaelis–Menten kinetics (Table 1): V_{max} = 0.137 ± 0.001 $\Delta Ca^{2+}i$ (μM × 22.10^4 $cells^{-1}$ × 180 s^{-1}) and K_m = 0.989 ± 0.027 mM, r^2 = 0.99 (N = 4). Amiloride decreased Ca^{2+} transport around 80% compared to control (one-way ANOVA, Fig. 1A/Table 1) showing a V_{max} = 0.028 ± 0.021 $\Delta Ca^{2+}i$ (μM × 22.10^4 $cells^{-1}$ × 180 s^{-1}) and a K_m = 2.857 ± 0.024 mM***. Verapamil was not different from control (Fig. 1B/Table 1) with V_{max} = 0.131 ± 0.007 $\Delta Ca^{2+}i$ (μM × 22.10^4 $cells^{-1}$ × 180 s^{-1}) and K_m = 0.765 ± 0.116 mM. Nifedipine showed values of V_{max} = 0.067 ± 0.007 $\Delta Ca^{2+}i$ (μM × 22.10^4 $cells^{-1}$ × 180 s^{-1}) and K_m = 0.231 ± 0.094 mM***, where K_m decreased around 23% compared to control (Fig. 1C/Table 1). Ouabain did not cause any change in intracellular Ca^{2+} in relation to control (Fig. 1D/Table 2) showing a V_{max} = 0.138 ± 0.012 $\Delta Ca^{2+}i$ (μM × 22.10^4 $cells^{-1}$ × 180 s^{-1}) and K_m = 0.799 ± 0.189 mM.

KB-R 7943 a Ca^{2+} influx inhibitor reduced V_{max} compared to control, but was not statistically significant, and K_m decreased around 20% (one-way ANOVA, Fig. 2A/Table 1) showing V_{max} = 0.076 ± 0.006 $\Delta Ca^{2+}i$ (μM × 22.10^4 $cells^{-1}$ × 180 s^{-1}) and K_m = 0.212 ± 0.059 mM***. Vanadate, on the other hand, increased intracellular Ca^{2+} in gill cells, and because maximum fluorescence (Fmax) was lower than intracellular fluorescence change due to vanadate, calibration resulted in negative intracellular calcium (Fig. 2B). Additionally

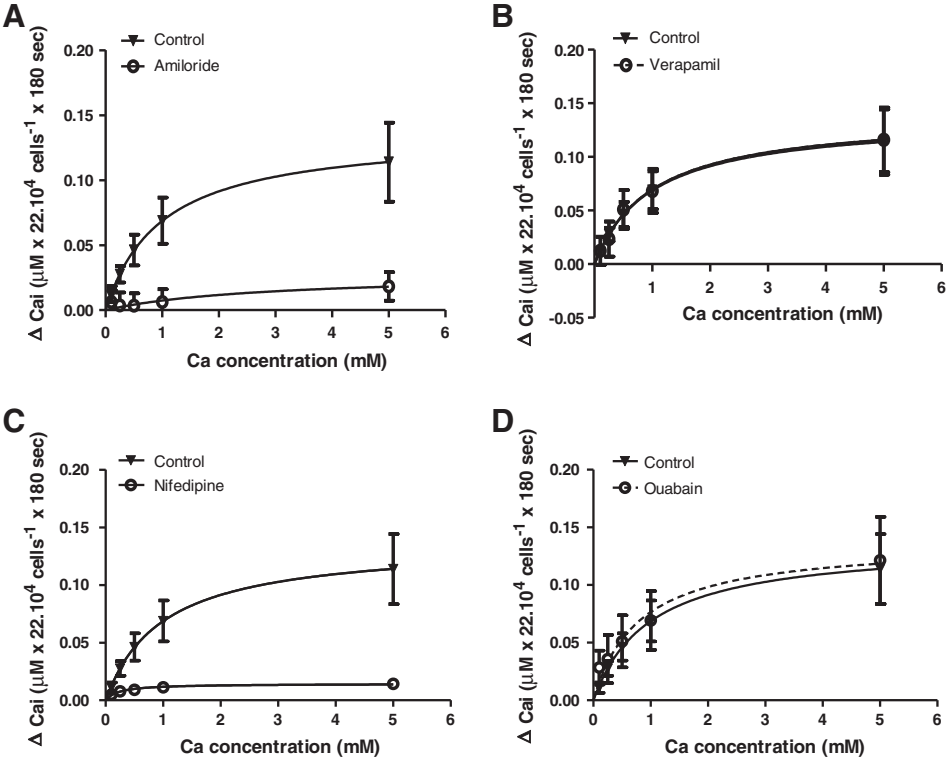

Fig. 1. Intracellular Ca^{2+} change (ΔCa^{2+} × 22 × 10^4 $cells^{-1}$ × 180 s^{-1}) (μM) in relation to different Ca concentrations (mM) for control (N = 4) and different inhibitors. A. Amiloride (200 μM), N = 4; B. Verapamil (100 μM), N = 4, C. Nifedipine (100 μM), N = 4; D. Ouabain (100 μM, N = 4). Means ± s.e.m. Statistical analyses are shown in Table 1.

Table 2
Kinetic parameters (mean ± s.e.m.; N = 4) for Ca^{2+} transporters for a freshwater crab (*Dilocarcinus pagei*; Sá et al., 2010) and a mangrove crab *Ucides cordatus*, living in variable salinity environments.

Dilocarcinus pagei			*Ucides cordatus*	
Gill cells	K_m (mM)	V_{max} ($\Delta Ca^{2+}{}_i$ μM × 22.10^4 cells^{-1} × 180 s^{-1})	K_m (mM)	V_{max} ($\Delta Ca^{2+}{}_i$ μM × 22.10^4 cells^{-1} × 180 s^{-1})
	0.42 ± 0.07	0.50 ± 0.02	0.99 ± 0.03	0.14 ± 0.00

Fig. 3B shows variation in fluorescence in relation to initial fluorescence to show Ca^{2+} accumulation inside the cells. The data is shown as arbitrary units of fluorescence change.

4. Discussion

We showed here that Ca^{2+} transport in *U. cordatus* gill cells, a euryhaline crab, follows Michaelis–Menten kinetics as seen for other crustaceans (Ahearn and Franco, 1993; Flik et al., 1994; Ahearn and Zhuang, 1996; Zhuang and Ahearn, 1996; Wheatly, 1999; Wheatly et al., 2001, 2002; Sá et al., 2009, 2010). Interestingly, a freshwater crab endemic to Brazil (*D. pagei;* Sá et al., 2010), showed Ca^{2+} transport kinetic parameters which were 2.5× of higher affinity (lower K_m) and 4× higher maximum velocities (V_{max}). This implies that transport rates for these freshwater crabs in their natural environment are more effective for transporting Ca^{2+} compared to the mangrove crab and reinforce that low Ca^{2+} concentration found in freshwater habitats is limiting for freshwater crabs. However, because intracellular Ca^{2+} is calculated indirectly and the values calculated are an approximation, the Ca^{2+} values for both crabs show some slight differences. Although both crabs are not phylogenetically related for a more effective comparison between them, results suggest that environmental constraints can shape the functionality of the plasma membrane transporters so that they are regulated close to levels of ions normally encountered in their environment.

As stated by Neufeld and Cameron (1993): 'Despite the importance of calcium in the life cycle of crustaceans, there is a notable lack of information on the cellular mechanism of active transport and it is clear that studies of calcium regulation in crustaceans should concentrate on this aspect'. In their work they mentioned that the mechanisms responsible for calcium regulation in crustaceans should include changes in the epithelial electrochemical gradient, permeability and active transport and are all important for Ca^{2+} uptake. Studying the rate of whole animal calcium uptake at various external Ca^{2+} concentrations, and assuming that it is roughly described by saturation kinetics, they found that organisms inhabiting low salinities have systems with higher affinities for Ca^{2+} (Neufeld and Cameron, 1993). Interestingly, the induction of augmented calcium transport capacity in fish acclimated to low calcium media in contrast to high calcium media has been demonstrated in the gills of rainbow trout (Perry and Wood, 1985), killfish (Mayer-Gostan et al., 1983), tilapia (Flik et al., 1986) and in tilapia larvae (Hwang et al., 1996). For the last work, this was seen through a 50% decrease in K_m and 25% increase in Jmax related to 40 times difference in environmental calcium.

An interspecific study with fiddler crabs, *Uca,* from the Gulf Coast of Mexico showed that specimens from either freshwater, brackish or hypersaline microhabitats showed physiological capabilities which were consistent with their habitat ion content and the limits in their osmoregulatory capabilities (Thurman, 2002). Another study comparing interspecific differences among stream insects related to cadmium (Cd) uptake showed that Cd transport in different species is related to increased number of Cd transporters (higher V_{max}) and not by changes in the affinity of the transporter (K_m). They did not attempt, however, to relate physiological parameters to differences in their microenvironment in relation to Cd, all of them being freshwater aquatic insects (Buchwalter and Luoma, 2005).

Comparing the effects of different inhibitors on Ca^{2+} transport rates, we saw a strong effect of vanadate, an inhibitor of Ca^{2+}-ATPase efflux, and its effects have already been reported in a number of works with cells or vesicles of crustaceans (Ahearn and Franco, 1993; Flik et al., 1994; Ahearn and Zhuang, 1996; Zhuang and Ahearn, 1996; Wheatly, 1999; Wheatly et al., 2001, 2002; Sá et al., 2009, 2010; Zanotto and Baptista, 2011). For plasma membrane Ca^{2+} channel inhibitors, we saw an effect of nifedipine and no effect of verapamil. Both are L-type Ca^{2+} channel inhibitors, and they inhibited epithelial Ca^{2+} channels in lobster (Ahearn and Zhuang, 1996; Sá et al., 2009) and shrimp (Zilli et al., 2003), but not in rainbow trout (Gagnon et al., 2007). Here, amiloride caused an 80% inhibition of net Ca^{2+} transport, decreasing V_{max} by 80% and increasing K_m by 40%. This is a known inhibitor of Na^+/Ca^+ exchange (NCX) and Na^+/H^+ exchange (Shetlar and Towle, 1989; Ahearn and Franco, 1993). KB-R, responsible for inhibiting Na influx in vertebrates (Pilitsis et al., 2001), showed an inhibition of Ca^{2+} transport, not seen in earlier work on intracellular Ca^{2+} net flux in the freshwater crab, *D. pagei* (Sá et al., 2010). However, a decreased Ca entry is seen in work with rat cerebral cortex (Pilitsis et al., 2001).

We did not see any effect of ouabain in the results reported here, and ouabain is known to inhibit Na^+/K^+-ATPase. It appears not involved directly with Ca^{2+} transport in these crabs, although it is responsible for generating a Na^+ gradient that can drive an exchanger of Ca^{2+} in crustaceans. Probably a mangrove crab that lives in a fluctuating salinity environment is able to cope with Na changes and the study reported here was performed in mixed gill cells. Earlier studies

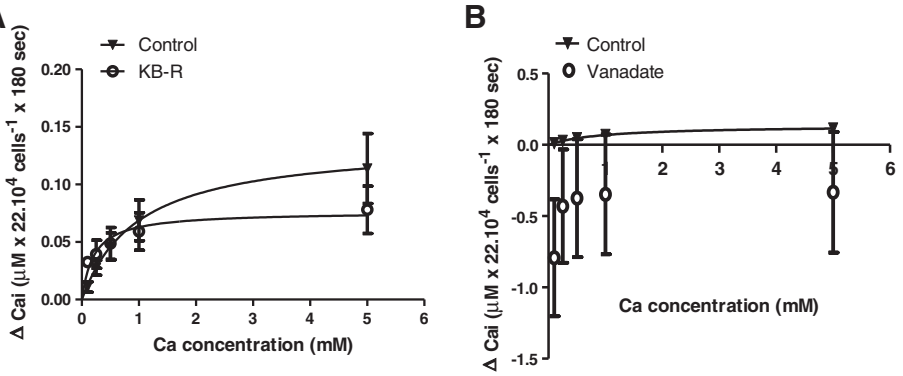

Fig. 2. Intracellular Ca^{2+} change ($\Delta Ca^{2+} \times 22 \times 10^4$ cells^{-1} × 180 s^{-1}) (μM) in relation to different Ca concentrations (mM) for control (N = 4) and different inhibitors. A. K-Br 7943 (50 μM), N = 4, B. Vanadate (10 mM), N = 4. Means ± s.e.m. Statistical analyses are shown in Table 1.

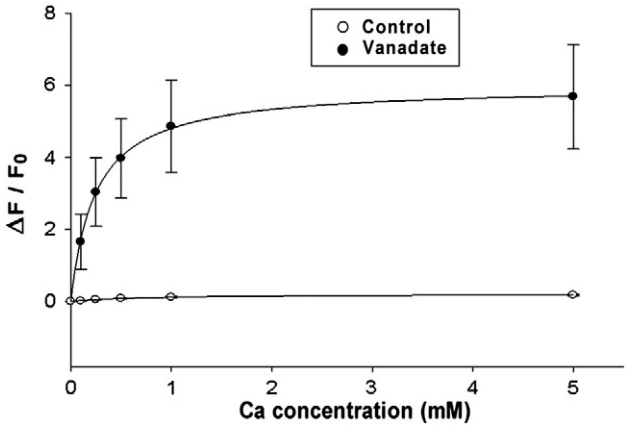

Fig. 3. Fluorescence change in relation to initial fluorescence (ΔF/Fo) as arbitrary units in relation to different Ca concentrations (mM) for control and vanadate treated cells (N = 4).

suggested that there are variations in Na influx and efflux in different gill filaments (Martinez et al., 1999).

Interestingly, *U. cordatus* maintains sodium hemolymph concentrations in the relatively narrow range from 300 to 390 mM in salinities up to 34‰, in spite of environmental salinity fluctuations, so that in more concentrated media *U. cordatus* is a strong hyporegulator (Santos and Salomão, 1985). In contrast to a freshwater crab, that is constantly accumulating ions from the dilute media in which they live, a functional strategy for Ca^{2+} uptake seems to reflect in the Ca transport kinetic parameters, as well as the effects of different inhibitors, which were more prevalent in a freshwater crab than seen here for *U. cordatus* (Sá et al., 2010).

In conclusion, crabs living in mangrove habitats, such as U. *cordatus*, are able to pump Ca with lower maximum velocities and lower affinity, compared to a freshwater crab *D. pagei* (Sá et al., 2010). Therefore, it seems that crustaceans displaying hyper-hypo ion regulation allow a greater transporter plasticity compared to a freshwater crab which is constantly hyper-regulating, and suggests avenues for further work by looking at Ca^{2+} transport kinetics of U. *cordatus* exposed to hypo or hyperosmotic media, a condition that mangrove crabs periodically encounter in their daily activities.

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