Bull. Environ. Contam. Toxicol. (1998) 61:629-636

Bulletin of Environmental Contamination and Toxicology

Effects of Cadmium on Gill Na,K-ATPase of the Estuarine Crab *Chasmagnathus granulata* (Decapoda, Grapsidae) During Postmolt: *In Vivo* and *In Vitro* Studies

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Received: 20 January 1998/Accepted: 5 October 1998

Adult and juvenile crabs of the species *Chasmagnathus granulata* live on the muddy flats of Samborombón bay, the external sector of the *Río de la Plata* estuary (Argentina), where they constitute a source of food for several fish species of commercial value. As a correlate to the fluctuating salinity of the environment, these estuarine crabs proved to be a strong ionic regulator species (Bromberg et al. 1995). This is mainly because of the gill Na,K-ATPase, whose activity allows a steady-state distribution of ions across the basolateral membrane to be maintained (Towle 1990).

Size growth of crabs implies molting. During this process, the resorption of the old exoskeleton takes place during the premolt stage; whereas the new cuticle is formed below (Passano 1960). During postmolt, on the other hand, the uptake of calcium into the organism is required for carapace calcification (Cameron 1989). Calcium influx is mainly driven by transport systems located in the gills. These include primary active transport through calcium ATPases and/or secondary active transport using the electrochemical gradient generated by Na,K-ATPase activity (Roer 1980; Towle and Mangum 1985).

In the estuary of *Río de la Plata,* cadmium was detected above the permissible levels in 82 % of the water samples analyzed (Comisión Administradora del Río de la Plata 1990). This might put crab osmoregulation at risk, since cadmium has been reported to inhibit Na,K-ATPase activity in homogenates and subcellular fractions from different tissues (Kinne-Saffran et al. 1993; Lijnen et al. 1991). Moreover changes in gill ultrastructure of crustaceans occurred during chronic exposure of individuals to sublethal concentrations of cadmium (Papathanassiou 1985). Thus, this study seeks to evaluate the impact of cadmium on branchial Na,K-ATPase activity from postmolt crabs. This work represents a first step in a global research on the effect of cadmium on the molting process of the crab *C. granulata.*

MATERIALS AND METHODS

Adult males of *Chasmagnathus granulata* (carapace width 28.0 ± 0.7 mm) were collected at the beach of *Faro San Antonio,* the southern end of

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Samborombón Bay (36°18'S and 56°48'W) during 1995-1996. Water temperature in the field ranges from 11.5°C in winter to 25.5°C in summer (Comisión Administradora del Río de la Plata 1990). Adult individuals follow an annual molting cycle, with crabs molting during April.

Once in the laboratory, crabs were acclimated for at least two weeks at 20 ± 1°C, to a photoperiod of 12L:12D and 30‰ water salinity. Twice a week, crabs were fed with chicken liver and rabbit food *ad libitum,* and all test solutions were renewed according to Rodríguez et al. (1992). In some experiments, molting was induced in crabs by eyestalk ablation. This was made at the base of their optic peduncles with fine scissors, and cauterized with heat. Eyestalk-ablated crabs start molting 20 days after ablation. Survival of ablated crabs to molting ranged from 80 to 100 %, from which 50 to 85 % remained alive for 18 days when individuals are sacrificed.

A static toxicological bioassay (American Public Health Association et al. 1992) was carried out to evaluate both the lethal and sublethal effects of cadmium. One day after crabs molted (stage A_2, according to Passano 1960) they were placed in individual 2 L glass jars containing 350 mL of 30 ‰ saline water and different cadmium concentrations (supplied as $CdCl_2$). Crabs were isosmotic to the salinity employed (Bromberg et al. 1995). Preliminary experiments using intact crabs proved that branchial Na,K-ATPase activity from 18 days postmolt crabs was 2.35-fold higher than that of intermolt individuals. Therefore, a postmolt period of 18 days (corresponding to stage B_2 of setal development) was selected for running all bioassays.

In order to determine the lethal concentration which killed 50 % of the crabs (LC_{50}, one day after molting intact crabs were exposed for 18 days to 0, 0.05, 0.10, 0.25 and 0. 50 mg/L Cd^{2+}. Mortality was recorded every 24 hr. A similar protocol was employed when analyzing the sublethal effect of cadmium. After 18 days of exposure, both eyestalk-ablated and intact postmolt crabs were sacrificed by quickly removing their carapace at 0-4 °C. Then, the 6^{th}, 7^{th} and 8^{th} gill pairs of each crab were excised, pooled into one sample and either immediately used (for oxygen consumption measurements, see below) or kept at -70°C until use (for measurements of apparent maximal Na,K-ATPase activity, see below).

The rate of oxygen consumption (VO_2) was measured in gill fragments isolated from eyestalk-ablated crabs that had been exposed to 0 (controls) or 0.25 mg/L of cadmium. Gill fragments were obtained by excising the three posterior pair of gills from each crab and subsequently cutting each gill filament at the middle. A closed-chamber respirometer containing a polarographic oxygen sensor was used. The electrode was calibrated in 30 ‰ saline water saturated with air. One millimolar ouabain was added after the cells had established a steady-state rate of respiration (about 5 min), and the change in respiration rate was monitored for 25 min. The difference in VO_2 before and after addition of ouabain was defined as ouabain-sensitive VO_2

(osVO_2), an estimate of Na,K-ATPase activity under physiological conditions (Schwarzbaum et al. 1992).

Preliminary experiments showed that neutralized solution of sodium cyanide (final concentration 2 mM) added before ending the run, suppressed more than 90 % of total oxygen consumption. Under the experimental conditions, VO_2 was constant for over 40 min. All measurements were performed under constant stirring at 25°C. Once the experiments were finished, gill fragments were kept frozen and homogeneized thereafter to determine proteins by the method of Lowry et al. (1951). The rate of oxygen consumption was expressed in nmol O_2/mg prot/min.

Apparent maximal Na,K-ATPase activity (Vmax) was assayed in gill homogenates and in gill membranes at 25°C by following the release of [γ-^{32}P]Pi from [γ-^{32}P]ATP (Richards et al. 1978). Gill homogenates were prepared with a Dounce homogenizer (2.5 % w/v) in a medium containing (in mM): 250 sucrose, 0.1 EDTA, 1 phenylmethylsulfonyl fluoride and 50 Imidazole-HCI (pH=7.4, 25°C). Homogenates were used for measuring enzyme activity as well as for preparing membranes as follows. Homogenate suspensions were centrifuged 5 min at 10,000 g. The resulting pellets (called here "membranes") were resuspended in 150 mM NaCl and 25 mM Imidazole-HCI (pH = 7.4, 25°C). Aliquots of homogenates and membranes were separated for protein determination by the method of Lowry et al. (1951).

The *in vivo* effect of cadmium on Vmax was evaluated in both intact and eyestalk-ablated crabs that had been exposed to varying levels of cadmium. Enzyme activity in homogenates was assayed by incubating an aliquot of gill homogenate (approx. 220 µg protein) for up to 45 min in assay medium (3 mM ATP, 1 µCi/mL [γ-^{32}P]ATP, 150 mM NaCI, 20 mM KCI, 3 mM $MgCl_2$, 0.2 mM EDTA, 25 mM Imidazole-HCI, pH=7.4 at 25°C). In all experiments, blanks for enzyme activity were determined in the presence of 1 mM ouabain. Specific enzyme activities are given in nmol Pi/mg protein/min.

The *in vitro* effect of cadmium on Vmax was analyzed in 18 days postmolt, eyestalk-ablated crabs unexposed to cadmium, by preincubating membranes for 40 min at different Cd^{2+} concentrations (from 0 to 100 mg/L of Cd^{2+}) before being used. Enzyme activity was measured by incubating about 120 µg of sample protein in assay medium B (similar to A, except that EDTA was absent). Care was taken to add sufficient Cd^{2+} in medium B so as to maintain the same [Cd^{2+}] as that used to preincubate the samples. In order to evaluate if the effect of cadmium on Na,K-ATPase activity was reversible, in separate experiments gill membranes were preincubated with 0 or 100 mg/L Cd^{2+} as above, and then Vmax was measured in the presence of 0 or 2 mM EDTA.

For statistical analysis a Probit test (Finney 1971) was used to estimate the LC_{50} and its 95% confidence limits, with Abbots correction for control

mortality. One-way and two-way ANOVA (when appropriate) were used to evaluate differences among mean values. In case of Vmax from gill membranes, non-linear regression was used to fit the following hyperbolic function to the data:

$$vi = \frac{Vm * IC_{50}}{IC_{50} + [Cd^{2+}]} \qquad Equation\ 1$$

where vi stands for Na,K-ATPase activity measured at different cadmium concentrations [$C\ d^{2+}$]. *Vm* and IC_{50} are fitting parameters of the equation as follows: *Vm* is the estimated value of *vi* at [$C\ d^{2+}$] = 0, whereas IC_{50} was estimated as the cadmium concentration yielding 50 % of *Vm.*

RESULTS AND DISCUSSION

Postmolt intact crabs showed a LC_{50} of 0.44 mg/L Cd_{2+} (95 % confidence interval: 0.22-0.88 mg/L Cd_{2+}). Accordingly, 0.10 and 0.25 mg/L Cd^{2+} were selected as valid concentrations for the remaining sublethal bioassays. Surviving crabs exposed to 0.5 mg/L Cd^{2+} were not considered, because of the strong selection towards the more resistant specimens at that concentration.

Figure 1 shows a typical measurement of the rate of oxygen consumption (VO_2) in gill fragments, from which osVO_2 was calculated.

In Table 1 it can be seen that exposure of crabs to cadmium neither affected VO_2 nor osVO_2 (P>0.05). Independently of the presence of cadmium, osVO_2 amounted to 50 % of VO_2.

Table 2 shows the effects of cadmium exposure of both intact and eyestalk-ablated postmolt crabs on Vmax. Cadmium seems to affect Na,K-ATPase activity in a dose dependent way, but such changes were not significant (P>0.05). Moreover, no significant differences were detected (P>0.05) between intact and eyestalk-ablated crabs.

In Figure 2, the effect of preincubating gill membranes *in vitro* with cadmium is evaluated. Na,K-ATPase activity was an hyperbolic function of [Cd^{2+}], with no apparent threshold for the effect of the cation on enzyme activity. The value of IC_{50} was 21.6 ± 3.3 mg/L Cd^{2+} and V_m was 32.9 ± 3.5 nmol Pi/mg prot/min.

Table 1. Effects of cadmium on gill VO_2 and osVO_2 from gill fragments of eyestalk-ablated postmolt crabs. Results are means (nmol O_2/mg prot/min) ± SE. (n= number of crabs).

Nominal [Cd^{2+}] (mg/L)	VO_2	osVO_2	n
0	24.1 ± 3.2	12.5 ± 2.1	9
0.25	22.1 ± 2.4	10.5 ± 1.1	10

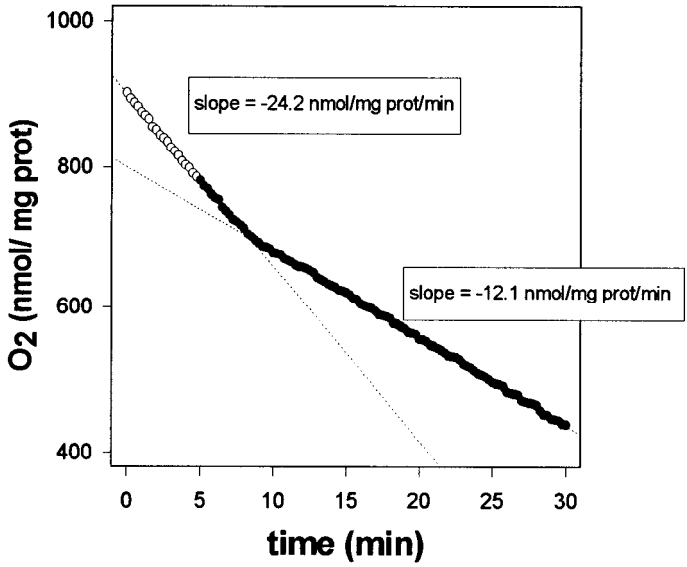

Figure 1. Oxygen content vs time (min) from freshly excised gill fragments. The slope of the curves represents the rate of oxygen consumption in the absence (O) or presence (●) of 1 mM ouabain. The difference in slopes represents $osVO_2$. Data are normalized to 1 mg of gill protein.

Irrespective of the presence of 2 mM EDTA in assay medium B, preincubation of membranes with 100 mg/L Cd^{2+} led to about 97% inhibition of Na,K-ATPase (n = 4). The lack of effect of EDTA was also corroborated when cadmium was absent in the preincubation medium, since after four independent experiments Na,K-ATPase activity amounted to 62.1 ± 12.5 nmol Pi/mg prot/min with EDTA, a value that is not signifcantly different (P>0.05) from 72.0 ± 11.8 nmol Pi/mg prot/min in the absence of EDTA.

Table 2. Effect of cadmium on apparent maximal Na,K-ATPase activity from gill homogenates of intact and eyestalk-ablated postmolt crabs. Results are means (nmol Pi/mg prot/min) ± SE. (n= number of crabs).

Nominal [Cd^{2+}] (mg/L)	App. maximal Na,K-ATPase activity			
	intact crabs	n	eyestalk-ablated crabs	N
0	30.6 ± 4.8	7	28.0 ± 2.8	10
0.10	34.4 ± 3.7	8	24.5 ± 1.8	11
0.25	23.1 ± 3.0	9	23.3 ± 1.8	13

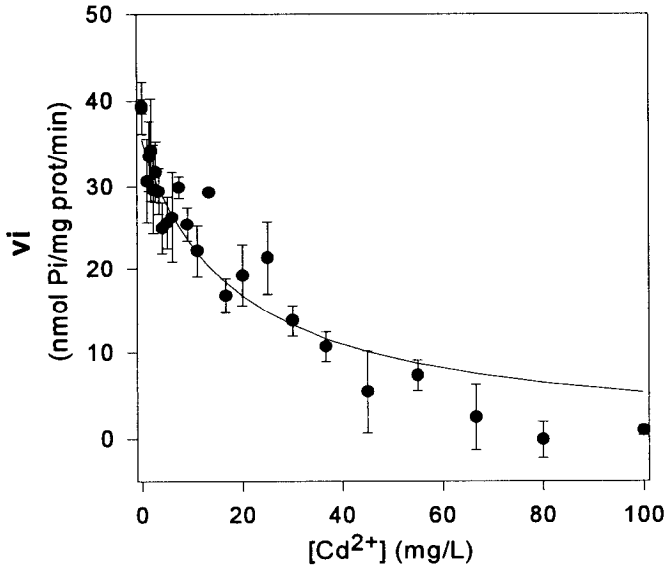

Figure 2. Na,K-ATPase activity (vi) vs nominal [Cd^{2+}] *in vitro,* in gills membranes from postmolt eyestalk-ablated crabs. Values are means (nmol Pi/mg prot/min) ± SE. The solid line represents the fit of a hyperbolic function to the data. (number of independent experiments = 4).

The present study clearly indicates that the Na,K-ATPase from gills of *C. granulata* is sensitive to Cd^{2+} inhibition *in vitro.* The IC_{50} for Cd^{2+} inhibition of the enzyme was reached at 21.6 ± 3.3 mg/L Cd^{2+} (Figure 2) a value in the same order of that reported in different vertebrate systems (Chetty et al. 1992; Kinne-Saffran et al. 1993).

Since the measurement of Na,K-ATPase activity was performed under optimal conditions of substrate and modulators, it represents the maximal capacity of the enzyme to hydrolyze ATP at 30 ‰ salinity (analog to Vm of Equation 1). Thus, the decrease of Vmax observed in Figure 2 results from cadmium inactivating the enzyme, probably by either complexing SH groups in active sites of the enzymes or by interfering with the formation of the enzyme substrate complex (Chetty et al. 1992). Furthermore, a chelator of divalent cations such as EDTA was ineffective in reversing the Cd^{2+} inhibition of Na,K-ATPase activity, so that the toxic effect of cadmium is irreversible (at least by EDTA).

The direct inhibitory action of Cd^{2+} on gill Na,K-ATPase is probably not specific to that enzyme, since divalent heavy metals exert their toxicity at the cellular level by several mechanisms (Lijnen et al. 1991). This is why when

analyzing the *in vivo* effects of cadmium, it can be seen that the LC_{50} for crabs is about 1/50 of the *in vitro* IC_{50} for gill Na,K-ATPase activity, so that much lower concentrations of cadmium are sufficient to strongly affect crab survival, when compared to the *in vitro* inhibition of Na,K-ATPase activity (Figure 2).

However, results of Table 2 show that *in vivo* exposure of crabs to sublethal concentrations of cadmium have no significant effect on Vmax, so that the capacity of the gills to drive the hydrolysis of ATP catalyzed by that enzyme remained unaffected. These results were obtained with both intact and eyestalk-ablated crabs, confirming that no effect of ablation is evident on enzyme activity.

Nevertheless, it could be argued that although the toxic action of cadmium on Na,K-ATPase does not affect Vmax, an inhibitory effect could have occurred *in vivo* under submaximal conditions. To further test this hypothesis, we also measured the $osVO_2$ of isolated gills from control and cadmium exposed crabs. When fragments from freshly excised gills are used, Na,K-ATPase functions with the physiological concentrations of substrate and modulators. This method has been successfully used to measure the physiological activity of the Na,K-ATPase in many tissues (see Schwarzbaum et al. 1992). According to our results, both the VO_2 (an estimate of the rate of ATP produced and O_2 consumed by oxidative metabolism) and $osVO_2$ (an estimate of Na,K-ATPase activity) were not affected by cadmium, so that the effective cadmium concentration achieved under these conditions do not compromise Na,K-ATPase activity or oxidative metabolism.

We conclude that the lethal effect of cadmium on *C. granulata* does not seem to be associated with gill Na,K-ATPase inhibition. The reasons for the inability of Cd^{2+} to decrease the *in vivo* activity of the Na,K-ATPase may be found in the binding of the cation to intracellular proteins, its sequestration in subcellular organelles, or both (Rainbow 1988; Sparla and Overnell 1990; Viarengo and Nott 1993).

Acknowledgments. This work was supported by a grant from the University of Buenos Aires (1994-97 scientific program). EM Rodríguez and PJ Schwarzbaum are career researchers from CONICET.

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