Cadmium induces both pyruvate kinase and Na+/H+ exchanger activity through protein kinase C mediated signal transduction, in isolated digestive gland cells of *Mytilus galloprovincialis* (L.)

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Summary

The present study investigates the transduction pathway mediated by cadmium in isolated digestive gland cells of mussel *Mytilus galloprovincialis*. The effects of cadmium treatment on a key glycolytic enzyme, pyruvate kinase (PK), and on Na+/H+ exchanger activity were examined. Cadmium (50 μmol l^{-1}) caused a significant elevation of intracellular pH (pHi) and a rise (176%) of Na influx relative to control values. The amiloride analogue, EIPA (20 nmol l^{-1}), a Na+/H+ exchanger blocker, together with cadmium, significantly reduced the effect of treatment by cadmium alone on both Na+ influx and pHi. In addition, PK activity was significantly increased after treatment with cadmium. PK activity was inhibited after treatment of cells with amiloride or EIPA together with cadmium. Moreover, phorbol-ester (PMA), a potent activator of protein kinase C (PKC), caused a significant rise in both pHi and PK activity, while staurosporine or calphostin C reversed both events. Adrenaline, isoprenaline and phenylephrine alone or together with cadmium also significantly increased the pHi and PK activity of isolated digestive gland cells. The latter effectors in combination with cadmium showed a synergistic effect on pHi and PK. These responses seem to be blocked by propranolol, metoprolol and prazosin. Our findings suggest a hormone-like effect of cadmium on digestive gland cells. The signal transduction pathway induced by cadmium involves the stimulation of PK, PKC and Na+/H+ exchanger in isolated digestive gland cells of *Mytilus galloprovincialis*.

Key words: mussel, *Mytilus galloprovincialis*, digestive gland, Na+/H+ exchanger, cadmium, pyruvate kinase, signal transduction.

Introduction

Heavy metals are ranked as highly toxic substances and are among the major contaminants of the marine environment. Cadmium represents one of the major aquatic pollutants. Cadmium is taken up by organisms, accumulates in the cytosol by forming metal–ligand complexes (Belcheva, 1988) and manifests its toxicity by affecting some enzymes of the antioxidant system (Bompart et al., 1991; Strubelt et al., 1996), and enzymes and metabolites of glucose metabolism (Soengas et al., 1996) and fatty acid biosynthesis (Steibert and Kokot, 1980). In addition, cadmium induces signal transduction cascades such as PKC, tyrosine kinase and casein kinase II (Adams et al., 2002). Cadmium also influences membrane functioning (Hussain et al., 1985; Kunimoto and Miura, 1988; Gupta and Chandra, 1991; Chelomin and Tyurin, 1992; Maloney et al., 1996).

Although cellular metabolism has been investigated in marine species (Almeida et al., 2001), the consequences of cadmium effects have been little studied (Watjen et al., 2001; Fabbri et al., 2003). There are a few data concerning the role of Hg, Cu, Zn and Cd on the regulation of metabolic rates in the cell and specifically on key glycolytic enzymes (Lai and Blass, 1984; Canesi et al., 1998, 2001; Huang and Tao, 2001; Viselina and Lukyanova, 2000). Nikinmaa (1983), Borgese et al. (1987) and Kaloyianni et al. (2000) reported that the activation of pyruvate kinase (PK), a key glycolytic enzyme, observed after adrenergic stimulation was related to Na+/H+ exchanger activity in vertebrate red cells. It was therefore of interest to investigate the effect of cadmium on Na+/H+ exchanger and PK activities in mussel cells.

The Na+/H+ exchanger is a membrane system involved in the coupled exchange of Na+ with H+ in a variety of eukaryotic cells. This system seems to be a major regulating element of intracellular pH (pHi) (Moolenaar et al., 1983; Bianchini and Pouyssegur, 1994) and cell volume control (Cala, 1983a,b). The activity of this cation exchanger is regulated by growth factors, hormones (Ceolotto et al., 1997; Rutherford et al., 1997; Kaloyianni et al., 1997, 2000, 2001; Sauvage et al., 2000; Konstantinou-Tegou et al., 2001; Bourikas et al., 2003), second messengers (Tse et al., 1993), neurotransmitters (Zange et al., 1990) and osmotic stress (Grinstein et al., 1992). This antiport is involved in signal transduction since intracellular pH changes have been closely related to signaling (Incerpi et

al., 1996). Furthermore, other transporters such as the Na^+-K^+-$2Cl^-$ cotransporter are related to cell signaling (Flatman, 2002). In mussel digestive gland cells no Na^+/H^+ exchanger function has been reported. The effect of heavy metals on Na^+/H^+ exchanger function has only recently been mentioned (Villela et al., 1999). To our knowledge, in invertebrate cells, Na^+/H^+ exchanger function has been reported (Zange et al., 1990; Willoughby et al., 1999; Giannakou and Dow, 2001) but not in relation to heavy metals.

Pyruvate kinase (PK) is one of the key enzymes of the glycolytic pathway (Storey and Storey, 1990) and catalyzes the conversion of phosphoenolpyruvate to pyruvate in one of the two ATP-producing steps in glycolysis. In mollusc tissues, pyruvate kinase activity is controlled by FDP, ATP and L-alanine, as well as ATP, cAMP and Ca^{2+} (Holwerda et al., 1989). In addition, molluscan pyruvate kinase is regulated by the phosphorylation/dephosphorylation process (Marie et al., 1979; Siebenaller 1979; Kiener and Westhead, 1980; Nakashima et al., 1982; Hakim et al., 1984; Holwerda et al., 1989; Carillo et al., 2001). Moreover, there are many physiological factors such as temperature, anaerobic conditions, tidal cycle, seasonal and ion concentration variations that can alter pyruvate kinase activity in molluscs (Cortesi and Carpene, 1981; Carpene et al., 1984; Hakim et al., 1984; Cortesi et al., 1985; Ibarguren et al., 1990; Simpfendorfer et al., 1997).

Recent data indicate that zinc and cadmium are involved in modulating signal transduction pathways (Hansson, 1996; Risso-de Faverney et al., 2001; Watjen et al., 2001). In mussels, there are reports on the effect of heavy metals on cell signaling (Canesi et al., 1998, 2000a, 2001; Viarengo et al., 2000), so we investigated the role of the toxic metal cadmium in isolated digestive gland cells of *Mytilus galloprovincialis*.

Since the Na^+/H^+ exchanger is affected by hormones (Borgese et al., 1987) and PK is also hormonally regulated in fish cells (Nikinmaa, 1983) it was of interest to investigate if the two proteins are influenced by common signal transduction pathways. The present study investigates the possible effects of cadmium on the Na^+/H^+ exchanger, as well as on pyruvate kinase (PK) activity in digestive gland cells of the mussel *Mytilus galloprovincialis*. Both PK and Na^+/H^+ exchanger were studied in relation to β_1, β_2 and α_1-adrenergic receptor stimulation and protein kinase C (PKC) activation, in order to clarify the transduction pathway induced by cadmium in the isolated digestive gland cells of *Mytilus galloprovincialis*.

Materials and methods
Animals
Mussels *Mytilus galloprovincialis* (L.) were collected from Kalamaria, located at the east side of Thermaikos Gulf (Thessaloniki, Greece). Results from our laboratory have shown that mussels collected from the same area accumulated cadmium, at a concentration of 27–$52\ ng\ g^{-1}$ dry mass of digestive gland, which is lower than the minimum levels

reported in mussels collected from other sites of Thermaikos Gulf (unpublished results from our laboratory). The site of mussel sampling is a less polluted site in an ecosystem with low levels of cadmium contamination, and therefore any cadmium accumulated does not interfere with the biochemical function of the mussels' tissues. Furthermore, the cadmium content measured in digestive gland of mussels in the present study was 40 times lower those measured in another study where the mussels were collected from areas considered to be non-polluted (Amiard et al., 1986).

Mussels, 5–6 cm long, were transferred from Kalamaria to the laboratory and maintained in static tanks containing filtered natural seawater (35–40% salinity) for 7 days at 15°C. The seawater was changed every 2 days. During the adaptation period, the animals were not fed. The concentrations of cadmium used in the present study ranged from 0.05 to $500\ \mu mol\ l^{-1}$, the lower concentration being close to that found in Thermaikos Gulf (EKTHE Oceanographic Institute, 1997).

Isolation of digestive cells
Digestive glands from 3–5 animals were cut into pieces and washed with buffer without added Ca and Mg (CMFS; 1100 mOsm, pH 7.3, containing $20\ mmol\ l^{-1}$ Hepes buffer, $500\ mmol\ l^{-1}$ NaCl, $12.5\ mmol\ l^{-1}$ KCl, $5\ mmol\ l^{-1}$ EDTA). Tissue samples were cut with scissors into small pieces and transferred to a flask containing 15 ml dissociating solution (0.01% collagenase type CLS IV, $175\ U\ mg^{-1}$, Biochrom AG, Berlin, Germany) in CMFS, followed by gentle stirring for 60 min at 15°C. After filtration of the cell suspension through $250\ \mu m$ and $60\ \mu m$ diameter nylon filters, the procedure as described by Canesi et al. (1997) was followed. Cell suspensions, maintained in Leibovitz L-15 medium (supplemented with $350\ mmol\ l^{-1}$ NaCl, $7\ mmol\ l^{-1}$ KCl, $4\ mmol\ l^{-1}$ $CaCl_2$, $8\ mmol\ l^{-1}$ $MgSO_4$ and $40\ mmol\ l^{-1}$ $MgCl_2$), were kept at 15°C for at least 3 h before being used for experiments. After the tissue treatment, cell viability was tested using Eosin exclusion and was about 83%. Subsequently, cell viability was tested after incubation with various concentrations of cadmium (0.05–$500\ \mu mol\ l^{-1}$) and 98% of the cells remained intact. Concentrations of cadmium higher than $500\ \mu mol\ l^{-1}$ resulted in a 50% cell death.

Isolated cells were incubated with 0.05–$500\ \mu mol\ l^{-1}$ of cadmium for 30 min in the presence or absence of inhibitors or activators as mentioned in the Results. Preliminary experiments showed that adrenaline at the range of $5\times10^{-9}\ mol\ l^{-1}$ caused maximum stimulation of pHi.

Pyruvate kinase activity determination
1 ml of cell suspension (approx. $10^6\ cells\ ml^{-1}$) was exposed to different concentrations of cadmium chloride ($CdCl_2$) and/or other substrates for 30 min. Samples were removed at 0 and 30 min. The cell suspension was centrifuged at 160 g for 5 min, washed with L-15 medium, and the precipitate representing the packed cells was lysed by the addition of 4 volumes of $20\ mmol\ l^{-1}$ imidazole/HCl buffer, pH 7.2 containing $10\ mmol\ l^{-1}$ EGTA, $10\ mmol\ l^{-1}$ EDTA,

0.1 mmol l^{-1} phenyl-methyl-sulfonyl-fluoride (PMSF) and 15 mmol l^{-1} β-mercaptoethanol. The suspension was subsequently sonicated for 6×10 s in an MS E Sonicator (Soniprep 150 MSE, TCP Inc., NJ, USA), followed by centrifugation at 2400 g for 30 min at 4°C. The supernatant (200–400 μl) was passed through a 5 ml column of Sephadex G-25, equilibrated in 40 mmol l^{-1} imidazole-HCl buffer (pH 7.1) containing 5 mmol l^{-1} EDTA, 15 mmol l^{-1} β-mercaptoethanol and 20% (v/v) glycerol, in order to remove low-molecular-mass metabolites (Helmerhost and Stokes, 1980). The columns were centrifuged for 1 min and the filtrates (100–150 μl) were used as a source of enzyme. PK activity was determined spectrophotometrically, as described by Ward et al. (1969). Optimal assays conditions for PK were 20 mmol l^{-1} imidazole-HCl (pH 7.2), 2 mmol l^{-1} ADP, 5 mmol l^{-1} MgCl, 20 mmol l^{-1} KCl, 0.15 mmol l^{-1} NADH, 1.5 mmol l^{-1} phosphoenol pyruvate (PEP) and 1 U ml^{-1} LDH. Protein content was determined using bicinchoninic acid (BCA) protein assay reagent protocol using bovine serum albumin (BSA) as standard (Sorensen and Brodbeck, 1986).

Enzyme activities are means ± S.D. for 7 experiments and are expressed as percentage (%) of control value. Enzyme activity of control cells was 5.23±1.04 nmol min mg^{-1} protein, which is in the same range as measured in a previous study (Canesi et al., 1999).

Determination of intracellular pH

The intracellular pH was measured using a modification of the experimental procedure as reported by Incerpi et al. (1996). Digestive gland cells suspended in physiological saline buffer (PS; 1100 mOsm, pH 7.3, containing 20 mmol l^{-1} Hepes buffer, 436 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} CaCl$_2$, 53 mmol l^{-1} MgSO$_4$ and 10 mmol l^{-1} glucose), were loaded with the fluorescent indicator 2′, 7′-bis (carboxyethyl)-5(6)-carboxyfluorescein tetra-acetoxymethylester (BCECF/AM; AppliChem, Inc., Darmstadt, Germany), which is highly lipid soluble, membrane permeable and readily cleaved by intracellular esterases.

Appropriate amounts of cells were incubated in PS buffer with BCECF/AM (1–2 μg 10^{-6} cells), (the volume of BCECF was taken from a stock solution and adjusted to the number of cells each time) at 15°C for 45 min in the dark. The isolated cells were then washed three times or more with the same medium to remove the fluorescent indicator and then resuspended in the same medium (2–3×10^6 cells/3 ml). When appropriate, various concentrations of cadmium chloride (0.05, 5, 50 and 500 μmol l^{-1}), 10 nmol l^{-1} phorbol-12-myristate-13-acetate (PMA; an activator of protein kinase C), 5.5 nmol l^{-1} adrenaline (L-epinephrine; an α- and β-adrenergic agonist), 1 μmol l^{-1} isoprenaline-HCl (a β-adrenergic agonist), 1 μmol l^{-1} phenylephrine-HCl (an α$_1$-adrenergic agonist), 20 nmol l^{-1} calphostin C (from *Cladosporium cladosporioides*; a specific inhibitor of protein kinase C), 1 μmol l^{-1} prazosin-HCl (a peripheral α$_1$-adrenergic antagonist), 1 μmol l^{-1} metoprolol-tartrate (a β$_1$-adrenergic antagonist), 20 nmol l^{-1} ethyl-*N*-isopropyl-amiloride (EIPA; a selective inhibitor of

Na$^+$/H$^+$ exchanger) and 10 μmol l^{-1} propranolol-HCl (an antagonist of β-adrenergic receptors), were added.

Fluorescence was measured while the suspension was continuously stirred using a magnetic stirrer at 20°C in a Perkin-Elmer (Connecticut, USA) LS 50B Fluorescence thermostatic spectrometer equipped with a thermostatic holder. Data were obtained as the ratio of the pH-sensitive excitation wavelength (495 nm) to the pH-insensitive excitation signal wavelength (440 nm), with the emission wavelength set at 530 nm.

Routinely, for each experiment, and also for the cell sample preparations used to evaluate pHi values, fluorescence was calibrated against pH. Cells were diluted in a series of standard-buffer solutions containing MES 30 mmol l^{-1} (pH 6), Hepes 30 mmol l^{-1} (pH 6.5) Mops 30 mmol l^{-1} (pH 7) and Tris 30 mmol l^{-1} (pH 8). Each buffer contained 440 mmol l^{-1} KCl and 1 mmol l^{-1} MgCl$_2$. Fluorescence was calibrated against pH as described by Thomas et al. (1979), using the polyether ionophore nigericin (6.7 mmol l^{-1}), which couples K$^+$ and H$^+$ gradients across the plasma membrane. High [K$^+$] solution in combination with the K$^+$/H$^+$ exchange ionophore nigericin leads to equilibration of extracellular K$^+$ with intracellular K$^+$ and pHi at the clamped extracellular pH. The calibration curve, done at the end of each experiment, was linear in the pH range 6–8 (r^2=0.99; typically y=3.13x–16.5). A standard curve was also conducted in the presence of cadmium and was the same as that in the absence of the metal. The background ratio of unlabelled cells was 6 times less than that of the labelled ones. The intracellular pH (pHi) was 7.4±0.01. The results are means ± S.D. from at least 7 experiments.

Measurement of ^{22}Na influx

Influx of ^{22}Na by the digestive gland cells was measured by a modification of a previously described method (Sauvage et al., 2000; Bourikas et al., 2003). After isolating the digestive gland cells, the suspension was centrifuged at 160 g for 5 min at 4°C. The packed cells were washed four times in a buffer free of Na$^+$ containing 10 mmol l^{-1} Tris/MES buffer (pH 6), 446 mmol l^{-1} KCl, 53 mmol l^{-1} MgSO$_4$, 35 mmol l^{-1} sucrose, 10 mmol l^{-1} glucose, 0.1 mmol l^{-1} ouabain, and finally the packed cells were suspended either in 10 mmol l^{-1} Tris/MES buffer (pH 6) or in 10 mmol l^{-1} Tris/Mops buffer (pH 8), both of which also contained 4,4-diisothiocyanatostilbene-2.2-disulfonic acid (DIDS; an inhibitor of anion exchanger) and 6-ethoxyzolamide (a carbonic anhydrase inhibitor), in order to avoid pHi disturbances, and bumetanide (a Na-K-2Cl$^-$ cotransport inhibitor), in order to avoid Na$^+$ transients (at final concentrations 0.125 mmol l^{-1}, 0.4 mmol l^{-1} and 0.01 mmol l^{-1}, respectively). The cells were allowed to resuspend in the above-mentioned buffer for 20 min.

Afterwards, the cells were centrifuged at 160 g for 5 min and the packed cells were finally suspended either in 10 mmol l^{-1} Tris/Mops buffer (pH 8) or in 20 mmol l^{-1} Tris/MES buffer (pH 6). Both these buffers contained 436 mmol l^{-1} NaCl, 53 mmol l^{-1} MgSO$_4$, 35 mmol l^{-1} sucrose, 10 mmol l^{-1} glucose, 0.1 mmol l^{-1} ouabain (an inhibitor of sodium pump,

in order to prevent Na^+ movement), 1 mmol l^{-1} iodoacetic acid (an inhibitor of glycolysis at the step catalysed by glyceraldehyde-P-dehydrogenase), 0.125 mmol l^{-1} DIDS (an anion exchanger inhibitor), freshly prepared antimycin A (final concentration 6 mg ml^{-1}), 0.4 mmol l^{-1} 6-ethoxyzolamide, 0.01 mmol l^{-1} bumetanide and labelled ^{22}Na (3.7 kBq/sample). When appropriate, cadmium chloride at final concentration of 50 µmol l^{-1}, calphostin C at 20 nmol l^{-1}, propranolol at 10 µmol l^{-1} and EIPA at 20 nmol l^{-1} were added immediately after the addition of the above buffer, and the cells incubated for 30 min. Then, the cell suspensions were transferred in plastic tubes containing 10 mmol l^{-1} Tris/Mops buffer (pH 8) or 20 mmol l^{-1} Tris/MES buffer (pH 6), plus 30% sucrose and gently layered over 0.2 ml of dibutyl-phthalate. Centrifugation of the cell suspension followed in order to collect the packed cells at the bottom of the tubes without the incubation buffer. A volume of 10 µl of the supernatant, without disturbing the dibutyl-phthalate liquid phase, was transferred in plastic vials for ^{22}Na counting (extracellular Na), while the rest was removed gently. Finally 100 µl of 30% perchloric acid (PCA) was added to the packed cells for cell lysis to occur and after centrifugation at 1600 g for 5 min the supernatant was transferred into plastic vials for ^{22}Na counting. Radioactivity was measured for 10 min per sample in a beta scintillation counter (LKB 1209 Rackbeta). Sodium influx, expressed as µmol Na^+ h^{-1} 10^{-6} cells, was calculated from the percentage of total radioactivity incorporated into the cells and the total concentration in the incubation buffer. Data were corrected for backflux of ^{22}Na. The intracellular sodium concentration measured in cells suspended in solution at pH 8 represents the total sodium influx, whereas intracellular sodium concentration measured in cells suspended in a solution at pH 6 represents the influx due to passive permeability alone. The difference between these two influxes represents the sodium influx stimulated by the pH gradient, and is expressed as the influx of Na^+ due to maximal activity of the Na^+/H^+ exchanger (V_{max}) (Delva et al., 1993; Sauvage et al., 2000).

Statistical analysis

For PK activity, pHi and Na^+ influx determination, statistical analysis was carried out using Instat 2 Software (Graphpad Instat, San Diego, CA, USA), Dunnet's test. The minimal level of significance chosen was $P<0.01$. The analyses were carried out using the STATISTICA statistical package (Microsoft Co., Thessalonika, Greece).

Results

Effect of cadmium on Na^+ influx and pHi of digestive gland cells

Treatment of isolated digestive gland cells of *Mytilus galloprovincialis* with various concentrations of cadmium chloride (0.05, 5, 50, 500 µmol l^{-1}) for 30 min, caused a significant elevation of pHi (Fig. 1). 50 µmol l^{-1} of cadmium chloride caused maximum increase in pHi, and was therefore used for all the experiments. The same concentration of heavy
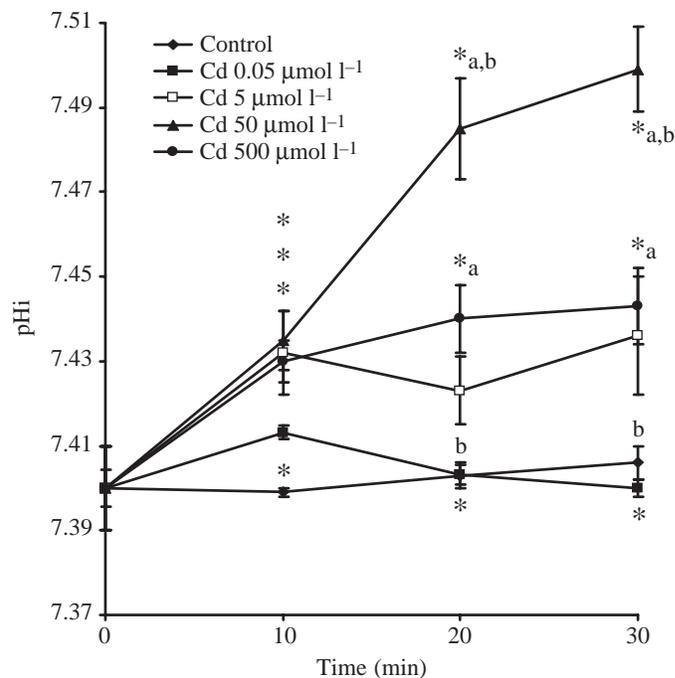

Fig. 1. The effect of different concentrations of cadmium on intracellular pH (pHi) of isolated digestive gland cells of *Mytilus galloprovincialis*. Values are means ± S.D. of at least 7 independent experiments. In each experiment, the tissue of four animals was used. *Significantly different after cadmium treatment from control value at the respective time point. Values that share the same letter (a, b) are statistically significant different from each other at the same time point; Dunnet's test, $P<0.01$.

metal has also been used in other studies investigating the effect of heavy metal on cell signaling (Canesi et al., 1998). The addition of 50 µmol l^{-1} cadmium also caused an increase of Na^+ influx as measured by ^{22}Na (Fig. 2), in digestive gland cells of *Mytilus*. Specifically, total Na^+ uptake in the cells increased from 1074 to 2967 µmol h^{-1} 10^{-6} cells after cadmium treatment. These results indicate a stimulation of the Na^+/H^+ exchanger by cadmium treatment of digestive gland cells of *Mytilus galloprovincialis*.

The influence of cadmium on pHi and ^{22}Na influx was inhibited by EIPA (20 nmol l^{-1}), (Fig. 2), a known selective inhibitor of the Na^+/H^+ exchanger. The transport system can also engage in Na^+ exchanges with a very low Na^+ transport rate, which is 6% of the rate of Na^+ transport in the Na^+/K^+ exchange reaction (Skou, 1988). Since most other sodium transporters are inhibited (see Materials and methods) the observed effect can be attributed to Na^+/H^+ exchanger inhibition. The increase of pHi under the influence of cadmium was not affected by DIDS, a Cl^-/HCO_3^- antiport inhibitor (results not shown).

Studies on vertebrate cells have shown that Na^+/H^+ activation is mediated by protein kinase C (PKC) activity (Kaloyianni et al., 2001; Bourikas et al., 2003). To investigate whether the effect of cadmium on pHi elevation of mussel cells was related to any metal-induced effect on protein kinase C
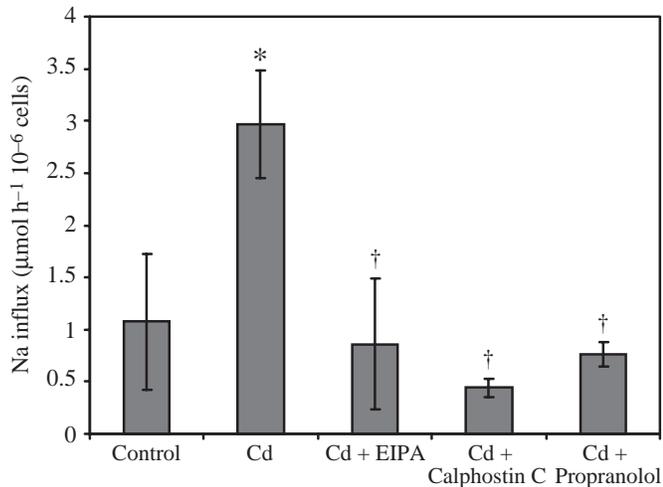
Fig. 2. Effect of cadmium and various effectors on Na$^+$ influx (μmol Na$^+$ h^{-1} 10^{-6} cells) in isolated digestive gland cells of *Mytilus galloprovincialis*. Cells were treated for 30 min either with cadmium at 50 μmol l^{-1} or cadmium (50 μmol l^{-1}) + 20 nmol l^{-1} EIPA (a selective inhibitor of Na$^+$/H$^+$ exchanger), or cadmium (50 μmol l^{-1}) + 20 nmol l^{-1} calphostin C (a specific inhibitor of protein kinase C), or cadmium (50 μmol l^{-1}) + 10 μmol l^{-1} propranolol (a β-adrenergic antagonist). See Materials and methods for details. Values are means ± S.D. of at least 4 independent experiments. In each experiment, the tissue of four animals was used. *Significant difference between control value and that observed after metal treatment; †significant difference between the value obtained after cadmium treatment with that obtained after cadmium + effector treatment; Dunnet's test, *P*<0.01.

Table 1. *Changes of pHi in isolated digestive gland cells after 30 min incubation of cells with 50 μmol l^{-1} cadmium plus various antagonists or EIPA and after incubation of the cells with each antagonist or EIPA alone*

	pHi		
	0 min	30 min	ΔpHi
Control cells	7.4±0.01	7.406±0.004[a]	0.006
CdCl$_2$	7.4±0.01	7.490±0.010[a]	0.090
CdCl$_2$+EIPA (20 nmol l^{-1})	7.4±0.01	7.399±0.060	−0.001
EIPA (20 nmol l^{-1})	7.4±0.01	7.400±0.005	0.000
CdCl$_2$+Propranolol (10 μmol l^{-1})	7.4±0.01	7.410±0.004	0.001
Propranolol (10 μmol l^{-1})	7.4±0.01	7.390±0.002	−0.001
CdCl$_2$+Prazosin (1 μmol l^{-1})	7.4±0.01	7.410±0.005	0.001
Prazosin (1 μmol l^{-1})	7.4±0.01	7.390±0.0007	−0.001
CdCl$_2$+Metoprolol (1 μmol l^{-1})	7.4±0.01	7.410±0.005	0.001
Metoprolol (1 μmol l^{-1})	7.4±0.01	7.410±0.003	0.001

[CdCl$_2$] = 50 μmol l^{-1}.

Values are means ± S.D. of at least 7 different experiments.

Values that share the same letter indicate statistically significant difference from each other; Dunnet's test (*P*<0.01). The value obtained after treatment with cadmium alone is significantly different from all values obtained in the presence of antagonists or EIPA alone and antagonists or EIPA together with cadmium; Dunnet's test (*P*<0.01).

mediated signal transduction, we tested the effects of: (1) the phorbol ester PMA, an activator of PKC and (2) calphostin C, a specific inhibitor of protein kinase C. PMA (10 nmol l^{-1}) treatment caused a significant rise in pHi after 20 min, reaching a plateau after 30 min of incubation (Fig. 3), suggesting that the antiport is influenced by PKC. In addition, the presence of calphostin C (20 nmol l^{-1}) together with cadmium resulted in a significant decrease in pHi value compared to that observed after treatment with cadmium alone (Fig. 3). Calphostin C together with cadmium also inhibited the increased influx of Na$^+$ in the digestive gland cells, relative to the Na$^+$ influx after treatment with cadmium alone (Fig. 2). The influence of cadmium on pHi as well as on Na$^+$ influx was inhibited by propranolol, an antagonist of β-adrenergic receptors, thus indicating that signal transduction induced by cadmium occurs *via* interaction with β-adrenergic receptors (Fig. 2, Table 1). β$_1$- and α$_1$-adrenergic receptor antagonists also counterbalanced the heavy metal effect on pHi rise (Table 1).

Effects of cadmium on PK activity

Cadmium added at micromolar concentrations to isolated digestive gland cells of *M. galloprovincialis* resulted in a time-dependent response in PK activity (Table 2). The dose-dependent response of cadmium showed that PK activity was maximally increased (11.69±2.08 nmol min^{-1} mg^{-1} protein) at

Table 2. *Effect of cadmium (50 μmol l^{-1}) on pyruvate kinase (PK) activity after treatment of isolated digestive gland cells for different periods of time*

Time (min)	PK activity (%)
0	100±19.90
5	133.783±15.80[b]
10	141.889±3.14[a,b]
15	142.767±6.22[a,b]
20	159.923±5.61[a,b]
30	222.125±17.92[a]

100% represents enzyme activity in the absence of 50 μmol l^{-1} CdCl$_2$ (5.23±1.04 nmol min^{-1} mg^{-1} protein). Values (% of control value) are means ± S.D. of at least 7 independent experiments. In each experiment, tissue from four animals was used.

[a]Significant difference between treated cells and the control cells; [b]significant difference between cells treated for different periods of time compared to cells treated with cadmium for 30 min; Dunnet's test (*P*<0.01).

50 μmol l^{-1} CdCl$_2$, followed by a decrease at 500 μmol l^{-1} CdCl$_2$ (8±1.75 nmol min^{-1} mg^{-1} protein), while 0.05 μmol l^{-1} of CdCl$_2$ showed only a small increase in PK activity (6.07±1.23 nmol min^{-1} mg^{-1} protein), in relation to control (5.23±1.04 nmol min^{-1} mg^{-1} protein), which represents PK activity in the absence of CdCl$_2$ (not shown). During the exposure period of 30 min, no significant changes in enzymatic activity were observed in control cells. To determine whether
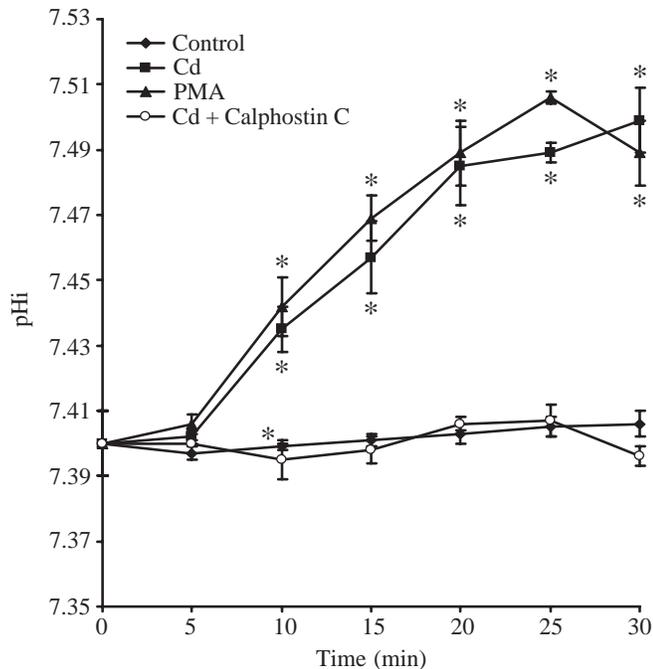
Fig. 3. The effect of cadmium (50 μmol l^{-1}) and/or various effectors on pHi of isolated digestive gland cells of *Mytilus galloprovincialis*. Cells were treated with PMA, a potent activator of protein kinase C, alone (10 nmol l^{-1}) or cadmium (50 μmol l^{-1}) alone, or + 20 nmol l^{-1} calphostin C (a specific inhibitor of protein kinase C). The results are mean ± S.D. of at least 7 independent experiments. In each experiment, the tissue of four animals was used. *Significant difference between the values obtained after treatment of the cells with PMA or with cadmium and the control value at the respective time point; Dunnet's test, *P*<0.01.

Fig. 4. Effect of cadmium alone and cadmium + Na$^+$/H$^+$ exchanger inhibitors on PK activity of isolated digestive gland cells of *Mytilus galloprovincialis*. 100% represents enzyme activity in the absence of cadmium (5.23±1.04 nmol min^{-1} mg^{-1} protein). Cells were treated for 30 min either with 50 μmol l^{-1} of cadmium alone, or 50 μmol l^{-1} cadmium + 10 μmol l^{-1} amiloride (an inhibitor of most plasma membrane Na$^+$ transport systems) or + 20 nmol l^{-1} EIPA (a selective inhibitor of Na$^+$/H$^+$ exchanger). PK activity in the presence of 20 nmol l^{-1} EIPA alone after 30 min of incubation was found to be 5.38±0.7 nmol min^{-1} mg^{-1} protein. The results (expressed as % PK activity of control value) are mean ± S.D. of at least 7 independent experiments. In each experiment, the tissue from four animals was used. *Significant difference between the value after cadmium treatment and the control value; †significant difference between the values after cadmium treatment with those observed after cadmium + inhibitors; Dunnet's test, *P*<0.01.

the effect of cadmium treatment on PK activity of mussel cells was related to a metal-induced effect on protein kinase C mediated signal transduction, we tested the effects of inhibitors in experiments similar to those employed for the Na$^+$/H$^+$ exchanger: (1) the phorbol ester PMA (an activator of PKC) and (2) staurosporine (antibiotic AM-2282, from *Streptomyces* sp.; an inhibitor of PKC) and calphostin C (a specific inhibitor of protein kinase C). Treatment of isolated digestive gland cells with 10 nmol l^{-1} PMA resulted in a significant increase of enzymatic activity, almost at the same range as observed after treatment with cadmium alone (Table 3). Additionally, staurosporine or calphostin C inhibited the cadmium effect on digestive gland cells (Table 4). The presence of propranolol (10 μmol l^{-1}) or prazosin (1 μmol l^{-1}) or metoprolol (1 μmol l^{-1}) together with cadmium caused an inhibition of PK increase (Table 4).

To investigate whether the metal induced effect on PK activity was related to Na$^+$/H$^+$ exchanger, the inhibitor of Na$^+$/H$^+$ exchanger and other sodium transporters, amiloride was used. It is noteworthy that treatment of isolated digestive cells with 10 μmol l^{-1} amiloride, together with cadmium, resulted in a significant decrease of the cadmium effect on enzymatic activity (Fig. 4). The same results were observed
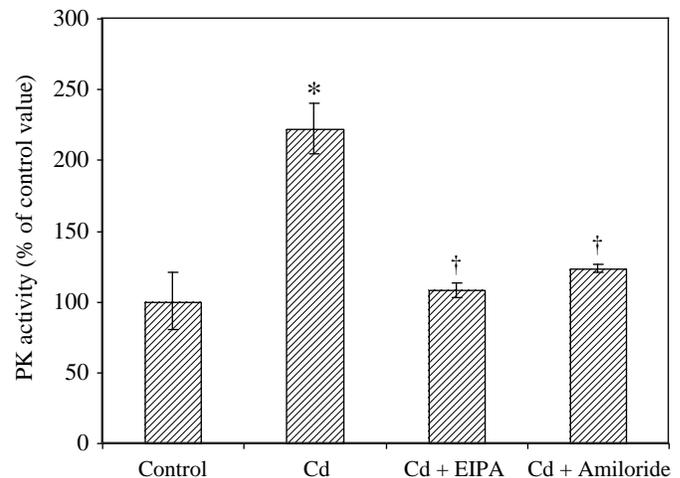
after treatment of cells with EIPA (20 nmol l^{-1}), which is a selective amiloride-type inhibitor of the Na$^+$/H$^+$ exchanger (Fig. 4). Treatment with each inhibitor alone, without cadmium, did not show any significant difference from enzymatic activity measured in control cells.

Effect of β- and α-agonists on pHi and PK activity

The effect of adrenaline on pHi change as well as on PK activity was tested. A significant rise in pHi was caused after adrenaline (5.5 nmol l^{-1}) treatment (Table 5). It is noteworthy that after adrenaline treatment, pHi reached even higher levels than those observed after cadmium treatment. After exposure of the cells to cadmium chloride together with adrenaline, the increased pHi observed was significantly higher than the value in control cells but significantly lower than the pHi rise obtained after incubation either with cadmium or with the agonist alone (Table 5). Therefore, the response of the cells in pHi increase after exposure to cadmium together with adrenaline seems to be synergistic and not additive.

Furthermore, PK activity was increased after adrenaline treatment of digestive gland cells (Table 3). The β- and α$_1$ agonists isoprenaline and phenylephrine, respectively, caused a significant rise of pHi and PK activity in isolated digestive

Table 3. *Effect of cadmium, various agonists and PMA on pyruvate kinase (PK) activity of isolated digestive gland cells of* Mytilus galloprovincialis

	PK activity (%)
Control cells	100±19.908
CdCl$_2$	222.125±17.920[a,b,c,d,e,f,g]
PMA	212.953±9.830[a,h]
CdCl$_2$+PMA	167.112±11.050[a,b,h]
Adrenaline	145.745±10.250[a,c,i]
CdCl$_2$+Adrenaline	119.741±10.930[d,i]
Isoprenaline	125.908±28.280[e]
CdCl$_2$+Isoprenaline	121.797±1.110[f]
Phenylephrine	216.797±16.640[a,k]
CdCl$_2$+Phenylephrine	155.327±13.030[a,g,k]

100% represents enzyme activity in the absence of cadmium (5.23 ± 1.04 nmol min^{-1} mg^{-1} protein).

Cells were treated for 30 min with 50 µmol l^{-1} CdCl$_2$, or 5.5 nmol l^{-1} adrenaline, or 1 µmol l^{-1} isoprenaline, or 1 µmol l^{-1} phenylephrine, or 10 nmol l^{-1} PMA, or cadmium together with each of the agonists or cadmium plus PMA. Values (% of control value) are means ± S.D. of at least 7 independent experiments. In each experiment, tissue from four animals was used.

[a]Significant difference between treated cells and control; values that share the same letter are significantly different from each other; Dunnet's test (*P*<0.01).

Table 4. *Pyruvate kinase (PK) activity after treatment of isolated digestive gland cells with different antagonists alone or together with cadmium*

	PK activity (nmol min^{-1} mg^{-1} protein)
Control (30 min)	5.230±1.040
CdCl$_2$	11.619±2.082[a]
CdCl$_2$+Prazosin (1 µmol l^{-1})	4.304±0.670[b]
CdCl$_2$+Metoprolol (1 µmol l^{-1})	2.889±0.450[a]
CdCl$_2$+Propranolol (10 µmol l^{-1})	5.378±0.600
CdCl$_2$+Staurosporine (20 nmol l^{-1})	7.770±0.580[a,b]
CdCl$_2$+Calphostin C (20 nmol l^{-1})	5.560±0.620
Prazosin (1 µmol l^{-1})	6.043±0.280
Metoprolol (1 µmol l^{-1})	3.540±0.500
Propranolol (10 µmol l^{-1})	5.490±0.630
Staurosporine (20 nmol l^{-1})	6.200±0.520
Calphostin C (20 nmol l^{-1})	4.800±0.300

[CdCl$_2$] = 50 µmol l^{-1}.

Pyruvate kinase activity (nmol min^{-1} mg^{-1} protein)

Values are means ± S.D. of at least 7 different experiments.

All values are significantly different from those obtained after treatment with cadmium alone (Dunnet's test, *P*<0.01). [a]Significant difference between values obtained from treated cells and the control value; [b]significant difference between cells treated with cadmium plus each antagonist value and cells treated with each antagonist alone; Dunnet's test (*P*<0.01).

Table 5. *Effect of cadmium (50 µmol l^{-1}), different agonists of adrenergic receptors and PMA on pHi of isolated digestive gland cells of* Mytilus galloprovincialis

	pHi		
	0 min	30 min	ΔpHi
Control cells	7.4±0.01	7.406±0.004	0.006
CdCl$_2$	7.4±0.01	7.490±0.010[a]	0.090
CdCl$_2$+Adrenaline	7.4±0.01	7.443±0.003[a,c]	0.043
Adrenaline	7.4±0.01	7.535±0.020[a,b]	0.135
CdCl$_2$+Isoprenaline	7.4±0.01	7.439±0.003[a,c]	0.039
Isoprenaline	7.4±0.01	7.440±0.004[a]	0.040
CdCl$_2$+Phenylephrine	7.4±0.01	7.434±0.002[a,c]	0.034
Phenylephrine	7.4±0.01	7.420±0.005[a]	0.020
CdCl$_2$+PMA	7.4±0.01	7.445±0.003[a,c]	0.045
PMA	7.4±0.01	7.489±0.010[a,b]	0.089

Cells were treated for 30 min either with 50 µmol l^{-1} of CdCl$_2$ or 5.5 nmol l^{-1} adrenaline, or 1 µmol l^{-1} isoprenaline, or 1 µmol l^{-1} phenylephrine, or 10 nmol l^{-1} PMA.

Values are means ± S.D. of at least 7 independent experiments. In each experiment, tissue from four animals was used.

[a]Significant difference between treated cells and control cells; [b]significant difference between values from cells incubated with cadmium plus each agonist and the value from cells incubated with each agonist or PMA alone; [c]significant difference between values from cells incubated with cadmium plus each of the agonists and those from cells incubated with cadmium alone; Dunnet's test (*P*<0.01).

gland cells (Tables 3, 5). Isoprenaline (1 µmol l^{-1}) caused an elevation of pHi, which was maximum after 15 min of treatment and decreased after 30 min, but was still significantly higher than the control. The same kinetics for PK activity were also observed after treatment with isoprenaline (results not shown). Similar to the pHi increase after adrenaline plus cadmium treatment, the response of the cells both in PK activity and on pHi after co-exposure to cadmium together with various agonists or PMA was synergistic (Tables 3, 5).

Discussion

The results of the present study show that micromolar concentrations of cadmium (50 µmol l^{-1}) play an important role in the elevation of pHi in isolated digestive gland cells of *Mytilus galloprovincialis* (Figs 1, 3, Tables 1, 5). Consistent with our results, Villela et al. (1999), reported that cadmium alters passive H$^+$ permeability in membrane vesicles of the eel. Concentrations of cadmium as high as 500 µmol l^{-1} seem to have smaller effects on the elevation of pHi. Furthermore, the effect of 50 µmol l^{-1} cadmium treatment on the digestive gland cells for 30 min, resulted in a significant rise (176%) of Na$^+$ influx relative to control values (Fig. 2). This increase in Na$^+$ influx together with the pHi increase after cadmium treatment reveals activation of the Na$^+$/H$^+$ exchanger in digestive gland cells of *Mytilus galloprovincialis*. Na$^+$ influx can also be observed by monitoring the function of the Na$^+$/Ca^{2+} exchanger. However, the latter exchanger may be inhibited by cadmium due to the closeness of the Cd^{2+} crystal ionic radius

to that of Ca^{2+} (Smith et al., 1987). The increase in influx of Na^+, together with the concomitant increase of pHi observed in the present study, point to Na^+/H^+ exchanger activation.

Further support for this interpretation is provided by the experiments using blockers of the exchanger. EIPA is a selective inhibitor of the Na^+/H^+ exchanger, which competes with sodium for binding to the exchanger (Clark and Limbird, 1991). At a concentration of 20 nmol l^{-1} it significantly reduces the effect of cadmium on both Na^+ influx and pHi increase, compared to the increase observed after treatment with cadmium alone. Therefore, the results show the existence of an amiloride-sensitive Na^+/H^+ exchanger in digestive gland cells of mussel *Mytilus galloprovincialis*, which is activated by cadmium.

A known stimulator of PKC, treatment by phorbol-esters produced similar pHi changes to those of cadmium, regarding pHi change in digestive gland cells. In accordance with the latter, the inhibitor of PKC calphostin C together with cadmium, diminished the stimulatory effects of cadmium on pHi increase as well as on Na^+ influx. From the results we could suggest that cadmium activates the exchanger by affecting the signaling pathway *via* activating protein kinase C. Our data is in accordance with other studies, where the stimulation of the Na^+/H^+ exchanger by hormones *via* protein kinase C was reported (Grinstein et al., 1985; Incerpi et al., 1994; Kaloyianni et al., 2001; Bourikas et al., 2003).

There are few data concerning the effect of heavy metals on the activity of key glycolytic enzymes (Canesi et al., 1998, 2000a, 2001). The present results demonstrate that 50 μmol l^{-1} of cadmium significantly activates the glycolytic enzyme PK after 30 min of treatment of digestive gland cells of *Mytilus galloprovincialis*. These data are consistent with the effect of zinc, insulin and growth factors on PK activity in digestive gland cells of mussels (Canesi et al., 1997, 1999, 2000b, 2001). Furthermore, after treatment of the cells with amiloride or EIPA, together with cadmium, a significant decrease of PK activity was observed (Fig. 4). It is known that amiloride inhibits most plasma membrane Na^+ transport systems, as epithelial Na^+ channel, the Na^+/H^+ exchanger and the Na^+/Ca^{2+} exchanger (Frelin et al., 1988; Kleyman and Cragoe, 1988). These data show that the stimulus from heavy metals passes to PK *via* Na^+/H^+ exchanger, or that PK activation depends on intracellular pH changes, caused by the Na^+/H^+ exchanger activity. Thus, the signal transduction pathway induced by cadmium leads to the activation of PK together or *via* Na^+/H^+ exchanger activation.

To characterize the signaling pathway involved in stimulation of PK in mussel digestive gland cells by cadmium treatment, the effects of staurosporine, calphostin C, and PMA on PK were investigated. We found a relationship between PKC activity and PK stimulation, after treatment of the isolated digestive gland cells with the heavy metal. In parallel with our results showing that cadmium uptake is related to the signaling pathway activating PKC as well as Na^+/H^+ exchanger or/and PK, the study of Adams et al. (2002) shows that cadmium induces signal transduction cascades such as PKC, tyrosine kinase and casein kinase II. Canesi et al. (2000a, 2001), reported that other heavy metals such as Zn can modulate the activity of the glycolytic enzymes phosphofructokinase (PFK) and pyruvate kinase (PK) through a tyrosine-mediated signal transduction pathway, while the heavy metals Hg and Cu interfere with Ca^{2+} mediated signaling in isolated digestive gland cells of mussel *M. galloprovincialis*. The involvement of Ca^{2+} in cadmium mediated signaling in the digestive gland cells of *M. galloprovincialis* is also probable since it is reported that cadmium impairs influx of Ca^{2+} in gills of *M. galloprovincialis* (Viarengo et al., 1993).

The signaling pathway induced by cadmium led us to hypothesise that the cell responses to cadmium treatment are similar to those observed after hormone treatment. Thus, the activity of PK and the change in pHi after adrenaline and isoprenaline treatment was investigated (Tables 3, 5). Specifically, adrenaline treatment of the digestive gland cells resulted in a significant increase of enzymatic activity as well an increase in pHi. The increases in pHi and PK activity were similar to β-adrenergic receptor activation in frog and human erythrocytes (Kaloyianni et al., 1997, 2000; Bourikas et al., 2003). The fact that cadmium, adrenaline and isoprenaline induce PK activation as well as increased pHi led us to investigate if cadmium also binds to β-adrenergic receptors. Treatment with propranolol, an antagonist of β-adrenergic receptors, together with the heavy metal, caused a significant decrease of PK activity and inhibition of the pHi and Na^+ increase (Fig. 2, Tables 1, 4). The inhibition of pHi and PK caused by prazosin and metoprolol, indicates that activation of α_1 and β_1 receptors after cadmium may also occur. From the results we could suggest that cadmium may exert its activation on digestive gland cells by interacting with β, β_1- and α_1-adrenergic receptors. Even though it is reported that cadmium is taken up by an inorganic anion exchanger and that cadmium transport across the apical membrane occurs not only *via* passive diffusion but also *via* a H^+ antiport system (Endo et al., 2000), in addition to its uptake by endocytosis and pinocytosis (Cossa, 1989; Endo et al., 1998), the use of adrenergic antagonists showed that β, β_1- and α_1- adrenergic receptors may also be functioning. In accordance with the latter, in oysters, an adrenaline mediated stress response has been recorded (Lacoste et al., 2001), which indicates the functioning of α- and β-adrenergic receptors. However, the mechanism of cadmium uptake in digestive gland cells of *Mytilus* remains to be elucidated.

In conclusion, under stress, cells adapt strategies for maintaining their intracellular homeostasis and function. Since stimulation or inhibition of PKC stimulates or inhibits both the Na^+/H^+ exchanger and PK activity, it seems reasonable to suggest that the heavy metal interacts with β, β_1- and α_1-adrenergic receptors, which then pass through PKC affecting or stimulating both Na^+/H^+ exchanger and PK. As a consequence of stimulating the activity of PK, glycolysis is accelerated in response to the increase demands for energy under stress. Increased glycolysis results in increased production of H^+, whose exit is facilitated by the already

activated Na$^+$/H$^+$ exchanger. On the other hand, it is noteworthy that the inhibition of Na$^+$/H$^+$ exchanger by EIPA results in inhibition of PK as well. These data suggest that either the heavy metal stimulus passes from PKC to PK *via* Na$^+$/H$^+$ exchanger or that PK activation depends on intracellular pH changes caused by Na$^+$/H$^+$ exchanger activity. These results support previous evidence that Na$^+$/H$^+$ exchanger is a regulator of intracellular signal transduction. Since Na$^+$/H$^+$ exchanger activity is regulated by a variety of stimuli, this antiport may contribute to the fine-tuning of several intracellular signal transduction pathways in the digestive gland cells of *Mytilus*. Further research is needed in order to clarify these mechanisms.

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