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Cytochemical localization and quantification of plasma membrane Ca^{2+}-ATPase activity in mollusc digestive gland cells

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SUMMARY

A cytochemical method allowing the localization and quantification of plasma membrane Ca^{2+}-ATPase (PMCA) in frozen sections obtained from digestive gland cells of *Mytilus galloprovincialis*, *Tapes tapes* and *Chamelea gallina*, is presented. The method utilizes lead as a trapping agent of PO_4^{2-} ions released by Ca^{2+}-ATPase activity. The amount of lead sulphide precipitate proportionally related to PMCA activity was quantified by a light microscopy digital imaging analysis system. The optimal assay conditions of Ca^{2+}-ATPase activity evaluated at pH 7.4 were: 200µM free Ca^{2+}, 200mM KCl, 2 mM ATP, and under such analysis conditions the enzyme showed a linear trend up to 60 min (at 20ºC). The PMCA activity was substrate specific: ADP was utilized only at a low rate (24% with respect to an equimolar ATP concentration), while glucose-6-phosphate and β-glycerophosphate were poorly hydrolyzed. The enzyme activity was strongly inhibited by sodium ortho-vanadate. Our detection of a Ca^{2+}-ATPase activity at nanomolar concentrations of free Ca^{2+} suggests that we have identified a plasma membrane Ca^{2+}-ATPase involved in Ca^{2+} homeostasis. The Ca^{2+}-ATPase was found to be localized in the basal part of the plasma membrane in the digestive gland cells of *Mytilus galloprovincialis* and *Tapes tapes*, but in the apical plasma membrane of *Chamelea gallina*. The possible implications of the different cellular distributions of PMCA activity is discussed.

INTRODUCTION

A variety of cellular functions are regulated by fluctuations of the cytosolic free calcium concentration [Ca^{2+}], mainly depending on receptor activation at the cell surface (Rasmussen and Barret, 1984; Van Os, 1987). Under physiological resting conditions, the cytosolic [Ca^{2+}] is kept at low values (around 10^{-7}M) by Ca^{2+} homeostasis systems involved in extrusion and compartmentalization activities (Carafoli, 1987).

Plasma membrane Ca^{2+}-ATPase (PMCA) plays an important role in the regulation of cytosolic free [Ca^{2+}], and it is assumed that in most cell types it represents the major high-affinity mechanism for Ca^{2+} extrusion to the extracellular fluid (Carafoli, 1991; Carafoli, 1994; Strehler, 1991). The biochemical and physiological functions of this Ca^{2+}-ATPase activity have been described in

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both vertebrate (Carafoli, 1987; Carafoli and Zurini, 1982; Enouf *et al.*, 1989; Gandhi and Ross, 1988; Saermark and Vilhardt, 1979; Trams and Lauter, 1978) and invertebrate cells (Pietrobon *et al.*, 1990; Viarengo *et al.*, 1991).

In addition, results concerning the enzyme localization in different tissues have been obtained by cytochemical methods. Several techniques involving the precipitation of calcium and lead salts ($Ca_3(PO_4)_2$ and $Pb_3(PO_4)_2$) have been applied to studies of Ca^{2+}-ATPase, Na^+/K^+-ATPase, alkaline phosphatase, etc. (Gomori, 1939; Padykula and Herman, 1955; Wachstein and Meisel, 1957; Ernst, 1972; Salama *et al.*, 1987; Mayahara *et al.*, 1980; Laborde *et al.*, 1990; Halbhuber *et al.*, 1992). A simplified technique for light and electron microscopy has also been developed for the detection of Ca^{2+}-stimulated ATPase activity in different vertebrate tissues (Ando *et al.*, 1981; Narain *et al.*, 1987; Ogawa *et al.*, 1986).

However, no attempts have been made to determine PMCA activity under optimal conditions. An important drawback concerns the use of biochemical assays at pH 9.0, while optimal PMCA activity peaks at pH 7.4 (Viarengo *et al.*, 1991; Stauffer *et al.*, 1995). This results in a very low ATPase activity and concomitant very low calcium transport through the plasma membrane.

In this paper, a cytochemical method suitable to localize and quantify the PMCA activity was developed. The use of image analysis allowed us to estabilish optimal analysis conditions at physiological pH (ionic strength, Ca^{2+}, Mg^{2+} and ATP concentrations etc.), to evaluate the enzyme activity, substrate specificity and the effects of the ATPase inhibitor vanadate.

The method was developed using frozen sections obtained from digestive glands of the marine mussel *Mytilus galloprovincialis* Lam, and applied to *Chamelea gallina* and *Tapes tapes* due to the lack of information on PMCA cytohistochemistry in molluscs. Moreover, recent studies have indicated that Ca^{2+}-ATPases may represent an important target for marine pollutants (Viarengo *et al.*, 1993).

The characteristic tendency to accumulate toxic chemicals has made filter feeding lamellibranch molluscs important bioindicators in biomonitoring programs, and PMCA evaluation could become an important new biomarker of stress in these organisms.

MATERIALS AND METHODS

Materials

All reagents were of highest grade (Sigma Chemical Co., St. Louis, MO): paraformaldehyde (pFA), adenosine-5'-triphosphate (ATP), adenosine-5'-diphosphate (ADP), glucose-6-phosphate (G6P), β-glycerophosphate, ethylene glycol-bis(β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA), lead acetate-citrate complex (LACA), sodium ortho-vanadate, calmodulin, levamisole, ouabain, tris(hydroxymethyl)-aminomethane (TRIS), malic acid, ammonium sulfide.

Animals

Specimens of *Mytilus galloprovincialis* Lam., measuring 4-5 cm in shell length, were collected at Palmaria (La Spezia, Italy). Animals were transferred to the laboratory and maintained for three days in an aquarium with filtered, aerated, artificial seawater at 16°C before use (La Roche *et al.*, 1970). In some experiments *Tapes tapes* and *Chamelea gallina* (sampled at Cesenatico, Italy) were also used.

Tissue Sampling

Digestive gland samples were mildly fixed in 1% paraformaldehyde, 3.5% w/v NaCl pH 8 for 30 min at 4°C. Subsequently, samples were washed twice in 0.2M Tris/maleate buffer, pH 7.4, flash-frozen for 40 seconds in N-hexane chilled with liquid N_2, and stored at -80°C. Cross sections (10µm) were obtained at -25°C in a HM505N Microm cryostat.

Localization of the Ca^{2+}-ATPase activity

Serial sections of mussel digestive gland were transferred onto glass slides and incubated for 30 min at 20°C in a medium consisting of 0.2 M Tris/maleate buffer, pH 7.4 (Viarengo *et al.*, 1991), 2 mM disodium salt ATP, 0.899 mM $CaCl_2$ (free $[Ca^{2+}]$=200 µM), 200 mM KCl, 8 mM levamisole, 0.2 mM ouabain, 10 mM $Pb(NO_3)_2$. The free $[Ca^{2+}]$ in the mixture was set by using a computer program described by Fabiato and Fabiato (1979). Control samples were incubated in a calcium-free medium containing 2 mM EGTA.

After incubation, the medium was removed and slides were rinsed in ice-cold 0.2 M Tris/maleate buffer, pH 7.4, for 10 min to remove the incubation medium and to stop the reaction. Finally,

slides were immersed in an ammonium sulfide-saturated water solution (1 min) in order to reveal the brown lead sulfide precipitate. Sections were then washed with distilled water, allowed to dry, and mounted in 20% (w/v) glycerol. All experiments were performed in quintuplicate.

The enzyme sensitivity to pH variations could not be tested by this technique due to a strong effect of pH on the amount of lead precipitate.

Quantification of the plasma membrane Ca^{2+}-ATPase activity

The quantification procedure is based upon the Lambert-Beer law, which relates the concentration of the chromophore (c) to the measured extinction or absorbance (A):

$A=\text{Log } I_0/I=k\cdot l\cdot c$

where I and I_0 are the intensities of transmitted and incident light, respectively, l is the path length of the light through the specimen, and k is a constant of maximum absorbance. The area is determined by image analysis of the video frame, and the absorbance in this area is measured.

Enzyme activity determination: A first examination of slides revealed a conspicuous accumulation of precipitate corresponding to the basal plasma membrane of digestive gland tubule cells. Hence, in order to obtain absorbance measurements of the precipitate present in the basal plasma membrane, we first sampled whole tubules on digital images (Fig. 1a). Then, we sampled internal areas representing tubule lumen, cell cytoplasm and apical plasma membrane (Fig. 1b) and finally we obtained the difference between a and b (a-b, Fig. 1c).

The Ca^{2+}-ATPase activity present in the basal plasma membrane was evaluated by comparison between the image calculated density of sample incubated in the presence of Ca^{2+}, and in the absence of Ca^{2+} and in the presence of EGTA (Viarengo *et al.*, 1991).

For each analysis we used five slides, each prepared from a different animal. For each slide a total of 20 images were sampled, hence obtaining a total of 100 optical density data for each experimental group.

Computer system: Images were recorded using a digital imaging system consisting of a Leitz microscope DM RB with a 40x objective, a Dage MTI camera and an analogue-digital converter. Images were digitized using the NIH Image software driving a SCION PCI video acquisition board (Scion Corp., Frederick, Maryland).

RESULTS

Localization of Ca^{2+}-ATPase

Tissue fixation for 30 minutes allowed sufficient preservation of the intracellular structures. Conversely, frozen sections that had not been fixed showed an enlargement of intercellular spaces and a decrease in cellular volume (data not shown). The Ca^{2+}-ATPase reaction product, transformed in lead sulphide, was readily discernible after a few minutes of incubation in standard reaction mixture, and was clearly visible as a dense precipitate in the basal part of the plasma membrane of digestive gland tubule cells (Fig. 1a). Sections incubated in a calcium-free medium showed only a minimal, diffuse precipitate, distributed both extra- and intracellularly (Fig. 1d).

Optimization of the biochemical parameters in the evaluation of Ca^{2+}-ATPase activity by a cytochemical assay

The Ca^{2+}-ATPase activity in the presence of a standard reaction mixture, containing 2 mM ATP and 200 µM free Ca^{2+}, showed a linear trend up to 60 min followed by a plateau (Fig. 2).

The plasma membrane Ca^{2+}-ATPase of mussel gland cells did not seem to be activated by calmodulin in the range 0.1-10 µg/ml (data not shown), similar to the enzyme activity present in mussel gill cells (Viarengo *et al.*, 1991), trout gill cells (Ma *et al.*, 1974) and rat hepatocytes (Carafoli, 1991).

The effect of increasing ATP concentration in the 10 µM-5 mM range on the Ca^{2+}-ATPase activity is shown in Fig. 3. The results demonstrate that the substrate saturation plateau is reached at 2mM ATP.

Moreover, the cytochemical assay showed a good PMCA response to temperature variations from 0°C to 10°C with a Q_{10} of ~ 2 with a progressive decrease in PCMA activity at higher temperatures (Fig. 4).

The effect of ionic strength on the Ca^{2+} stimulated-ATPase activity was measured in the presence of different concentrations of KCl (Fig. 5), finding optimal values in the range of 100-200 mM KCl.

The effect of increasing Mg^{2+} concentrations (1µM-5mM) on the ATPase activity was measured in an assay mixture without added Ca^{2+}. The ATPase activity was optimal at a Mg^{2+} concentration of 1mM. In the presence of 1 mM $MgCl_2$, the addition of 200 µM $CaCl_2$ significantly raised the enzyme activity (Fig. 6).

The PMCA activity as a function of free Ca^{2+} concentration was obtained in the absence of added

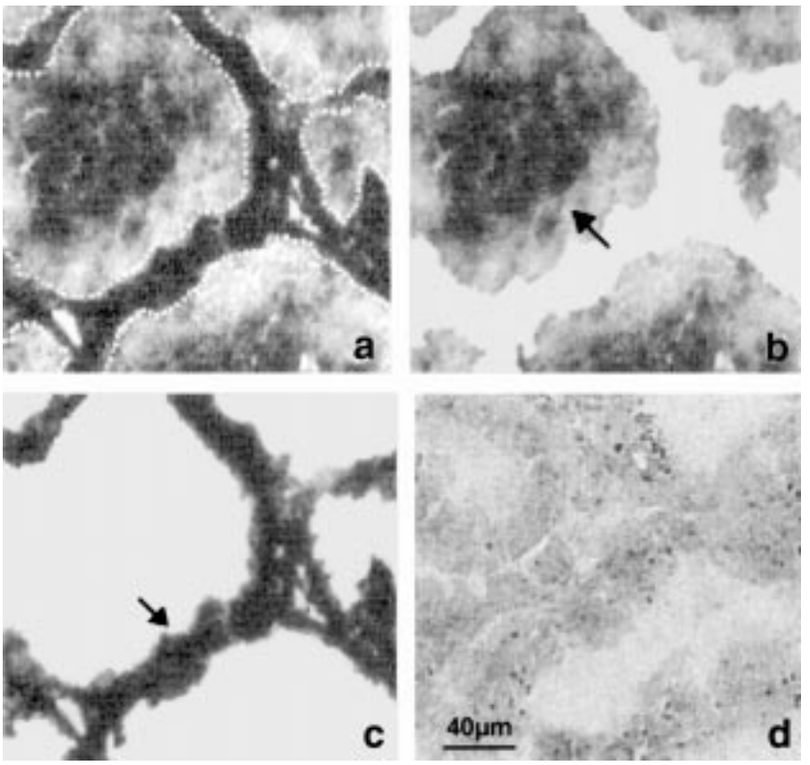

Fig. 1 - Images of digestive gland tubules of *Mytilus galloprovincialis* showing Ca^{2+}-ATPase-specific lead sulfide precipitate (arrows). **(a)**Image of digestive gland after incubation with lead sulfide for 30 minutes. **(b)**Original image shown in a after negative selection of basolateral membrane area's. **(c)**Selected basolateral membranes showing high amounts of lead sulfide precipitate. **(d)**Sample incubated in the absence of Ca^{2+} and in the presence of EGTA.

Mg^{2+}. The Ca^{2+}-ATPase appeared highly sensitive to increasing free [Ca^{2+}]. In the presence of 200nM free Ca^{2+} a stimulation of activity was already visible and reached a maximum at a concentration of 200µM free Ca^{2+} (Fig. 7).

The Ca^{2+}-stimulated phosphatase activity was tested in the presence of different substrates such as ATP, ADP, G6P, β-glycerophosphate, each added at an equimolar concentration. ATP appeared (Fig. 8) to be the preferential substrate but ADP was also hydrolyzed, although to a lesser extent (24.4% of maximum hydrolysis obtained with ATP). By contrast, G6P and β-glycerophosphate were poorly hydrolyzed.

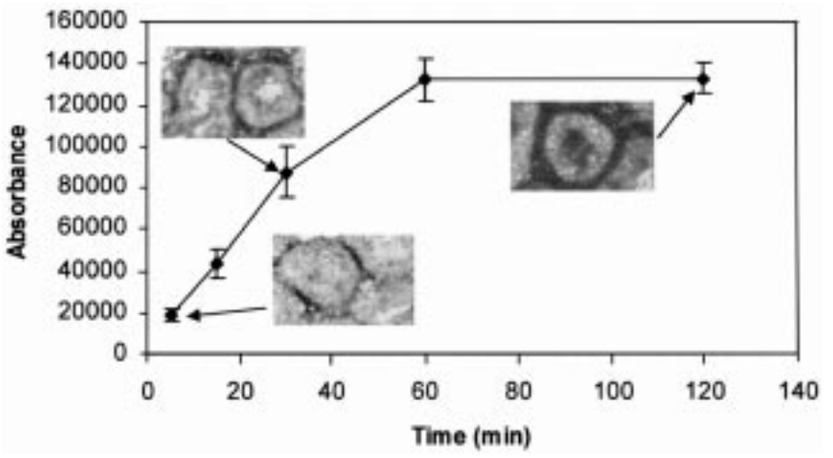

Fig. 2 - Time course of mussel digestive gland PMCA activity. Experiments were carried out in the presence of 200µM Ca^{2+} using the standard reaction medium as described in the Materials and Methods. In this and the following figures, values are the mean ± SD from n=100 measurements.

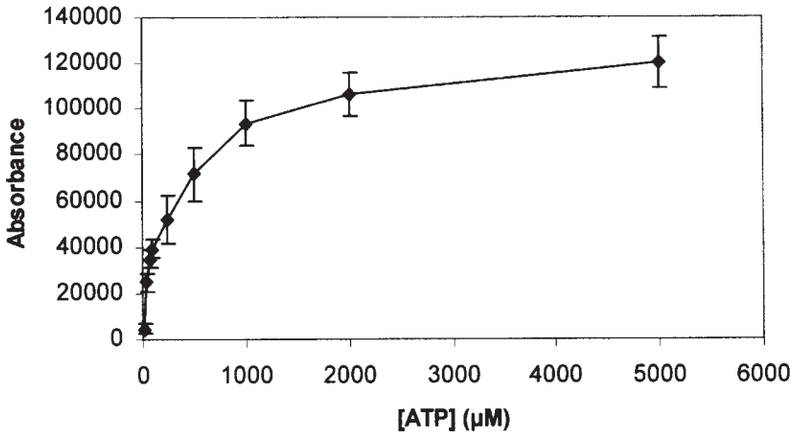

Fig. 3 - Ca^{2+}-ATPase activity at various ATP concentrations in the presence of 200µM Ca^{2+}. Enzyme activity was evaluated as described in Materials and Methods.

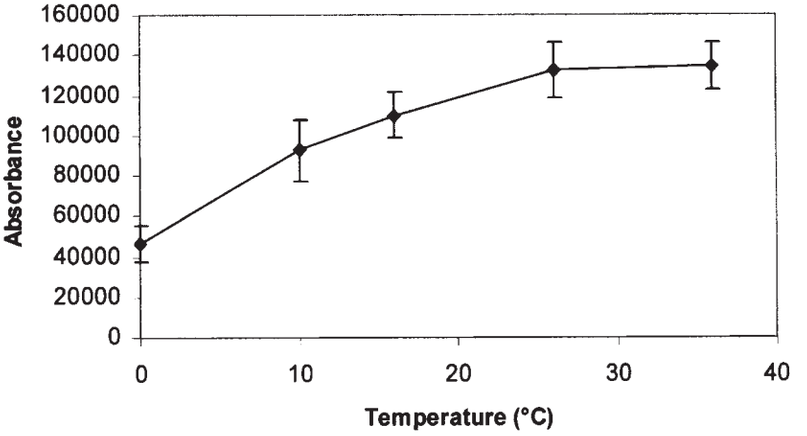

Fig. 4 - Temperature dependence of PMCA activity. Experiments were carried out in the presence of 200µM free Ca^{2+} as described in Materials and Methods.

Sodium ortho-vanadate is a well-known inhibitor of ATPase activity related to transmembrane ion transport (Carafoli, 1987). Fig. 9 shows that in our assays the mussel PMCA exhibited a high sensitivity to vanadate. The Ca^{2+}-ATPase activity was significantly reduced at a concentration of 200 µM vanadate and totally inhibited at 1 mM.

We have applied this method to identify and localize the plasma membrane Ca^{2+}-ATPase activity in two other molluscs: *Tapes tapes* and *Chamelea gallina*. Similar to what was found in *Mytilus galloprovincialis* (Fig. 10a), the Ca^{2+}-ATPase activity in Tapes tapes is present in the basal membrane of the tubular digestive cells (Fig. 10b), while in *Chamelea gallina* the enzyme is located in the apical portion (Fig. 10c).

DISCUSSION

So far, cytochemical methods to detect Ca^{2+}-dependent-ATPase activities have utilized alkaline buffers with pH values ranging from 8 to 9 (Padykula & Herman, 1955; Ando *et al.*, 1981). This is mainly due to the fact that Pb^{2+} salts (Ando *et al.*, 1982; Chayen *et al.*, 1981; Ernst, 1972; Mayahara *et al.* 1980; Wachstein and Meisel, 1957) or Ca^{2+} salts (Halbhuber *et al.*, 1996; Padykula and Herman 1955), both of which are used to trap the PO_4^{2-} ion released by ATP hydrolysis, are efficiently precipitated at alkaline pH. However, biochemical data clearly indicate that the Ca^{2+}-dependent ATPase activity involved in transport of calcium cations from the cytosol to

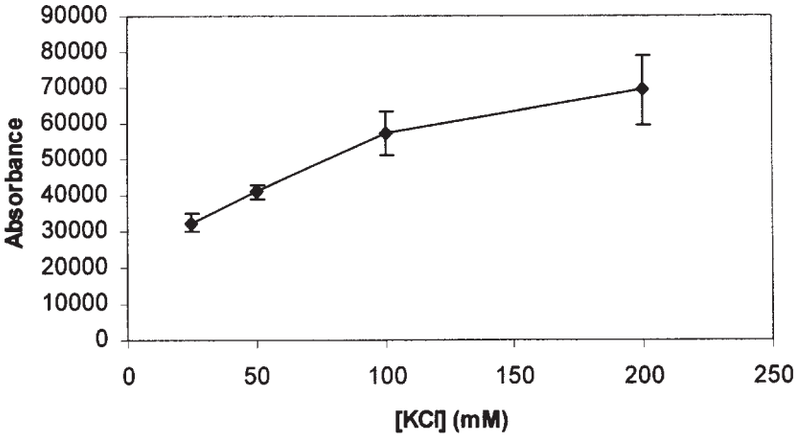

Fig. 5 - Effect of increasing KCl concentrations on the Ca^{2+}-ATPase activity. The experiments were carried out as described in Materials and Methods.

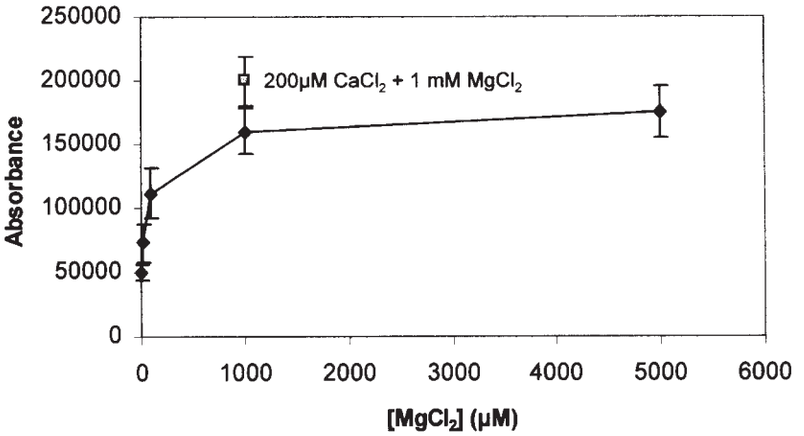

Fig. 6 - Effect of increasing Mg^{2+} concentration on the ATPase activity. The enzyme activity was evaluated in the absence (■) or in the presence (◆) of free Ca^{2+} using the standard reaction mixture described in Materials and Methods.

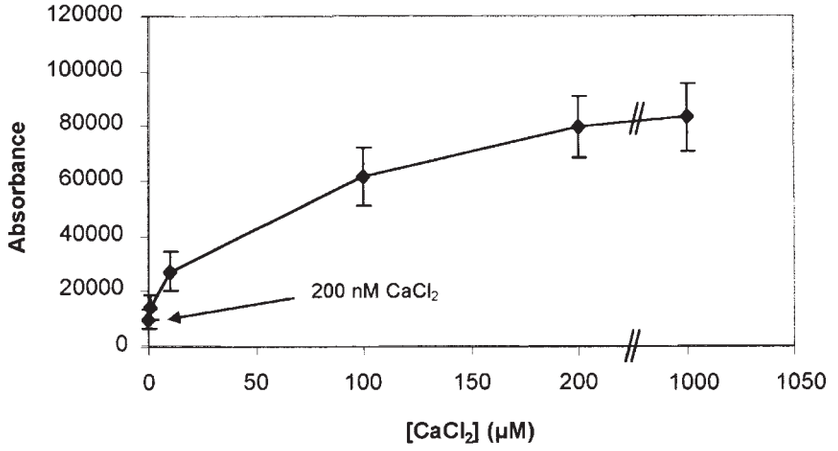

Fig. 7 - Effect of increasing free $[Ca^{2+}]$ on the ATPase activity. The experiment was carried out in the absence of Mg^{2+} and the enzyme activity was evaluated as described in Materials and Methods.

the external medium is optimal between pH 7.2-7.4 and extremely low at pH values higher than 8 (Stauffer *et al.*, 1995). This is also true for the Ca^{2+}-ATPase activity present in the plasma membrane of mussel cells (Viarengo *et al.*, 1991).

In order to obtain a sensitive cytochemical tech-

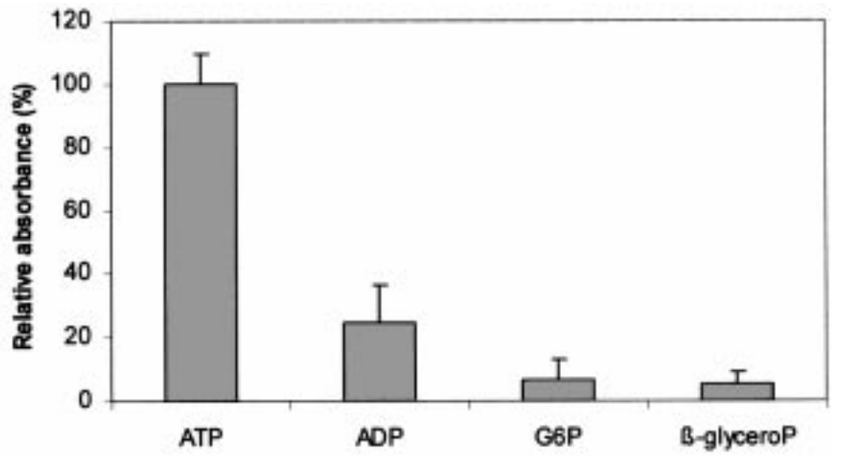

Fig. 8 - Substrate specificity of mussel PMCA. Data are expressed as percentage of the hydrolysis of different substrates with respect to ATP. The different substrates were added to a standard reaction mixture at equimolar concentrations.

nique providing for both a correct biochemical characterization of the enzyme and its localization, a method was developed to detect, at physiological pH, the Ca^{2+}-ATPase activity present in the plasma membrane of the digestive gland of mussels. The cytochemical procedure was carried out on frozen sections, using a mild fixation with paraformaldehyde to preserve tissue morphology and to increase membrane permeability. This resulted in a uniform permeation of the different components of the Ca^{2+}-ATPase reaction mixture, allowing equal enzyme activity rates in all the digestive gland cells in the section.

$Pb(NO_3)_2$ was used as capturing agent to optimize enzyme localization. Such a technique is largely based on the theory that phosphate ions, delivered by enzymatic hydrolysis of organic phosphates, are trapped in "status nascendi" at their site of formation by Pb^{2+} cations present in the medium to form a highly insoluble precipitate. Our data demonstrate that the Ca^{2+}-dependent ATPase activity, as revealed by a homogeneous precipitate of lead sulfide, is located at the basal part of the plasma membrane of tubule cells of mussel digestive gland.

Such enzymatic activity is highly sensitive to increasing Ca^{2+} ion concentrations, whereas in a Ca^{2+}-free medium only a diffuse phosphate precipitate can be observed. Our data suggest that this method might be both sensitive and specific in demonstrating Ca^{2+}-dependent ATPase activity in the plasma membrane of mussel digestive gland cells.

On the contrary, LACA, a lead complex widely used as a phosphate capturing agent (Gomori, 1939; Padykula and Herman, 1955; Wachstein and Meisel, 1957; Ernst, 1972; Salama *et al.*, 1987; Mayahara *et al.*, 1980; Laborde *et al.*, 1990; Halbhuber *et al.*, 1992), produced poor results in our assays. LACA required longer incubation periods (up to 3 hours) than $Pb(NO_3)_2$, and also produced

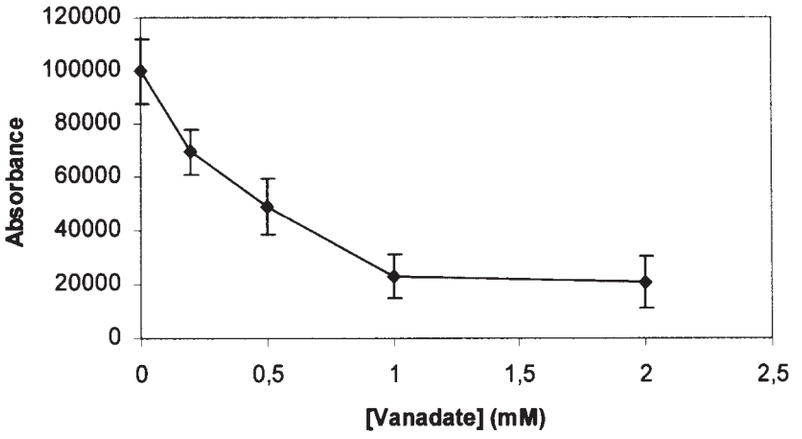

Fig. 9 - Inhibition of Ca^{2+}-ATPase activity by vanadate, evaluated in the presence of 200µM free Ca^{2+}. Data yield an IC50 of ~300µM.

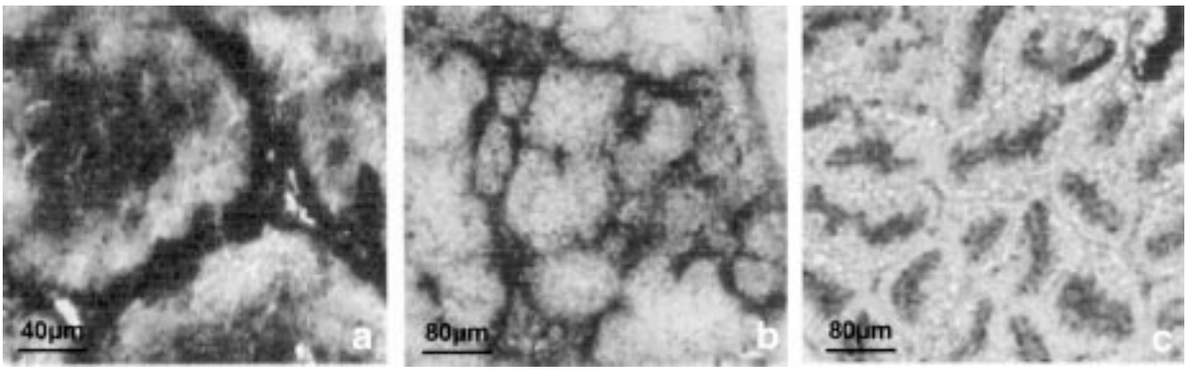

Fig. 10 - Localization of Ca^{2+}-ATPase after incubation with lead sulfide. In *Mytilus galloprovincialis* (a) and Tapes tapes (b), the Ca^{2+}-ATPase is mainly localized in the basolateral membrane of the digestive gland cells. In *Chamelea gallina* (c), the enzyme is localized in the apical membrane. Images were obtained as described under Materials and Methods.

an increase in background staining, with atypical localization, i.e. in the lysosomal vacuolar system (Pons, unpublished data).

Quantification of the Ca^{2+}-ATPase-specific lead precipitate after image analysis enabled the design of a reproducible and sensitive semiquantitative cytochemical method, suitable to evaluate variations in enzymatic activity.

In addition, under optimal assay conditions (200 µM free Ca^{2+}, 200 mM KCl, 2 mM ATP, pH 7.4) a linear increase in Ca^{2+}-ATPase activity was detected for up to 60 min. The Ca^{2+}-stimulated enzymatic activity is a true ATPase, preferentially hydrolysing ATP over ADP. Moreover, other phosphorylated substrates, such as G6P and β-glycerophosphate, are essentially not utilized by the enzyme.

Similar to other cation translocases described in vertebrate and invertebrate cells, the enzyme is highly inhibited by micromolar concentrations of vanadate, a specific inhibitor of cation pumps.

Comparison of our cytochemical results with previous biochemical data (Viarengo *et al.*, 1991) demonstrates a good correlation between the biochemical properties of the Ca^{2+}-ATPase identified in purified plasma membranes of mussel gill cells and the cytochemical behaviour of the enzyme present in the basal membrane of digestive gland cells.

Unlike in human red blood cells, Ca^{2+} stimulated-ATPase activity in mussel digestive glands, trout gills, rat hepatocytes (Lin, 1985) and corpus luteum plasma membranes (Verma and Penniston, 1981), can also be activated by Ca^{2+} ions in the absence of exogenous Mg^{2+}.

In situ ATP hydrolysis in mussel gland tubule plasma membrane preparations can be enhanced by the addition of Mg^{2+}.

The data showing that, the Ca^{2+}-ATPase activity present in mussel glands, like the typical transmembrane Ca^{2+}-transport ATPase (Ghijsen *et al.*, 1980), is stimulated by a wide range of free Ca^{2+} concentrations (starting at 200 nM), suggests that this Ca^{2+}-ATPase may be involved in Ca^{2+} homeostasis (Carafoli, 1987).

Various studies on the toxic effects of pollutants have clarified that certain chemicals may alter Ca^{2+} homeostasis by inhibiting PMCA activity (Orrenius, 1989; Viarengo and Nicotera, 1991), leading to enhanced intracellular [Ca^{2+}] levels. Therefore, our "cytobiochemical" method, allowing both localization and quantification of the PMCA activity, seems useful to evaluate the effects of environmental stress on Ca^{2+}-dependent signal transduction pathways. Accordingly, it might be used as an "early warning" biomarker in biomonitoring programs.

The application of this technique in other molluscs has demonstrated a striking difference in the Ca^{2+}-ATPase localization. *Tapes tapes* showed a basolateral distribution similar to the mussel, whereas in *Chamelea gallina* the enzyme activity was localized to the apical membrane. This latter localization might increase, at least in part, the susceptibility of *Chamelea gallina* to environmental stressors. In fact, as the adsorption of nutrients and pollutants mostly occurs on the apical side of the digestive gland cells, this area of the cell (i.e. the apical membrane) is the first target for pollutants that enter the cell. This fact could render the organism more sen-

sitive to environmental stressors, and in part explains the high mortality of *Chamelea gallina* in specific coastal areas of the Adriatic sea in recent years. Preliminary data indicate that in stressed animals (as judged by a battery of biomarkers such as lysosomal membrane stability, DNA damage, lysosomal lipofuscin accumulation, etc.) living in polluted areas, a strong decrease of Ca^{2+}-ATPase activity in digestive gland cells is usually observed.

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REFERENCES

Ando T., Fujimoto K., Mayahara H., Miyajima H., and Ogawa K.: A new one-step method for the histochemistry and cytochemistry of Ca^{2+}-ATPase activity. Acta Histochem. Cytochem. *14*, 707-726, 1981.

Ando T., Mayahara H., Fujimoto K., Miyajima H., and Ogawa K.: Ultracytochemical localization of Ca^{2+}-ATPase activity on the epitelial cells of rat trachea. Acta Histochem. Cytochem. *15*, 812, 1982.

Carafoli E., and Zurini M.: The Ca^{2+}-pumping ATPase of plasma membranes. Purification, reconstitution and properties. Biochim. Biophys. Acta *31*, 683(3-4), 279-301, 1982.

Carafoli E.: Intracellular calcium homeostasis. Annu. Rev. Biochem. *56*, 395-433, 1987.

Carafoli E.: Calcium pump of the plasma membrane. Physiol. Rev. *71*, 129-153, 1991.

Carafoli E.: Biogenesis: plasma membrane calcium ATPase: 15 years of work on the purified enzyme. FASEB J. *8(13)*, 993-1002, 1994.

Chayen J., Frost G.T.B., Doods R.A., Bitsensky L., Pitchfork J., Baylis P.H., and Barmet R.J.: The use of a hidden metal captive reagent for the measurement of Na^+/K^+-ATPase activity: A new concept in cytochemistry. Histochemistry *71*, 533-541, 1981.

Enouf J., Bredoux R., Bordeau N., Sarkadi B., and Levy-Toledano S.: Further characterization of the plasma membrane- and intracellular membrane-associated platelet Ca^{2+} transport systems. Biochem. J. *15*, 263(2), 547-52, 1989.

Ernst S.A.: Transport adenosine triphosphatase cytochemistry. II. Cytochemical localization of ouabain-sensitive, potassium-dependent phosphatase activity in the secretory epithelium of the avian salt gland. J. Histochem. Cytochem. *20(1)*, 23-28, 1972.

Fabiato A., and Fabiato E.: J. Physiol. (Paris) *75*, 463-505, 1979.

Gandhi C.R., and Ross D.H.: Characterization of a high-affinity Mg^{2+}-independent Ca^{2+}-ATPase from rat brain synaptosomal membranes. J. Neurochem. *50(1)*, 248-56, 1988.

Ghijsen W.E.M., De Jong H.R., and Van Os C.H.: Dissociation between Ca^{2+}-ATPase and alkaline phosphatase activities in plasma membranes of rat duodenum. Biochim. biophys. Acta *599*, 538-551, 1980.

Gomori G.: Microtechnical demonstration of phosphatase in tissue sections. Proc. Soc. Exp. Biol. Med. *42*, 23-26, 1939.

Halbhuber K.J., Feuerstein H., Möller U., and Möller M.: Modified cerium-based and Gomori-based cerium methods for light microscopic phosphatase histochemistry: The cerium-perhydroxide-diaminobenzidine-nickel (Ce-H_2O_2-DAB-Ni and Ce/Ce-H_2O_2-DAB-Ni) two-step procedures. Acta Histochem. *92*, 87-103, 1992.

Halbhuber K.J., Scheven C., Jirikowski G., Feuerstein H., and Ott U.: Reflectance enzyme histochemistry (REH): visualization of cerium-based and DAB primary reaction products of phosphatases and oxidases in cryostat sections by confocal laser scanning microscopy. Histochem. Cell Biol. *105*, 239-249, 1996.

Laborde K., Bussieres L., De Smet A., Dechaux M., and Sachs C.: Quantification of renal Na^+/K^+-ATPase activity by image analysing systems. Cytometry *11(8)*, 859-868, 1990.

La Roche G., Eisler R., and Tarzwell C.M.: Bioassay procedure for oil and oil dispersant toxicity evaluation. J. Water Pollut. Control Fedn. *42*, 1982-1988, 1970.

Lin S.H.: The rat liver plasma membrane high affinity (Ca^{2+}-Mg^{2+})-ATPase is not a calcium pump. J. Biol. Chem. *260(20)*, 10976-10980, 1985.

Ma S.W.Y., Shami Y., Messer H.H., and Copp D.H.: Properties of Ca^{2+}-ATPase from the gill of rainbow trout (Salmo gairdneri). Biochim. biophys. Acta *345*, 243-251, 1974.

Mayahara H., Fujimoto K., Ando T., and Ogawa K.: A new one-step method for the cytochemical localization of ouabain-sensitive potassium-dependent p-nitrophenylphosphatase activity. Histochemistry *67*, 125-138, 1980.

Narain S., Fujimoto K., and Ogawa K.: Ca^{2+}-ATPase activity in the gastric parietal cells of rat during perinatal development. Acta Histochem. Cytochem. *20*, 57, 1987.

Ogawa K.S., Fujimoto K., and Ogawa K.: Ultracytochemical studies of adenosine nucleotidases in aortic endothelial and smooth muscle cells – Ca^{2+}-ATPase and H^+, K^+-ATPase. Acta Histochem. Cytochem. *19*, 601, 1986.

Orrenius S., McConkey D.J., Bellomo G., and Nicotera P.: Role of Ca^{2+} in toxic cell killing. Trends Pharmacol. Sci. *10(7)*, 281-5, 1989.

Padykula H.A., and Herman E.: Factors affecting the activity of adenosine triphosphatase and other phosphatases as measured by histochemical techniques. J. Histochem. Cytochem. *20*, 375-380, 1955.

Pietrobon D., Di Virgilio F., and Pozzan T.: Structural and functional aspects of calcium homeostasis in eukaryotic cells. Eur. J. Biochem. *193(3)*, 599-622, 1990.

Rasmussen H., and Barret P.Q.: Calcium messenger system: an integrated view. Physiol. Rev. *64*, 938-984, 1984.

Saermark T., and Vilhardt H.: Isolation and partial characterization of magnesium ion- and calcium ion-dependent adenosine triphosphatase activity from bovine brain microsomal fraction. Biochem. J. *181(2)*, 321-30, 1979.

Salama A.H., Zaki A., and Eisenmann D.R.: Cytochemical localization of Ca^{2+}-Mg^{2+} adenosine triphosphatase in rat incisor ameloblasts during enamel secretion and maturation. J Histochem. Cytochem. *35(4)*, 471-482, 1987.

Stauffer T.P., Guerini D., and Carafoli E.: Tissue distribution of the four gene products of the plasma membrane Ca^{2+} pump. A study using specific antibodies. J. Biol. Chem. *270(20)*, 12184-90, 1995.

Strehler E.E.: Recent advances in the molecular characterization of the plasma membrane Ca^{2+} pump. J. Membr. Biol. *120*, 1-15, 1991.

Trams E.G., and Lauter C.J.: A comparative study of brain Ca^{2+}-ATPases. Comp. Biochem. Physiol. B. *59(3)*,191-4, 1978.

Van Os C.H.: Transcellular calcium transport in intestinal and renal epithelial cells. Biochimica et Biophysica Acta *906*, 195-222, 1987.

Verma A.K., and Penniston J.T.: A high affinity Ca^{2+} stimulated and Mg^{2+}-dependent ATPase in rat corpus luteum plasma membrane fractions. J. Biol. Chem. *256(3)*, 1269-1275, 1981.

Viarengo A., and Nicotera P.: Possible role of Ca^{2+} in heavy metal cytotoxicity. Comp. Biochem. Physiol. C. *100(1-2)*, 81-4, 1991.

Viarengo A., Pertica M., Mancinelli G., Damonte G., and Orunesu M.: Biochemical characterization of the plasma membrane Ca^{2+} pumping ATPase activity present in the gill cells of *Mytilus galloprovincialis* L. Comp. Biochem. Physiol. *100B*, 753-758, 1991.

Viarengo A., Mancinelli G., Pertica M., Fabbri R., and Orunesu M.: Effects of heavy metals on the Ca^{2+}-ATPase activity present in gill cell plasma-membrane of mussels (*Mytilus galloprovincialis* Lam.). Comp. Biochem. Physiol. *106C*, 655-660, 1993.

Wachstein M., and Meisel E.: Histochemistry of hepatic phosphatases at physiologic pH. With special reference to the demonstration of bile canaliculi. Am. J. Clin. Pathol. *27*, 13, 1957