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Mercury- and copper-induced lysosomal membrane destabilisation depends on $[Ca^{2+}]_i$ dependent phospholipase A2 activation

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Abstract

Heavy metals are environmental pollutants able to produce different cellular effects, such as an alteration of Ca^{2+} homeostasis and lysosomal membrane destabilisation. The latter is one of the most used stress indices in biomonitoring programs. Recently, it has been demonstrated that cytosolic calcium increase can modulate lysosomal membrane destabilisation via activation of Ca^{2+} -dependent phospholipase A2 (cPLA2). The aim of this work was to investigate the possible involvement of Ca^{2+} -activated PLA2 in lysosomal membrane destabilisation induced by heavy metals in mussel haemolymph cells. We have studied the effects of Hg^{2+} and Cu^{2+} on free cytosolic calcium using Fura2/AM-loaded cells and lysosomal membrane destabilisation using neutral red (NR) staining. Hg^{2+} induced a $[Ca^{2+}]_i$ rise from 100 to 780 nM in 30 min, and a lysosome destaining of 70% after 60 min that indicates destabilisation of lysosomal membranes. Both effects were reduced in a Ca^{2+} -free medium, suggesting a cause-effect relationship. Exposure to Cu^{2+} produced the same effects, but with an intensity of about 50% respect to Hg^{2+} . Metal-induced lysosomal destabilisation was also reduced in cells pre-exposed to a specific Ca^{2+} -dependent cPLA2 inhibitor (AACOCF3). Conversely, haemocyte pretreatment with a Ca^{2+} -independent PLA2 inhibitor (bromo-enol-lactone (BEL)) did not prevent the destabilizing effect of heavy metals on lysosomes. Exposure to heavy metals also produced an increase in lysosomal volume of 1.8–2-folds, that was prevented by pre-incubation with AACOCF3 but not with BEL. These data indicate an involvement of cPLA2 in lysosomal membrane destabilisation induced by heavy metals.

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Keywords: Heavy metals; Intracellular calcium; Lysosomal membrane stability; Phospholipase A2; BEL; AACOCF3

1. Introduction

The involvement of intracellular calcium mobilisation in heavy metal cytotoxicity was investigated

in several studies (Viarengo and Nicotera, 1991; Nathanson et al., 1995; Hechtenberg et al., 1996). Heavy metals can affect calcium homeostasis systems through direct interaction (Viarengo, 1994; Verbost et al., 1989; Zhang et al., 1990) or by induction of oxidative processes (Suzuki and Ford, 1991; Burlando et al., 1997). A sustained increase of cytosolic free calcium, $[Ca^{2+}]_i$, initiates a signaling cascade leading

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to the activation of phospholipase A2 and C, endonucleases and proteases (Nicotera and Orrenius, 1998; Trump, 1996), and the expression of *c-fos*, *c-jun* and *c-myc* (Trump and Berezsky, 1996).

Another known cytotoxic effect of metal cations is the alteration of the morphology and functionality of lysosomes, cellular organelles which accumulate metal cations in non-toxic forms (Moore, 1991). When the heavy metal content exceeds the capacity of the lysosome, damage and leakage occur, with release of hydrolytic enzymes into the cytoplasm leading to severe cell damage (Deckers et al., 1980; Viarengo et al., 1981; Moore et al., 1984). These processes provide the basis of the use of lysosomal membrane stability as a general biomarker of environmental stress (Moore et al., 1984; Viarengo et al., 2000). Lysosomal membrane stability is an extremely sensitive stress index related to a variety of organic and inorganic xenobiotics, as well as physical environmental stressors, such as low temperature (Camus et al., 2000).

Membrane destabilisation has been associated with fusion of lysosomes to produce enlarged organelles involved in the autophagy process (Lowe, 1988). Lysosome volume changes are related to non-specific manifestations of sublethal injury due to pollutant exposure. Hence, the lysosome/cell ratio is also used as a general stress index in biomonitoring analyses (Lowe et al., 1981; Marigomez et al., 1989; Cajaraville et al., 2000).

Metal cation damage of lysosomal membranes has been the object of various studies (Viarengo et al., 2000; Pourahmad et al., 2001; Eaton and Qian, 2002) but the mechanism of injury is still unclear. However, it has been recently demonstrated that cytosolic Ca^{2+} -dependent phospholipase A2 (cPLA2) has a role in lysosomal activation by 17β -estradiol (Burlando et al., 2002). The elevation of $[\text{Ca}^{2+}]_i$ is a prerequisite for PLA₂ activation (Clark et al., 1991) and it is possible that this process contributes to the Ca^{2+} cytotoxicity process (Schievella et al., 1995). It has been shown that in mammals cPLA2 can cause membrane degradation, changes in plasma and mitochondrial membrane bioenergetics and permeability (Zhao et al., 2001), and lysosomal membrane destabilisation (Mukherjee et al., 1997). These effects, together with increased production of lysophospholipids, arachidonic acid, eicosanoids, platelet activating factors, and reactive oxygen species, have been implicated in

destructive cellular processes due to cPLA2 in kidney, heart, intestine and the central nervous system (Sapirstein et al., 1996). Recent studies in mussel haemolymph cells demonstrated that heavy metals alter both the intracellular calcium level (Viarengo et al., 1994) and lysosomal membrane functionality (Viarengo et al., 2000).

Marine mussels are used as environmental sentinels in pollution biomonitoring (Moore, 1991) due to their capability of contaminant accumulation by filter feeding. Haemocytes represent the main component of the mussel immune system and have a highly-developed lysosomal system. These cells can be easily obtained from mussel haemolymph in a non-destructive manner (Moore et al., 1996). In this work, $[\text{Ca}^{2+}]_i$ variations in Fura2-loaded haemocytes exposed to Hg^{2+} or Cu^{2+} were evaluated by image analysis and correlated to the effects on lysosomal membrane stability in living cells using the neutral red (NR) cytotoxicity test (Lowe et al., 1992; Lowe and Pipe, 1994). Moreover, a possible involvement of cPLA2 in lysosomal membrane destabilisation was investigated by using AACOCF3, a specific Ca^{2+} -dependent cPLA₂ inhibitor, or bromoenol-lactone (BEL), a Ca^{2+} -independent PLA₂ inhibitor, which have already been used also on invertebrate cells (Ford et al., 1999; Canesi et al., 2002).

2. Materials and methods

2.1. Chemicals

Digitonin, neutral red, bromoenol-lactone: (*E*)-6-(bromomethylene)tetrahydro-3-(1-naphthalenyl)-2H-pyran-2-one (BEL), poly-L-lysine and Sigmacote were obtained from Sigma Chemical Co. (St. Louis, MO); arachidonyl trifluoromethyl ketone (AACOCF3) was obtained from Calbiochem (La Jolla, CA); Fura2/AM was from Molecular Probes (Eugene, OR); all other reagents were of analytical grade.

2.2. Solutions

Artificial sea water: 473 mM NaCl, 18 mM Na_2SO_4 , 16 mM MgCl_2 , 6.2 mM KCl, 5 mM CaCl_2 , 1.5 mM NaHCO_3 , 0.045 mM NaF, 0.56 mM KBr, 0.32 mM H_3BO_3 , 0.048 mM $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, pH 8.0.

Physiological saline: 20 mM Hepes, 436 mM NaCl, 53 mM MgSO₄, 10 mM KCl, 10 mM CaCl₂, pH 7.3.

Loading buffer: 30 mM Hepes, 0.5 mM sucrose, 2.5 mM MgCl₂, 2.5 mM CaCl₂, 125 mM NaCl, 2.5 mM KCl, pH 7.3.

2.3. Animals

Mussels (*Mytilus galloprovincialis* Lam.) with a shell length of 4–5 cm were obtained from a local farm (La Spezia, Italy) and acclimated for 3 days in an aquarium with aerated, recirculating artificial seawater at 15 °C.

2.4. Haemolymph cell collection

Mussel haemolymph (1 ml) was withdrawn from the posterior adductor muscle with a hypodermic syringe containing an equal volume of artificial seawater. The needle was then removed and the syringe content was put into a siliconised (Sigmacote) eppendorf tube.

2.5. Evaluation of lysosomal membrane stability and of lysosomal volume

Aliquots of 40 µl of haemocytes suspension were dispensed onto microscope coverslips pre-treated with 2 µl poly-L-lysine (1:10 in distilled water) to improve cell-to-substratum adhesion. Coverslips were incubated for 30 min in a light-proof humidity chamber at 15 °C, to allow for cell settling. Excess suspension was then drained off from the coverslip and 40 µl of a 5% neutral red stock solution (20 mg/ml in DMSO) in artificial seawater was added. After 15 min, excess dye was washed out and 40 µl of artificial seawater was added. At regular intervals, coverslips were inspected under an Olympus IMT-2 inverted microscope equipped with a CCD CUE video camera, and images were recorded by a Dage MTI camera and digitised by the CUE2 imaging system (Galai Production Ltd., Israel). After image recording coverslips were transferred back to the humidity chamber. A number of fields were recorded on the same coverslip during the microscopic capture of images.

Digitised images allowed the evaluation of NR retention time within lysosomes. The optical density (OD) of 30–60 cells, randomly selected on each of four coverslips from different cell preparations, was

measured by first tracing the cell contour and then recording the average OD within the selected area. An estimate of the ratio between the total cell volume and the overall volume of lysosomes was obtained by measuring whole cell areas and overall lysosomal areas in 30–60 different cells selected as described above, using an OD threshold function in the CUE2 imaging system.

2.6. [Ca²⁺]_i measurements

Aliquots of 40 µl of haemolymph suspension were settled on coverslips as described above, incubated with loading buffer containing 4 µM Fura2/AM for 30 min at 15 °C, rinsed with buffer to remove extracellular dye, and bathed with 40 µl of physiological saline ± metal. Cells were then observed under an Olympus IMT-2 inverted microscope equipped with an IMT-2-RFL fluorescence attachment (Olympus Optical Co., Germany) and with an MTI SIT 68 intensified camera (Oatencourt Ltd., England). Images were acquired every minute using the CUE2 RMS 4.0 imaging system (Galai Production Ltd., Israel). Background fluorescence was subtracted before analysis.

Fura2 calibration was achieved by the equation of Grynkiewicz et al. (1985):

$$[\text{Ca}^{2+}]_i = \frac{K_d(F - F_{\min})}{F_{\max} - F} \frac{\text{Sf}_2}{\text{Sb}_2}$$

where K_d = 135 nM, F_{\max} and F_{\min} are the maximum and minimum fluorescence intensities, measured after cell treatment with 50 µM digitonin and 5 mM EGTA, respectively, and Sf_2/Sb_2 is the ratio between the excitation efficiencies of free probe and Ca²⁺-bound probe at 380 nm.

2.7. Statistics

Data were analysed by the ANOVA and Dunnet's tests using the Instat Software Package (GraphPad, San Diego, CA).

3. Results

Haemolymph cells exposed to Hg²⁺ (50 µM) showed a time-dependent increase of cytosolic calcium concentration reaching the value of about

800 nM after 30 min from an average basal level of about 78 nM (Fig. 1A). In order to evaluate the possible influence of Ca^{2+} on lysosomal membrane stability, cells were treated with Hg^{2+} in a Ca^{2+} -free medium, obtaining a drastic reduction in the $[\text{Ca}^{2+}]_i$ variation induced by the metal (Fig. 1A). A similar result was observed after exposure to Cu^{2+} (50 μM)

in the presence of extracellular Ca^{2+} , but with a lower Ca^{2+} increase compared to Hg^{2+} (Fig. 1A).

Lysosomal membrane destabilisation was evaluated by staining cells with the lysosomotropic dye neutral red. Exposure to Hg^{2+} of NR-stained cells reduced staining to about 30% of the control after 60 min. (Fig. 1B). In cells exposed to Hg^{2+} in a

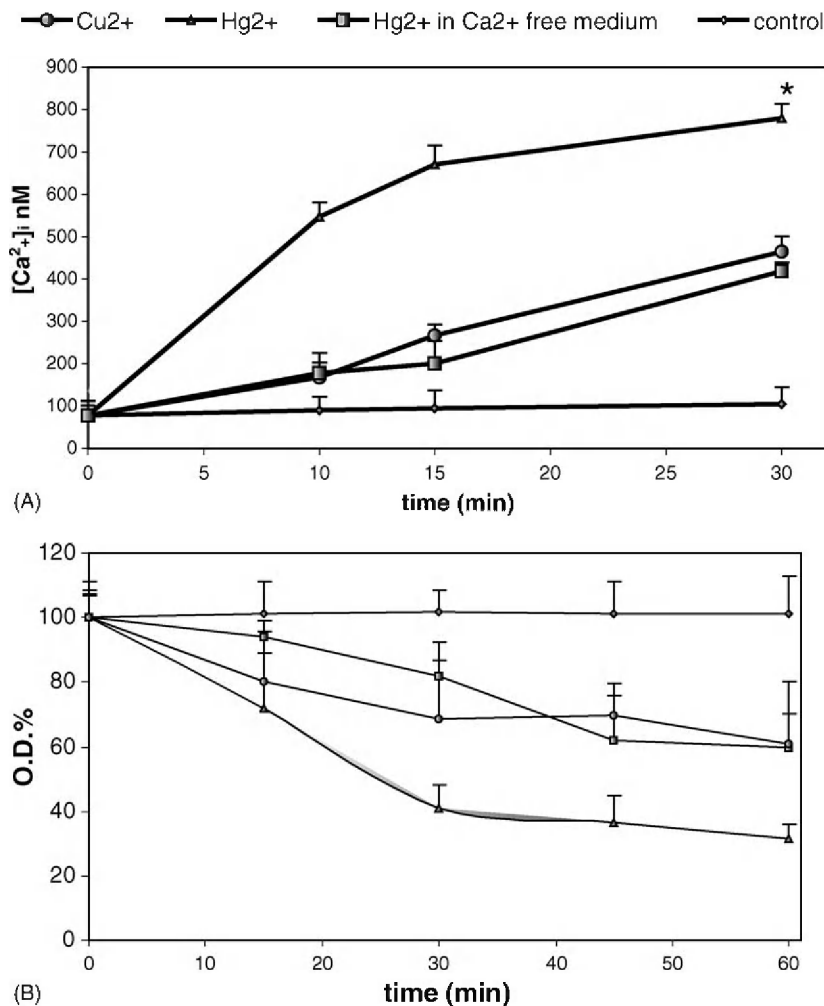


Fig. 1. Effects of Hg^{2+} or Cu^{2+} on the $[\text{Ca}^{2+}]_i$ and lysosomal membrane stability in mussel haemocytes. (A) Cells treated with 50 μM Hg^{2+} show a significant ($P < 0.01$) $[\text{Ca}^{2+}]_i$ rise in 30 min. Exposure of haemocytes to 50 μM Hg^{2+} in a Ca^{2+} -free medium or to 50 μM Cu^{2+} in the presence of Ca^{2+} induced a lower but significant ($P < 0.01$) variation in $[\text{Ca}^{2+}]_i$ level. Data are means \pm S.D. of Ca^{2+} measurements in 15 different cells. Statistical comparisons (Dunnett's test) were made on endpoint values at 30 min. (B) 50 μM Hg^{2+} induced a significant ($P < 0.01$) decrease of lysosome optical density in cells stained with neutral red. Haemocytes treated with 50 μM Cu^{2+} or 50 μM Hg^{2+} in a Ca^{2+} -free medium showed a lower but significant rate of lysosome destaining ($P < 0.01$). Data are means \pm S.D. ($n = 30$) expressed as percent of control optical density. Statistical comparisons (Dunnett's test) were made on endpoint values at 60 min.

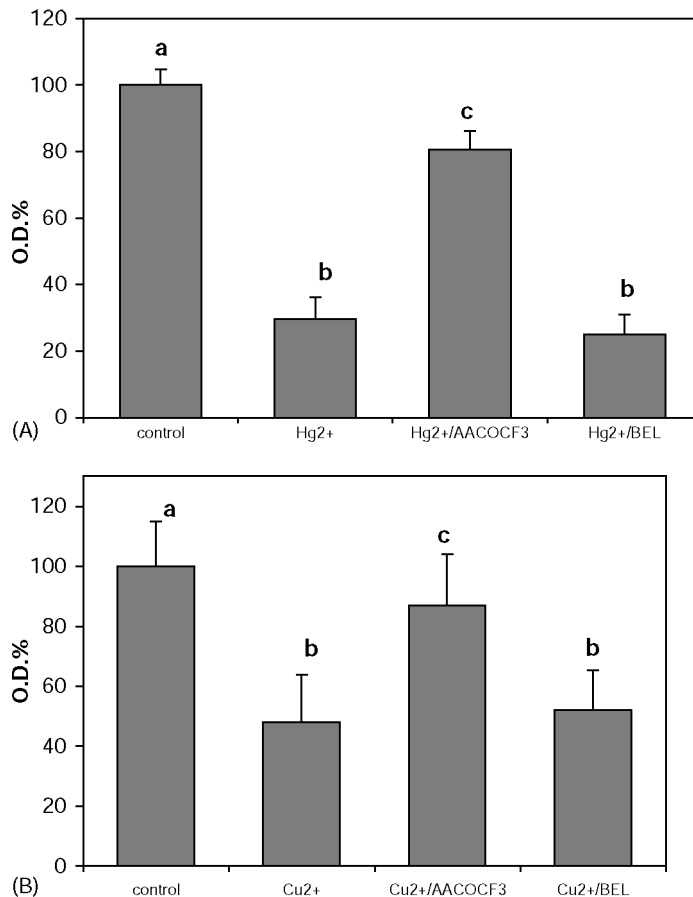


Fig. 2. Endpoint values (60 min) of lysosome NR staining in mussel haemocytes exposed to different treatments. Hg²⁺ or Cu²⁺ produced a significant lysosome destaining ($P < 0.01$). (A) Pre-incubation with 10 μ M AACOCF3 for 10 min prior to Hg²⁺ partially prevented the lysosome destaining induced by the metal. Pre-treatment with 20 μ M BEL for 10 min before Hg²⁺ had no effect. (B) The use of inhibitors with Cu²⁺ gave the same results. Data are means \pm S.D. ($n = 60$) expressed as percent of control optical density. Different letters to bars indicate significant differences according to Bonferroni ($P < 0.01$).

Ca²⁺-free medium, the NR staining of lysosomes was reduced to about 60% of the control at 60 min. The same percentage of OD reduction was observed after haemocyte treatment with Cu²⁺ in the presence of external Ca²⁺ (Fig. 1B).

In order to evaluate the role of Ca²⁺-dependent cPLA2 in lysosome injury, 60 min-endpoint values of optical density in NR-stained cells were used. Haemocytes pre-exposed to 10 μ M AACOCF3 before Hg²⁺ treatment showed values of optical density significantly higher than those of cells exposed only to the metal in a medium containing calcium (Fig. 2A). The pre-incubation of haemocytes

with a Ca²⁺-independent PLA2 inhibitor (BEL) at a concentration of 20 μ M did not prevent the effect of Hg²⁺ on lysosomal membrane stability (Fig. 2A).

Haemocytes preincubated with AACOCF3 prior to 50 μ M Cu²⁺ exposure showed optical density values higher than those of cells exposed only to Cu²⁺ (Fig. 2B). Conversely, no significant differences were observed in haemocytes pre-incubated with BEL before Cu²⁺ treatment with respect to the cells exposed only to the metal (Fig. 2B). Pre-treatment with AACOCF3 of Fura2-loaded haemocytes prior to Hg²⁺ did not interfere with [Ca²⁺]_i variations, since the increase

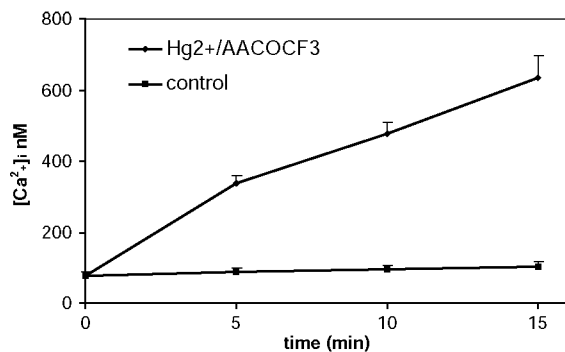


Fig. 3. Effect of AACOCF3 pre-incubation prior to Hg²⁺ on [Ca²⁺]_i showing that the drug does not prevent the Ca²⁺ rise induced by the metal. Data are means ± S.D. of 10 Ca²⁺ measurements.

was quite comparable with that of cells exposed to Hg²⁺ only (Fig. 3, see also Fig. 1).

Considering the possible correlation between membrane lysosomal destabilisation and changes in lysosomal volume density, lysosomal volume variations were analysed after metal cation treatments. These results were expressed as the increase of the ratio between the overall volume of lysosomes and the total cell volume after 60 min. Both Hg²⁺ and Cu²⁺ increased the lysosomal volume. Pre-treatment with AACOCF3 reduced or prevented the effect of heavy metals (Fig. 4). Conversely, pre-treatment with BEL did not affect the metal-induced lysosome enlargement (Fig. 4).

4. Discussion

The results of our study confirm that micromolar concentrations of Hg²⁺ and Cu²⁺ are able to rapidly destabilise lysosomal membranes of mussel haemolymph cells (Viarengo et al., 2000). Data concerning Ca²⁺ variations strongly suggest that lysosomal destabilisation depends on cytosolic Ca²⁺ increase. Treatment of cells with Hg²⁺ in a Ca²⁺-free medium reduced the [Ca²⁺]_i increase suggesting that the metal induces both Ca²⁺ entry and intracellular Ca²⁺ release. In Hg²⁺ treated cells, the depletion of external calcium prolonged NR retention time. Accordingly, also in cells exposed to Cu²⁺ the Ca²⁺

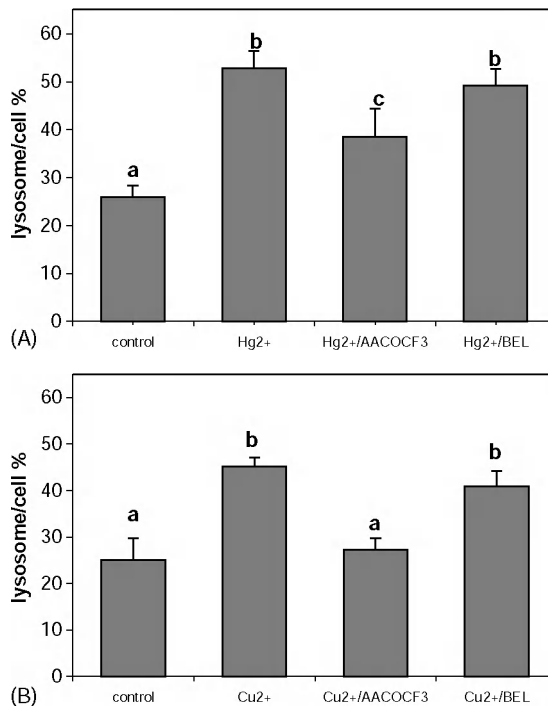


Fig. 4. Effects of Hg²⁺ (A) or Cu²⁺ (B) on lysosome size. Digital imaging evaluation of the lysosome/cell volume ratio shows an increase in the overall lysosomal volume after 60 min exposure to either 50 μM Hg²⁺ or 50 μM Cu²⁺ ($P < 0.01$). In cells pre-exposed to 10 μM AACOCF3 Hg²⁺ induced a lower lysosome volume increase, while Cu²⁺ had no effect at all. Pre-incubation with 20 μM BEL did not prevent the lysosome volume increase. Data of lysosome/cell ratio are expressed as percent values and represent means ± S.D. ($n = 60$). Different letters to bars indicate significant differences according to Bonferroni ($P < 0.01$).

increase was lower and the dye retention time was longer. Hence, the complex of data suggests a possible link between [Ca²⁺]_i rise and lysosomal membrane destabilisation.

Recently, it has been suggested that Ca²⁺-dependent cPLA2 has an essential role in 17β estradiol-induced lysosomal membrane destabilisation (Burlando et al., 2002). Therefore, it is possible that cPLA2 activity may also contribute to lysosomal membrane injury exerted by heavy metals. The pre-treatment with AACOCF3 of haemocytes exposed to Hg²⁺ or Cu²⁺ indicated that the inhibition of Ca²⁺-dependent cPLA2 reduced the destabilisation of the lysosome membranes, whereas the inhibitor of Ca²⁺-independent

cPLA2 (BEL) had no effect. The effect of AACOCF3 was entirely due to the impairment of Ca^{2+} -dependent cPLA2 activity and not to an interference of the drug with $[\text{Ca}^{2+}]_i$ increase. However, the non-complete disappearance of metal effects in the presence of AACOCF3 suggests that a mechanism different from Ca^{2+} -dependent PLA2 activation could also play a minor role in lysosome membrane destabilisation. In recent studies it has been demonstrated that Hg^{2+} and Cu^{2+} are able to increase intralysosomal pH, probably by interacting with the membrane proton pump (Viarengo et al., 2000) or by inducing lipid peroxidation (Freitas et al., 1996), thus leading to a decrease in lysosomal membrane fluidity (Myers et al., 1991).

A reduced lysosomal stability is accompanied by lysosome morphological changes (Moore et al., 1987), such as an increase in size associated with organelle fusions (Lowe et al., 1981). Our results indicate that Hg^{2+} and Cu^{2+} produce lysosome enlargement and this effect is in part or totally prevented by the inhibition of Ca^{2+} -dependent cPLA2 with AACOCF3. This indicates that lysosome fusion in mussel haemocytes exposed to heavy metals depends on cPLA2 activity. This is consistent with previous findings concerning a role of PLA2 in lysosome/endosome fusion (Mayorga et al., 1993). However, it must be considered that following AACOCF3 treatment, Hg^{2+} was still able to produce a small increase in lysosome size, thus suggesting that some other minor mechanism could be involved in this process. It is known that $[\text{Ca}^{2+}]_i$ increase triggers the formation of large lysosomes in human neutrophils and in fibroblasts, probably through the involvement of calcium binding proteins of the annexin family (Bakker et al., 1997, Mayorga et al., 1994).

In conclusion, our study indicates a pivotal role of Ca^{2+} -activated cPLA2 in the lysosomal membrane destabilisation induced by Hg^{2+} and Cu^{2+} . This is the same mechanism that has been previously reported for the effect of estradiol on lysosomes (Burlando et al., 2002), and therefore our data lead towards a unifying explanation for the effects of toxicants and hormones on the lysosomal system. At the same time, we also provide an interpretation for the cellular mechanisms which are at the basis of one of the most sensitive and versatile biomarkers used in the evaluation of the stress syndrome.

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