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Article *in* Journal of Biological Research - Bollettino della Società Italiana di Biologia Sperimentale · January 2010

DOI: 10.4081/jbr.2010.4435

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Can Endocrine Disrupters Interfere With Ca^{2+} Homeostasis In Invertebrate Cells ?

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Keywords: ***EDs, Mytilus, Cytosolic [Ca^{2+}], Ca^{2+}-ATPases, Hemocytes, Gills, Digestive gland.***

Abstract

A wide range of environmental chemicals have been shown to alter the endocrine system of both wildlife and humans. There is increasing evidence that many of these endocrine disruptors (EDs), in particular estrogenic chemicals, can rapidly affect cellular Ca^{2+} homeostasis and signaling in mammalian systems. In this work, in vitro and in vivo data are summarised on the effects of different compounds known or suspected as EDs on Ca^{2+} homeostasis in a marine invertebrate, the blue mussel Mytilus spp. Both synthetic estrogens and different EDs (DES, BPA, NP, PCB congeners, etc.) rapidly increased cytosolic $[Ca^{2+}]$ in mussel hemocytes, as evaluated by FURA2 single cell fluorescence microscopy. The observed $[Ca^{2+}]$ increase was unaffected by the antiestrogen Tamoxifen and was due to either increased Ca^{2+} influx or release from intracellular stores, depending on the compound. Moreover, different EDs, including the brominated flame retardant TBBPA (tetrabromo bisphenol A) induced a dose-dependent inhibition of the plasma membrane Ca^{2+}-ATPase (PMCA) activity from mussel gills in vitro, this supporting a direct effect on membrane pumps. The in vitro effects of EDs were observed at concentrations generally higher than those of E_2. However, in vivo, mussel exposure to environmental concentrations of Bisphenol A (BPA) and of the polybrominated diphenyl ether TBDE-47 resulted in large inhibition of PMCA activity in the digestive gland. The results indicate that, in invertebrate like in mammalian systems, interference with Ca^{2+} homeostasis may represent a significant mode of action of a variety of EDs .

1. Introduction

A wide range of environmental chemicals can alter the endocrine system of both wildlife and humans (1 , 2 , 3). Many of these compounds, or Endocrine Disruptors (EDs) have been shown to act through interference with the synthesis, metabolism and mode of action of steroid hormones, in particular of the natural estrogen 17β-estradiol (E_2), and are thus considered as environmental estrogens; these include both synthetic estrogens (like DES, 17α-ethynyl estradiol, mestranol, etc) and estrogenic chemicals or xenoestrogens (alkhylphenols, polychlorinated byphenyls-PCBs, dioxins, various pesticides and herbicides, etc.). These compounds can bind to intracellular Estrogen Receptors (ERs), leading to modulation of gene expression; the estrogenic potency of most xenoestrogens based on receptor affinity is weak (at least 1000 fold lower) than that of E_2 (1 , 2 , 3). However, accumulating evidence indicates that xenoestrogens can also interfere with the rapid, non genomic modes of action of E_2, through both ERs present in the plasma membrane and receptor-independent mechanisms, and lead to modulation of cell signalling through kinase- and Ca^{2+}-mediated pathways (4 , 5 , 6 , 7 , 8). In particular, modulation of cytosolic [Ca^{2+}] seems to represent a preferential target for the rapid effects of nM-low μM concentrations of xenoestrogens in several mammalian cell types through involvement of Ca^{2+}channels, ryanodine-sensitive calcium stores, G protein coupled receptors and AMPc (5 , 9 , 6 , 10 , 7). Since [Ca^{2+}] represents an important messenger in mediating the action of hormones and neurotransmitters (11), alterations in [Ca^{2+}] homeostasis may not only represent a significant target for ED toxicity, but could alter the physiological responses of cells to endogenous hormones.

EDs have been found in freshwater, estuarine and marine environments (12), thus representing a potential hazard for aquatic species, including invertebrates (13). Bivalves are sessile, filter-feeding molluscs that can represent a primary target for EDs, due to their high bioaccumulation and low biotransformation potential for contaminants (14). The effects of both natural estrogens and xenoestrogens have been previously investigated in the marine bivalve *Mytilus galloprovincialis* Lam.. E_2 has been shown to affect the function of mussel immune cells, the hemocytes, through rapid, non genomic activation of kinase pathways involving both cytosolic kinases and transcription factors (15 , 16). Moreover, different synthetic estrogens and EDs (including PCBs, alkylphenols, brominated flame retardants) affected the hemocyte function by interfering with different components of E_2-activated kinase pathways (17, 18 , 19 , 20 , 21). The effects of these compounds on immune function and signaling were similar *in vitro*, at short incubation times (mins), and *in vivo*, in the hemocytes of mussels treated with these compounds for 24 hrs (15 , 19).

In mussel hemocytes, E_2 also induced rapid increases in cytosolic [Ca^{2+}] (22 , 15); the effect of E_2 was due to activation of a Ca^{2+} influx and was unaffected by the antiestrogen Tamoxifen (15). Although E_2-mediated Ca^{2+} signaling seemed to play a minor role in mediating the effects of the hormone on the hemocyte function, these results suggested that also in mussels EDs, including xenoestrogens, may act through interference with cellular Ca^{2+} homeostasis. In this work, data are summarised on the effects of different known or suspected EDs on Ca^{2+} homeostasis of mussel cells obtained in different experimental conditions *in vitro* and *in vivo*. The effects on cytosolic [Ca^{2+}] were evaluated by single cell fluorescence microscopy in mussel hemocytes loaded with Fura2/AM.

The in vitro effects on plasma membrane Ca^{2+}-ATPase (PMCA) activity isolated from mussel gills were also investigated. The possible in vivo effects on PMCA were also evaluated in the digestive gland of mussels (*Mytilus edulis* L.) exposed to environmental concentrations of the alkylphenol Bisphenol A (BPA) or to the polybrominated diphenylether TBDE-47.

2. Materials and Methods

2.1 Animals

Mussels (*Mytilus galloprovincialis* Lam.) 4–5 cm long, were obtained from SEA (Gabicce Mare, PU) and kept for 1–3 days in static tanks containing artificial sea water (ASW) (1 l/mussel) at 16°C. Sea water was changed daily.

2.2 Single cell $[Ca^{2+}]_i$ measurements in mussel hemocytes

Hemolymph was extracted from the posterior adductor muscle of 8–20 mussels, using a sterile 1ml syringe with a 18 G1/2" needle. With the needle removed, hemolymph was filtered through a sterile gauze and pooled in 50 ml Falcon tubes at 4 ◦C. Hemocyte monolayers were prepared as previously described (15, 18). Single cell $[Ca^{2+}]_i$ measurements were carried out as previously described (23, 15). Aliquots of 40 μl of hemolymph were settled on glass cover-slips for 20 min at 16°C, rinsed and loaded with 4 μM Fura2/AM as previously described. $[Ca^{2+}]_i$ measurements were carried out with an Olympus IMT-2 inverted microscope equipped with an IMT2-RFL fluorescence attachment (Olympus Optical, Germany) and with a MTI68 intensified camera (Oatencourt, England). Images were acquired at 15 sec intervals using the CUE2 RMS 4.0 imaging system (Galai Production, Israel). Background fluorescence was subtracted before analysis. Free $[Ca^{2+}]$ was calculated following Grynkiewicz et al. (1985) (24). Intracellular calibration was performed at the end of each experiment adding the ionophore ionomycin (10 μM) and excess EGTA to obtain maximal and minimal fluorescence ratios, respectively, as previously described (23 , 15). Data are the mean ± SD of at least five experiments, each involving at least 10-12 cells.

2.3 Determination of gill plasma membrane Ca^{2+}-ATPase (PMCA) activity

Plasma membrane preparation: mussel gills were rapidly dissected and homogenised in 0,32 sucrose, 1 mM $MgCl_2$, 20 mM Hepes buffer, pH 7.2, and plasma membranes were separated in a sucrose density gradient as described in Viarengo et al. (1991) (25). Aliquots of plasma membranes (about 70 μg protein) were incubated at 19°C for 30 min with different concentrations of E_2 and estrogenic chemicals and the Ca^{2+}-ATPase specific activity (expressed as μmoles Pi/mg protein/hr) was determined as described by Viarengo et al. (1991) (25) through evaluation of released inorganic phosphate in the presence of 200 μM free Ca^{2+}, estimated according to Fabiato and Fabiato (1979) (26) and 0,2 mM ouabain, pH 7.2. Data, expressed as percent specific activity with respect to control values, are the mean±SD of 4 experiments in triplicate.

2.4 In vivo exposure to BPA and TBDE-47

Experiments were carried out within the activities of the EU BEEP project (Biological Effects of Environmental Pollution in Marine Coastal Ecosystems) (WP1) (27). Blue mussels (*M. edulis*), collected at a pristine site at Førlandsfjorden, Norway, were acclimated in a continuous flow-through system supplied with filtered seawater, at 10–12 °C, 34‰, and then exposed for 7-21 days to 50 ppb (0.2 μM/l) of bisphenol A (BPA) or for 21 days to 5 ppb (0.01 μM/l) of tetrabromodiphenylether-47 (TBDE-47) (27). Acetone was used as a vehicle at maximal concentrations of 0.0002 % (w/v). Mussels were fed every second day with a mix of *Isochrysis* and *Rhodomonas* algae. Exposure concentrations were selected on the basis of previously reported LC50 values that were divided by a factor of 100 and are close to concentrations described in the environment (27). At the end of exposure, digestive glands were sampled from 20 (4x 5) animals from each experimental group, cut into small pieces (4-5

mm length), and mildly fixed in 1% paraformaldehyde. 3,5% NaCl pH 8 for 30 min at 4°C. Samples were then washed in 0.2 M Tris/maleate buffer pH 7.4, flash-frozen for 40 sec in N-exane chilled with liquid N_2 and stored at -80 °C.

2.5 Cytochemical determination of PMCA activity in digestive gland tissue sections

Ca^{2+}-ATPase activity (PMCA) in tissue sections was evaluated by the cytochemical method described by Pons et al. (2002) (28). Serial cross sections (10 □m) were cut using a HM350 Microm microtome (Walldorf, Germany), transferred onto glass slides and incubated for 30 min at 20°C in a medium containing 6 h at room temperature in a medium containing 0.2 M Tris/maleate buffer pH 7.4, 2 mM disodium salt ATP, free Ca^{2+} 200 □M (estimated according to Fabiato and Fabiato, 1979) (26) □□□200 mM, 8 mM levamisole, 0.2 mM ouabain, 10 mM $Pb(NO_3)_2$. Negative 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 and immersed in ammonium sulphide-saturated water solution (1min) in order to reveal the brown lead sulfide $Pb_3(PO4)_2$ precipitate. Sections were washed in distilled water, allowed to dry and mounted in 20% (w/v) glycerol. Sections were observed using a DM RB Leitz microscope (Leitz Wetzlar, Germany) equipped with a Dage MTI camera and analogue-digital converter (DAGE-MTI Inc., Michigan City, IN) and images were quantified by an image analysis system (NIH Image-Scion Image software, version 1.59) and data were expressed as percent optical density with respect to controls.

2.6 Statistics

Data are mean±SD of at least 4 experiments in triplicate and were analysed by the non-parametric Mann-Whitney U test.

2.7 Chemicals

Fura2/AM was from Molecular Probes (Eugene, OR); ionomycin was from Calbiochem (La Jolla, CA). All other reagents were of analytical grade and were puraches from Sigma (Milan-Italy).

3. Results

3.1 Effect of EDs on cytosolic [Ca^{2+}]in mussel hemocytes

Fig. 1 data are reported on the effects of the synthetic estrogen DES and of the estrogenic chemicals, the alkylphenols NP and BPA, on cytosolic [Ca^{2+}] as determined by fluorescence microscopy in Fura2/AM loaded hemocytes. All compounds, at the concentration of 5 μM, induced a small but not significant rise in [Ca^{2+}] (data not shown). However, DES, at the concentration of 25 μM, caused a rapid and transient rise in cytosolic [Ca^{2+}], with a maximal 2,5-fold increase with respect to control cells (206±12 nM at 150 sec from DES addition; C=72.5±8.0 nM; $P \leq 0.05$) (Fig. 1A); higher concentrations (100 μM) induced an higher and persistent rise in [Ca^{2+}]. The DES-induced [Ca^{2+}] transient was unaffected in the presence of extracellular EGTA, or by cell pre-treatment with the antiestrogen Tamoxifen. As shown in Fig. 1B and 1C, similar and transient increases in cytosolic [Ca^{2+}] were also observed with 25 μM NP or BPA and higher concentrations induced a persistent rise in [Ca^{2+}]. Also the effects of NP and BPA were unaffected by EGTA or Tamoxifen.

PCB congeners that were shown to rapid affect kinase-mediated signalling in mussel hemocytes (16) were tested their possible effects on cytosolic [Ca^{2+}], and the results are reported in Fig. 2. Low concentrations (3 μM) of the non-coplanar PCB congeners P47 (2,2',4,4'-tetrachlorobiphenyl) and P153

(2,2',4,4',5,5'-hexachlorobiphenyl) induced a slow and significant rise in [Ca^{2+}], that was persistent for up to 15 min, reaching values of 190±8 nM and 179±12 nM, respectively, corresponding to a two-fold increase with respect to controls (P≤0.05) (Fig. 2A). Higher concentrations (30 µM) induced higher and steeper rises in [Ca^{2+}]. Similar effects were observed with the coplanar congener P77 (3,3',4,4'-tetrachlorobiphenyl) (data not shown). The source of the PCB-induced [Ca^{2+}] rise was investigated in more detail and representative results obtained with the congener P47 are reported in Fig. 2B. Addition of EGTA did not affect the PCB-induced rise in [Ca^{2+}] observed within 5-10 min; however, in the presence of EGTA, the effect of P47 became transient, with the level of [Ca^{2+}] returning to control values at 15 min. Moreover, a similar effect was observed in hemocytes pre-treated with the channel blocker Verapamil. The results suggest that the effect of low concentrations of P47 was due to an early release of Ca^{2+} from intracellular stores followed by an influx from the extracellular medium through voltage-dependent Ca^{2+}-channels. Again, the effect was not prevented by Tamoxifen (data not shown).

3.2 In vitro effects of EDs on gill plasma membrane Ca^{2+}-ATPase activity (PMCA)

The possible *in vitro* effects of different EDs on the Ca^{2+}-ATPase activity present in the plasma membranes (PMCA) of mussel gills were investigated and the results are reported in Fig. 3. Purified plasma membrane fractions (about 70 µg/protein) were incubated for 30 min with different concentrations of EDs and the Ca^{2+}-ATPase activity was evaluated as µmoles Pi released/mg protein/hr as previously described (25). The effects of the natural estrogen E_2 were first evaluated and the results are reported in Fig. 3A. Incubation of gill plasma membranes with E_2 decreased the Ca^{2+}-ATPase activity; however, the effect was significant only at higher concentrations (1-2 nmoles/mg protein) (-28 and -26% with respect to controls, respectively, P≤0.05) and did not further increase at higher E_2 concentrations (data not shown). DES, NP and BPA induced a similar concentration-dependent decrease in Ca^{2+}-ATPase activity (Fig. 3B) although at higher concentrations; significant effects were recorded significant from 0,5 µmoles/mg protein (-23% with BPA, -36% with DES, and -46% with NP; P≤0.05); maximal inhibition was observed at 2 µmoles/mg membrane protein (from -50% with BPA and -60% with DES and NP; P≤0.05).

PCB congeners induced a stronger inhibition of Ca^{2+}-ATPase activity (Fig. 3C); in particular, both P47 and P153 at 50 nmoles/mg protein induced a 50% decrease in enzyme activity (P≤0.05) and almost complete inhibition was observed with both compounds at 250 nmoles/mg protein. Finally, the possible effect of a brominated BPA derivative, the flame retardant tetrabisphenol A (TBBPA), a strong activator of E_2-mediated cell signaling in mussel hemocytes (20 Canesi et al., 2005b) was evaluated. TBBPA induced a dramatic decrease in Ca^{2+}-ATPase activity (Fig. 3D): 40% inhibition was observed at concentrations as low as 1 nmole/mg protein (P≤0.05) and complete inhibition at 50 nmoles/mg protein.

3.3 In vivo effects of BPA and TBDE-47 on digestive gland Ca^{2+}-ATPase activity

Mussels were exposed for 7 and 21 days to sublethal concentrations of BPA (50 µg/l/animal, corresponding to 0,2 µM) or of BDE-47 (5 µg/l/animal, corresponding to 0.01 µM), in the course of experiments performed within the EU BEEP project (27). Cytochemical evaluation of Ca^{2+}-ATPase activity was carried out in the digestive gland and the results are shown in Fig. 4. As

previously described (28), Ca^{2+}-ATPase activity in the digestive gland cells of control animals was mainly localized in the basal part of the plasma membrane (not shown). Ca^{2+}-ATPase staining decreased in response to exposure to both BPA and BDE-47. Quantification by image analysis of stained tissue sections revealed a large inhibition of the Ca^{2+}-ATPase activity induced by exposure to BPA for 7 and 21 days (-60 and -80% with respect to controls, respectively, $P \leq 0.05$). Exposure to TBDE-47 for 21 days almost abolished Ca^{2+}-ATPase activity.

Discussion

In mammalian cells EDs have been demonstrated to rapidly affect Ca^{2+} homeostasis, due to modulation of intracellular Ca^{2+} release and of different types of Ca^{2+} channels, inhibition of Ca^{2+}-ATPases (29 , 30 , 5 , 9 , 10 , 6 , 7 , 31). The results here presented demonstrate that different EDs increased cytosolic $[Ca^{2+}]$ in mussel hemocytes and inhibited gill PMCA activity. Significant effects were observed with different compounds at different concentrations and generally higher that those of the natural estrogen E_2. Moreover, *in vivo* mussel exposure to environmental concentrations of the estrogenic chemical BPA and of the brominated flame retardant TBDE-47 significantly decreased PMCA activity in the digestive gland. Although the effects and modes of action on cytosolic $[Ca^{2+}]$ and PMCA activity in different cells and tissues were not fully investigated in detail for each compound, these represent the first data indicating that EDs can affect Ca^{2+} homeostasis in invertebrate cells. Overall, the results support the hypothesis that interference with the cellular systems involved in regulating cytosolic $[Ca^{2+}]$ may represent a significant mode of action for a wide variety of EDs also in these organisms.

The synthetic estrogen DES and the estrogenic alkylphenols BPA and NP induced a rapid and transient increase in cytosolic $[Ca^{2+}]$ in mussel hemocytes similar to that observed with concentrations 1000 times lower of the natural estrogen E_2 (15). Similarly, DES, BPA and NP were shown to interfere with kinase-mediated cell signalling at concentrations 1000 times higher than those of E_2 (18). The source of the $[Ca^{2+}]$ rise induced by these compounds was mainly intracellular, since it was unaffected by EGTA (this work), whereas the effect of E_2 involved activation of a Ca^{2+} influx (15). The time course and amplitude of the $[Ca^{2+}]$ increase induced by DES, NP and BPA were similar to those previously observed in TM4 rat Sertoli cells with similar concentrations of BPA and NP (32). On the other hand, in other mammalian cell types DES, BPA and NP were shown to increase cytosolic $[Ca^{2+}]$ at concentrations comparable with those of E_2 (5 , 6 , 10). In mussel hemocytes, higher concentrations of DES, BPA and NP led to a sustained rise in Ca^{2+} similar to that induced by E_2; again, such an effect was observed at concentrations about 1000 times higher than that of the natural estrogen. Since persistent increases in $[Ca^{2+}]$ induced by high E_2 were associated with increased lysosomal protein catabolism indicating autophagic processes (22), also high concentrations of EDs may result in Ca^{2+}-mediated cytotoxicity. The effects of both E_2 and EDs on cytosolic $[Ca^{2+}]$ were not prevented by Tamoxifen, this suggesting that classical ERs were not involved. Similarly, in different breast cancer cell lines expressing both ERα and ERβ, ERβ only, or none, the antiestrogen ICI 182780 did not affect the rapid $[Ca^{2+}]$ rise induced by different EDs (6).

We have previously shown that different PCBs, and in particular *ortho*-substituted congeners, in the low µM range, affect the immune function of mussel hemocytes through disregulation of MAPK signalling (17). The results here reported show that, in the same experimental conditions, the di-*ortho* substituted PCBs P47 (2,2',4,4'-tetrachlorobiphenyl) and P153 (2,2',4,4',5,5'-hexachlorobiphenyl) induced significant changes in cytosolic [Ca^{2+}]. These effects were observed at concentrations about 10 times lower than those of DES, BPA and NP; however, the PCB-induced increase in [Ca^{2+}] was slow and persistent, showing a distinct time course with respect to that observed with E_2 or other EDs. The rise in [Ca^{2+}] induced by 3 µM P47 was mediated by two components: a Ca^{2+} release from intracellular stores was apparently responsible for the earlier phase of the [Ca^{2+}] rise, followed by an influx of [Ca^{2+}] from the extracellular medium; such an influx was prevented by cell pre-treatment with Verapamil, indicating the involvement of voltage-gated Ca^{2+} channels. These data are in line with those obtained in mammalian cells: *ortho*-PCBs have been shown to increase [Ca^{2+}] levels by inhibiting microsomal and mithocondrial [Ca^{2+}] buffering and Ca^{2+} extrusion process in neuronal cells (33) and both *ortho*- and *non ortho*- PCB congeners affected [Ca^{2+}] in bovine myometrial cells (34).

In different mammalian cell types, synthetic estrogens like DES and estrogenic alkylphenols such as BPA and NP have been shown to act through direct interaction with membrane Ca^{2+} transport systems, in particular of Ca^{2+}-ATPases (35 , 36 , 32 , 37). EDs have been demonstrated to inhibit Ca^{2+}-ATPases also in fish (38 , 39). The results here presented demonstrate that the plasma membrane Ca^{2+}-ATPase (PMCA) activity of mussel gills represents a target for different EDs *in vitro*. A significant decrease in PMCA activity was observed with the natural estrogen E_2 at 1-2 nmoles/mg protein; however, higher concentrations did not result in stronger effects. The synthetic estrogen DES and the estrogenic alkylphenols BPA and NP also decreased the enzyme activity; an inhibition comparable to that induced by E_2 was observed although at concentrations 250 times higher (0,25-0,5 µmoles/mg protein). The PCB congeners tested proved to be stronger inhibitors of PMCA activity; in particular, these compounds were effective from 25 nmoles/mg protein and resulted in dramatic inhibition (-80%) of enzyme activity at 250 nmoles. *Ortho*-substituted non coplanar PCBs have been shown to alter microsomal calcium transport in mammalian brain by direct interaction with ryanodine receptors (40 ; 41). Finally, when we tested the possible effects of the brominated BPA derivative TBBPA, a 40% decrease in PMCA activity was observed at concentrations as low as 1 nmole/mg protein, and complete inhibition at 50 nmoles. TBBPA belongs to Brominated flame retardants (BFRs) a large groups of compounds added to or applied as a treatment to polymeric materials (plastics, textiles, electronic equipment) to prevent fires: TBBPA is the most important individual BFR used in industry (41); TBBPA and its derivatives have been found in human blood samples and in wildlife species (42 , 43). In mammalian cells, TBBPA can act as a cytoxicant, neurotoxicant, thyroid hormone agonist and has a weak estrogenic activity (43 , 44). In mussel hemocytes, TBBPA in the low µM range has been shown to stimulate the immune function through rapid activation of kinase-mediated pathways common to those of E_2 (20). The results here presented demonstrate that, among the compounds tested, TBBPA was the most powerful inhibitor of gill PMCA, suggesting that Ca^{2+}-mediated cytotoxicity may represent a significant mode of action for brominated flame retardants, as

recently demonstrated in cerebellar granule cells (45 , 46). The effects of EDs on *Mytilus* PMCA demonstrate that, as in mammalian cells, EDs can directly interact with membrane Ca^{2+} transport systems. Different EDs may affect the movement of hydrophobic ions across lipid bilayers or specifically interact with different Ca^{2+}-ATPases (35 , 36 , 40 , 32 , 47 , 48). Further research is needed in order to clarify the mechanisms of action of individual EDs on mussel gill PMCA.

Overall, the results presented indicate that EDs can affect Ca^{2+} homostasis in *Mytilus*. The effects of most EDs on cytosolic [Ca^{2+}] and PMCA activity were observed at concentrations generally higher than those of E_2; in particular, the effects on cytosolic [Ca^{2+}] showed different time courses and involvement of distinct mechanisms for individual compounds. On the other hand, significant effects of BPA were observed in mussel hemocytes and digestive gland *in vivo* at longer exposure times (24 hrs) were observed at concentrations comparable to that of E_2 (19 , 49). However, when comparing the potency of EDs with that of E_2, it must be considered that in vivo estrogenic chemicals are generally more stable than E_2 and can accumulate in the tissues, and circulating EDs may not be sequestered by plasma steroid-binding proteins; therefore, unmodulated action of these compounds on target tissues and cells may occur.

The results obtained *in vivo* demonstrate that exposure to environmental concentrations of the estrogenic alkhylphenol BPA and of the brominated flame retardant TBDE-47 resulted in large inhibition of PMCA activity in mussel digestive gland. The results confirm that EDs can affect Ca^{2+} homeostasis in mussel tissues and that PMCA represent a significant target for both BPA and brominated flame retardants this estrogenic chemicals in these organisms. The effects observed in vivo may be related not only to a direct effect of these compounds on enzyme activity, but also on PMCA expression, and may also involve ER-dependent mechanisms. ER-like receptors have been identified in *Mytilus* (50), and significant changes in gene expression have been observed in mussels exposed to E_2 (51) and to BPA (49). Research is in progress to evaluate the possible effects of both natural and environmental estrogens on PMCA activity and expression in mussel tissues.

Legend to Figures

Fig. 1 – Effects of different EDs on cytosolic [Ca^{2+}] in Fura2/AM loaded hemocytes.
A) DES: Control (**---**); 25 μM DES (•);100 μM DES (▲); Tamoxifen/25 μM DES (○); EGTA/25 μM DES (□);
B) NP : Control (---); 25 μM NP (•);100 μM NP (▲); Tamoxifen/ 25 μM NP (○); EGTA/ 25 μM NP (□) ;
C) BPA: Control (---); 25 μM BPA (•);100 μM BPA (▲); Tamoxifen/25 μM BPA(○) ; EGTA/25 μM BPA(□) ;
Data are representative of at least 4 experiments, each involving at least 10-12 cells.

Fig. 2 – Effects of the *ortho*-substituted PCB congeners P47 (2,2',4,4'-tetrachlorobiphenyl) and P153 (2,2',4,4',5,5'-hexachlorobiphenyl) on cytosolic [Ca^{2+}] in Fura2/AM loaded hemocytes.
A) Control (---) ; 3 μM P47 (△) ; 3 μM P153 (□); 30 μM P47 (▲); 30 μM P153 (■).
B) Control (---) ; 3 μM P47 (△) ; EGTA/3 μM P47 (■) ; Verapamil/ 3 μM P47 (▲).
Data are representative of at least 4 experiments, each involving at least 10-12 cells.

Fig. 3 – Effects of different EDs on mussel gill plasma membrane Ca^{2+}-ATPase (PMCA) activity. Data, expressed as μmoles Pi released/mg protein/hr, are reported as % of control values. A) 17β-estradiol (E_2); B) DES, NP and BPA; C) P47 and P153; D) TBBPA. Data are the mean ± SD of at least four experiments in triplicate.

Fig. 4 – Cytochemical determination of Ca^{2+}-ATPase activity in digestive gland sections of mussels exposed to the estrogenic chemical BPA (0,2 μM) for 7 and 21 days or TBDE-47 for 21 days. Changes in Ca^{2+}-ATPase activity (Optical densities, OD) were quantified by digital imaging of tissue sections and expressed as % of control values . * = P≤0,05. Mann-Whitney U test.

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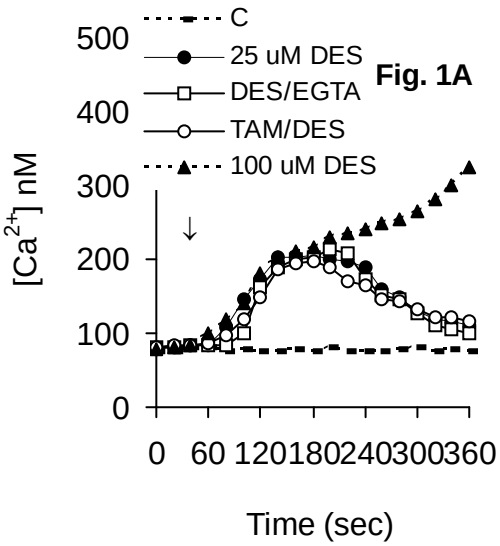


Fig. 1A

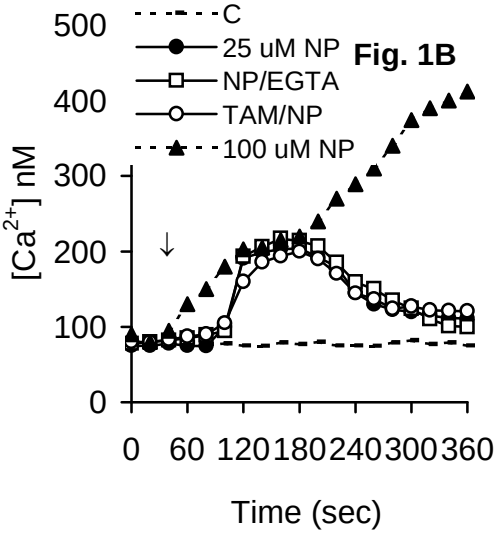


Fig. 1B

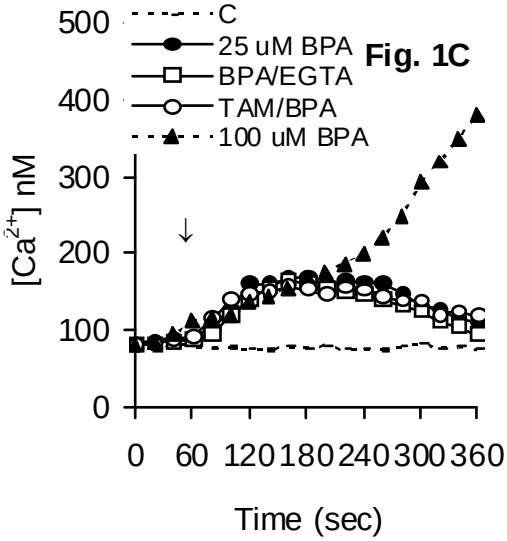
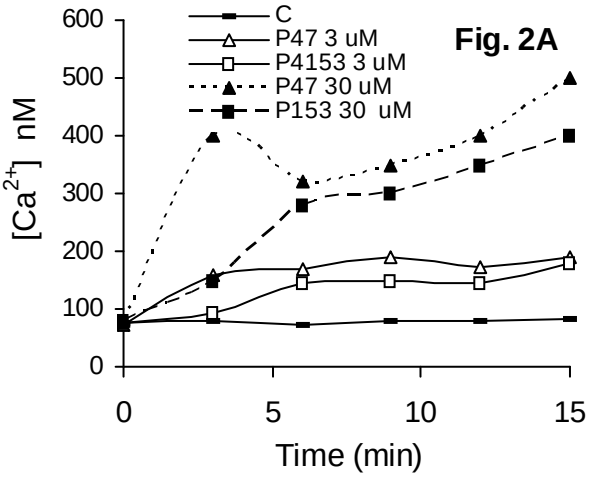
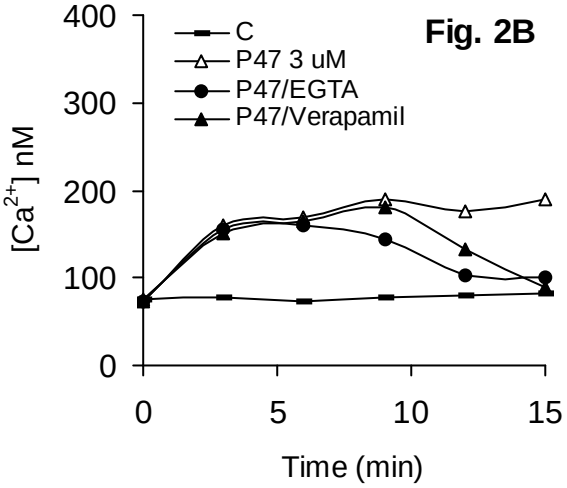
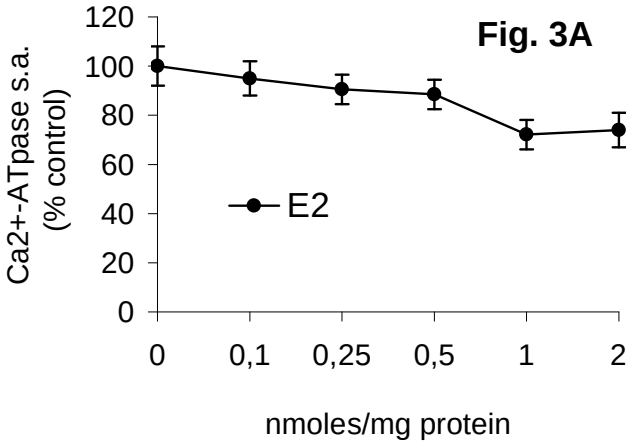
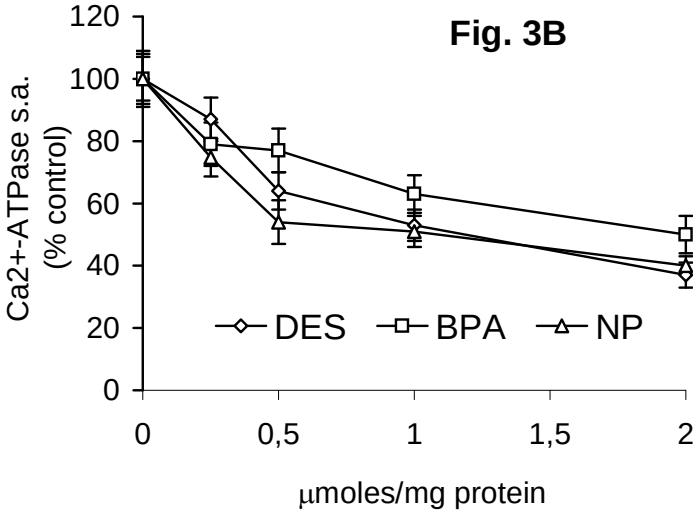


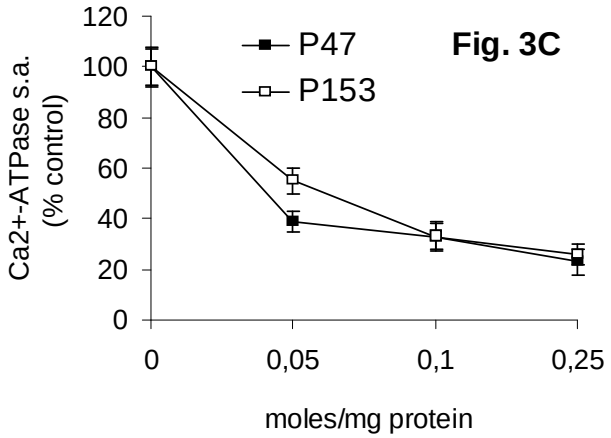
Fig. 1C

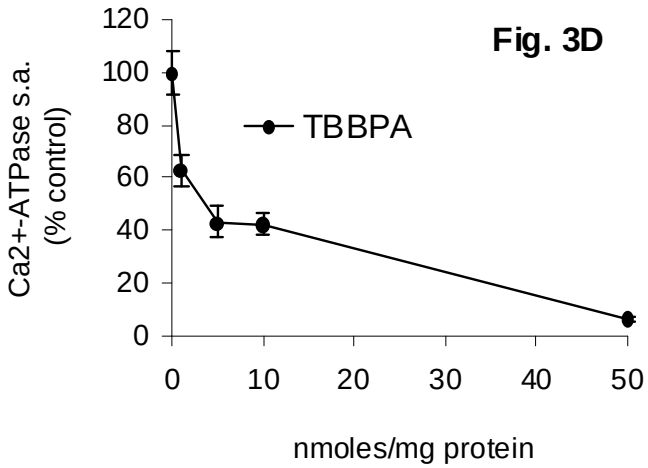












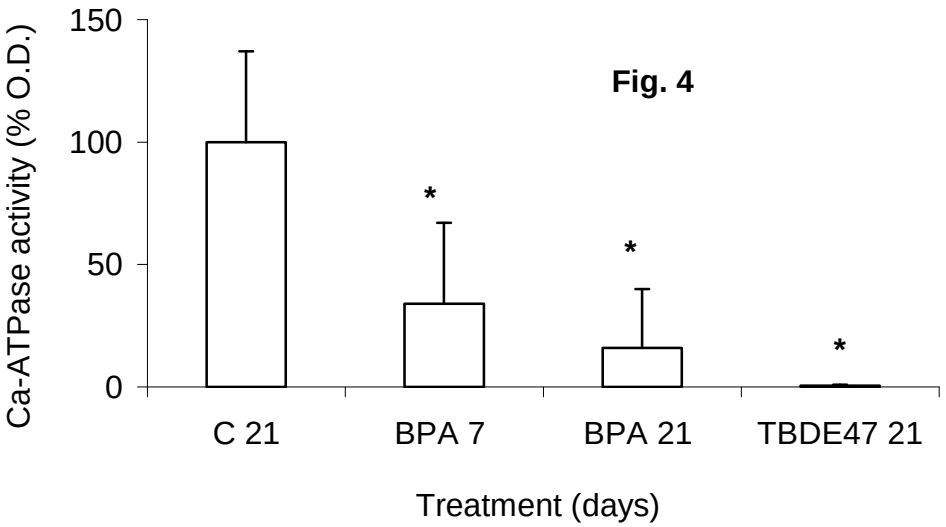


Fig. 4