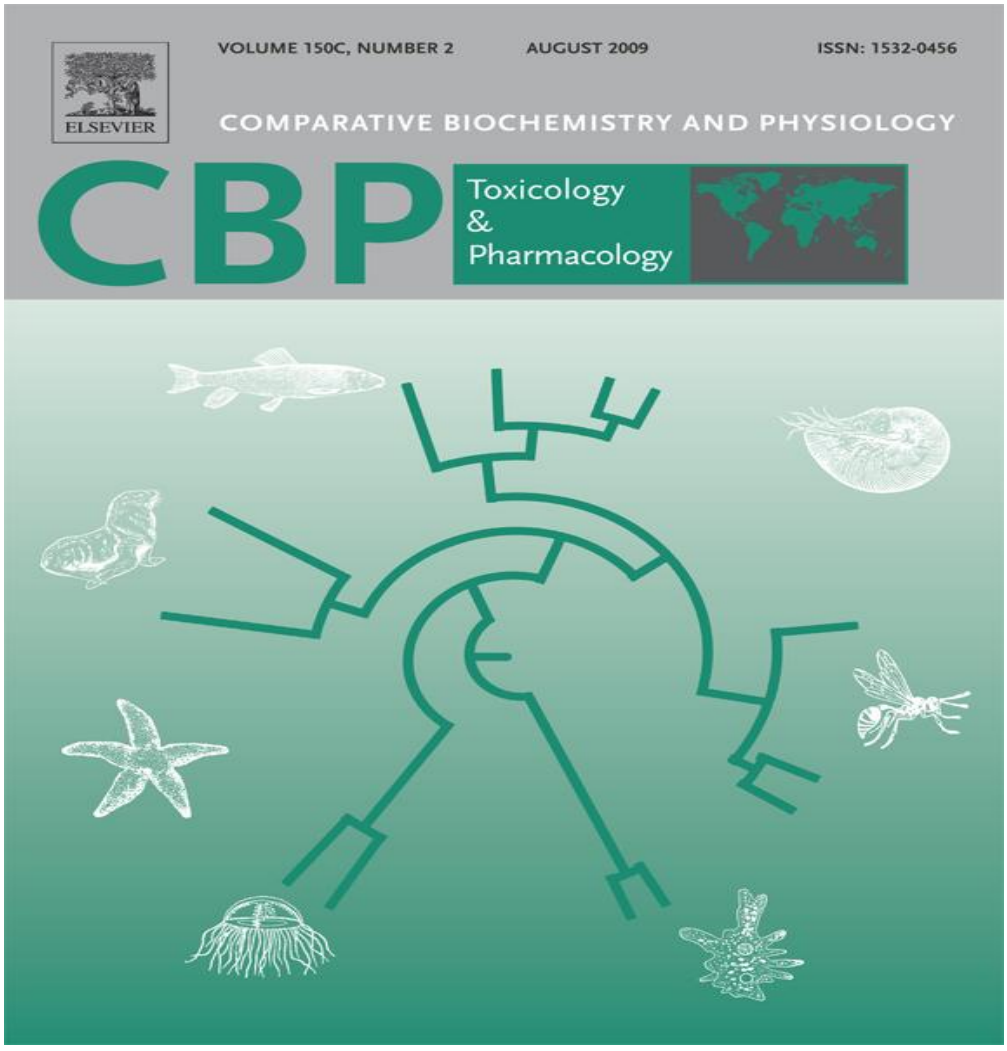

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Measuring Ca^{2+}-signalling at fertilization in the sea urchin *Psammechinus miliaris*: Alterations of this Ca^{2+}-signal by copper and 2,4,6-tribromophenol

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ABSTRACT

During fertilization, eggs undergo a temporary rise in the intracellular concentration of free Ca^{2+} ions. Using the membrane permeable acetoxymethylester of the fluorescent calcium indicator dye Fura-2, Fura-2 AM, the Ca^{2+}-signal at fertilization was not detectable in eggs of the sea urchin *Psammechinus miliaris*. However, after treatment of the eggs with Fura-2 AM in combination with MK571, an inhibitor for multidrug resistance associated proteins, clear Ca^{2+}-signals at fertilization could be measured without microinjection of the dye. We used this methodology to detect possible alterations of Ca^{2+}-signalling at fertilization by exposure of eggs to environmental pollutants. For this purpose, the heavy metal copper, the bromophenol 2,4,6-tribromophenol, the organic compound bisphenol A and the polycyclic aromatic hydrocarbon phenanthrene were tested for their potential to inhibit fertilization success of *P. miliaris*. Copper and 2,4,6-tribromophenol showed a dose-dependent effect on fertilization rates of *P. miliaris* and significantly inhibited fertilization at 6.3 µM Cu^{2+} and 1 µM 2,4,6-tribromphenol. Bisphenol A significantly inhibited fertilization success at 438 µM while phenanthrene had no effect up to 56 µM. 6.3 µM copper and 100 µM 2,4,6-tribromophenol significantly increased the Ca^{2+}-signal at fertilization. This alteration may contribute to the reduced fertilization rates of *P. miliaris* after exposure to copper and 2,4,6-tribromophenol.

1. Introduction

Free calcium ions are essential second messengers in cells from their origin at fertilization throughout their entire lifespan (Carafoli, 2002). Disruption of cellular Ca^{2+} homeostasis appears to mediate the toxicity of many chemicals (Nicotera et al., 1992). Sustained increase in intracellular Ca^{2+} can provoke cytotoxic mechanisms in various cells and tissues by activation of Ca^{2+}-dependent enzymes, alterations of the cytoskeleton, mitochondrial damage, and by the activation of irreversible catabolic processes which may ultimately result in cell death (Nicotera et al., 1992; Stohs and Bagchi, 1995; Nicotera and Orrenius, 1998). A diverse range of natural and anthropogenic chemicals such as divalent heavy metal ions, bromophenols, bisphenol A as well as polycyclic aromatic hydrocarbons have been shown to interfere with cellular Ca^{2+} signalling (e.g. Büsselberg et al., 1990; Davila et al., 1995; Stohs and Bagchi, 1995; Nielsen et al., 2003; Wozniak et al., 2005).

At fertilization, eggs undergo an increase in intracellular Ca^{2+} beginning at the point of sperm–egg fusion and crossing the egg to the antipode in a wave-like fashion (Santella et al., 2004; Whitaker, 2006). This calcium wave is the first event at fertilization triggering the quiescent egg into metabolic activity by posttranslational activation of enzymes, exocytosis of cortical granules for formation of the fertilization membrane and resumption of the cell cycle (Covian-Nares et al., 2004; Santella et al., 2004).

In the following paragraph a selection of chemicals interfering with cellular Ca^{2+} signalling and homeostasis are presented: Cu^{2+} is an essential metal ion required for metabolic processes in all eukaryotes but can reach toxic levels in aquatic environments (Bryan and Langston, 1992; Stohs and Bagchi, 1995; Zorita et al., 2006). Cu^{2+} has been shown to alter Ca^{2+} signals in developing embryos of the macroalgae *Fucus serratus* (Nielsen et al., 2003). Bromophenols are industrially produced flame retardant intermediates and wood preservatives (Howe et al., 2005) which also occur naturally in the marine environment in algae (Whitfield et al., 1999) as well as in fish and invertebrates (Boyle et al., 1992; Fielman et al., 2001). Recently, bromophenols such as 2,4,6-tribromophenol have been shown to disturb cellular Ca^{2+}-signalling in neuroendocrine cells (Hassenklöver et al., 2006). Bisphenol A, an important key monomer in the production of polycarbonate plastics and epoxy resins, and endocrine disruptor, affects Ca^{2+} homeostasis by provoking Ca^{2+} influx via Ca^{2+} channels in mammalian tumor cell lines (Wozniak et al., 2005). Further, in goldfish bisphenol A significantly altered plasma Ca^{2+} levels (Suzuki et al., 2003). Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants contained in petroleum hydrocarbons and formed during combustion of fossil fuels and other products (Latimer and Zheng, 2003). PAHs and its metabolites have been shown to alter Ca^{2+}-associated signalling

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pathways in immune (Davila et al., 1995) and nonimmune cells (Barhoumi et al., 2006) as well as in isolated membrane vesicles of mammalian skeletal muscles (Pessah et al., 2001).

Sea urchins are widely used to study the cellular events at fertilization (Santella et al., 2004; Whitaker, 2006). Further, some natural and anthropogenic chemicals have been tested on Ca^{2+} homeostasis in sea urchin eggs (Walter et al., 1989; Pesando et al., 1991, 1996; Girard et al., 1997). Thereby, the permeability of the plasma membrane to Ca^{2+} and other ions as well as the accumulation and release of sequestered Ca^{2+} were assessed (Pesando et al., 1991, 1996; Girard et al., 1997). Walter et al. (1989) investigated the Ca^{2+} content and uptake of Ca^{2+} as well as the role of mitochondrial damage in sea urchin eggs upon exposure to mercury chloride.

In sea urchins and some other organisms the calcium wave represents a single event which is followed by a few minor rises in the intracellular concentration of Ca^{2+} ions (Stricker, 1999). The mechanisms by which the sperm triggers Ca^{2+} release at fertilization are still under debate (Santella et al., 2004). In the most established model the sperm is believed to introduce a sperm factor into the egg promoting the formation of inositol-1,4,5-triphosphate ($InsP_3$) which initiates the activating Ca^{2+} wave (Jaffe et al., 2001; Santella et al., 2004). Studies indicate that in sea urchins there are two further messengers of Ca^{2+} signalling: nicotinic acid adenine dinucleotide phosphate (NAADP) and cyclic ADP ribose (cADPr) giving the fertilization calcium wave a boost and longevity (Steinhardt et al., 1977; Whitaker, 2006). Steinhardt et al. (1977) and Schmidt et al. (1982) have shown that the Ca^{2+} is released from intracellular stores, whereby later $InsP_3$ and cADPr were identified for mobilizing Ca^{2+} from the endoplasmic reticulum (reviewed by Galione, 1994, Jaffe et al., 2001). In contrast, NAADP is known to induce Ca^{2+} release from lysosomes (Churchill et al., 2002).

Calcium signals are mostly measured using fluorescent calcium indicator dyes (Whitaker, 2006). The ratiometric fluorescent dye Fura-2 has already been used for measuring the calcium wave at fertilization in eggs of the sea urchin *Lytechinus pictus* (Poenie et al., 1985; Swann and Whitaker, 1986) as well as in ascidians and mammals (Hyslop et al., 2001; Carroll et al., 2003). In general, the dyes are microinjected into the eggs. Indeed, Fura-2 is also available as membrane permeable acetoxymethylester Fura-2 AM. After crossing the membrane Fura-2 AM is quickly hydrolyzed by intracellular esterases to produce membrane impermeable Fura-2. Previously, the inhibitor for multidrug resistance associated proteins (MRP) MK571 has been shown to enhance uptake of fluorescent dyes in animal cells (Manzini and Schild, 2003; Bickmeyer et al., 2008) as well as in diatoms (Scherer et al., 2008). MRPs are efflux transporters of the ATP Binding Casette (ABC) superfamily actively transporting and sequestering endogenous and exogenous compounds (Holland and Blight, 1999; Leslie et al., 2001). In marine invertebrates MRPs have been demonstrated to be expressed in marine bivalve mollusks as well as in sea urchins (Hamdoun et al., 2004; Lüdeking et al., 2005).

The aim of the present study was to test if chemicals may alter the calcium wave at fertilization in sea urchins. For this purpose, the heavy metals Cu^{2+} and Pb^{2+}, the bromophenol 2,4,6-tribromophenol, bisphenol A, and the polycyclic aromatic hydrocarbon phenanthrene

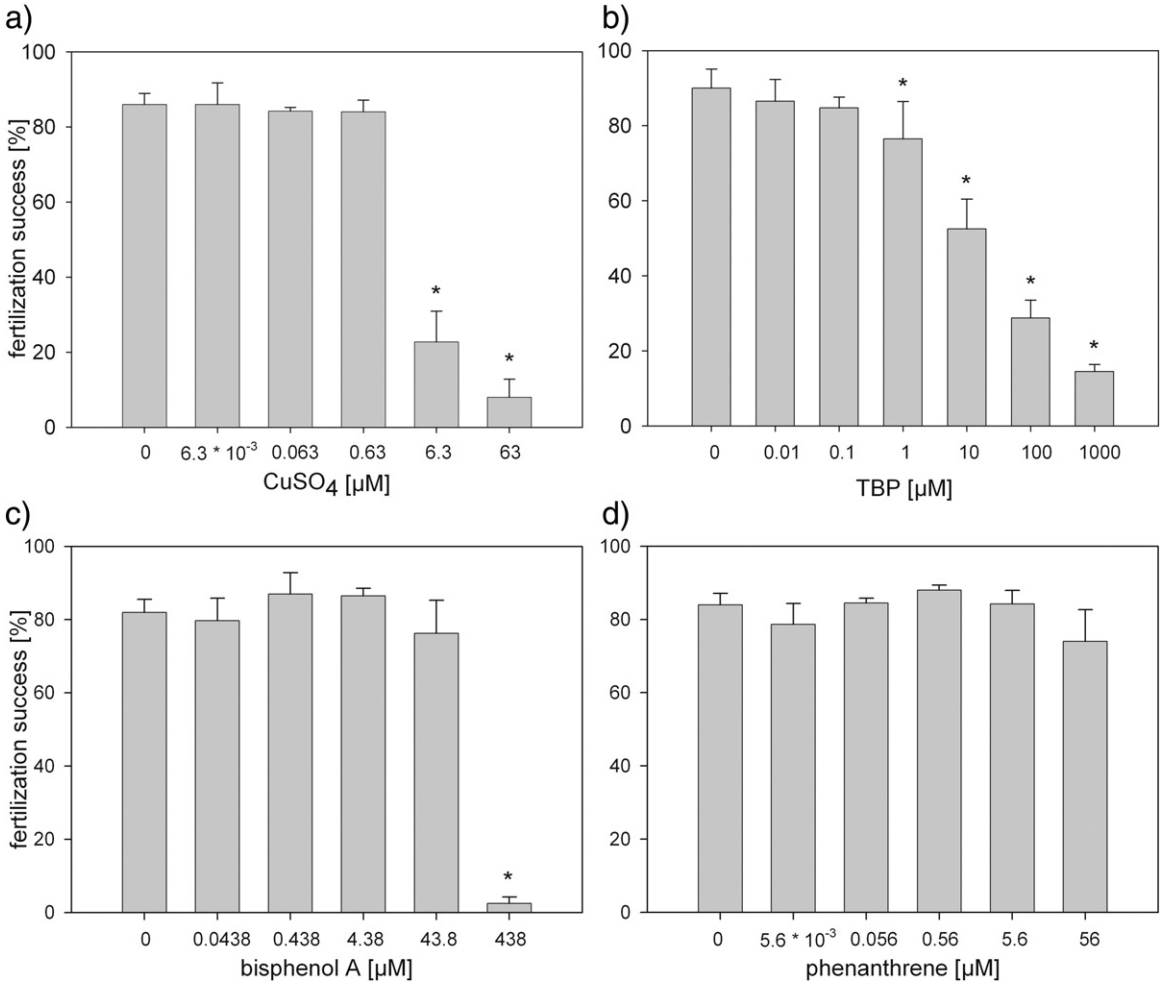

Fig. 1. Fertilization success of *Psammechinus miliaris* during exposure to set concentrations [µM] of a) copper sulfate, b) 2,4,6-tribromophenol (TBP), c) bisphenol A and d) phenanthrene. Asterisks indicate significant differences in comparison to controls ($CuSO_4$: one-way ANOVA $p<0.001$, Dunnett's test $p<0.05$; TBP: one-way ANOVA, $p<0.001$, Dunnett's test $p<0.05$; Bisphenol A: one-way ANOVA $p<0.001$, Dunnett's test $p<0.05$; Phenanthrene: Kruskal–Wallis ANOVA on ranks $p=0.074$).

were tested for their potential to inhibit fertilization success of the sea urchin *Psammechinus miliaris*. We measured the calcium wave at fertilization using Fura-2 AM and the MRP inhibitor MK571.

2. Materials and methods

2.1. Animal collection and maintenance

P. miliaris were collected by fishing subtidal populations close to the Island Sylt (Germany) by a beam trawl with the FK Uthörn in April 2008. Sea urchins were transported to the Biological Institute Helgoland where they were kept in running sea water at ambient temperature until use.

2.2. Collection and processing of gametes

P. miliaris were induced to spawn by injection of 0.5 mL 0.5 M KCl. Sperm was collected from the aboral pore of each individual using a syringe fitted to a needle and stored 'dry' on ice until use. Female sea urchins were induced to spawn in the same way, but eggs were released directly into artificial sea water. Gametes were collected during the first 20 min of spawning. The quality of eggs was assessed microscopically on the basis of uniformity of shape and size.

2.3. In vitro fertilization assays

The following chemicals were tested on fertilization success of *P. miliaris*: copper sulfate ($CuSO_4 \times 5H_2O$), 2,4,6-tribromophenol (TBP), bisphenol A and phenanthrene. 1000× concentrated stock solutions were prepared in distilled water (copper sulfate), DMSO (phenanthrene, bisphenol A), or methanol (2,4,6-tribromophenol). Concentration ranges of test substances were selected according to His et al. (1999), King and Riddle (2001) and Fernández and Beiras (2001) for copper, Hassenklöver and Bickmeyer (2006) and Hassenklöver et al. (2006) for TBP, Roepke et al. (2005) and Kiyomoto et al. (2006) for bisphenol A and Steevens et al. (1999) and Pillai et al. (2003) for phenanthrene. Eggs and sperm were obtained as described above but eggs were released in general purpose medium 2 (GP 2: 360 mM NaCl, 24.8 mM Na_2SO_4, 8.2 mM KCl, 0.74 mM KBr, 0.09 mM $Na_2B_4O_7 \times 10H_20$, 46.7 mM $MgCl_2 \times 6H_2O$, 11.9 $CaCl_2 \times 2H_2O$, 0.08 mM $SrCl_2 \times 6H_2O$, 2.02 mM $NaHCO_3$) (instead of ASW) which had been aerated for 24 h (U.S. EPA, 1993; Caldwell et al., 2002). During fertilization assays it is essential to keep the concentrations of sperm and eggs constant in the different treatments and replicates. In preliminary experiments (data not shown) *in vitro* fertilization without exposing the sperm was not satisfactory since washing and counting of the eggs took too long and resulted in low egg quality with insufficient fertilization rates in controls. Therefore, eggs as well as sperm – though only for a short period – were exposed to the test substances during fertilization assays. Thus, reduced fertilization success may be the result of toxicity on both types of gametes.

Eggs from three to four females were pooled and sperm from two to three males were pooled (U.S. EPA, 1993). Approximately 200 unfertilized eggs of the pooled egg suspension were stocked in 1 mL medium in polystyrene 24 well microplates. Eggs were incubated at 18 °C either with the test substances at set concentrations or with the respective controls (distilled water, DMSO, or methanol). Solvents had

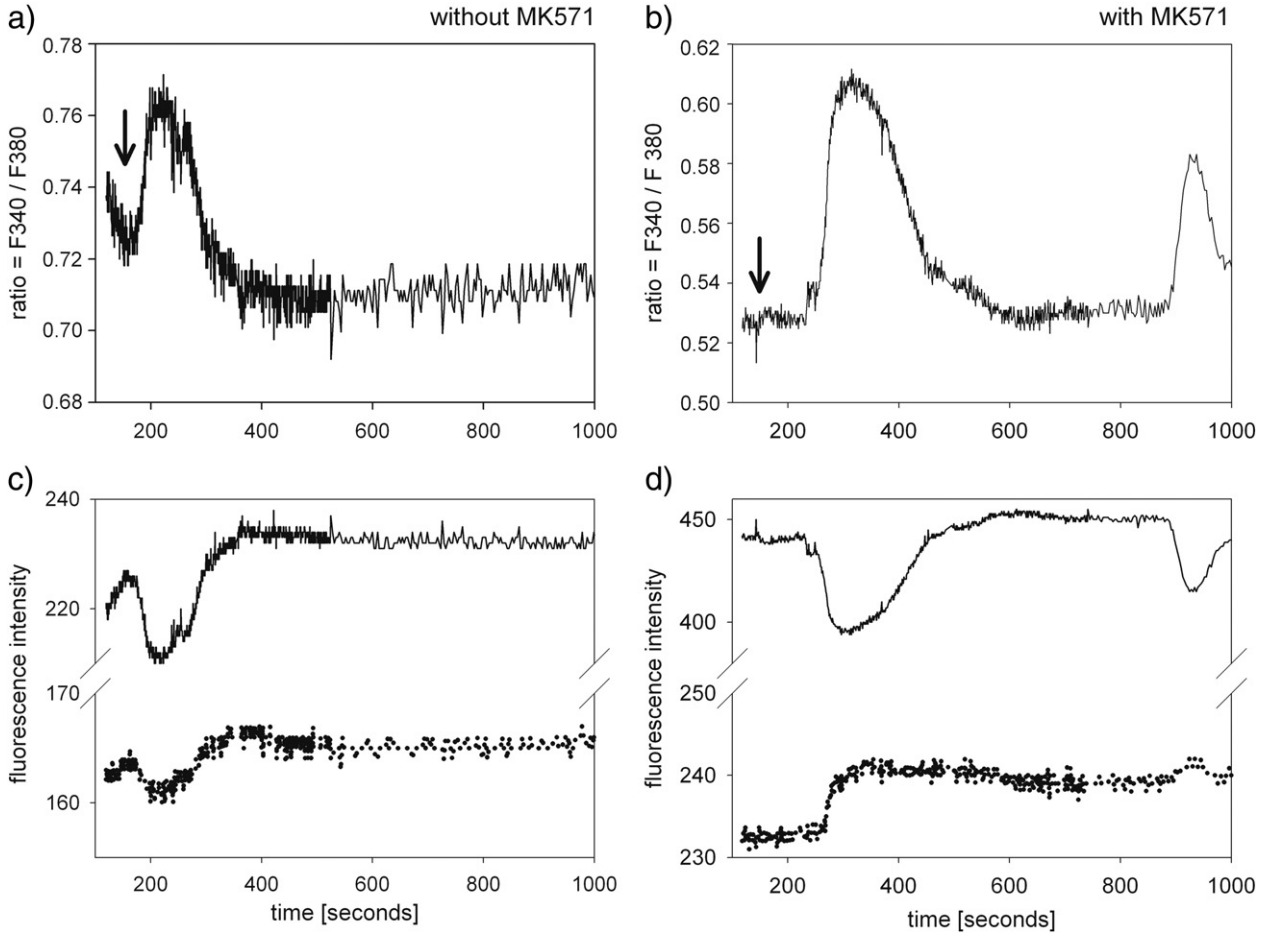

Fig. 2. Changes of the ratio F340/F380 (upper panel) and corresponding fluorescence intensities at 340 nm (dotted line) and 380 nm (solid line) excitation (lower panel) in single eggs of *Psammechinus miliaris* at fertilization. Eggs were incubated with a) 10 µM Fura-2 only and b) 10 µM Fura-2 and 50 µM MK571. Arrows indicate point of adding sperm. Note a second Ca^{2+} rise approximately 400 s after the first peak in b).

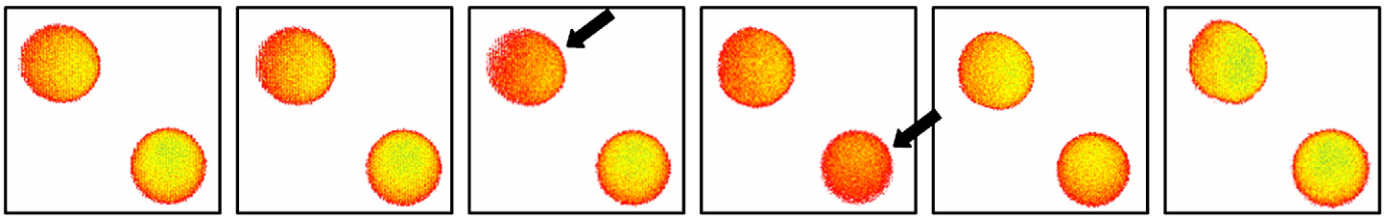

Fig. 3. The ratio of F340/F380 which represent changes of intracellular Ca^{2+} in two eggs of *Psammechinus miliaris* during fertilization (from left to right). Note changes in the ratio F340/F380 with maximal ratio changes indicated by arrows. Shown are pseudocoloured relative fluorescence images of eggs incubated with 10 µM Fura-2 and 50 µM MK571 with high fluorescence intensities in red and low in green, respectively.

a final concentration of 0.1% in all wells. Four replicate incubations were run per treatment. After 60 min, 10 µL of sperm suspension in GP2 were added to give a final sperm concentration of 2.5×10^6 mL^{-1} (Caldwell et al., 2002). Sperm had been allowed to activate in GP2 approximately 10 min prior to use. The plates were gently agitated for 30 s to increase sperm/egg encounters and incubated for 15 min. Fertilization was stopped by adding 100 µL 4% formaldehyde in ASW. The final concentration of formaldehyde is sufficient to stop fertilization (U.S. EPA, 1993) which was evident by amotile sperm and the absence of multi-cellular embryos in the wells. Fertilization success was determined using an inverted microscope (Axiovert 25, Zeiss, Germany) and was defined as elevation of the fertilization membrane observed at 100× magnification. 100 eggs per well were counted and the number of unfertilized eggs were recorded.

2.4. Fluorometric measurement of intracellular Ca^{2+} levels at fertilization

For measuring Ca^{2+} signals at fertilization, gametes were obtained as described above but for each test eggs from one female and sperm from one male were used (gametes were not pooled). Eggs were incubated with artificial seawater (ASW: 460 mM NaCl, 10.4 mM KCl, 55 mM $MgCl_2 \times 6H_20$, 11 mM $CaCl_2$, 15 mM Hepes-Na; pH 7.5) containing either 10 µM Fura-2 AM or 10 µM Fura-2 AM with 50 µM MK571 for 60 min at room temperature. Eggs were washed three times with ASW. They were fertilized by adding sperm which was activated by dilution of dry sperm in ASW immediately prior to use.

Fluorescence of eggs during *in vitro* fertilization was monitored by an imaging system (Visitron, Puchheim, Germany) with a CCD camera

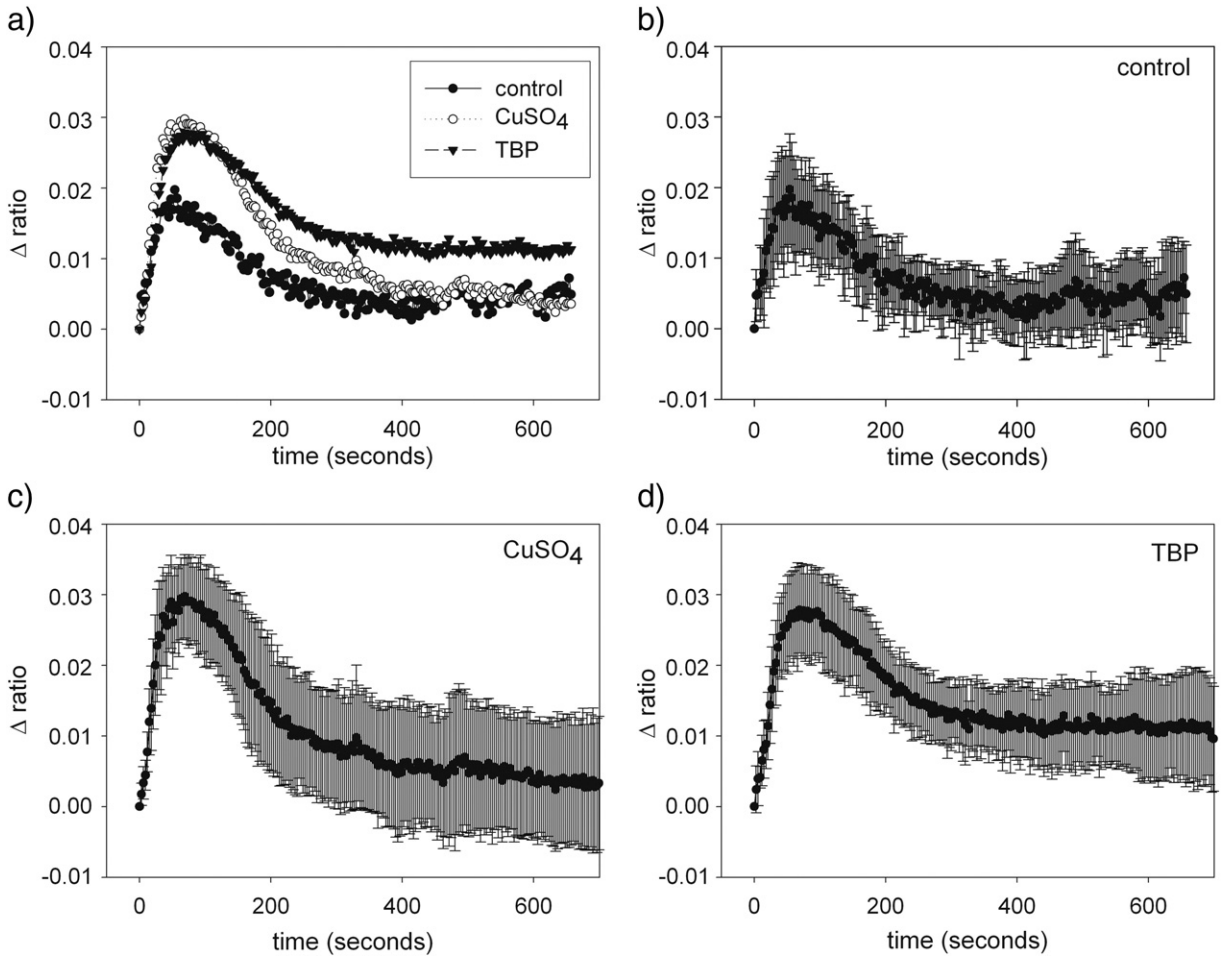

Fig. 4. Mean changes in the ratio of F340/F380 at fertilization in control eggs ($N = 8$ eggs) and in eggs incubated with 6.3 µM $CuSO_4$ ($N = 9$) and 100 µM TBP ($N = 9$), respectively. To obtain Δ ratio values data were subtracted with the respective ratio of F340/F380 for each eggs at the beginning of the fertilisation wave. a) mean Δ ratio for the three treatments, b), c), d) mean Δ ratio values with standard deviations for controls, $CuSO_4$- and TBP-treated eggs, respectively. Data are obtained from three separate experiments per treatment with 2 to 4 eggs each.

(Coolsnap) mounted on an inverted microscope (Axiovert 100, Zeiss). Two to four eggs were measured simultaneously by using the 'region of interest' function of the software (Metafluor, Meta Imaging Series). Fluorescence was obtained through an UV objective (NeoFluar 20x, Zeiss). Data were obtained from division of two images, one obtained at 340 nm, the other at 380 nm excitation. Obtained ratio values were not converted to intracellular Ca^{2+} concentrations.

2.5. The effects of Cu^{2+} and TBP on Ca^{2+}-signalling in eggs

Gametes were obtained as described for the fluorometric measurements. In contrast to *in vitro* fertilization assays, counting of eggs is not necessary during fluorometric measurement of intracellular Ca^{2+} levels. Therefore, eggs were washed prior to addition of sperm so that only the eggs were exposed to the tested chemicals.

First, eggs were incubated in medium (GP2) with 10 µM Fura-2 AM and 50 µM MK571 for 60 min and washed three times with GP2. Test substances (6.3 µM Cu^{2+} or 100 µM TBP) were added and fluorescence was recorded as described above. Next, it was tested whether the test substances lead to alterations of the calcium wave at fertilization of *P. miliaris*. To avoid interference of the MRP inhibitor on the toxicity of the test substances, eggs were treated with the test substances first and incubated with Fura-2 AM and MK571 afterwards. In detail, eggs were incubated with test substances (6.3 µM Cu^{2+} or 100 µM TBP) for 60 min, washed three times with GP2, and then incubated with 10 µM Fura-2 AM and 50 µM MK571 for 60 min and washed again three times with GP2. Eggs were fertilized with 10 µL sperm suspension (dry sperm diluted 1:50 immediately prior to use) and fluorescence was recorded. Two to four eggs were recorded simultaneously and at least three experiments were run per treatment. Incubation of eggs and tests were performed at 18 °C.

2.6. Statistics

Statistical tests were run with Sigma Stat 3.0 (SPSS Inc.). Residuals were tested for normality and variance homogeneity. Data for fertilization rates were arc sin square root transformed and a one-way ANOVA with a Dunnett's test as post hoc test was run as described by the U.S. EPA (1993) for sea urchin fertilization tests. In case the data were not normally distributed a Kruskal–Wallis ANOVA on ranks was used. The Ca^{2+} signals (the Δ $ratio_{max}$ and the time needed to reach maximal changes in the Ca^{2+} signal) were analysed with a one-way ANOVA and a Dunnett's test. The significance level was set at $p<0.05$. Note that in Sigma Stat 3.0 the *p*-values for the Dunnett's test are unavailable. The software only indicates if the *p*-value is above or below the significance level of 0.05.

3. Results

3.1. In vitro fertilization assays

In Fig. 1 the fertilization rates of *P. miliaris* after exposure to the test substances are presented. Exposure to Cu^{2+} significantly reduced fertilization success of *P. miliaris* at the two highest concentrations (6.3 and 63 µM, one-way ANOVA, $p<0.001$). 63 µM Cu^{2+} significantly decreased the fertilization rate by 76% in comparison to controls. TBP significantly inhibited fertilization success of *P. miliaris* at 1 µM (one-way ANOVA, $p<0.001$). Exposure to 1000 µM TBP, the highest concentration tested, significantly inhibited the fertilization rate by 75% in comparison to controls. 438 µM bisphenol A significantly reduced fertilization success by 97% in comparison to controls. (one-way ANOVA, $p<0.001$). Indeed, for bisphenol A no effects were found at concentrations lower than 438 µM. Phenanthrene showed no effect on fertilization success at concentrations of up to 56 µM (Kruskal–Wallis ANOVA on ranks, $p=0.074$).

3.2. Fluorometric measurement of intracellular Ca^{2+} levels at fertilization

In the experiments, eggs of *P. miliaris* were successfully fertilized by adding activated sperm, which was checked visually after each experiment by elevation of the fertilization membrane. However, after incubation with Fura-2 AM no calcium signals could be observed in the eggs during fertilization ($N=8$ eggs measured in three separate experiments). In some cases, an increase in the ratio F340/F380 was recorded. Indeed, close inspection of the corresponding fluorescence intensities at 340 and 380 nm excitation shows that the ratio changes are due to decreasing intensities at both wavelengths induced by cell movement during fertilization (see Fig. 2a). These changes cannot be regarded as alterations in intracellular Ca^{2+} since the fluorescence spectrum of Fura-2 does not shift in opposite directions with an increase at 340 and a decrease at 380 nm.

In contrast, after incubation with Fura-2 AM and MK571 clear calcium waves could be observed in the eggs upon fertilization (Fig. 3) ($N=8$ eggs measured in 4 separate experiments). The changes in the ratio of F340/F380 clearly correspond to changes in intracellular Ca^{2+} since the fluorescence intensity increases at 340 nm and decreases at 380 nm excitation (Fig. 2b). The ratio of F340/F380 changed by 0.033 ± 0.02 in comparison to the resting ratio level before fertilization ($N=8$). The calcium waves reached their maximum after 95 ± 33 s and lasted 351 ± 130 s ($N=8$).

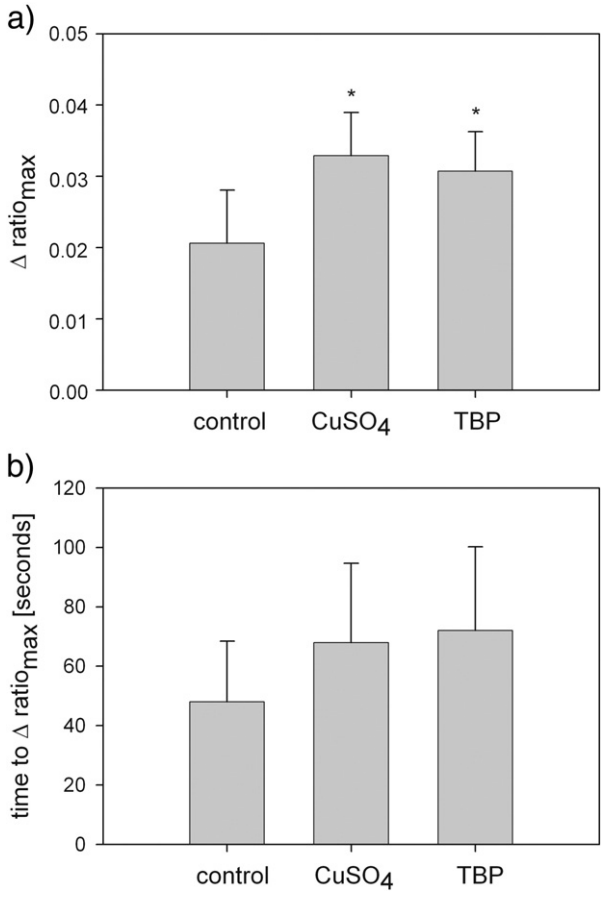

Fig. 5. a) Maximal changes in the ratio F340/F380 at fertilization in control ($N=8$ eggs), $CuSO_4$- ($N=9$) and TBP-treated ($N=9$) eggs. Asterisks indicate significant differences in comparison to controls (one-way ANOVA, $p<0.001$, Dunnett's test $p<0.05$). b) The time [s] needed to reach maximal changes in the ratio F340/F380 at fertilization in control, $CuSO_4$- and TBP-treated eggs. No significant differences were found in comparison to controls (one-way ANOVA, $p=0.143$). Data are obtained from three separate experiments per treatment with 2 to 4 eggs each.

In one egg, a second Ca^{2+} rise was observed 10 min after the first peak (Fig. 2b) which was around 70% smaller and with 177 s shorter than the first peak with 371 s.

3.3. The effects of Cu^{2+} and TBP on Ca^{2+}-signalling in eggs

For the following experiments 6.3 µM copper sulfate and 100 µM TBP were selected which inhibited fertilization success by 65% and 61%, respectively, in comparison to controls. Neither 6.3 µM Cu^{2+} nor 100 µM TBP induced detectable calcium signals in unfertilized eggs (data not shown). After incubation of eggs with Cu^{2+} and TBP the fertilizing sperm still induced a Ca^{2+} wave. In Fig. 4 mean relative changes in the ratio of F340/F380 at fertilization, Δ ratio, obtained after subtracting the resting ratio of F340/F380 prior to fertilization are shown in the different treatments. Eggs treated with Cu^{2+} and TBP exhibited alterations of the fertilization calcium wave: The maximal changes in the ratio F340/F380 (the Ca^{2+} peak) were significantly higher in Cu^{2+}- and TBP-treated eggs in comparison to controls (Fig. 5a). In TBP-treated eggs the Δ ratio remained above the resting ratio prior to fertilization within 600 s after fertilization whereas in control and Cu^{2+}-treated eggs the Δ ratio fell to resting levels approximately 400 s after fertilization. In addition, the Δ ratio showed a high variance in Cu^{2+}-treated eggs after the fertilization Ca^{2+} wave. Indeed, the time needed to reach the maximal changes in the ratio was not different in the treatments (Fig. 5b).

In eggs of all treatments, small postfertilization Ca^{2+} rises could be observed within 12 min postfertilisation (control: 15 postfertilization waves, Cu^{2+}: 5, TBP: 12). In controls up to four and in Cu^{2+}- and TBP-treated eggs up to two postfertilization Ca^{2+} waves were recorded, respectively.

4. Discussion

So far, studies on the fertilization Ca^{2+} wave have focused on the North American sea urchin species *Lytechinus pictus* and *Strongylocentrotus purpuratus*. Though, Genazzani et al. (1999) have demonstrated that egg homogenates of *P. miliaris* share the same Ca^{2+} release mechanisms as *L. pictus* and *S. purpuratus* with the $InsP_3$, the cADRP and the NAADP pathways. In *L. pictus*, the Ca^{2+} wave at fertilization is reported to reach its peak after approximately 20 s (Poenie et al., 1985, Swann and Whitaker, 1986), while in the present study, the Ca^{2+} rise in *P. miliaris* takes approximately 95 s to reach its maximum. However, the duration of the Ca^{2+} transients are comparable with approximately 5 min in *L. pictus* measured by Poenie et al. (1985) and in *P. miliaris* in the present study.

Different developmental stages of marine invertebrates are known to be affected differently by chemicals or polluted water samples with fertilization being either more or less sensitive than embryonic and larval development (e.g. Kobayashi, 1980, 1990; Gopalakrishnan et al., 2008). By comparing toxicity of chemicals on different life stages exposure times towards test substances often differ and make a direct comparison difficult. In the present study, fertilization success of *P. miliaris* was inhibited by bisphenol A only at the highest concentration (438 µM) tested. Phenanthrene did not affect fertilization rates of *P. miliaris* up to 56 µM. Yet, these values are above critical concentrations reported to affect sea urchin embryonic and larval development (Table 1). Furthermore, the effective concentration of phenanthrene is above its 'safe' level of <=29 nM developed by several countries for aquatic organisms (Law et al., 1997). To our knowledge, no maximum permissible value has been set for bisphenol A in the aquatic environment.

We show that fertilization success of *P. miliaris* was significantly affected at 6.3 µM Cu^{2+}. The same concentration increased the calcium wave at fertilization indicating disturbance of Ca^{2+} homeostasis in the eggs by exposure to Cu^{2+}. Early life history stages of marine invertebrates may be particularly sensitive to elevated copper concentrations (Kobayashi, 1980, 1990; Bielmyer et al., 2005, Table 1). Generally, divalent heavy metals are known to affect cellular calcium homeostasis (Stohs and Bagchi, 1995). Exposure of sea urchin eggs towards the heavy metal mercury chloride increased calcium influx and calcium content in a time and dose-dependent manner resulting in disturbance of mitochondrial function and finally cell death (Walter et al., 1989). In sperm of the mussel *Mytilus edulis* treated with 3.3 mM Cu^{2+} Ca^{2+} levels significantly decreased in mitochondria and acrosomes indicating an increase in the ionic permeability of organelle membranes and possibly resulting in an increase in cytosolic Ca^{2+} (Earnshaw et al., 1986). Ca^{2+} channels on the sperm plasma membrane have also been proposed to be affected after paternal exposure of sea urchins to the heavy metal Cd^{2+} (Au et al., 2001). Further, Cu^{2+} has been shown to alter Ca^{2+} signals in developing embryos of the macroalgae *Fucus serratus*: Moderate Cu^{2+} concentrations (422 nM) inhibited generation of cytosolic Ca^{2+} signals in response to hypoosmotic shock whereas high Cu^{2+} concentrations (2.11 – 8.44 µM) elevated cytosolic Ca^{2+} (Nielsen et al., 2003).

Next to Cu^{2+}, TBP significantly decreased fertilization success of *P. miliaris* in a dose-dependent manner in our study. Recently, bromophenols have been shown to inhibit larval survival and metamorphosis of the sea urchin *Strongylocentrotus nudus* (Agatsuma et al., 2008, Table 1). In the present study, TBP significantly decreased fertilization success of *P. miliaris* at 10 µM. Moreover,

Table 1
Toxicities of test substances on different species of sea urchins (Echinoidea).

Test substance	Species	Effective concentration	Duration of exposure	Developmental stage	Effect	Reference
Bisphenol A	*Strongylocentrotus purpuratus*	1 µM	96 h	Embryos–larvae	EC50 for normal development	Roepke et al. (2005)
	Hemicentrotus pulcherrimus	≥10 µM	Directly after until up to 48 h postfertilization	Embryos–larvae	Suppression of development	Kiyomoto et al. (2006)
	Hemicentrotus pulcherrimus	≥10 µM	12 h after fertilization	Embryos–larvae	No effect on development	Kiyomoto et al. (2006)
Copper	*Heliocidaris erythrogramma*	≥4 nM		Sperm–eggs	Reduced fertilization success, (most sensitive among five tested sea urchin species)	Kobayashi (1980)
	Diadema antillarum	44 nM	40 h	Embryos–larvae	EC50 for abnormal development	Bielmyer et al. (2005)
	Paracentrotus lividus	≥0.25 µM	48 h	Embryos–larvae	Inhibition of growth	Fernández and Beiras (2001)
	Paracentrotus lividus	1.1 µM	48 h	Embryos–larvae	EC50 for complete development	
Phenanthrene	*Lytechinus variegatus*	≥6 nM	2 h	Eggs–embryos	Inhibition of embryo development	Steevens et al. (1999)
	Lytechinus anemensis	≥1 µM	n.st.	Embryos	Disruption of axial development	Pillai et al. (2003)
2,4,6-Tribromophenol	*Strongylocentrotus nudus*	≥3 nM	1 h	Larvae	Inhibition of metamorphosis	Agatsuma et al. (2008)
	Strongylocentrotus nudus	≥30 nM	24 h	Larvae	Inhibition of swimming activity	Agatsuma et al. (2008)
	Strongylocentrotus nudus	150 nM	24 h	Larvae	Mortality	Agatsuma et al. (2008)

Presented are the tested chemicals, the tested species, the effective concentration, the duration of the exposure, the tested life stage(s), the observed effect, as well as the reference. n. st. = not stated.

exposure of eggs to 100 µM TBP significantly increased the Ca^{2+} wave at fertilization indicating alterations of calcium signalling by TBP. An increase in intracellular Ca^{2+} partly by Ca^{2+} release from intracellular stores after exposure to TBP has already been shown in neuroendocrine (PC12) cells (Hassenklöver et al., 2006). In a subsequent study, Hassenklöver and Bickmeyer (2006) show that TBP selectively reduced calcium channel currents in PC12 cells with a half-maximal concentration of 28 ± 18 µM. Additionally, this effect increased with ongoing exposure time (Hassenklöver and Bickmeyer, 2006). We, therefore, may strongly underestimate the efficacy of TBP.

In sea water samples from the German Bight up to 6 ng L^{-1} TBP (equivalent to 0.02 nM) are reported while the highest level of all identified bromophenols was 74 ng L^{-1} (Reineke et al., 2006). Higher levels of bromophenols are found in marine sediments and macroalgae: In estuarine sediments from the Rhone river up to 3.7 mg kg^{-1} TBP were found and in the marine macroalgae *Ulva lactua* up to 1.6 mg kg^{-1} TBP have been detected (Howe et al., 2005). Regarding the environmental levels of Cu^{2+}, concentrations range from 0.2 to 2.6 µg L^{-1} dissolved Cu^{2+} (equivalent to 3 to 41 nM) in the North Sea. Indeed, at point sources up to 600 µg L^{-1} dissolved Cu^{2+} (equivalent to 9 µM) can be found (Bryan and Langston, 1992). The effective concentrations of Cu^{2+} and TBP tested in this study are, therefore, higher than relevant aqueous concentrations in the marine environment, except for Cu^{2+} at point sources. Regarding TBP, exposure and uptake by sea urchins need to be further investigated in regard to high TBP contamination of sediments in their habitats and the high TBP levels of macroalgae which may serve as food source as already proposed by Agatsuma et al. (2008). The effective concentrations of Cu^{2+} are above the European safe level of 41 nM Cu^{2+} in marine waters (European Copper Institute, 2008). Indeed, it should be considered that during measuring the Ca^{2+} signal at fertilization eggs were not exposed to the tested chemicals to avoid interference of the MRP inhibitor with the test substances. Effective concentrations of Cu^{2+} and TBP on Ca^{2+} signalling at fertilization in *P. miliaris* are, therefore, likely to be lower than the tested concentrations of 6.3 µM Cu^{2+} and 100 µM TBP, respectively.

In contrast to the effects on the Ca^{2+} wave at fertilization, we were not able to detect induction of Ca^{2+} signals by exposure of unfertilized eggs to Cu^{2+} and TBP. Indeed, the low dye loading of the eggs in comparison to eggs microinjected with indicator dyes in conjunction with the large size of the eggs limits detectability and resolution of intracellular Ca^{2+} changes in these cell types.

We demonstrate that Cu^{2+} and TBP affect calcium signalling at fertilization in sea urchin eggs. This may contribute to the reduced fertilization success of *P. miliaris* exposed to Cu^{2+} and TBP, respectively. Disturbance of Ca^{2+} channels and homeostasis by heavy metals have previously been suggested to affect acrosome reaction and motility of invertebrate sperm (Earnshaw et al., 1986, Au et al., 2001). Since sperm motility is directly correlated with fertilization success (Au et al., 2002) further studies are needed to investigate the effects of Cu^{2+} and TBP on sperm motility of sea urchins. Next to fertilization, early development is regulated by Ca^{2+} (Whitaker, 2006) and may be prone to disturbance of calcium homeostasis by TBP and Cu^{2+}. Increased Ca^{2+} levels can trigger the release of hormones from secretory granules but they can also initiate signalling cascades by activation of kinases which may rapidly affect embryonic development. Sustained increase in intracellular Ca^{2+} may further activate Ca^{2+}-dependent degradative enzymes, compromise mitochondrial function and cytoskeletal organization, and ultimately result in cell death.

In the present study, we successfully visualized the calcium wave at fertilization in eggs of sea urchins without using methods risking injury of the cell membrane: In eggs of *P. miliaris* incubated with Fura-2 AM and the MRP inhibitor MK571 we observed clear Ca^{2+} signals at fertilization. Still, it has to be considered that transport inhibitors may reduce fertilization-evoked Ca^{2+}-signals in sea urchin eggs (Davis et al., 2008). In previous studies, Fura-2 was microinjected into eggs of the sea urchin *Lytechinus pictus* to measure the Ca^{2+} rise at fertilization (Poenie et al., 1985; Swann and Whitaker, 1986). Simple incubation of eggs with Fura-2 AM has already been performed to investigate Ca^{2+} signals in mouse eggs (Hyslop et al., 2001). However, we could not observe Ca^{2+} signals in sea urchin eggs incubated with Fura-2 AM only. Similarly, Stricker et al. (1992) mentions that in eggs of the sea urchin *Lytechinus pictus* incubated with acetoxymethylesters of the calcium indicator dyes fluo-3 and calcium green no Ca^{2+} wave at fertilization could be detected.

MK571 is a specific blocker for MRP transporters and its application has been suggested to facilitate loading of animals cells (Manzini and Schild, 2003; Bickmeyer et al., 2008) as well as diatoms (Scherer et al. 2008) with calcium indicator dyes. Eggs and embryos of the sea urchin *Strongylocentrotus purpuratus* are known to express efflux transport activity (Hamdoun et al., 2004). Hamdoun et al. (2004) have shown that the efflux activity is relatively low in unfertilized eggs but is dramatically upregulated within 25 min postfertilization possibly by translocation of transporters in vesicles to the plasma membrane (Hamdoun et al., 2004). In sea stars, immunocytochemistry revealed that MRP-like proteins are localized throughout the cytoplasm in oocytes and translocated to the periphery during oocyte maturation (Roepke et al., 2006). After fertilization, eggs of the sea urchin Lytechinus pictus microinjected with the calcium indicator fluo-3 show higher fluorescence intensities in the cortex than in the centre of the cell. (Stricker et al., 1992). Possibly, in the absence of MK571 Fura-2 is extruded from the cytoplasmic spaces just underneath the plasma membrane by MRP transporters. Changes in intracellular Ca^{2+} which may primarily occur in these cellular regions may, therefore, not be detected when MRP transporters are active.

As demonstrated in the present study, decreasing fluorescence intensities of Fura-2 at 340 and 380 nm excitation induced by cell movements may result in an increasing ratio of F340/F380. This rise in the ratio of F340/F380 may be misinterpreted as an increase in intracellular Ca^{2+} concentration. Indeed, Fura-2 changes its fluorescence spectrum upon binding of Ca^{2+} ions in opposite ways: at 340 nm the intensity increases whereas at 380 nm it decreases (Grynkiewicz et al., 1985). The observed decrease in the fluorescence intensity of Fura-2 is due to gross movement of the eggs: Movement of cells is known as major artefact in imaging techniques using fluorescent dyes (Tsien et al., 1985; Silver et al., 1992). In the present study, attacking sperm caused slight movement of the eggs during *in vitro* fertilization. Moreover, eggs changed their shape upon fusion with sperm which is also known from other microscopic studies (Schatten, 1981; Stricker et al., 1992).

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