



Characterization of functional activity of ABCB1 and ABCC1 proteins in eggs and embryonic cells of the sea urchin *Echinometra lucunter*

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Synopsis

ABC transporter (ATP-binding-cassette transporter) proteins have been strongly associated with the phenomenon of multidrug resistance in cancer cells. Furthermore, their physiological expression has been studied in many organisms, including bacteria, fungi, plants and vertebrate or invertebrate animals. Their widespread expression through the evolution demonstrates their relevance to the survival of living things. In the present study, we characterized the functional activity of ABCB1 and ABCC1 proteins in gametes and embryonic cells of the sea urchin *Echinometra lucunter*. The ABC transporter proteins' functional activity was up-regulated post-fertilization. Eggs and spermatozoa of *E. lucunter* accumulated more C-AM (calcein acetoxymethyl ester), a fluorescent substrate of ABCB1 and ABCC1 proteins, than embryonic cells. Verapamil, reversin 205 and indomethacin were able to increase C-AM influx in eggs and embryos. However, verapamil and reversin 205 were more efficient than indomethacin, suggesting a predominance of ABCB1 protein over ABCC1 protein activity. Multidrug resistance modulating agents, at the concentration range that inhibited ABC transporter proteins, did not block the embryonic development until blastula stage. However, inhibition of ABCB1-mediated efflux by reversin 205 circumvented resistance of embryos to the antimitotic vinca alkaloid vinblastine. Embryonic development was more efficiently blocked when colchicine was previously added to eggs than to embryos 5 min after fertilization. This set of results suggests that these proteins act as a fundamental biochemical barrier conferring a protective physiological role against toxic xenobiotics in *E. lucunter* embryos.

Key words: ATP-binding-cassette transporter (ABC transporter) protein, *Echinometra lucunter*, efflux pump, gamete, embryonic development, sea urchin

INTRODUCTION

Since the description of P-gp [P-glycoprotein; MDR1 (multidrug resistance 1) or ABCB1] in 1976 [1], ABC transporters (ATP-binding-cassette transporters) have drawn the attention of the scientific community. Some members of the ABC transporter superfamily are associated with the phenomenon of MDR in human tumour cells. MDR is one of the major obstacles for the therapeutic success of cancer treatment [2]. First described in bacteria [3], ABC transporter proteins have been identified in a wide range of organisms, such as fungi, protozoans, plants, vertebrate animals or invertebrate animals [4–7]. The advance of molecular biology techniques and mapping of genomes allowed the identification of ABC transporter genes in several species. However,

the understanding of the biological role of these proteins requires more profound and wide investigations, allowing a better understanding of the physiological processes that are mediated by this fascinating family of proteins.

ABC transporter proteins constitute the largest family of transmembrane proteins. These proteins mediate the transmembrane transport of several compounds or ions, including hormones, sterols, lipids, phospholipids, oligopeptides, nucleotides, heavy metals, chloride ions and xenobiotics [8]. ABC transporters are ATPases found in the plasma membrane [i.e. ABCB1/P-gp, ABCC7/CFRT (cystic fibrosis transmembrane conductance regulator), ABCC1/MRP (multidrug resistance protein) and ABCC8/SUR (sulfonylurea receptor)] and in the endomembrane system [i.e. ABCB2/TAP1 (antigen peptide transporter 1), ABCB3/TAP2, PGH1 (P-gp homologue 1), ABCD2/ALDP

Abbreviations used: ABC transporter, ATP-binding-cassette transporter; C-AM, calcein acetoxymethyl ester; FSW, filtered sea water; MDR, multidrug resistance; MFI, mean of fluorescence intensity; NBD, nucleotidebinding domain; P-gp, P-glycoprotein.

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(adrenoleukodystrophy protein), ABCD3/PMP70 (peroxisomal membrane protein 70)] and MTABC (mitochondrial ABC transporter) of eukaryotic cells, being also expressed in the prokaryotic plasma membrane (i.e. Nor-A, Sav1866 and MsbA) [9–12]. ABC transporter proteins are characterized by the presence of two NBDs (nucleotide-binding domains), which show highly conserved motifs (Walker A and B) that bind and hydrolyse ATP [13]. A functional ABC transporter contains at least two NBDs, which are responsible for ATP hydrolysis that drives the pump activity of the proteins, and two transmembrane domains.

Some drugs modulate ABC transporter protein activity causing an increase in intracellular substrate concentration. These drugs are known as MDR modulators and include many substances that are not pharmacologically or chemically related, such as cyclosporin A, verapamil, trifluoperazine, quinine, propranolol, reversin 205, indomethacin, probenecid, MK-571 and others [14]. These drugs have been used as pharmacological tools in the investigation of ABC transporter protein activity in a wide range of biological models.

Most of the studies of ABC transporter protein expression or functional activity have been focused on somatic cells. Investigations concerning the physiological role of ABC transporter proteins in gametes or embryonic cells are still recent and incipient. Mengerink and Vacquier [15] were the first to describe the expression of an ABC transporter protein in animal gametes. These authors identified a glycoprotein in the plasma membrane of sea urchin spermatozoid with high homology to human ABCA3 protein, which is involved in the transport of phospholipids and cholesterol. ABC transporter proteins have also been described in the development of marine echiuroid worm, terrestrial nematode roundworm and mouse [16–19]. Recent works have characterized functional activity of ABC transporter proteins during the early embryonic development of sea urchin and sea star [7,20]. The mapping of the sea urchin *Strongylocentrotus purpuratus* genome revealed 65 genes encoding ABC transporter proteins [21]. This set of results opens a promising perspective for the investigation of the physiological role of ABC transporters in the fertilization and early embryonic development processes.

Cell membrane fusion of male gamete and egg is the crucial event in the formation of new embryos, which leads to remarkable physiological changes in the female gamete. Opening of ion channels, alterations in the membrane electrical potential, increase in cytosolic calcium ion levels, rise in intracellular pH and modulation of structural and enzymatic proteins are events that surround the post-fertilization, and are responsible for the beginning of cell division and the formation of a new organism [22,23]. Several proteins and carbohydrates have been described as essential to establish the interaction between the spermatozoid and the egg needed for fertilization. However, alterations in egg plasma membrane after fertilization are still not fully understood. Egg membrane undergoes severe structural and functional modifications allowing the establishment of adequate conditions that support the early stages of development. Free-living cells are fully exposed to chemical stress and ABC transporter proteins play a key role in the protection against xenobiotics under these conditions [24]. Animals in which fertilization and early devel-

opment occur in a 'free-living cell system-like' manner also need a biochemical mechanism to protect the developing organism against xenobiotics.

In the present study, we characterized the functional activity of ABCB1 and ABCC1 proteins in the early embryonic development of *Echinometra lucunter*, a widespread sea urchin that inhabits the tropical and temperate waters of the Atlantic Ocean.

MATERIALS AND METHODS

Animal capture and maintenance

Adult sea urchins (*E. lucunter*) were collected from the Atlantic Ocean at the João Pessoa coast of Brazil (7°09'S, 34°47'O). Animals were transported to the laboratory in plastic containers filled with local sea water. Animals were washed with FSW (filtered sea water) and placed in a reservoir with 4 litres of FSW per animal, under constant oxygen supply. Animals were maintained for a maximum period of 15 days. Animal capture was authorized by IBAMA (Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis; authorization code number 11545-1).

Sex identification and egg fertilization

Animals were exposed to 12 V a.c. until gametes were visible at aboral surface. Male gametes were identified as white secretion and females were identified as orange secretion. Complete spawning was induced by an intracoelomic injection of 0.5 M KCl (2–3 ml) and collection of gametes was limited to 15 min after injection. The sperm was collected with a glass Pasteur pipette and kept at 4°C until use (not more than 5 days). Eggs were collected directly in FSW and after decantation they were washed twice in 500 ml of fresh FSW. Egg suspension was adjusted to 1×10^4 cells/ml. Dry sperm was diluted to 1:5000 in FSW for the fertilization procedure. The increase in fertilization membrane was monitored with a light microscope. Embryos were used in all assays only at 5 min post-fertilization.

C-AM (calcein acetoxymethyl ester) accumulation assay

C-AM (Sigma, St Louis, MO, U.S.A.) is a non-fluorescent substrate of ABC transporter proteins and its intracellular accumulation is inversely proportional to ABC transporter protein activity. C-AM is converted into fluorescent calcein by cellular esterases. The fluorescent dye is retained in the cell as it is not an ABC transporter protein substrate. In this way, a high fluorescence is indicative of low ABC transporter protein activity and a low fluorescence is a sign of a high transport activity. For the C-AM accumulation assay, 1×10^4 eggs or embryos/ml were incubated with 250 nM of the dye and cultured in sterile 24-multiwell plates at $26 \pm 2^\circ\text{C}$ protected from light. At required time intervals, aliquots were analysed by fluorescence microscopy (Olympus BX41 equipped with a mercury arc lamp; $\lambda_{\text{ex}} = 488 \text{ nm}$) or confocal microscopy (Leica TCS SPII-AOBS equipped with an ArKr 488 nm laser;

Leica Microsystems). Fluorescence microscopy images were acquired with Sony Cyber-Shot H3 (Carl Zeiss lens), 0.25 s exposure time, $F = 3.5$ diaphragm aperture and ISO 800. All images were captured with a $\times 40$ objective (unless otherwise indicated). Fluorescence intensity was analysed with Image J software [NIH (National Institutes of Health), Bethesda, MD, U.S.A.] and values are expressed as either fluorescence arbitrary units (a.u.) or MFI (mean of fluorescence intensity).

Effects of MDR modulators on intracellular calcein accumulation in eggs and embryos

Eggs or embryos (1×10^4 eggs or embryos/ml) were treated with the MDR modulators verapamil, reversin 205 and indomethacin for 20 min and incubated with 250 nM C-AM at different time intervals in sterile 24-multiwell plates at $26 \pm 2^\circ\text{C}$ protected from light. MDR modulators were added 10 min after fertilization. Fluorescence analysis was performed as described above. All MDR modulators were purchased from Sigma.

Kinetics of intracellular calcein accumulation in embryos

Embryos (1×10^4 embryos/ml) were incubated with 250 nM C-AM for 60 min. C-AM was added 5 or 30 min after fertilization. Calcein fluorescence intensity was analysed by fluorescence microscopy at 5, 10, 20, 40 and 60 min after fluorochrome addition.

Embryonic development assay

Fertilization was performed as described above and 1×10^4 embryos/ml were incubated with drugs and cultured in sterile 24-multiwell plates at $26 \pm 2^\circ\text{C}$ protected from light. At particular time intervals, 200 μl of embryos was fixed with the same volume of 4% formaldehyde and stored at 4°C . Analyses of embryonic developmental stages (2–4-cell or morula stage) were performed with a light microscope at $\times 400$ magnification. A total of 100 embryos were evaluated for each sample. Negative controls contained the same amount of vehicle as that used at the highest drug concentration assayed. All experiments were performed in triplicate and repeated at least three times. For colchicine antimetabolic assays, embryos (5 min post-fertilization) or eggs (10 min before sperm addition) were treated with the drug at different concentrations and cultured in sterile 24-multiwell plates at $26 \pm 2^\circ\text{C}$.

Effect of reversin 205 on the resistance to vinblastine

At 5 min after fertilization, embryos were incubated with reversin 205 (1 or 10 μM) for 10 min and then treated or not with different vinblastine concentrations. After different periods, 200 μl of embryos was fixed with the same volume of 4% formaldehyde and stored at 4°C , and analysis was performed as previously described. All experiments were performed in triplicate and repeated at least three times.

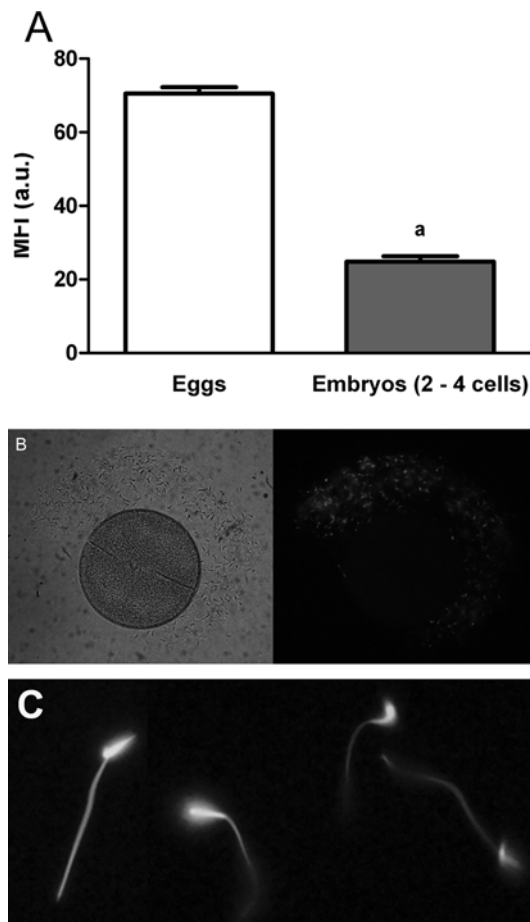


Figure 1 Intracellular calcein accumulation in eggs and embryos (A) Results shown are MFI (\pm S.E.M.) for 40–50 individual eggs or embryos incubated with C-AM (250 nM). Results shown are representative of three independent experiments performed in triplicate. 'a' indicates $P < 0.001$ compared with control (Student's t test). (B) Left panel: a 2–4-cell embryo surrounded by spermatozoa (light microscopy). Right panel: a dim 2–4-cell embryo surrounded by bright spermatozoa (fluorescence microscopy). (C) Higher magnification of fluorescent spermatozoa (fluorescence microscopy; $\times 100$). All pictures show images of representative samples.

RESULTS

Intracellular calcein accumulation in gametes and embryonic cells

Gametes and embryonic cells were exposed to C-AM for the same time interval. Embryonic cells accumulated C-AM to a lower extent than gametes. C-AM accumulation in eggs was 2.8 times higher than that observed in 2–4-cell embryos (Figure 1A). This difference was also observed in male gametes, which accumulate more C-AM than embryos (Figure 1B). Figure 1(C) shows bright spermatozoa surrounding a 2–4-cell embryo.

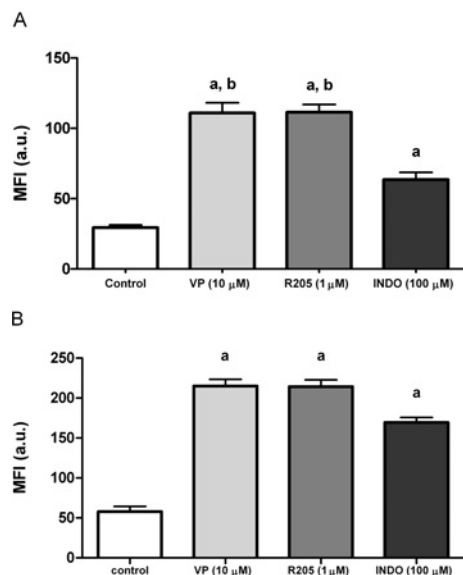


Figure 2 Effects of MDR modulators on intracellular calcein accumulation in embryos

Results shown are MFI (\pm S.E.M.) for 40–50 individual embryos in the 2–4-cell stage (A) or the early blastula stage (B) incubated with C-AM (250 nM) and treated or not with verapamil (VP), reversin 205 (R205) and indomethacin (INDO). Results shown are representative of three independent experiments performed in triplicate. 'a' indicates $P < 0.001$ compared with the control. 'b' indicates $P < 0.001$ compared with the indomethacin group (one-way ANOVA followed by the Tukey's test).

Effects of MDR modulators on intracellular calcein accumulation in embryos

To investigate if the differences in intracellular calcein accumulation between gametes and embryonic cells were due to ABC transporter protein activity, embryos were incubated with C-AM and treated with MDR modulator agents. Verapamil (10 μM), reversin 205 (1 μM) and indomethacin (100 μM) were able to increase intracellular calcein accumulation in *E. lucunter* embryonic cells ($P < 0.001$). Calcein MFI of untreated 2–4-cell embryos was 29.43 ± 1.87 . ABCB1 modulators induced a steep rise in calcein MFI. Calcein MFI was 110.90 ± 7.29 and 111.50 ± 5.09 when embryos were treated with verapamil and reversin 205 respectively. Indomethacin also enhanced calcein MFI but to a lesser extent than observed on ABCB1 modulators treatment (63.57 ± 5.09) (Figure 2A). All modulators were also able to increase calcein accumulation in early blastula embryos. Calcein fluorescence intensity was 3.7 times higher when embryos were incubated with verapamil or reversin 205, while the increase of calcein MFI with indomethacin treatment was 2.9 times higher when compared with the untreated group (Figure 2B).

Effects of MDR modulators on intracellular calcein accumulation in eggs

We also investigated ABC transporter protein activity in *E. lucunter* female gametes. Eggs previously treated with verapamil (10 μM), reversin 205 (1 μM) and indomethacin (100 μM) showed an increase in intracellular calcein accumulation

(Figure 3A). Eggs treated with reversin 205 or verapamil showed the same level of fluorescence intensity as that observed in embryos treated with the same modulators. This pattern was not observed in eggs treated with indomethacin (Figure 3B). A cortical distribution of fluorescent calcein was observed by confocal microscopy in untreated eggs (Figure 4A). This distribution profile was lost when eggs were treated with the ABCB1 protein modulator agent reversin 205 (Figure 4B).

Effect of egg or embryo treatment on the resistance to colchicine

To study the role of ABC transporter protein activity in the resistance to xenobiotics, eggs or embryos (5 min after fertilization) were exposed to colchicine at different concentrations and the early embryonic development was monitored by light microscopy. Colchicine is a natural compound that impairs cell division. This drug acts in mitosis preventing the progression to anaphases and subsequent cell cycle continuation. Our results showed that colchicine was more effective in blocking the first cell division when eggs were exposed to this chemotherapeutic agent before fertilization ($IC_{50} = 15.11 \mu\text{M}$) than when colchicine was added to embryos ($IC_{50} = 105.60 \mu\text{M}$) (Figure 5).

Kinetics of intracellular calcein accumulation in embryos

To investigate if ABC transporter protein activity was established promptly after fertilization, two staining protocols were developed. C-AM was added to embryos 5 or 30 min after fertilization and MFI was assessed at different time intervals. Our results showed that embryos accumulated more calcein when the dye was added just 5 min after fertilization. One hour after fluorochrome addition, MFI was 134.70 (calcein added 5 min after fertilization) or 28.80 (C-AM added 30 min after fertilization) (Figure 6). These values represent a difference of 4.68 times in intracellular calcein accumulation according to the moment of adding the dye. A plateau was reached 20 min after calcein addition for both protocols.

Reversin 205 circumvents vinblastine resistance in embryonic cells

Vinblastine, an antimetabolic drug that binds to tubulin and inhibits microtubule assembly, is a well-known ABC transporter protein substrate. To investigate the effect of ABCB1 protein-mediated efflux on the embryotoxic activity of vinblastine, embryos were previously incubated with reversin 205 and treated with vinblastine at different concentrations. Reversin 205 increased embryos' sensitivity to vinblastine antimetabolic properties. The progression to the 2–4-cell stage was strongly blocked when vinblastine was associated with reversin 205. Vinblastine (100 and 200 nM) itself had no effect on the progression to the first two cell divisions. However, its association with reversin 205 (10 μM) powerfully blocked the development, notably at 200 nM, where no embryo reached the 2–4-cell stage (Figure 7A). Combination of vinblastine with the ABCB1 protein modulator produced a typical dose–response pattern. Similar results were observed

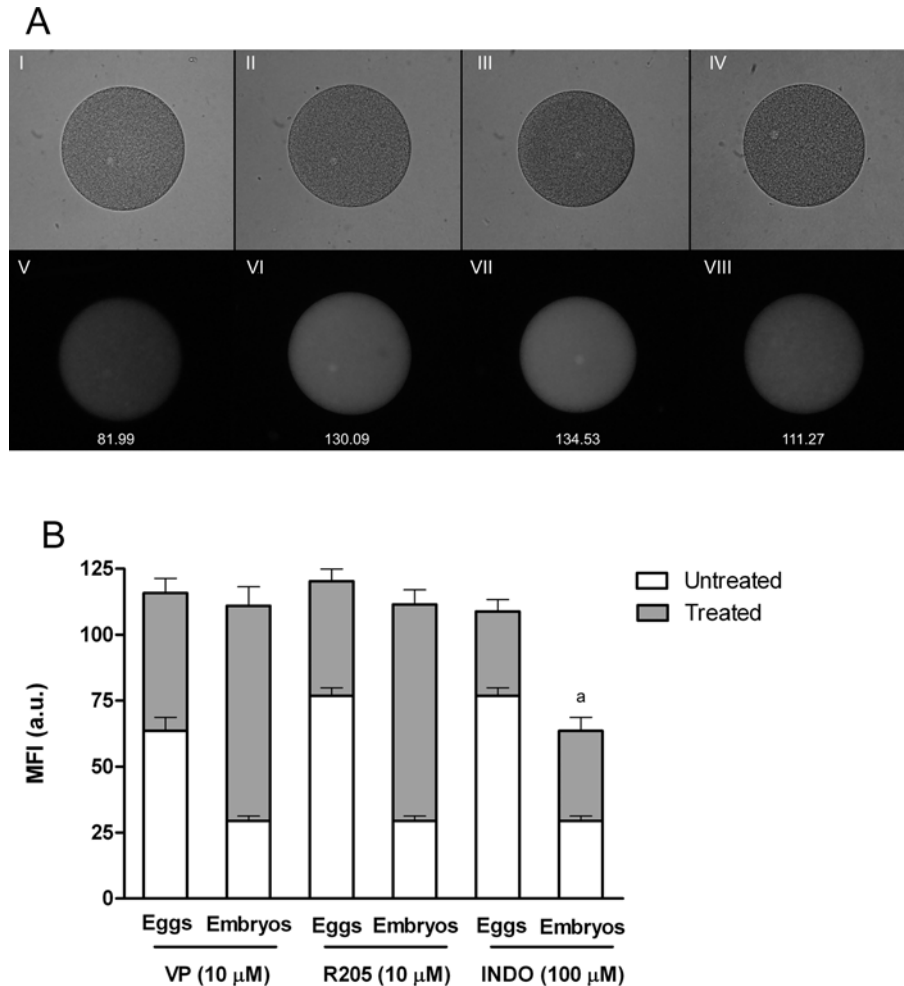


Figure 3 Effects of MDR modulators on intracellular calcein accumulation in eggs

(A) Eggs were incubated with C-AM (250 nM) and treated or not (I, V) with verapamil (II, VI), reversin 205 (III, VII) and indomethacin (IV, VIII). Numbers indicate the MFI of each cell. All pictures show light microscopy (I, II, III, IV) or fluorescence microscopy (V, VI, VII, VIII) images of representative samples. (B) Results shown are MFI (\pm S.E.M.) for 40–50 individual eggs or 2–4-cell embryos incubated with C-AM (250 nM) and treated or not with verapamil (VP), reversin 205 (R205) or indomethacin (INDO). Results shown are representative of three independent experiments performed in triplicate.

regarding the progression to the morula stage. However, in the presence of reversin 205 (10 μM), vinblastine was able to completely block the progression to morula stage at the lowest concentration (100 nM) (Figure 7B). Reversin 205 alone had no effect on embryonic development.

DISCUSSION

The history of ABC transporter proteins has been strongly associated with the phenomenon of MDR in cancer cells as well as in bacteria. Since the study by Schinkel et al. [37], the focus has been changed and the physiological role of this intriguing family of proteins has been investigated. In chemical stressing ambient liv-

ing beings have developed mechanisms to guarantee cell integrity [24]. This feature has been described in multicellular as well as in unicellular organisms. Most of the studies of ABC transporter protein activity have been developed in somatic cells. Only a few studies were performed in both gametes and embryonic cells. The relevance and understanding of the biological role of ABC transporter proteins in gametes and somatic cells are crucial to the comprehension of the processes that regulate protein expression and physiological functions. In the present study, we characterized the functional activity of ABCB1 and ABCC1 proteins in the sea urchin *E. lucunter* gametes and embryonic cells.

The utilization of ABC transporter protein fluorescent substrates has been extensively used to study the activity of these proteins in a wide range of cell types [25–29]. Using an efflux assay based on intracellular C-AM accumulation, we observed that embryonic cells of *E. lucunter* accumulated less C-AM than

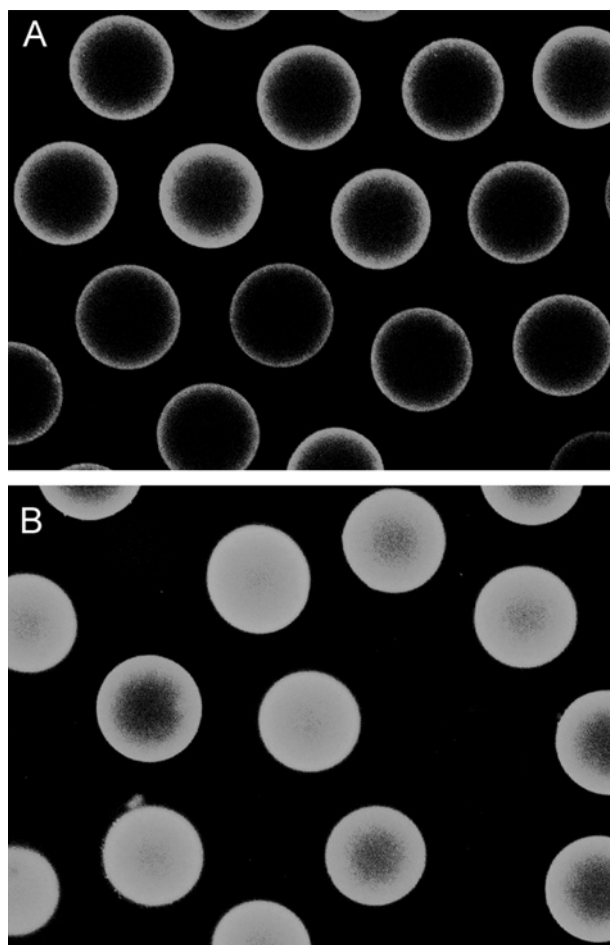


Figure 4 Cortical distribution of fluorescent calcein in eggs

Eggs were incubated with C-AM (250 nM) and treated or not (A) with reversin 205 (B). Panels show confocal images through the middle plane of cells.

gametes (Figure 1). Eggs accumulated 2.8 times more C-AM than 2–4-cell embryos. Due to the reduced cell volume it was not possible to measure, appropriately, fluorescence intensity in spermatozoa. However, Figure 1(B) shows a dim 2–4-cell embryo surrounded by many fluorescent male gametes. To investigate if the reduced C-AM accumulation in embryos could be attributed to ABC transporter protein activity, we incubated *E. lucunter* embryos with three well-known MDR modulators: verapamil, reversin 205 and indomethacin. The first two are ABCB1 protein modulators of the first and third generation respectively; indomethacin is an ABCC1 modulator. Our results showed a significant increase in intracellular C-AM accumulation in embryos previously treated with all modulators. However, verapamil and reversin 205 induced a higher increase in C-AM accumulation than indomethacin (Figures 2A and 2B). All MDR modulators, at the concentration range that inhibited ABC transporter proteins, did not block embryonic development and did not induce any morphological changes in em-

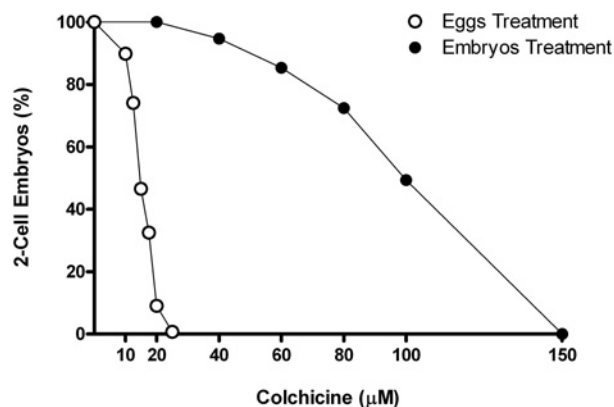


Figure 5 Effect of egg or embryo treatment on the resistance to colchicine

Eggs or embryos (5 min post-fertilization) were incubated with different colchicine concentrations. Two-cell embryos (%) represent the percentage of embryos that completed the first cell division.

bryos. These findings suggest that ABCB1 protein activity is more outstanding than ABCC1 protein activity in *E. lucunter* embryonic cells. Similar results were obtained by Roepke et al. [7] studying MDR protein expression in sea star oocytes. Hamdoun et al. [20] had previously described the presence of ABC transporter activity in gametes and embryonic cells of the sea urchin *S. purpuratus*. However, these authors obtained a more pronounced increase in C-AM accumulation using verapamil and reversin 205 than we achieved in *E. lucunter* embryos with the same modulators. Whereas C-AM accumulation increased approx. 4-fold when *E. lucunter* embryos were incubated with verapamil (10 µM) or reversin 205 (1 µM), verapamil and reversin 205, at the same concentrations, increased C-AM intracellular accumulation in *S. purpuratus* embryos by approx. 20 and 30 times respectively [20]. These findings suggest a different degree of ABCB1 expression in embryonic cells between the species studied.

Furthermore, we investigated the pattern of resistance to vinblastine, a vinca antimitotic alkaloid and a well-known ABCB1 and ABCC1 substrate. Our results showed that *E. lucunter* embryos were more sensitive to cell division blocking caused by vinblastine than *S. purpuratus* embryos. The full blockade of the first cell division in *E. lucunter* was achieved with 1 µM vinblastine (results not shown), while the same extent of blockade was achieved with only 10 µM vinblastine in *S. purpuratus* embryos (10 times more resistant) [20]. The higher sensitivity to vinblastine may be directly associated with a lower ABC transporter protein activity in *E. lucunter* embryos when compared with *S. purpuratus* embryos.

E. lucunter eggs also showed ABCB1 and ABCC1 activity since all modulators were able to increase C-AM accumulation. The same pattern of modulation, obtained in embryo assays, was also observed in eggs: ABCB1 modulators verapamil and reversin 205 induced a higher increase in C-AM accumulation than indomethacin (Figure 3A). Moreover, embryos treated with reversin

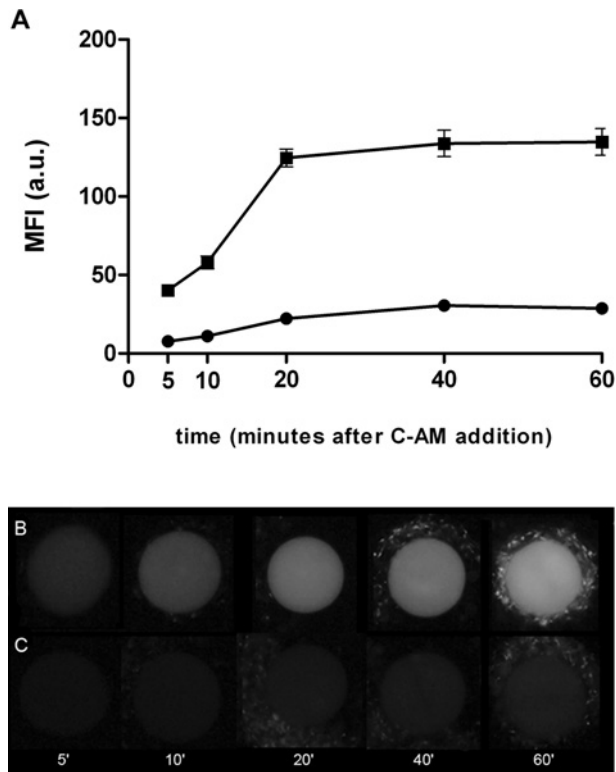


Figure 6 Kinetics of intracellular calcein accumulation in embryos

(A) Results shown are MFI (\pm S.E.M.) for 40–50 individual embryos incubated with C-AM at 5 min (■) or 30 min (●) post-fertilization. Fluorescence microscopy images of embryos treated with C-AM at 5 min (B) or 30 min (C) post-fertilization are shown. Numbers indicate time (minutes) post-fertilization.

205 or verapamil showed the same level of fluorescence intensity observed in eggs treated with the same modulators (Figure 3B), which suggests no difference in the level of saturation associated with the passive transport among these two cell types. The same pattern of staining was obtained by Hamdoun et al. [20] in the study of *S. purpuratus*. Our studies on the intracellular calcein accumulation in eggs, using confocal microscopy, revealed a cortical distribution of fluorescent calcein in *E. lucunter* eggs, suggesting that egg esterases are compartmentalized in vesicles (cortical granules) and not diffused into the cytosol (Figure 4A). Eggs treated with reversin 205 showed a marked increase in C-AM influx but still preserve a cortical-like fluorescence pattern, whereas the centre of the cell remained dim or slightly bright (Figure 4B).

Intracellular C-AM accumulation assay demonstrated that eggs have a lower transporter activity than embryos. Hence, we incubated eggs or embryos with different concentrations of colchicine, another well-known ABC transporter protein substrate, and monitored the first cell division. Figure 5 shows that embryo development was more sensitive to colchicine when the eggs were exposed to the drug before fertilization. Colchicine was 7 times more effective when the eggs were previously treated with the

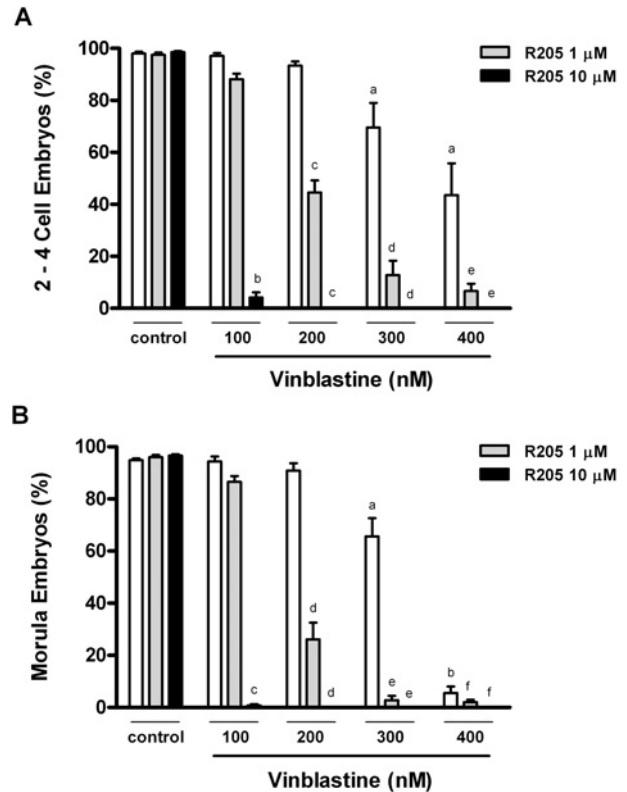


Figure 7 Reversin 205 circumvents vinblastine resistance in embryonic cells

Embryos were incubated with vinblastine (100, 200, 300 or 400 nM) and treated or not with 1 or 10 μ M reversin 205 (R205). '2–4-cell embryos (%)' and 'Morula embryos (%)' represent the percentage of embryos that reached respective stages of embryonic development. Results represent three independent experiments performed in triplicate. (A) 'a' indicates $P < 0.01$ compared with the control. 'b' indicates $P < 0.001$ compared with vinblastine 100. 'c' indicates $P < 0.001$ compared with vinblastine 200. 'd' indicates $P < 0.001$ compared with vinblastine 300. 'e' indicates $P < 0.001$ compared with vinblastine 400 (one-way ANOVA followed by the Tukey's test). (B) 'a' indicates $P < 0.01$ compared with the control. 'b' indicates $P < 0.001$ compared with the control. 'c' indicates $P < 0.001$ compared with vinblastine 100. 'd' indicates $P < 0.001$ compared with vinblastine 200. 'e' indicates $P < 0.001$ compared with vinblastine 300. 'f' indicates $P < 0.001$ compared with vinblastine 400 (one-way ANOVA followed by the Tukey's test).

drug, indicating a clear change in the profile of ABC transporter protein activity after fertilization that was responsible for reducing colchicine access to the intracellular compartment. Hamdoun et al. [20] suggested that this change may be attributed to the massive exocytosis that occurs after gamete membrane fusion, as cytochalasin-D and latrunculin-A, two cytoskeleton-disrupting agents, block the beginning of ABC transporter protein activity induced by fertilization. In this case, ABC transporter proteins may be expressed in vesicle membranes and are addressed to the cell membrane after the exocytosis of the cortical granules is induced by calcium wave.

According to the resistance profile of colchicine, we used another approach to investigate the establishment of ABC transporter protein activity after fertilization. Thus C-AM was added



to the embryos 5 or 30 min after fertilization, and intracellular C-AM accumulation was evaluated for 60 min. Our results showed a clear difference in the content of fluorescent calcein depending on the time of C-AM addition (Figure 6). C-AM accumulation was 5 times greater when the dye was added just 5 min after fertilization, which suggests that the pumps require a lag interval to be functionally active in the cell membrane. This delay may be due to protein conformational changes that occur in the cell membrane after exocytosis. Although the involvement of PKC (protein kinase C) in the regulation of ABCB1 activity is still a matter of debate [30], several works suggest that ABC transporter protein activity may be up-regulated by phosphorylation promoted by enzymes involved in cell signalling as PKA or PKC [31–35]. Our results also demonstrated that intracellular fluorescent calcein content became stable 20 min after the addition of C-AM independently of ABC transporter protein activity, which may be related to the time needed for a complete conversion of C-AM into fluorescent calcein.

Vinblastine, a chemotherapeutic drug that binds to tubulin and inhibits the cell cycle, has been extensively used in studies involving MDR proteins in cancer cells. These studies demonstrated that ABC transporter protein activity is closely related to the efflux of the chemotherapeutic agent. Our results showed that reversin 205 (1 and 10 μ M) was able to sensitize embryos to vinblastine antimetabolic effects (Figures 7A and 7B). In addition, reversin 205 itself did not block early embryonic development. Verapamil and indomethacin, at the same concentration that was able to increase intracellular C-AM accumulation, did not affect embryonic development as well (results not shown).

Considering that modulation of ABCB1 or ABCC1 protein activity did not impair early embryonic development, our results suggest that the biological role of these proteins in gametes and embryonic cells is to protect these cells against chemical stress and these proteins are not implicated in the transport of intrinsic factors responsible for the progression of cell division or embryonic development. Sawicki et al. [19] studying murine embryonic development demonstrated that ABCB1 and ABCG2 proteins are functionally active only in morula stages. The prompt activity of ABCB1 and ABCC1 proteins in two-cell embryos of the sea urchin may be attributed to the external fertilization that exposes embryos to a chemically hostile environment. Our present study suggests that changes in activity of MDR-associated ABC transporter proteins during development are a general phenomenon in the sea urchin. This protective physiological role has been described for many other organisms, from humans (i.e. endothelial cells that constitute the blood–brain barrier, epithelial cells of the intestine or tubular cells of the kidney) to unicellular organisms, such as protozoa (trypanosomatids), fungi or bacteria [36–39].

ACKNOWLEDGEMENT

We thank Rubens Lima do Monte Neto for the sea dive and the introduction to the amazing world of sea urchins and Dr Ierecê Lucena Rosa (Laboratório de Peixes, Ecologia e Conservação/

Universidade Federal da Paraíba, João Pessoa, Brazil) for the filtered sea water.

FUNDING

This work was supported by Programa Institucional de Bolsas de Iniciação Científica (PIBIC)/Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (undergraduate fellowships to E.T. and A.L. de M.C.).

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Received 11 June 2009/21 July 2009; accepted 19 August 2009

Published as Immediate Publication 19 August 2009, doi 10.1042/BSR20090081
