



Short communication

# The sea urchin embryo as a model for studying efflux transporters: Roles and energy cost

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## Abstract

We describe the use of the sea urchin as a model for studying efflux transporters and estimating energy cost for the cytotoxin protective system provided by these transporters. The unfertilized egg has low transport activity, which increases to a new steady state shortly after fertilization. Activity results from *p*-glycoprotein (*p*-gp) and MRP type transporters which protect the embryo from cytotoxic drugs that can disrupt cell division or induce apoptosis. The energy cost is estimated from a novel use of calcein-AM as a substrate; keeping 0.25  $\mu$ M substrate levels out of the cell utilizes only 0.023% of steady state respiration.

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It is well appreciated that efflux transporters, such as members of the *p*-gp and MRP families, provide a first line of defense against natural and anthropogenic contaminants by preventing toxicants from entering the cell or actively removing toxicants from within the cytoplasm (Leslie et al., 2001; Smital et al., 2004). The study of these transporters is made difficult, however, by the challenge of assaying efflux activity as well as the absence of suitable marine models.

In the past, our laboratory has used the fluorescent substrate rhodamine to evaluate efflux activity in mussel gill tissue (e.g., Cornwall et al., 1995). Problems with this substrate and model system are three-fold: (1) rhodamine washes out of the cells during the

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measurement (2) there is large variability between individual animals (Luckenbach and Epel, 2005) and (3) tissue activity varies seasonally (Keppler and Ringwood, 2001). Here, we describe the utilization of calcein-AM as a model substrate and the sea urchin embryo as a convenient aquatic model organism; their combined use allows reproducible measurements of transporters with no fluorescent dye washout problems and little seasonal variation. We find protective roles in protecting the embryo from toxicants and apoptosis and show for the first time that energy costs for this protection mechanism are extremely low, utilizing less than 0.1% of steady state respiration.

Protective roles of the transporter against vinblastine were tested by exposing embryos at 45 min after fertilization (after steady-state transporter changes are completed, see Hamdoun et al., 2004) to the indicated vinblastine concentrations in the presence of 5  $\mu\text{M}$  MK571 (MRP inhibitor) or 5  $\mu\text{M}$  PSC833 (specific inhibitor of *p*-glycoprotein). Cell division status was then scored by microscopy at 145 min after fertilization. Protective roles against apoptosis were assessed by adding 1  $\mu\text{M}$  etoposide to embryos at the 4-cell stage in the presence or absence of verapamil (a *p*-gp inhibitor) and following development and apoptosis for 24 h. Apoptosis was assessed by the TUNEL assay at the indicated times.

The efflux transport assay used for assessing ATP utilization is based on the use of Calcium-AM as a substrate for both *p*-glycoprotein and MRP transporters (Essodaigui et al., 1998). Embryos are incubated in 250 nM calcein-AM. If this non-fluorescent calcein-AM enters the cytoplasm, the AM ester group is hydrolyzed, resulting in production of fluorescent calcein. Calcein is not a substrate for the transporter and remains trapped within the cell as a fluorescent marker and index of transporter activity. Accumulation of calcein is measured in vivo with quantitative fluorescence microscopy or in cell extracts on a microplate reader using 488 excitation/520 emission (see Hamdoun et al., 2004 for details).

There is little efflux activity in the unfertilized egg but activity is rapidly established beginning at 25 min after fertilization (Hamdoun et al., 2004). This transport activity protects the embryos against toxicants such as the microtubule depolymerizing drug vinblastine whose effect is easily measured as inhibition of cell division. As seen in Fig. 1(A), the 50% inhibition for vinblastine is about 3  $\mu\text{M}$ . However, if efflux transporter activity is inhibited with either MK571 (an MRP inhibitor) or PSC833 (a *p*-glycoprotein inhibitor) the effective concentration is decreased 10-fold.

A similar lowering of effective concentration is seen for induction of apoptosis by the topoisomerase inhibitor etoposide (DNA damage inducing apoptosis). As seen in Fig. 1(B), 1  $\mu\text{M}$  etoposide has no effect on apoptosis when embryos are exposed to this drug from the 4-cell stage and followed for 24 h. As seen, there is also no effect of the *p*-glycoprotein inhibitor verapamil. However, if the two drugs are combined, there is a large increase in apoptosis.

The energy cost of the transport activity can be estimated by measuring how much calcein-AM is excluded from the cell by efflux transporter activity. Once transport activity is established at 25 min after fertilization, little calcein-AM penetrates the cell. However, if one inhibits transport activity as with MK571 and PSC833 at concentrations that give maximal inhibition, there is no impedance to calcein entry as the efflux transporter cannot pump the calcein out of the cell. The amount of calcein that can now enter the cell provides an indication of how much of the calcein is actually excluded by the respective transporters, which is also a quantitative measure of activity.

## Inhibition of Transporters In the Presence of Toxins Leads to Increased Cell Division Abnormalities and Apoptosis

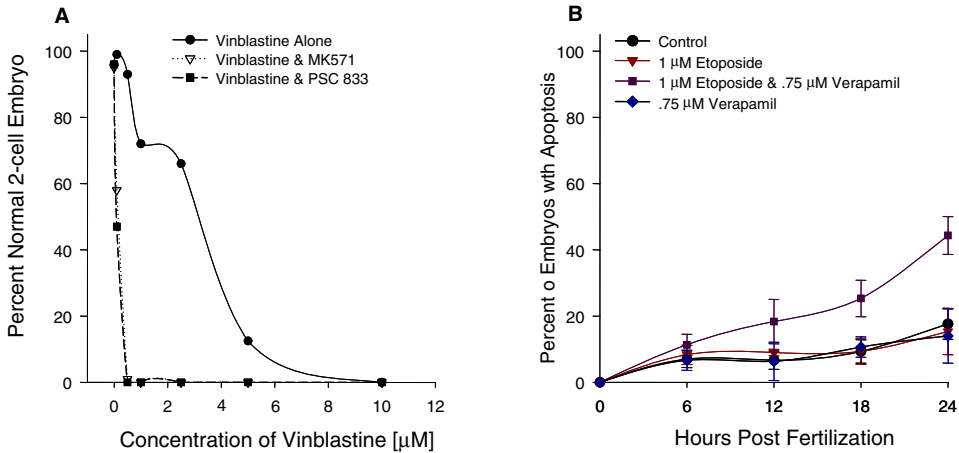


Fig. 1. (A) Efflux transporters protect sea urchin embryos from cytotoxins. Cell division in control embryos is 50% inhibited at 4  $\mu\text{M}$  vinblastine. If efflux transporters are inhibited with 5  $\mu\text{M}$  MK571 (MRP inhibitor) or 5  $\mu\text{M}$  PSC833 (*p*-gp inhibitor) the effective concentration of vinblastine is now less than 1  $\mu\text{M}$ . (B) Apoptosis induced by 1  $\mu\text{M}$  of the topoisomerase inhibitor etoposide has no effect on apoptosis during sea urchin development. In the presence 0.75  $\mu\text{M}$  verapamil (which does not affect apoptosis) apoptosis is increased such that over 40% of embryos exhibit some apoptotic cells as opposed to about 15% embryos with some apoptotic cells in the control.

After creating standard fluorescence curves for calcein, we precisely calculated the molarity of the calcein that entered the cells when activity was inhibited between the 2- and 4-cell stage; this amount is then equivalent to the calcein-AM that is excluded by the cell when the transporter is functioning. Using an 0.1% embryo suspension in the presence of 250 nM calcein-AM and an assumption of two ATP molecules consumed for each substrate molecule transported (Rao and Nuti, 2003), we estimate that each embryo consumes  $3.9 \times 10^8$  molecules of ATP per minute for the combined *p*-gp and MRP efflux transporter activity. Translating this into respiratory equivalents, this amount of ATP represents 0.023% of total respiration activity at this stage. This compares with the 60–70% of energy that is estimated for maintaining Na/K concentrations in the cytoplasm as established by Na/K ATPase in the sea urchin embryo (Leong and Manahan, 1997).

The conclusion then is that these transporters are highly efficient and that the transporters' protective role is achieved with an extremely small energy expenditure of the cell. In terms of the cells' energy budget for toxicant protection, this number does not take into account the cost of synthesis of these transporters. Nor does this estimate account for energy used in secondary transformation of any toxicants that can bypass the activity of the efflux transporters and thus gain access to the cytoplasm.

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