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Short communication

Inhibition of multidrug resistance transporters in the diatom *Thalassiosira rotula* facilitates dye staining

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Abstract

Cells are protected by multidrug resistance transporters, which remove potentially harmful chemicals entering the cells from the environment or originating endogenously from the cellular metabolism. Multidrug resistance transporters have not been investigated so far in marine eukaryotic algae like diatoms. We investigated the uptake of a calcium-sensitive dye, Fura 2 acetoxymethylester (AM), by the marine diatom *Thalassiosira rotula* in the presence and absence of substances known to inhibit multidrug resistance transporters (ATP-binding cassette transporters, ABC). Three inhibitors known to block transporters in living organisms were tested in the marine diatom *T. rotula*. We applied verapamil, which blocks multidrug resistance P-glycoprotein (MDR1), probenecid as an inhibitor of organic anion transport and the specific inhibitor of multidrug resistance-associated protein (MRP), MK571, obtaining positive results with the highly specific MK571. This leads to the assumption that the cells of *T. rotula* possess MRP transporters. Marine diatom cells can now be loaded by incubation with a calcium-sensitive dye, which facilitates measurements of cellular calcium signals without using methods risking injury of the cell membrane. This opens an avenue for investigation on diatom calcium signalling and perhaps how they process environmental signals.

Keywords: MK571; ABC transporter; Fura 2; Probenecid

1. Introduction

Cells protect themselves by several defence mechanisms against harmful chemicals from the environment or from toxic endogenic compounds of the cellular metabolism, for example oxygen radicals. One line of defence is the active, energy consuming, extrusion of these compounds by ATP-binding cassette transporters (ABC transporters). The multidrug resistance P-glycoprotein transporters (MDR1) have been shown to have a broad spectrum of hydrophobic substrates [10]. The MRP transports a variety of neutral hydrophobic compounds and additionally organic anions (for review: [2]). Another transporter belonging to the group of ABC transporters is for example the breast cancer resistance protein (BCRP) [3].

Information from the extracellular solution is often detected and processed by membrane receptors and other proteins, translating the information into second messenger cascades in the cell. Intracellular free Ca^{2+} is a well-investigated cellular second messenger in eukaryotic organisms and a key actor in regulatory functions, playing a major role in triggering various cellular responses in animal and plant cells. More recently it has been described in marine algae [4] as a possible stress surveillance system [17]. Calcium signal measurements have been carried out in plant cells. However, the methods for loading the cells with calcium dyes are comparably difficult techniques such as patch clamp or pressure injection [16,6] or molecular techniques as aequorin over-expression based measurements [4,14]. It is known since several years in mammalian cells that acetoxymethylester (AM) derivatives of various fluorescent

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indicators are actively extruded by the multidrug transporter (MDR1/ P-glycoprotein) in mammals [10]. The dye extrusion is blocked by competing substrates and inhibitors of the multidrug transporter. The hydrophilic free acid forms of the indicators are not exported by MDR1 [10]. In tadpoles' olfactory receptor neurons (ORNs) Manzini and Schild [13] showed the modulation of dye extrusion by inhibitors of ABC transporters.

As unicellular marine organisms in particular are directly exposed to the aquatic environment containing harmful xenobiotics we assumed that marine diatoms should have a similar protection or defence system against harmful or cytotoxic substances. As this has never been investigated it was our aim to incubate the marine diatom *Thalassiosira rotula* with a membrane-permeable dye as indicator. As inhibitors of cellular extrusion mechanisms we used MK571, which is a specific inhibitor of MRP (multidrug resistance-associated proteins) [7,1], probenecid P36400, which is a known inhibitor of organic anion transport [15], and verapamil, an inhibitor of P-glycoprotein [5,12,1]. Verapamil additionally is a Ca^{2+} channel blocker [11] which is used to treat angina, migraine or high blood pressure and can even prevent a rapid heart rate in humans.

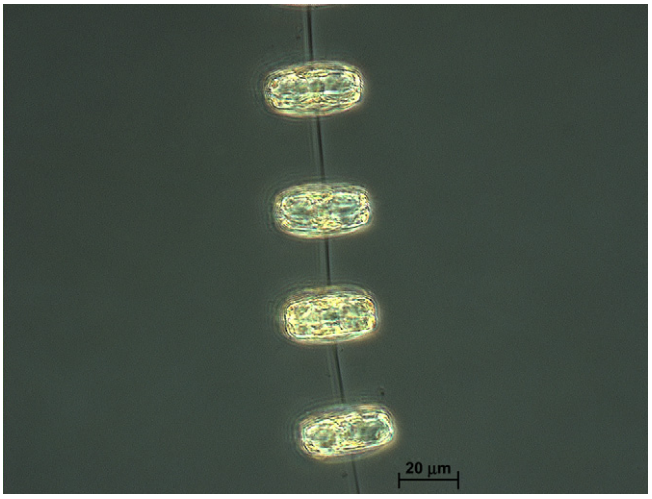

Fig. 1. *Thalassiosira rotula*. Scale bar 20 µm.

2. Material and methods

T. rotula cells were isolated in March 2004 and in August 2005 both from Helgoland Roads in the German Bight. The cultures were kept in F/2 + silicate medium in a culture

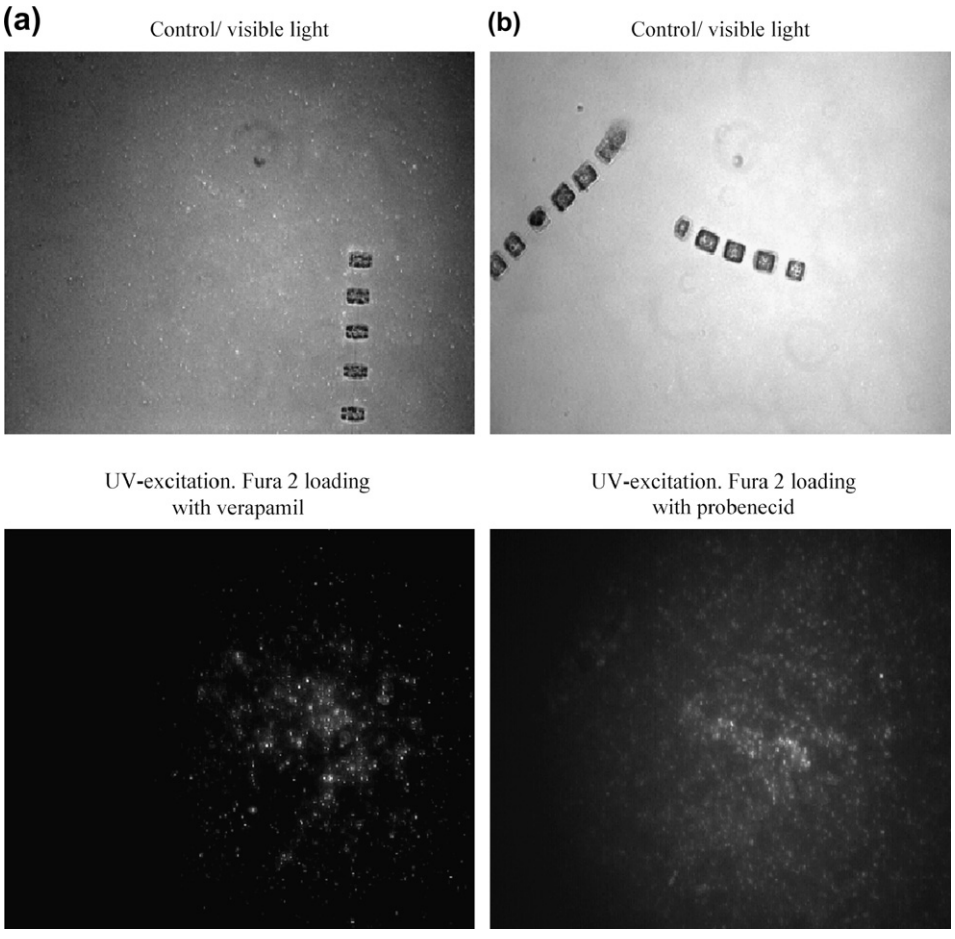

Fig. 2. *Thalassiosira rotula* incubated for 2 h with Fura 2/AM. (a) In the presence of verapamil and (b) in the presence of probenecid. UV excitation at 380 nm, emission at 510 nm.

Table 1
Dye loading of *Thalassiosira rotula* in the presence of multidrug resistance transporter inhibitors (N = number of experiments including each several individual cells)

	T. rotula	
MK571	$N = 31$	Effective concentrations ≥ 50 μM
Verapamil	$N = 14$	Concentrations tested ≤ 160 μM − without effect
Probenecid	$N = 14$	Concentrations tested ≤ 8 mM − without effect

room with a 12 h dark/light cycle and at temperature of 15 °C. The F/2 medium was made up with artificial seawater. The total salinity was about 31.6, by adding guaranteed nutrient-free sea salt (hw Meersalz professional, Wiegandt GmbH, Krefeld, Germany) to 1 L of Millipore water. Then the F/2 nutrients plus silicate were added according to Guillard and Ryther [8].

2.1. Imaging

The fluorescence of the cells was monitored by a polychromator system (Visitron, Puchheim, Germany) and a CCD camera (Coolsnap cs) mounted on an inverted microscope (Zeiss Axiovert 100). Fluorescence was detected using a UV objective (Zeiss NeoFluar 20×).

2.2. Chemicals

The dye used to load the cells was Fura 2 acetoxymethylester (Fura 2/AM, Sigma), a vital dye which is a UV-excited Ca^{2+} indicator [9]. The esterification of the carboxyl groups makes the molecule lipophilic and therefore it can cross the cell membrane. The ester bonds are split in the cytosol due to cytosolic esterases and Fura 2/AM accumulates in the cell as formaldehyde, acetate and free acid. This free acid binds to calcium and is excitable with UV-light and thus functions as a calcium indicator. The samples were incubated by one multidrug resistance transporter inhibitor and Fura 2 for 2 h at room temperature in the dark before measurement.

MK571 (Cayman Chemical Company 2005) was used in concentrations from 10 μM up to 365 μM dissolved in DMSO (final concentration <1%) and F/2 before it was added to the algae. DMSO in concentrations of lower than 1% showed no effect in control experiments. MK571 was stored at −20 °C.

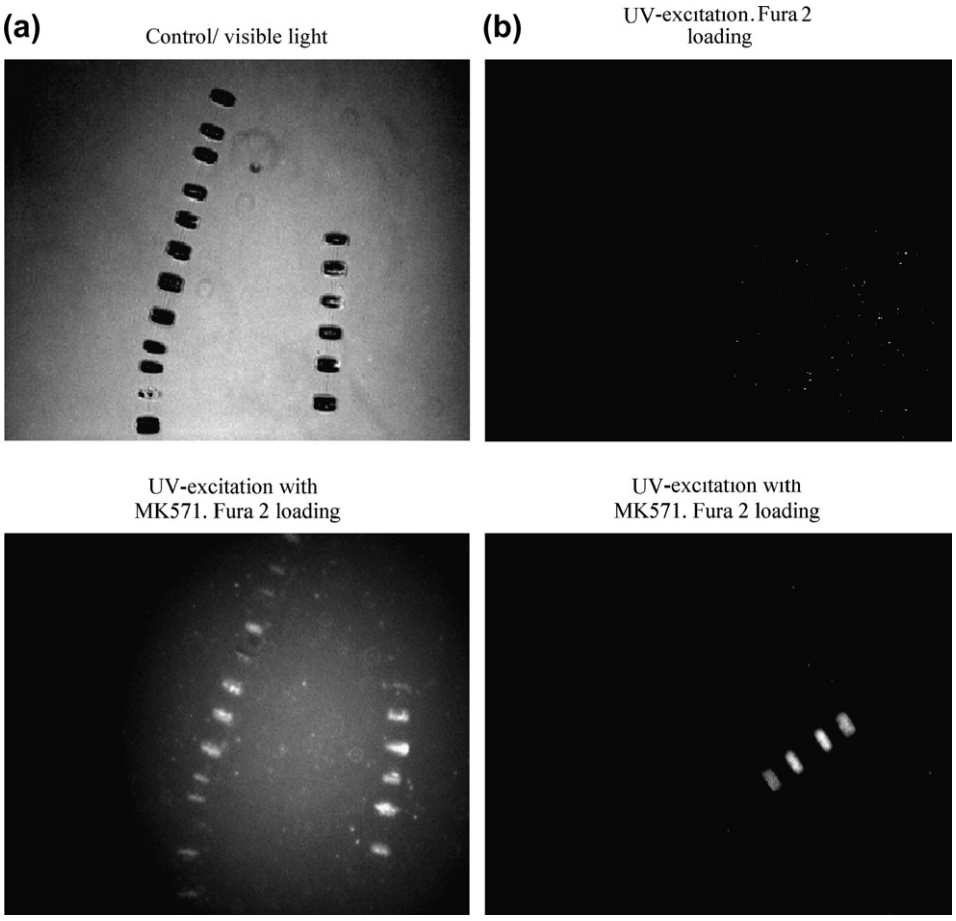

Fig. 3. *Thalassiosira rotula* incubated for 2 h with Fura 2/AM. (a) In the presence of MK571 and (b) Fura 2 incubation without other compounds and in the presence of MK571. UV excitation at 380 nm, emission at 510 nm.

Probenecid (P36400, Molecular Probes) was dissolved in Hanks' balanced salt solution (HBSS). Final concentrations ranged from 2.5 mM to 8.3 mM.

Verapamil HCl (Sigma–Aldrich, Germany) is a Ca^{2+} channel blocker [11]. Verapamil was used in concentrations of 50–160 μM.

3. Results

At the investigation outset we found the finding that cells from the two strains of the marine diatom *T. rotula* (Fig. 1) isolated from the Helgoland Roads in March and August could not be loaded by incubation with the acetoxymethylester derivative of the calcium indicator dye Fura 2/AM (Fig. 2a). We tried to incubate and load the cells with Fura 2/AM in the presence of verapamil, probenecid and MK571 known to inhibit more or less specific multidrug resistance transporters. The experiments were repeated several times (see Table 1) always obtaining comparable results. Verapamil (50–160 μM) and probenecid (≤8 mM) showed no effect on diatom fluorescence (Fig. 2a and b). Verapamil and probenecid possibly also facilitated the staining of the bacteria surrounding *T. rotula* cells but this needs to be further investigated. The dye loading of the diatoms only was successful when MK571 was present in concentrations of or higher than 50 μM. Ten micromolar was not effective in facilitating Fura 2 loading of the cells (Fig. 3a and b).

4. Discussion

The failure to load the cells with the calcium-sensitive dye Fura 2/AM led to the idea to block multidrug resistance transporters, if present. The approach used by Manzini and Schild [13] demonstrated dye loading of tadpole olfactory receptor neurons by incubation only in the presence of transporter blockers. They showed that organic anion transporters MRP and MDR1/ P-glycoprotein were responsible for dye removal.

We show here that especially the MRP transporters seem to be responsible for dye removal, assuming that MK571 is specific for this transporter in diatoms. The application of verapamil, which inhibits MDR1/ P-glycoprotein, showed no effect. This was similar to probenecid as an organic anion transporter inhibitor. Homolya et al. [10] and Manzini and Schild [13] showed that ester dyes as Fura 2 are removed by MDR1 transporters. This seems not to be the case in the diatom *T. rotula* which gives rise to the hypotheses that they do not possess MDR1 transporters. The MRP transporter in diatoms seems to bind organic anions, in our case Fura 2 in its free anionic form after hydrolyzation of the ester bond and – as MRP transporter expels lipophilic organic molecules too – the Fura 2/AM. Despite its efficacy as inhibitor of MRP transporters, probenecid showed no effect in our experiments indicating target site differences between algae and for example vertebrate transporters. Surprisingly the most specific inhibitor available of MRP [7,1] is effective in the diatom *T. rotula*.

In addition to the first investigation on microalgae (diatoms) our approach now enables the comparably easy investigation of cellular signalling using membrane-permeable Fura 2/AM and possibly other dyes.

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