Uptake of the fluorescent indicator atebrin into acidic vacuoles in the halotolerant alga Dunaliella salina

Meira Weiss and Uri Pick

Department of Biochemistry, Weizmann Institute of Science, P.O. B. 26, Rehovot 76100, Israel

Received 30 March; accepted 25 June 1991

Abstract. The fluorescent indicator atebrin (3-chloro-9-(4-diethylamino-1-methylbutyl)-7-methoxy-acridine) is taken up by Dunaliella salina cells at alkaline external pH and accumulates in acidic vacuoles. The uptake is unaffected by light, by photosynthetic inhibitors, by protonophores or by ionophores; however, the dye can be released by amines, indicating that it is specifically accumulating in acidic vacuoles. Amines induce a biphasic enhancement of atebrin fluorescence – a fast phase, accompanied by redistribution within the cell, consistent with release of the dye from the vacuoles to the cytoplasm, and a slow phase, correlated with release of atebrin from the cells. These results are interpreted to indicate a slow equilibration of atebrin across the plasma membrane and a fast equilibration across the vacuolar membrane. Part of the dye cannot be released by the amines, and appears to be internally bound. Atebrin uptake is inhibited by cholesteryl hemisuccinate and is stimulated by lysophosphatidylcholine, indicating that modification of the lipid composition of the plasma membrane affects the permeability to atebrin. Analysis of the pH dependence of atebrin uptake indicates that the dye enters the cells by fluid-phase permeation. Different stresses enhance the rate of atebrin uptake and release, indicating that they modify plasma-membrane structure or composition. Atebrin may serve as a specific marker for acidic vacuoles, as an indicator for amine uptake, and as a probe for subtle changes in the permeability of the plasma membrane.

Key words: Amine uptake – Atebrin (uptake, release) – Dunaliella – Plasma membrane permeability – Vacuole (acidic)

Introduction

Vacuoles fulfill a large number of functions in yeast and plant cells (for review, see Boller and Wiemken 1986).

Abbreviations: Atebrin = 3-chloro-9-(4-diethylamino-1-methylbutyl)-7-methoxy-acridine, DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; SF-6847 = 3,5-di(iso)butyl-4-hydroxybenzylidenemalonitriile

The central vacuoles in plants are the major reservoir of water, inorganic ions, metabolites and macromolecules, and play an important role in pH homeostasis and in compartmentation of Na⁺, Cl⁻ and Ca²⁺. Vacuoles have a major role in cell growth and in providing a static support to the cell. In addition, vacuoles of plants and yeasts are the equivalent of lysosomes in animal cells, and contain a whole array of hydrolytic and degradative enzymes. Their special biochemical and physiological status stems from their acidic pH, which is generated by two H⁺ pumps in the vacuolar membranes – a nitrate-sensitive H⁺-ATPase and a pyrophosphatase (Rea and Sanders 1987).

Most plants and macroalgal cells contain one central vacuole which occupies most of the cell's volume; it arises from fusion of smaller vacuoles during the development of the cell. In contrast, unicellular algae, such as Chlorella and Chlamydomonas, possess several small vacuoles which occupy a small fraction of the cellular volume. The existence of acidic vacuoles in unicellular algae was deduced from 31P-nuclear magnetic resonance experiments, which demonstrated a alpι peak corresponding to acidic compartments in Chlorella and in Dunaliella (Kuchitsu et al. 1987, 1989); from electron micrographs combined with X-ray microanalysis which demonstrated the existence of polyphosphate granules inside vacuolar structures in Chlorella (Peverly et al. 1978) and Scenedesmus (Tillberg and Rowley 1989); and from special stains which accumulate in acidic vacuoles. The advantage of the latter is that they provide a technique to visualize and to follow changes in the morphology and pH of acidic vacuoles in vivo. Several methods for visualization of acidic vacuoles using fluorescent probes have been developed. They include fluorescent amines such as quinacrine (atebrin) or chloroquine, which diffuse across the membrane and accumulate in acidic compartments (Allison and Young 1964; Weisman et al. 1987; Kuchitsu et al. 1987); impermeable fluorescent polymers, such as fluorescein isothiocyanate-dextran, which are encapsulated by endocytosis in yeast cells (Makarov 1985); and endogenous fluorophores (Weisman et al. 1987). Utilization of fluorescent amines for staining of acidic vacuoles in photosynthetic cells may be hampered by inhibition of photosynthesis and accumula-
tion of the dye in the intrathylakoid space of the chloroplast. However, since previous studies with algae were aimed at cytochemical identification of the vacuoles, the toxicity and selectivity of the probes with respect to chloroplast thylakoids have been ignored (Kuchitsu et al. 1987, 1989).

In this paper we describe the utilization of the fluorescent acridine dye atebrin (3-chloro-9-(4-diethylamino-1-methylbutyl)-7-methoxy-acridine) for labeling acidic vacuoles in the halotolerant alga *Dunaliella salina*. We demonstrate that the dye accumulates selectively in the acidic vacuoles and does not enter the chloroplast, that it can be released by amines and that it is taken up by fluid-phase permeation. Possible utilization of this dye to follow subtle changes in the permeability of the plasma membrane and in the mechanism of amine uptake is suggested.

**Material and methods**

Atebrin uptake and release from *D. salina* cells. *Dunaliella salina* cells were cultured in 0.5 M NaCl medium as previously described (Chitlaru and Pick 1989). Incubation with atebrin was performed in cell suspensions containing 5·10^8 to 10^9 cells/ml, 20 mM 3-amino-3-(hydroxymethyl)-1,3-propanediol(tris)-Cl, pH 9; 5 mM KCl; 5 mM MgCl_2; 0.5 M NaCl and 3 µM atebrin hydrochloride at 24°C. For atebrin-release experiments, cells were preloaded for 20 min as described above, centrifuged for 10 min at 750 · g and resuspended in fresh medium without atebrin.

Atebrin release measured by centrifugation through silicone oil. Samples containing 200 µl concentrated *D. salina* cells, (2·10⁷ cells/ml) which were preloaded with atebrin as described above and suspended in the above mentioned buffer, were applied to 0.4-ml microfuge tubes containing 100 µl silicone oil (AP-100; Wacker, München, FRG). Cells were separated by centrifugation for 45 s (12000 rpm) in an Eppendorf (Eppendorf, Gerätebau, FRG) 5412 microfuge. Samples of 100 µl of the upper phase were removed for determination of extracellular atebrin. For determination of intracellular atebrin, the tips of the tubes containing cell pellets were cut, the pellets were extracted by vigorous mixing in 0.5 ml water, and reextracted in 0.5 ml of 1 M NaBr. The extracts were combined and 0.5-ml samples were taken for determination of intracellular atebrin. Atebrin fluorescence of all samples was measured in 2 ml containing 50 mM Tris-Cl, pH 9, as described below.

**Measurements of fluorescence and oxygen evolution.** Atebrin fluorescence was measured in a Perkin-Elmer (Norwalk, Conn., USA) MPF 44A spectrofluorimeter with the excitation and emission wavelengths set at 359 and 505 nm, respectively. Illumination was provided by a 150-W halogen-lamp projector filtered through a Schott & Gen. (Mainz, FRG) RG-645 cut-off filter. Oxygen evolution was measured at 23°C in a 2.5 ml water-cooled cell, of a Rank oxygen electrode illuminated as described above. The measuring media were identical to the atebrin-uptake medium.

**Microscopic techniques.** Cytochemical visualization of atebrin-loaded cells was performed in a Zeiss (Oberkochen, FRG) III RS microscope equipped with an Epi-fluorescence condenser and Osram (München, FRG) high-pressure mercury lamp (HBO 50 W). Cells were loaded with 3 µM atebrin as described above, washed in atebrin-free medium, concentrated to 5·10^8 to 10^9 cells/ml and fixed with 0.02% glutaraldehyde for 5–10 min on glass slides coated with diethylaminoethyl dextran (Sigma Chemical Co., St. Louis, Mo., USA). Fluorescent cells were excited by a 300- to 420-nm ultraviolet filter and viewed through a 500-nm cutoff filter. Photographs were taken with a Kodak TMAX P-3200 film (Eastman-Kodak, Rochester, N.Y., USA). For electron microscopy, cells were fixed and stained with uranyl acetate as previously described (Bental et al. 1990).

**Special chemical.** Bafilomycin A was a generous gift from K.H. Altendorf, Osnabrück, FRG.

**Results**

**Atebrin uptake and localization in *Dunaliella salina*.** Incubation of *D. salina* cells with atebrin at pH 9 results in uptake of most of the dye into the cells, as can be followed by the decrease in fluorescence of the atebrin in the medium (Fig. 1). The figure also demonstrates that the time courses of fluorescence quenching and of the amount of atebrin which is taken up by the cells are closely correlated. Atebrin uptake results in staining of cytoplasmic vacuoles in the cells, which can be viewed in a fluorescence microscope (Fig. 2A). The vacuoles are concentrated in the apical part of each cells, which is not occupied by the chloroplast. Their number varies from 5 to 30 per cell and their typical diameter is 0.5–1.0 µm. A few cells however may contain only two to four larger vacuoles of about 2 µm diameter. These vacuoles resemble in size, structure and distribution the vacuoles seen in electron micrographs of fixed cell slices (Fig. 2C).

Since atebrin is an amine which accumulates in isolated thylakoids in the light, is a known uncoupler of photophosphorylation (Kraayenhof 1970), and has been utilized to estimate intrathylakoidal pH (Schuldiner et al. 1972), it seemed of importance to determine if in the intact *Dunaliella* cells atebrin also accumulates in the...