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Rapid Report

Activation of plant plasma membrane H^+-ATPase by the non-ionic detergent Brij 58

Esmeralda Ibarz [a,1], Michael G. Palmgren [a,2], Javier Palazón [b], Maria-Teresa Piñol [b], Ramón Serrano [a,*,3]

[a] *European Molecular Biology Laboratory, Heidelberg, Germany*
[b] *Departamento de Fisiologia Vegetal, Facultad de Farmacia, Barcelona, Spain*

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Abstract

Plasma membrane vesicles were purified from tobacco callii and the modulation of H^+-ATPase by detergents was investigated. The nonionic detergent Brij 58 not only activated ATP hydrolysis (2-fold) but also proton pumping (more than 4-fold). Triton X-100, within a more limited concentration range, produced a similar effect. The simultaneous activation of ATP hydrolysis and proton pumping is not compatible with current interpretations of effects of nonionic detergents on the H^+-ATPase based on latency of the enzyme and opening of vesicles.

Keywords: ATPase, H^+-; Plasma membrane; Tobacco calli; Detergent activation

1. Introduction

The central role played by the plasma membrane H^+-ATPase in the physiology of plant cells [1,2] suggest the existence of regulatory mechanisms which match the activity of this electrogenic proton pump to changing physiological demands. Physiological studies have pointed to regulation of the H^+-ATPase by auxin [3,4], fusicoccin [5], light [6,7] and osmotic stress [8,9], but the detailed signal transduction pathways are unknown.

The carboxyl terminus of fungal and plant plasma membrane H^+-ATPases seems to function as an autoinhibitory domain which could be displaced by perturbations of the enzyme triggered by either detergents or physiological modulators [10–15]. Glucose in yeast [10,12], fusicoccin in plants [14,15] and the ionic detergents lysophosphatidic acid in yeast [16] and lysophosphatidylcholine in plants [11,14,15,17,18] seem to activate the H^+-ATPase by displacing its carboxyl terminus. Non-ionic detergents such as Triton X-100 [16,18,19] and Brij 58 [14,15,18] were postulated to just open membrane vesicles and exposing latent ATPase molecules. However, in the course of our studies on the regulation of tobacco callii ATPase [20] we have observed that Brij 58, and to a lesser extent Triton X-100, activate simultaneously ATP hydrolysis and proton pumping. This is not compatible with opening of vesicles.

Callus cultures were stablished from the basal part of central leaf veins of *Nicotiana tabacum* L. cv. Petit Havana SR1 and pieces of 0.04 g were inoculated in fresh medium with hormones and grown for two weeks as described [20]. Callii were homogenized and a crude membrane fraction obtained by differential centrifugation as described [20]. Plasma membranes were purified from crude membranes by sucrose gradient centrifugation [21]. The plasma membranes were isolated at the interface between sucrose layers of 33 and 46% (w/w). Cytochrome-*c* oxidase (mitochondrial marker), NADPH–cytochrome-*c* oxidoreductase (endoplasmic reticulum marker) and Triton X-100-stimulated UDPase (Golgi marker) were assayed as described by Briskin et al. [22]. One unit (U) of enzymatic activity is the amount of enzyme catalyzing the

* Corresponding author. Valencia, Spain. Fax: +34 6 3877859.
[1] Present address: Max-Planck-Institut für Zuchtungsforschung, Carl-von-Linné-Weg 10, 5000 Köln 30 (Vogelsang), Germany.
[2] Present address: Department of Plant Biology, Royal Veterinary and Agricultural University, 40 Thorvaldsensvej, 1870 Frederiksberg, Copenhagen, Denmark.
[3] Present address: Instituto de Biologia Molecular y Celular de Plantas, UPV-CSIC, Universidad Politecnica, Camino de Vera 14, 46022 Valencia, Spain.

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conversion of 1 μmol of substrate per min. Protein was assayed by the method of Bradford [23] using the Bio-Rad dye reagent concentrate and bovine γ-globulin as standard. ATP hydrolysis was measured by adapting a described method [21] to microtiter plates. Final volume was 60 μl and the reaction was stopped with 100 μl of the P_i reagent. Incubations was for 30 min at 37°C and the reaction was linear for at least 40 min. Buffer composition was 50 mM 4-morpholinoethanesulfonic acid (pH 6.5 with Tris), 5 mM $MgCl_2$, 100 mM KNO_3, 5 mM sodium azide and 0.3 mM ammonium molybdate. Nitrate, azide and molybdate are inhibitors of vacuolar ATPase, mitochondrial ATPase and acid phosphatase, respectively. Inhibition of measured activities by inhibitors of plasma membrane H^+-ATPase such as diethylstilbestrol (0.16 mM) or vanadate (0.16 mM) was more than 90%. Acid phosphatase activity was measured in the same conditions as plasma membrane H^+-ATPase but with 2 mM *p*-nitrophenyl phosphate as substrate and without molybdate in the buffer. One unit of activity (U) corresponds to 1 μmol of P_i liberated per min.

Intravesicular acidification was measured by the quenching of fluorescence of 9-amino-6-chloro-2-methoxyacridine [21] measured with an SLM 800 spectrofluorometer. Buffer composition was 10 mM 4-morpholinoethanesulfonic acid (pH 6.5 with KOH), 5 mM $MgSO_4$, 50 mM KNO_3 (to inhibit vacuolar ATPase and depolarize the membranes), 15 μg/ml oligomycin (to inhibit mitochondrial ATPase) and 1 mM ATP. The concentration of vesicles was 0.1 mg protein/ml. The specificity of the assay was tested by inhibition with vanadate (0.2 mM).

Plasma membrane vesicles of high purity were prepared from tobacco callii (Table 1). The contaminating activities of marker enzymes for other organelles such as mitochondria, endoplasmic reticulum and Golgi were greatly reduced by the sucrose gradient purification step. The activities of mitochondrial and vacuolar ATPases were not significant in callus plasma membrane preparations because the inhibitions observed by either azide or nitrate were less than 10%. On the other hand, callus plasma membranes contain substantial activities (0.05–0.1 U/mg) of acid phosphatase(s) sensitive to molybdate. These enzymes hydrolyze ATP and *p*-nitrophenyl phosphate and do not require divalent cations for activity. Plasma membrane H^+-ATPases do not hydrolyze *p*-nitrophenyl phosphate and require Mg^{2+} for activity [24,25]. Therefore it is very important that the assay for callus plasma membrane H^+-ATPase is made specific by the inclusion of molybdate as phosphatase inhibitor.

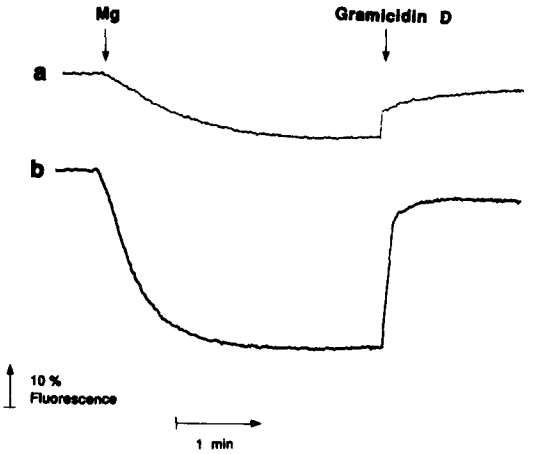

Fig. 1. Brij 58 activates proton pumping in plasma membrane vesicles from tobacco calli. Curve a: no detergent. Curve b: 0.13 mg/ml Brij 58. The concentration of vesicles was 0.13 mg protein/ml. The additions of magnesium (5 mM; to start the reaction) and of gramicidin D (1.5 μg/ml; to dissipate the proton gradient) are indicated by arrows. The experiment was repeated three times with similar results.

The callus plasma membrane H^+-ATPase has the typical features of plant H^+-ATPases [1,2]. Its pH optimum is 6.5, with only 30% of activity at pH values of 5.5 and 7.5. The K_m for ATP (0.7 mM) is intermediate between that of H^+-ATPases from tobacco roots (1.2 mM) and leaves (0.1 mM) (Ibarz, E. and Serrano, R., unpublished data).

Non-ionic detergents such as Brij 58 and Triton X-100 activate ATP hydrolysis and proton pumping in callus plasma membrane vesicles. This is more apparent with Brij 58, which produces up to 5-fold activation of proton pumping at concentrations of the same order than membrane protein (Fig. 1). With a protein concentration of 0.1 mg/ml, a titration of the detergent effects indicates that at concentrations of up to 0.03–0.05 mg/ml both Brij 58 and Triton X-100 activate ATP hydrolysis and proton pumping to similar extents (2-fold, Figs. 2A and 2B). Lysophosphatidylcholine, even at these very low concentrations, inhibits proton pumping while strongly activating ATP hydrolysis (5-fold, Fig. 2C). At higher detergent concentrations Triton X-100 is also inhibitory for proton pumping while ATP hydrolysis remains unaffected (Fig. 2B). On the other hand, higher concentrations of Brij 58 further

Table 1
Protein and marker enzyme activities of membranes from tobacco calli

	Protein	H^+-ATPase		Cytochrome-*c* oxidase		NADPH–cyt-*c* reductase		UDPase	
	(mg)	mU/mg	%	U/mg	%	U/mg	%	U/mg	%
A	1.00	15	100	21.5	100	3.1	100	11	100
B	0.13	110	92	6.7	3.9	0.5	2	0.8	1

Results of a typical purification starting with 1.25 g (fresh weight) of tissue are shown. The experiment was repeated twice with similar results. A: crude membranes. B: purified plasma membranes.

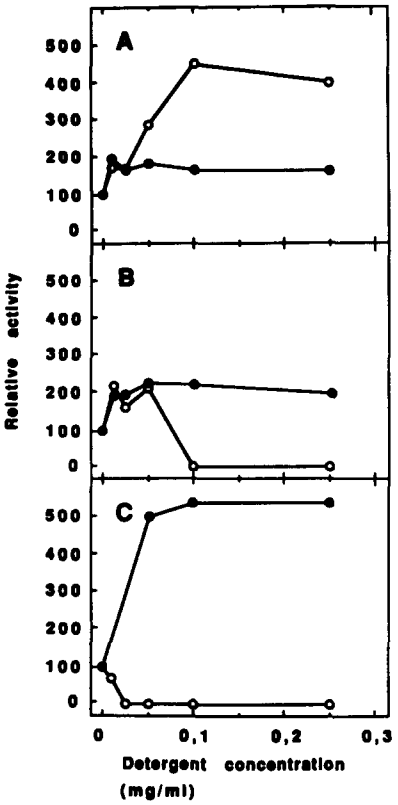

Fig. 2. Differential effects of Brij 58 (A), Triton X-100 (B) and lysophosphatidylcholine (C) on ATP hydrolysis (closed symbols) and proton pumping (open symbols) in plasma membrane vesicles from tobacco calli. The concentration of vesicles was 0.1 mg protein/ml. The activity in the absence of detergent is taken as 100%. The absolute values were 30–40 mU/mg for ATP hydrolysis and 10–12% quenching per min for proton pumping. The experiment was repeated twice with similar results.

activate proton pumping (up to 5-fold) while ATP hydrolysis remains unaffected. Proton pumping in the presence of Brij 58 remains fully sensitive to vanadate, demonstrating that it corresponds to plasma membrane H^+-ATPase. Although lysophosphatidylcholine activates proton pumping in oat root plasma membrane vesicles [17], the inhibitory effect observed in callus plasma membranes may reflect the uncoupling activity of fatty acids resulting from its hydrolysis by some plant tissues [18]. Accordingly, the addition of lysophosphatidylcholine at 0.1 mg/ml to callus vesicles dissipates a previously generated proton gradient.

Our observation that proton pumping and ATP hydrolysis are simultaneously increased by Brij 58 would suggest that this detergent directly interacts with the callus ATPase in a similar way that lysophosphatidylcholine acts in other systems. The H^+-ATPase activated by Brij 58 can still be activated by lysophosphatidylcholine [11,14,15], suggesting that both detergents act by different mechanisms. Similar observations with Brij 58 have been made with spinach leaf plasma membranes by Johansson, F. and Larsson, C. (University of Lund, personal communication) and therefore this phenomenon seems to be of general occurrence.

We have discarded some obvious explanations. For example, Brij 58 could trigger vesicle fusion and then amplify the acridine fluorescence signal. Measurement of vesicle size by photon correlation spectroscopy [26] with a Malvern Autosizer model IIc (Malvern Instruments, UK) results in values for control and Brij 58-treated vesicles of 360 ± 70 nm and 230 ± 100 nm, respectively (mean $\pm$ standard deviation). Therefore, Brij 58 does not induce vesicle fusion. Alternatively, Brij 58 could improve the sealing of the vesicles. However, measurement of the time-course of decay of the pH gradient after inhibition of the ATPase by vanadate shows no effect of the detergent on passive proton permeability.

At present we consider two possible explanations. Brij 58 (polyoxyethylene 20 cetyl ether; $C_{16}E_{20}$) is a detergent with very low critical micelle concentration (about 0.5 μg/ml) and with an hydrophillic-lipophilic balance very unfavourable for membrane solubilization [27,28]. The lack of inhibition of proton pumping by the tested concentrations of this detergent suggest that Brij 58 is not capable of opening membrane vesicles. Therefore, it is likely that Brij 58 neither solubilizes nor open plant plasma membranes. It may insert into the membranes and bind to membrane proteins such as the ATPase, which could be modified. Alternatively, Brij 58 could cause a reorientation of right-side out vesicles, exposing its latent ATPase. This mechanism has been previously proposed for the effect of Triton X-100 on plant membranes [29]. In this case, however, ATP hydrolysis and proton pumping should be stimulated to a similar extent. As indicated in Fig. 2, this mechanism could explain the activation observed at low concentrations of Brij 58 and Triton X-100. At higher concentrations of Brij 58 only proton pumping is further stimulated and this is more compatible with the first explanation.

When plasma membrane vesicles of mixed orientation are digested with trypsin in the presence of Brij 58, the C-terminus is removed from all H^+-ATPase molecules [15]. In the absence of Brij 58, however, the C-terminus is only removed from a fraction of H^+-ATPase molecules, as expected from the presence of right-side out vesicles with the C-terminus of the H^+-ATPase not exposed to the external solution [11,14]. Therefore Brij 58 seems to somehow increase the accessibility of the extravesicular solution to the internal C-terminus of the H^+-ATPase in right-side out vesicles. This is compatible with reorientation of the vesicles.

Further work is needed to clarify this unexpected effect of non-ionic detergents on proton pumping by plant H^+-ATPase. In particular, the reorientation hypothesis should be tested by proper assessment of vesicle sidedness [16,29] and Brij 58 should be tested on H^+-ATPase deleted of its terminal regulatory domain at the protein [11] or gene [13] levels. In any case, our results demonstrate that Brij 58 cannot be used to assess the latency of plant plasma membrane H^+-ATPase. Similar conclusions concerning the

use of Triton X-100 in latency studies have been reported [30].

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