Vacuolation induced by VacA toxin of *Helicobacter pylori* requires the intracellular accumulation of membrane permeant bases, Cl^- and water

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Abstract The protein vacuolating toxin A (VacA) of *Helicobacter pylori* converts late endosomes into large vacuoles in the presence of permeant bases. Here it is shown that this phenomenon corresponds to an accumulation of permeant bases and Cl^- in HeLa cells and requires the presence of extracellular Cl^-. The net influx of Cl^- is due to electroneutral, Na^+, K^+, $2Cl^-$ cotransporter-mediated transport. Cell vacuolation leads to cell volume increase, consistent with water flux into the cell, while hyper-osmotic media decreased vacuole formation. These data represent the first evidence that VacA-treated cells undergo an osmotic unbalance, reinforcing the hypothesis that the VacA chloride channel is responsible for cell vacuolation. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Cell vacuolation; Cl^- transport; Na^+, K^+, $2Cl^-$ cotransporter; VacA anion channel; *Helicobacter pylori*

1. Introduction

The protein vacuolating toxin A (VacA; 95 kDa), secreted by virulent strains of *Helicobacter pylori*, is implicated in the pathogenesis of human diseases such as gastritis and gastroduodenal ulcer [1,2].

After acidic pH activation, VacA inserts into the cell plasma membrane to form an oligomeric, anionic selective channel [3–6], which can also conduct urea [7]. In the presence of membrane permeant bases, such as NH_3, acid-activated toxin induces many cytoplasmic vacuoles derived from late endosomes in cultured cells [8–11]. According to a proposed model [3,4,6] endocytosed VacA channels would increase the permeability of late endosome membranes to anions, stimulating the proton pumping activity of the co-localized electrogenic vacuolar-type ATPase (V-ATPase). This would drive the lumenal accumulation of NH_4^+ and thus, for osmotic reasons, of water. According to this possibility, vacuolation results from the dilatation of normal endosomal vesicles under an increased endo-lumenal osmotic pressure. To test this hypothesis, the cellular accumulation of weak bases, Cl^- and water was measured upon VacA treatment. Cl^- concentration and osmolarity of the extracellular medium and the plasma membrane Cl^- permeability were also manipulated. Results indicate an essential role of osmotic unbalance in VacA-induced vacuolation, supporting the direct involvement of the VacA anionic channel.

2. Materials and methods

2.1. Toxin and reagents

VacA was purified from CCUG 17874 *H. pylori* strain cultures and activated before use as described [12,13]. $^{36}Cl^-$ (1 μCi/μmol), [^{14}C]urea (57 μCi/μmol), [1(3)-^{3}H]glycerol (3 Ci/mmol) and (methyl-[^{3}H])-methylamine hydrochloride (48 Ci/mmol) were purchased from Amersham Pharmacia Biotech (UK). Synthetic diphytanoyl-phosphatidylcholine (purity >99%) was from Avanti Polar Lipids (Alabaster, AL, USA). Bumetanide and furosemide were from Sigma (Milan, Italy). Na-gluconate, K-gluconate, Mg-(gluconate)$_2$ and Ca-(gluconate)$_2$ and type V bovine serum albumin (BSA) were from BDH-Merck Eurolab srl (Milan, Italy).

2.2. Cell culture and intoxication with VacA

HeLa cells were grown on plastic at 37°C in a humidified 5% (v/v) CO_2 atmosphere in Dulbecco's modified Eagle medium (DMEM) containing 10% (v/v) fetal calf serum (FCS) and gentamicin. Trypsin–EDTA (ethylenediamine *N*,*N*,*N*′,*N*′-tetraacetic acid) detached cells were seeded on 96- or 12-well trays at the density of 25 000 cells/cm^2 and cultured for 2 days before experiments. Pre-activated VacA (pH 2.0) was diluted in DMEM, containing 2% (v/w) FCS at 37°C and further incubated with cells in a humidified 5% (v/v) CO_2 atmosphere for 4 h at 37°C. In some experiments cells were treated as specified in the legends with phosphate-buffered saline (PBS)/BSA pH 7.4 (135 mM NaCl, 6.5 mM Na_2HPO_4, 3.5 mM KCl, 1.5 mM KH_2PO_4, 5 mM glucose, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.02% BSA, pH 7.4) plus or minus 10 mM NH_4Cl or with PBS/BSA/gluconate pH 7.4 (135 mM Na-gluconate, 6.5 mM Na_2HPO_4, 3.5 mM K-gluconate, 1.5 mM KH_2PO_4, 5 mM glucose, 0.5 mM Ca-(gluconate)$_2$, 0.5 mM Mg-(gluconate)$_2$, 0.02% BSA, pH 7.4) plus or minus NH_4-gluconate pre-warmed at 37°C. Sometimes media contained 0.5–200 μM bumetanide or furosemide or 20–100 mM mannitol.

2.3. Cell vacuolation and urea efflux rate

Cell vacuolation was determined as neutral red uptake (NRU) as described [13]. To assay urea efflux, cells intoxicated with VacA in DMEM plus 2% FCS were equilibrated at 37°C with [^{14}C]urea (1.5 μCi/ml) for 1 h, rapidly washed twice with PBS/BSA pH 7.4 containing 200 μM bumetanide or furosemide at 0°C, and further incubated for the following 30 min, in the same medium at 0°C. The rate of urea cell efflux was measured by counting the radioactivity collected from the extracellular medium and expressed as percent release per min of urea present within cells at the zero time [7].

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Abbreviations: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; NKCC, Na^+, K^+, $2Cl^-$ cotransporter; NRU, neutral red uptake; PBS, phosphate-buffered saline; VacA, vacuolating toxin A; V-ATPase, vacuolar-type ATPase

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2.4. Cell uptake of $^{36}Cl^-$, [^{3}H]methylamine, [^{14}C]urea and [^{3}H]glycerol

Cells were treated with no toxin or with VacA in DMEM, 2% FCS plus or minus 10 mM methylamine in the presence of $^{36}Cl^-$ (5 μCi/ml) or [^{3}H]methylamine (20 μCi/ml) for 4 h. After three washes with the same ice-cold medium devoid of radiolabeled probes cells were dissolved in 0.5% SDS and the amount of radioactivity present in cells was counted by liquid scintillation. In the case of glycerol uptake, cells were treated with VacA for 3 h in DMEM, 2% FCS, 10 mM NH_4Cl and further incubated in the same medium containing [^{3}H]glycerol (7 μCi/ml) for 1 h at 0°C. After three washes with ice-cold PBS/BSA pH 7.4, cells were dissolved in 0.5% SDS and the amount of radioactivity present in cells was counted by liquid scintillation. In some experiments cells were incubated for 1 h at 0°C with DMEM, 2% FCS, 10 mM NH_4Cl, without carbonate, or with the same medium plus 100 mM mannitol or diluted with distilled water in order to obtain a net decrease of 60 mOsm/l in the presence of [^{3}H]glycerol or [^{14}C]urea. After washing with the same media and dissolution by 0.5% SDS, cell-associated radioactivity was determined as above.

2.5. Measurement of VacA-induced ion current in planar lipid bilayers

Experiments were conducted as previously described [3,6]. The medium was 0.5 M KCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM K-HEPES, pH 7.2, in both chambers. Diphytanoyl-phosphatidylcholine was used as membrane lipid. Low pH (2.0) activated VacA (2–4 nM) was added to the *cis* compartment and current was determined with or without addition to either the *cis* or the *trans* side of inhibitors (bumetanide or furosemide 0–500 μM).

3. Results

3.1. Cell vacuolation corresponds to an increased cellular content of permeant bases and Cl^-

Membrane permeant bases, such as NH_3, are required for VacA-induced vacuolation [15]. [^{3}H]Methylamine was used in order to test if VacA determines an increased accumulation of these molecules within acidic compartments. Vacuolation led to an increased [^{3}H]methylamine cellular uptake (Fig. 1). The amount of $^{36}Cl^-$ also increased in VacA-treated cells only in the presence of weak bases, when vacuolation occurred. Taking into account the isotopic dilution of the two tracers in the extracellular medium, the molar ratio of Cl^- and methylamine turned out to be close to 1:1.

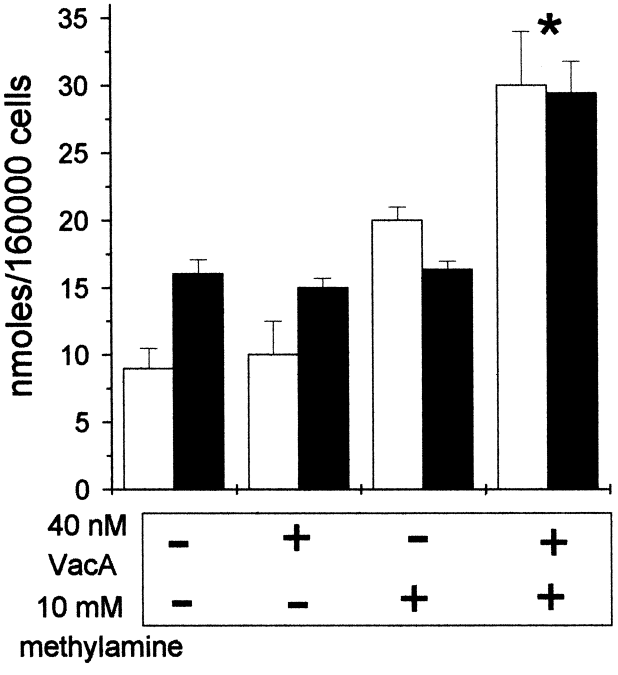

Fig. 1. Cell uptake of methylamine and Cl^- induced by VacA. HeLa cells were treated as indicated with VacA and/or methylamine for 4 h in the presence of either [^{3}H]methylamine (20 μCi/ml; open columns) or $^{36}Cl^-$ (5 μCi/ml; solid columns). Data are the mean of three experiments run in duplicate and bars are ±S.E.M. The asterisk indicates the presence of cell vacuolation.

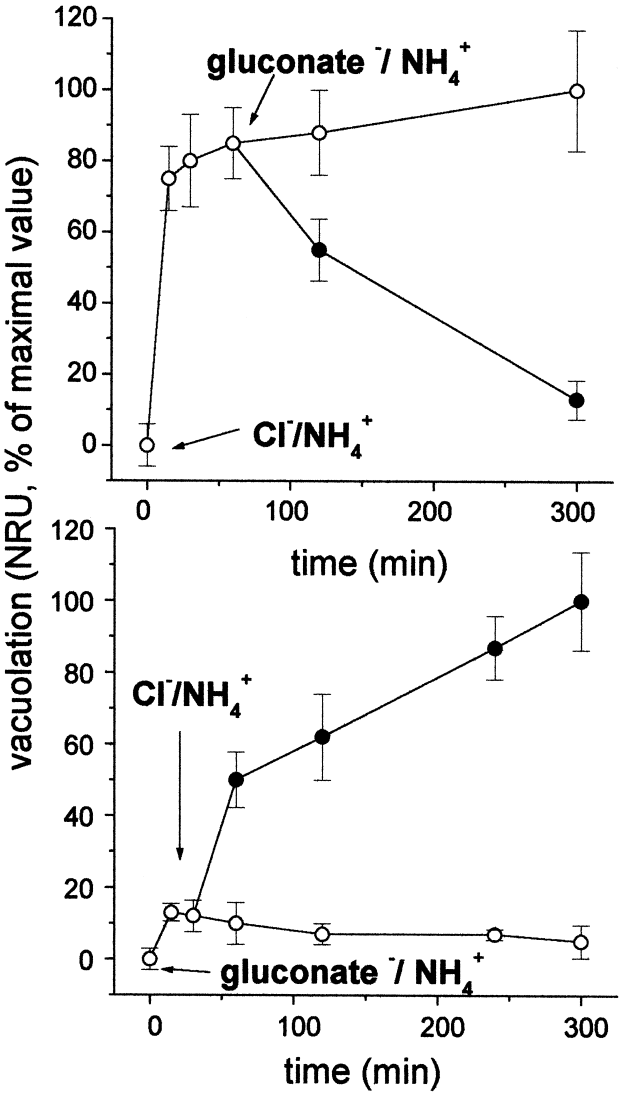

Fig. 2. Effect of extracellular Cl^- deprivation on cell vacuolation induced by VacA. HeLa cells intoxicated for 4 h with 40 nM VacA were washed and further incubated with PBS/BSA plus 10 mM NH_4Cl (Cl^-/NH_4^+) or with a medium resembling PBS/BSA where Cl^- was replaced by gluconate, plus 10 mM NH_4-gluconate ($gluconate^-/NH_4^+$), when specified. Cell vacuolation was determined by NRU at the indicated times. Data, expressed as percent of the maximal value within the same experiment, are the mean of three experiments run in triplicate and bars represent ±S.E.M.

3.2. Extracellular Cl^- is required for cell vacuolation induced by VacA

To test the role of chloride anions in the mechanism of vacuole formation, without affecting the preliminary steps of VacA cell interaction, we took advantage from the observation that vacuoles may be induced by the addition of NH_4Cl to the extracellular medium subsequent to VacA treatment [14]. Indeed, in Fig. 2A it is shown that after treatment of

HeLa cells for 4 h with VacA, vacuolation was promptly induced by addition of NH_4Cl. However, the substitution of normal medium with one where Cl^- was replaced by gluconate reversed cell vacuolation. Consistently, cell vacuolation was scarce when VacA intoxication was followed by incubation with PBS/BSA/gluconate plus 10 mM NH_4-gluconate and developed upon reintroduction of Cl^- containing PBS/BSA medium plus NH_4Cl (Fig. 2B).

3.3. Inhibition of the Na^+, K^+, $2Cl^-$ cotransporter (NKCC) decreases vacuole development

NKCC is the major entry pathway of Cl^- into HeLa cells

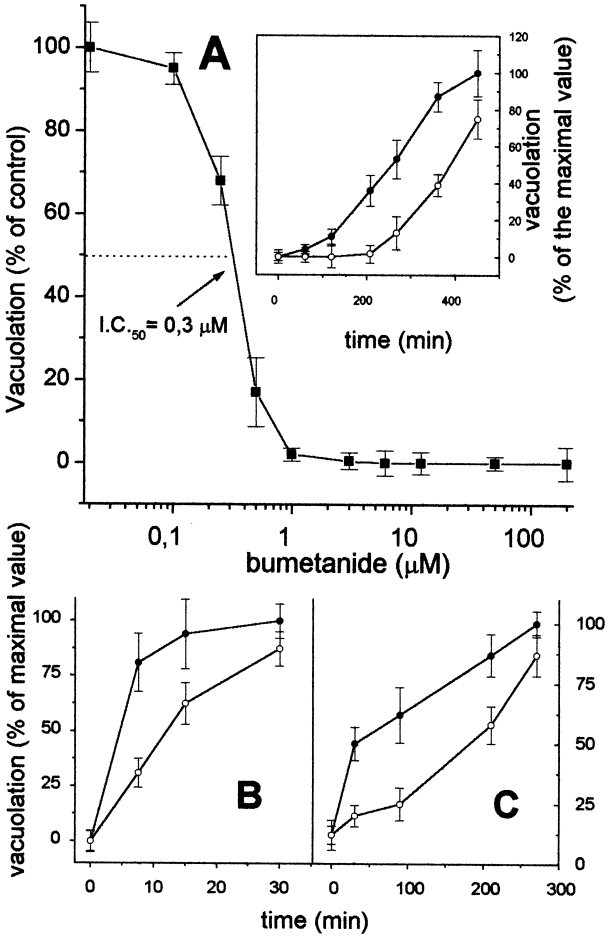

Fig. 3. Effect of bumetanide on the vacuolating activity of VacA. HeLa cells were treated with 40 nM VacA in PBS/BSA plus 10 mM NH_4Cl for 3 h in the presence of the different doses of bumetanide (A), or treated with 40 nM VacA for different times in the absence (solid symbols) or in the presence (open symbols) of 200 μM bumetanide (inset of A), and their vacuolation was quantified by NRU. B: Cells were intoxicated with VacA (40 nM) in PBS/BSA with no NH_4^+, washed and further incubated in PBS/BSA plus 10 mM NH_4Cl in the absence (solid symbols) or in the presence (open symbols) of 200 μM bumetanide. C: After VacA treatment as above, cells were further incubated in Cl^- free gluconate/BSA medium with no NH_4^+ for 30 min. Thereafter cells were placed in PBS/BSA plus 10 mM NH_4Cl in the absence (solid symbols) or in the presence (open symbols) of 200 μM bumetanide. Cell vacuolation was quantified by NRU at the indicated time and expressed as percent of control samples (treated with toxin alone) in A or as percent of the maximal effect within the same experiment in the inset of A and in B and C. Data are the mean of at least three experiments run in triplicate and bars are ± S.E.M.

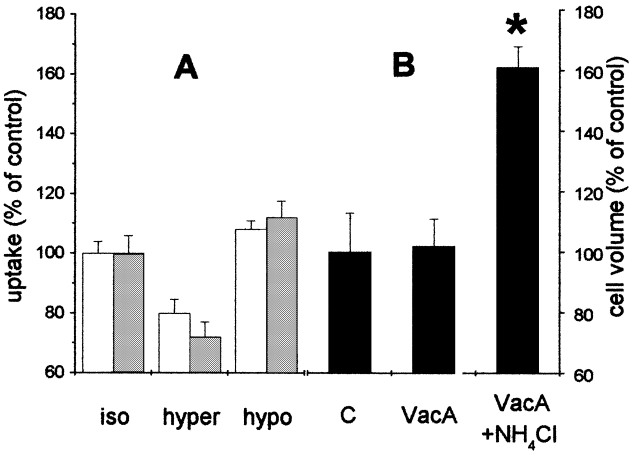

Fig. 4. Effect of VacA-induced vacuolation on cell volume. A: The uptake of [^{14}C]urea (white columns) or of [^{3}H]glycerol (gray columns) was measured in HeLa cells incubated with iso-osmotic, hyper-osmotic and hypo-osmotic media at 0°C for 60 min. B: Cells were treated with no toxin or with VacA (40 mM) in iso-osmotic conditions, in the presence or absence of 10 mM NH_4Cl. After 4 h cell volume was determined with the assay shown in A, using [^{3}H]glycerol. Data, expressed as percent of control cells (incubated with no toxin), are the mean of three experiments run in triplicate. Bars represent ± S.E.M. Asterisk indicates the condition leading to vacuolation.

[15]. The antagonists of NKCC such as bumetanide [16] inhibited HeLa vacuolation due to VacA (Fig. 3), without affecting Cl^- and urea conduction by the VacA pore (not shown). Similar results were obtained with the related inhibitor furosemide (not shown). Inhibition of V-ATPase-supported lumenal acidification was ruled out since basal uptake of the acidotropic dye neutral red was not modified by this drug (not shown). To exclude the possibility that bumetanide inhibited VacA binding, endocytosis and transport to late endosomes, VacA was again pre-incubated in the absence of weak bases, and vacuolation triggered by adding NH_4Cl [14]. Also in this case the kinetics of NH_4Cl-triggered vacuolation were inhibited by bumetanide (Fig. 3B). If cells were pre-incubated with the Cl^- free medium, prior to assay, bumetanide inhibition upon Cl^- reintroduction was more evident (Fig. 3C). Bumetanide only slowed down vacuolation: after a sufficient time NRU reached a nearly maximal value, in agreement with the existence of other Cl^- entry pathways into the cell [15].

3.4. Cell vacuolation results in volume cell increase and is inhibited by hyper-osmolarity

The osmotic model of vacuole formation predicts a net water movement from the extracellular space into the cytosol and then into acidic compartments. Variations of the cell volume were therefore determined by adapting to glycerol the method based on the equilibrium distribution of urea [17]. This was necessary since the membrane permeation of urea but not of glycerol is affected by VacA [7]. Hyper-osmotic or hypo-osmotic media induced variations of glycerol cell uptake (performed at 0°C, to avoid metabolic incorporation) identical to those of urea and compatible with known cell volume modifications in these conditions [18]. By using this assay, the volume of fully vacuolated cells was shown to increase

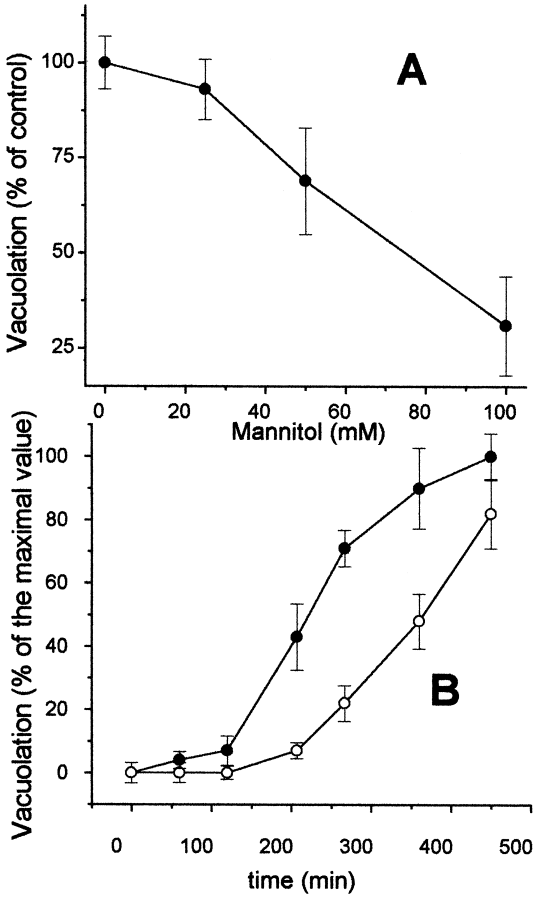

Fig. 5. Effect of hyper-osmotic conditions on cell vacuolation due to VacA. HeLa Cells were incubated for 4 h with VacA (40 nM) in PBS/BSA plus 10 mM NH_4Cl, in the presence of increasing doses of mannitol (A) or with 40 nM VacA in PBS/BSA plus 10 mM NH_4Cl in the absence (solid symbols) or in the presence (open symbols) of 100 mM mannitol for different time intervals (B). Cell vacuolation was then determined by NRU and expressed as percent of control cells (no mannitol) in A or as percent of the maximal value in B. Data are the mean of at least three experiments run in triplicate and bars are ± S.E.M.

about 60% with respect to controls, suggesting a strong water influx (Fig. 4).

The implication of water in vacuolation was also indicated by the inhibitory effect of media made hyper-osmotic by the addition of the membrane non-permeant molecule mannitol (Fig. 5).

4. Discussion

This study concerns the mechanism underlying cell vacuolation due to VacA. This protein forms transmembrane pores able to conduct Cl^- and also urea [3–7]. Pharmacological evidence suggests that the VacA channel is essential for vacuole development [3,4,6] and an osmotic model of vacuole formation, compatible with this and other experimental evidence [3], has been formulated:

1. VacA channels formed in the cell plasma membrane are endocytosed and transported to late endosomes endowed with V-ATPase.
2. The increase of anion (Cl^-) conduction by the endosomal membrane enhances V-ATPase-mediated proton transport into the lumen of these vesicles.
3. Permeant weak bases (NH_3), when present in the medium, accumulate in the VacA-, V-ATPase-positive vesicles by combining with H^+, to form membrane non-permeant cations (NH_4^+), which are poorly conducted by the VacA channel [3].
4. The net accumulation of NH_4Cl which results creates an osmotic pressure in the endosome lumen higher than that present in the cell cytosol. This determines a water flux from the cytosol to the endosome interior accounting for its swelling and cell vacuolation.

Given the considerable volume occupied by the vacuolar compartments, it is reasonable that ions and water lost by the cytoplasm are replaced by an equivalent amount entering from the extracellular space.

The data presented in this study are consistent with the predictions of this model. In fact, not only is the development of vacuoles accompanied by a comparable increase of cell content of permeant bases and Cl^-, but it also requires Cl^- anions in the medium.

Consistently, hampering of Cl^- transmembrane flow by specific inhibitors of the NKCC decreased the kinetics of vacuole generation. This may seem unexpected, since VacA itself permeabilizes the plasma membrane to Cl^-. It must be remembered however that the transport mediated by the channel is electrogenic, and that the increase in Cl^- permeability leads to depolarization [4]. Persistence of depolarization requires a continuing VacA-mediated efflux of anions (and accompanying cations), which must be replaced via electroneutral, NKCC-mediated influx. NKCC inhibition is therefore expected to lead to a reduction of $[Cl^-]_{cyt}$, which presumably accounts for the lower rate of vacuole formation in the presence of bumetanide.

Cells are also shown to increase their volume upon vacuolation, a condition only compatible with an increased water content. Inhibition of vacuolation by hyper-osmotic media, which determine a water efflux from cells, further points to the importance of water fluxes in this phenomenon. Altogether the obtained data fulfil the predictions of the osmotic model of vacuolation, further supporting the essential role of the anion selective VacA channel in this process.

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