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The vacuolating toxin of *Helicobacter pylori* mimicks the CFTR-mediated chloride conductance[1]

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Abstract **Cystic fibrosis (CF) is caused by defects of the CF transmembrane conductance regulator (CFTR), which acts both as an anion-selective channel and as a regulator of other proteins. The relative contribution of these two functions in CF disease is debated. The toxin VacA forms channels with properties similar to those of the CFTR, and we report here that it can insert into the membrane of various cells originating from respiratory epithelia, generating a chloride conductance comparable to that produced by activation of the CFTR. VacA may therefore become a valuable tool in the study of CF pathogenesis.**
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Key words: VacA; Cystic fibrosis; Cystic fibrosis transmembrane conductance regulator; Chloride channel; Respiratory epithelium; *Helicobacter pylori*

1. Introduction

The cystic fibrosis (CF) transmembrane conductance regulator (CFTR) is a Cl^- channel of the apical membrane of epithelial cells, the activity of which is regulated by phosphorylation and intracellular nucleotides [1]. It also functions as a regulator of other membrane proteins, such as the Na^+-selective epithelial channel ENaC [2]. Mutations of the CF gene can result in defective synthesis, processing and function of CFTR [3], causing disease in various organs by incompletely understood mechanisms.

In particular, the pathogenesis of CF lung disease, which is characterized by recurrent infection, inflammation and lung destruction [1], is intriguing. Two major hypotheses have been debated [4–8]. One view holds that in CF there is a decrease of the depth of the airway surface liquid (ASL), while its composition remains constant. This would interfere with ciliary beating and movement of the mucus. The model emphasizes the role of the CFTR as an inhibitor of ENaC. Other researchers have obtained data indicating that the salinity of ASL is increased in CF. This would decrease the effectiveness of antimicrobial peptides and allow chronic colonization by bacteria such as *Pseudomonas aeruginosa*. In this scheme the major role of CFTR is considered to be in chloride conduction, i.e. as a channel. The matter of ASL salinity has proven difficult to resolve. Recent results indicate that both ASL composition and depth are the same in normal and CF subjects [9–12], and suggest that CFTR deficiency may impact most on secretory gland cells [11,13]. Understanding whether CF lung pathogenesis is linked to the role of the CFTR as a chloride channel or as a channel regulator (or both) will be the key to rational strategies for the treatment of CF. Indeed, one of the therapeutic possibilities being actively explored envisions substitution of CFTR function by activation of other endogenous chloride channels (e.g. [14–16]) or via incorporation of artificial anion channels into the membrane of CF cells (e.g. [17]).

VacA [18] is a toxin secreted by the human pathogen *Helicobacter pylori*, which colonizes the mucous layer covering the gastric mucosa and is associated with diseases such as chronic gastritis and ulcerations [19,20]. VacA, which can be routinely collected from culture supernatants, forms oligomeric, anion-selective, slightly voltage-dependent, low-conductance channels upon insertion into artificial [21–23] as well as cell membranes, including the apical membrane of frog gastric epithelium [24,25]. Upon internalization, these pores alter the permeability to Cl^- of the late endosomal membrane, resulting in the formation of vacuoles if permeant bases are present. Since *H. pylori* generates ammonia by splitting urea, in the stomach this phenomenon is part of the events leading to erosion of the mucosa. If regulatory aspects are not considered, the biophysical properties of the VacA channel are similar to those of the CFTR: both form nearly ohmic, anion-selective, low-conductance pores. Sensitivity to the toxin, measured in vacuolation assays, differs markedly from cell type to cell type [26,27], for reasons still unclear but having mainly to do with differences in binding and internalization efficiency.

The toxin thus offers promise as a tool to clarify some key mechanistic aspects of CF. As a chloride channel with a completely different structure, but comparable biophysical properties, upon insertion into the apical portion of epithelial cells it may be expected to partly substitute for CFTR if the main task of the latter is to conduct anions, but not if the CFTR is mainly a regulator acting via protein–protein interactions. As a step toward such utilization, we report here that VacA can

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[1] Dedicated to the memory of Rossella Rolfini.

Abbreviations: ASL, airway surface liquid; BEGM, bronchial epithelial growth medium; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; DMEM, Dulbecco's minimum Eagle's medium; FCS, fetal calf serum; NPPB, 5-nitro-2-(3-phenylpropylamino) benzoic acid

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indeed incorporate efficiently into the membrane of various cells obtained or derived from respiratory epithelia, and that it can conduct chloride currents with whole-cell amplitudes in the range of those elicited by CFTR stimulation by pharmacological means.

2. Materials and methods

2.1. Materials

All reagents and medium components were purchased from Sigma (Milan, Italy) unless otherwise stated. 5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) was from Research Biochemicals International (Natick, MA, USA). Geneticin was from Gibco Invitrogen S.R.L. (Milan, Italy), LHC-9 from Biofluids (Rockville, MD, USA), bronchial epithelial growth medium (BEGM) from Bio Whittaker (Walkersville, MD, USA). VacA was from strain CCUG 17874, isolated as previously described [28]. The toxin was activated by preincubation at pH 2, 37°C for 8 min before addition to cells.

2.2. Cells

The following human respiratory epithelial SV40-immortalized cell lines were grown in serum-free LHC-9 medium under a 5% CO_2 atmosphere: BEAS-2B, a non-CF bronchial line [29]; CFT^+/2CF Meo^-/2CFSMEo^-, a tracheobronchial submucosal glandular line from a CF patient with the ΔF508 mutation [30]; NFT/56FHTE8o^-, a non-CF fetal tracheal line [31]; CFT^-/CFNP9o^-, a nasal polyp line from a CF patient expressing the ΔF508 mutation [30]. Primary cultures of respiratory epithelial cells from nasal polyps and bronchial tissue were obtained as described [32] and grown in LHC-9. The mouse mammary epithelial C127 transformed cells stably expressing CFTR wild-type (wt) or CFTR ΔF508 [33] were grown in Dulbecco's minimum Eagle's medium (DMEM)+10% fetal calf serum (FCS) (v/v) containing 200 µg/ml geneticin. HeLa cells were grown in DMEM plus 10% FCS, penicillin (100 U/ml) and streptomycin (100 µg/ml).

2.3. Vacuolation protocol

Cells were seeded, at 10^5 cells/ml in 12- or 24-well trays and allowed to grow for 24 h at 37°C. They were then rinsed and further incubated, with or without 16 nM VacA, in the following media: BEAS-2B, CFT^+, NFT, primary bronchial cells, primary polyp cells: BEGM+5 mM NH_4Cl; HeLa, CFT^-, C127: minimum essential medium plus 23 mM $NaHCO_3$, 5 mM NH_4Cl, 2% FCS. The effects of the toxin were documented by photographs (Zeiss IM35 microscope; 32× objective) approximately 4 h from the start of the incubation, after loading the cells with neutral red as described [24].

2.4. Patch-clamp experiments

Cells were grown on glass coverslips, transferred to the patch-clamp chamber, washed with 150 mM NaCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM HEPES/Na, pH 7.3 and exposed to acid-preactivated VacA for about 30 min at room temperature in the same medium. Whole-cell seals were then established. The pipette solution was 110 mM (polyp cells, Fig. 2D) or 125 mM (others) CsCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM HEPES/Cs, 4 mM ATP/Na, pH 7.3 (CsOH). This composition prevented activation of volume- or Ca^{2+}-activated channels, as established by control experiments in the absence of VacA. Current conduction was recorded applying 300-ms voltage steps from $V_{hold} = -40$ mV (20-mV intervals; 15-s intervals between pulses). For maximal stimulation of the CFTR in transfected C127 cells (Fig. 2A,B), the pipette solution was supplemented with 100 µM cAMP (alternatively, the bath medium contained 100 µM 8-bromo-cAMP), 100 µM 3-isobutyl-1-methylxanthine, 1 mM ATPγS and 1 U/ml protein kinase A (active subunit).

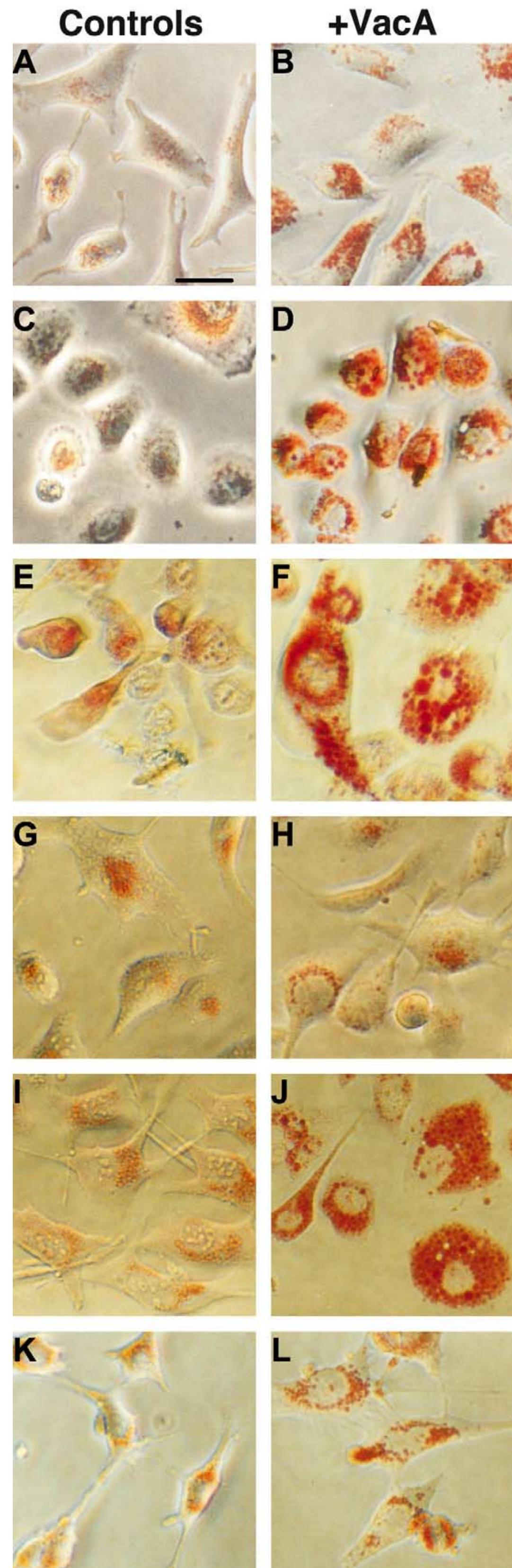

Fig. 1. Vacuolating effects of VacA on airway epithelial cells. Left column: controls. Right column: VacA-treated cells. Bar: 50 µm. A,B: HeLa cells (reference line). C,D: Nasal polyp (homozygous CFTR ΔF508; primary culture). E,F: Bronchial explant (CFTR wt; primary culture). G,H: CFT^+/2CF Meo^-/2CFSMEo^- cells. I,J: NFT/56FHTE8o^-. K,L: C127 (expressing CFTR wt). VacA-treated cells where incubated for about 4 h at 37°C in the presence of 16 nM toxin and 5 mM NH_4Cl, while controls received only NH_4Cl. For details see Section 2.

3. Results

VacA is known not only to discriminate between different cell types, but also to induce vacuolation only in a cell type-, toxin and permeant base concentration- and time-dependent fraction of even a presumably clonal population. Also, vacuoles develop with different kinetics in different single cells. We tested whether the toxin would act on airway epithelial cells, either from primary cultures or from established immortal lines, by visual assessment of toxin-induced vacuolation, compared to that of HeLa cells in parallel experiments. Fig. 1 shows representative images. Primary cultures of human nasal polyp cells expressing CFTR wt or homozygous CFTR ΔF508, and bronchial cells (CFTR wt) vacuolated to an extent comparable to or greater than that of HeLa cells, with a portion of cells responding sluggishly to the toxin. The sensitivity to the toxin of a few tested immortalized respiratory epithelial cell lines varied. Two bronchial cell lines (BEAS-2B and CFT^{+}) proved quite resistant, while a fetal tracheal line (NFT) and one derived from a polyp (CFT^{-}) were approximately as sensitive as HeLa cells. Murine C127 cells expressing CFTR wt showed a mixed behavior, as if consisting of two distinct populations. The cells that reacted to VacA – approximately 60% – did so markedly, forming prominent vacuoles, while the toxin had very little effect on resistant cells.

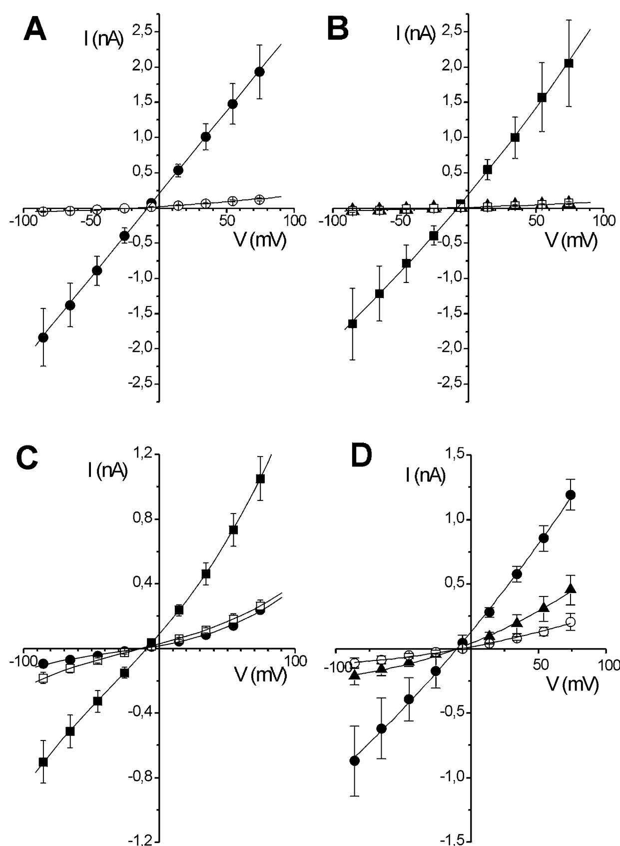

Fig. 2. Electrophysiological behavior. Averaged *I*/*V* plots from patch-clamp experiments in the whole-cell configuration. Error bars: S.E.M. A: C127 cells transfected with the functional human CFTR gene. Open circles: controls (no treatment; $n=5$); solid circles: CFTR, maximally activated ($n=8$). B: Mock-transfected C127 cells. Open squares: controls (no treatment; $n=6$); solid triangles: the unaffected subpopulation of VacA-treated cells ($n=5$); solid squares: VacA-affected cells ($n=7$). When present, VacA was 53 nM. C: CFT^{-} cells. Solid circles: controls ($n=7$); solid squares: VacA-treated ($n=7$); open squares: VacA-treated after the addition of 100 μM NPPB ($n=6$). When present, VacA was 16 nM. D: Nasal epithelial cells (primary culture of explanted polyps). Data from wt and cf cells were pooled. Open circles: controls ($n=8$); solid triangles: an arbitrary subset of VacA-treated cells ($I_{-46\ \mathrm{mV}} > -200$ pA; $n=4$); solid squares: the remainder of the VacA-treated cells ($n=3$). When present, VacA was 10 nM.

Vacuolation is believed to be a consequence of pore formation by VacA. Thus, we expected the cell lines which underwent vacuolation to also exhibit VacA-induced currents in whole-cell patch-clamp experiments, in analogy to the behavior of HeLa cells [24]. This was indeed the case. Fig. 2 presents results from C127 (expressing CFTR wt) (A,B), CFT^{-} (C) and primary culture polyp (D) cells. The whole-cell conductance of VacA-treated cells depends on toxin concentration and on exposure time as well as on the cell type.

With C127 cells the results of patch-clamp experiments could be divided into two sets: cells exhibiting no increase of the whole-cell current level with respect to controls (5/12), and cells showing instead a marked difference (7/12; see Fig. 2B). The presence of a sensitive and an insensitive population agrees with the results of vacuolation experiments. A comparison with the average *I*/*V* plot obtained under maximal stimulation of the CFTR (Fig. 2A) showed that intoxication by VacA can lead to current densities as high as or higher than any due to the CFTR. In the case of CFT^{-} cells (Fig. 2C) all VacA-exposed cells had clearly higher whole-cell currents than the controls. This current was sensitive to the well-known VacA inhibitor NPPB [34,35], added after the first few rounds of recording. Primary culture polyp cells displayed an intermediate behavior: all cells were apparently affected by VacA, but to variable extents. In Fig. 2D the data have been arbitrarily grouped into two sets depending on whether the current intensity measured at $V=-46$ mV was higher or lower than -200 pA.

4. Discussion

The experiments presented above confirm that VacA can be considered to be a 'portable chloride channel', capable of inserting into the plasma membrane of human epithelial cells, albeit with quantitative variations depending on the cell line and on the individual cell. The conductance increases caused by the toxin obviously depend on the concentration applied, and can be in the same range as those produced by CFTR stimulation. In the absence of permeant bases, VacA induces little cell sufferance. Thus, it might be used to alter the permeability to anions of model or explanted epithelia, to verify the effect of variations of this parameter, in itself, on the depth and composition of the superficial aqueous layer, mucus production and bacterial colonization.

The selectivity of toxin action depends on incompletely understood factors, mainly binding of the toxin to the cell surface. In turn, differences in binding characteristics are thought to impact on membrane insertion, pore formation and cell vacuolation. Different sensitivities by clonal cells are tentatively ascribed to differences in receptor expression. Toxin isoform characteristics may also contribute to specificity [22]. Once these aspect become clearer, it might be possible to engineer specific toxin forms affecting only some cell types.

The CFTR is believed to have functions in intracellular

compartments as well as in the apical cellular membrane, and indeed an estimated 50% is localized in intracellular compartments [36]. Roles have been proposed in the regulation of mucin expression and of membrane traffic, and in the maintenance of a proper pH gradient across the membrane of endosomes, secretory vesicles and the *trans*-Golgi network (e.g. [36–40]). Like the CFTR, VacA has the potential to alter pH homeostasis in intracellular compartments, both by providing an electrophoretic transport pathway for charge-balancing anions and by facilitating HCO_3^- diffusion. Its action is, however, as far as is known, restricted to the endosomal system. A comparison between its effects and those of CFTR re-introduction in model cellular systems may therefore also help to clarify whether alterations of the pH of some compartments may or may not have an effect on the chosen readout.

In summary, VacA may be a useful tool for the elucidation of the different roles of CFTR in the pathogenesis of CF lung disease, thanks to (a) biophysical properties similar to those of CFTR, (b) the ability to insert into the plasma membrane of respiratory cells and (c) its presence in the endosomal compartment. As more is learned about the structure–function relationships of the toxin and its routing upon internalization, molecular modifications may become feasible which would increase its usefulness by introducing specific channel properties and targeting. The use of toxins thus ought to be considered along with gene therapy, the activation of other endogenous chloride channels and the exogenous administration of chloride-conducting peptides as a possible therapeutic tool for CF.

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