Cellular Microbiology (2002) **4**(7), 397–409

The *Vibrio cholerae* haemolysin anion channel is required for cell vacuolation and death

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Summary

Several strains of *Vibrio cholerae* secrete a haemolytic toxin of 63 kDa, termed *V. cholerae* cytolysin (VCC). This toxin causes extensive vacuolation and death of cells in culture and forms an anion-selective channel in planar lipid bilayers and in cells. Here, we identify inhibitors of the VCC anion channel and show that the formation of the anion channel is necessary for the development of the vacuoles and for the cell death induced by this toxin. Using markers of cell organelles, we show that vacuoles derive from different intracellular compartments and we identify the contribution of late endosomes and of the *trans*-Golgi network in vacuole biogenesis.

Introduction

The Gram-negative bacterium *Vibrio cholerae* is an intestinal non-invasive pathogen that adheres to the apical side of the small intestine (Salyers and Whitt, 1994) thereby inducing cholera, an acute watery diarrhoea disease of humans (Kaper *et al*., 1995). *Vibrio cholerae* produces several virulence factors that are involved in colonization and adhesion and also releases protein toxins that co-operate to induce the alteration of ion and water fluxes across the intestinal epithelium, which is the basis of the massive loss of fluids characteristic of cholera (Kaper *et al*., 1995). Cholera toxin enters into epithelial cells and elevates the cAMP level, which affects the functioning of the Cystic Fibrosis Transmembrane Regulator (CFTR) chloride channel and of other cellular proteins (Hogenauer *et al*., 2000). The zona occludens toxin alters the tight junctions that control the paracellular permeability of the polarized epithelial monolayer (Marinaro *et al*., 1999). The *V. cholerae* heat-stable toxin causes rapid fluid accumulation after activation of the guanylyl cyclase and the influx of Ca^{2+} ions across the plasma membrane (Visweswariah *et al*., 1992; Hoque *et al*., 2001).

In addition, many *V. cholerae* strains secrete a haemolytic toxin of 63 kDa, termed *V. cholerae* cytolysin (VCC) or haemolysin (Honda and Finkelstein, 1979; Ramamurthy *et al*., 1993; Dalsgaard *et al*., 1995). VCC causes lysis of vertebrate erythrocytes and cultured cells (Honda and Finkelstein, 1979; Zitzer *et al*., 1997a). It also increases the vascular permeability of rabbit skin and it is lethal to mice (Yamamoto *et al*., 1984).

VCC forms transmembrane anion-selective pentameric channels with a diameter of 0.9–1.0 nm, leading to osmotic swelling of the cell and lysis (Krasilnikov *et al*., 1992; Zitzer *et al*., 1995, 1997a; 1999). The membrane-permeabilizing action of VCC has been documented in planar lipid bilayers and liposomes (Krasilnikov *et al*., 1992; Zitzer *et al*., 1993; Menzl *et al*., 1996). A pathogenic role for VCC in enteritis is suggested by the observation that the purified protein elicits fluid accumulation in rabbit ileal loops (Zitzer *et al*., 1993). Furthermore, a cytocidal action of VCC on human intestinal cells was attributed to VCC oligomerization and pore formation on cell membranes (Zitzer *et al*., 1997b; 1999). Recently, extensive cell vacuolation of HeLa and Vero cells was shown to be induced by *V. cholerae* filtrates and the vacuolating activity was linked to VCC (Coelho *et al*., 2000; Mitra *et al*., 2000). Such cell vacuolating effect was reminiscent of that induced by the VacA cytotoxin of *Helicobacter pylori* (Cover and Blaser, 1992; Montecucco and Rappuoli, 2001; Morbiato *et al*., 2001). On the other hand, some differences among the cellular vacuoles induced by the two toxins were apparent. *Vibrio cholerae* cytolysin-induced vacuoles were found to uptake the weak base dye, neutral red, indicating that they are acidic like VacA-induced vacuoles. However, the specific vacuolar ATPase proton pump inhibitor bafilomycin A1, which is able to inhibit vacuole formation in VacA treated cells, did not prevent vacuole formation in VCC exposed cells (Cover *et al*., 1993; Papini *et al*., 1993; Coelho *et al*., 2000; Mitra *et al*., 2000; Figueroa-Arredondo *et al*., 2001).

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Table 1. Effect of anion channel inhibitors on VCC channel and cell vacuolation.*

	Vacuolation		Channel activity	
Inhibitor	Working concentration (μM)	Inhibitory effects	Reversibility	$IC_{50,Rev}$ (μM)
DIDS	100–2000	Yes	Rev. + irrev.	76 ± 1
SITS	100–2000	Yes	rev. + irrev.	600 ± 50
IAA-94	25–400	No	Rev.	850 ± 70
NPPB	25–400	No	Rev.	>1000
Niflumic Acid	25–500	No	Rev.	ND
Flufenamic Acid	25–500	No	Rev.	ND
NPA	25–400	No	Rev.	ND
Glybenclamide	10–50	No	ND	ND

*VERO cells were treated with VCC 40 pM for 24 h in the presence of increasing concentrations of inhibitor (in the indicated range) to determine inhibitory effects on the vacuolation process. Extension of vacuolation was determined as neutral red uptake. Inhibition of VCC channel activity was assayed by planar lipid bilayer measurements as described in the *Experimental procedures*. ND, not determined.

These results stimulated us to extend our previous studies on the mechanism of cell vacuolation induced by VacA (Montecucco *et al*., 2001) to the VCC-induced cell vacuolation with the aim that their comparison would provide further insights on the mechanism of cell vacuolation induced by both toxins and on the membrane traffic processes involved in vacuole biogenesis (Schiavo and van der Goot, 2001). Unlike other studies, we have used highly purified VCC preparations and we provide strong evidence that the VCC channel activity is essential for

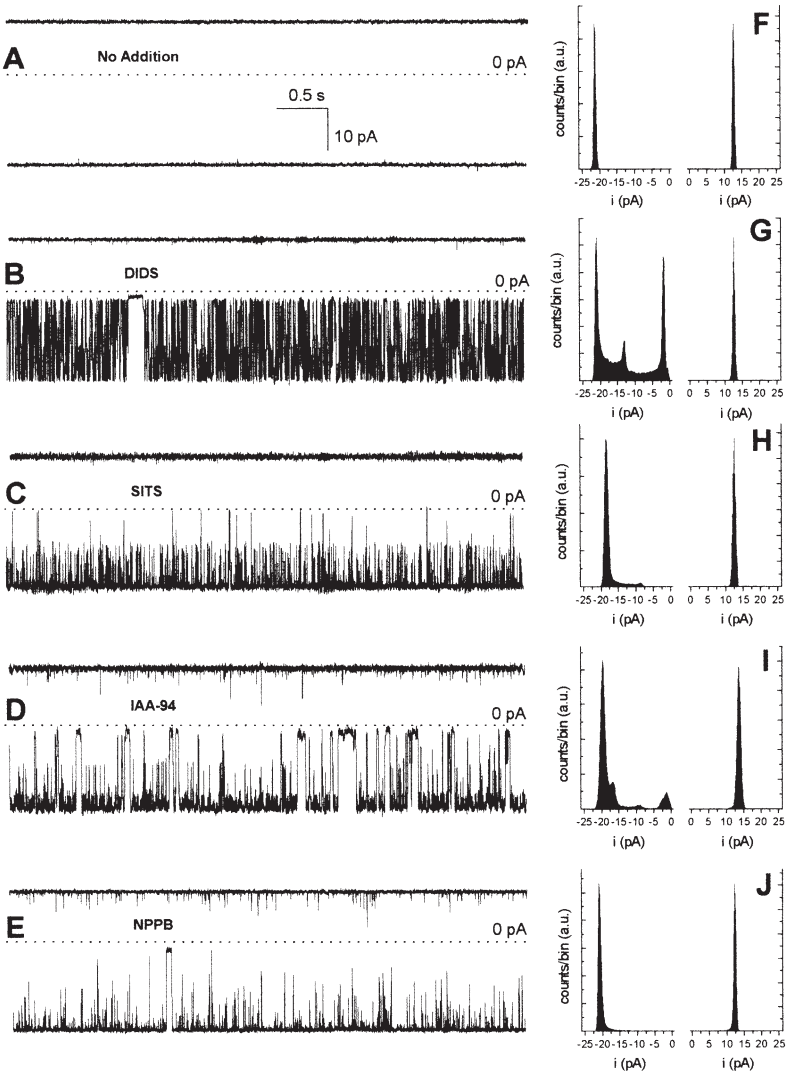

Fig. 1. Effect of anion channel inhibitors on the VCC channel in planar lipid bilayers. Representative current recordings and all-point current amplitude histograms illustrating the behaviour of VCC pores in the absence of any addition (A) and in the presence of 50 μM DIDS (B), 50 μM SITS (C), 200 μM IAA-94 (D) and 200 μM NPPB (E) in the *cis* compartment. Voltage, +50 (upper traces) or −50 (lower traces) mV. Filtering, 0.5 KHz; digitizing, 2.5 KHz. (F–J): amplitude histograms obtained from longer segments of the recordings shown to their left.

vacuolation and for cell death to take place. In addition, we report the identification and electrophysiological characterization of DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid) and SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid) as good inhibitors of the VCC anion channel. We also provide evidence that late endosomes and Golgi apparatus contribute to vacuole formation.

Results

Effect of anion channel inhibitors on the VCC channel in planar lipid bilayers

VacA was previously shown to induce cell vacuolation via its anion-selective channel activity localized mainly at the level of late endosomal compartments (Iwamoto *et al*., 1999; Tombola *et al*., 1999a; Montecucco and Rappuoli, 2001; Morbiato *et al*., 2001). An order of potency was determined for panel of anion channel inhibitors on VacA inserted planar lipid bilayers and the same order of inhibitory activity was found in the inhibition of vacuole formation in cells (Tombola *et al*., 1999b). To test if VCC anion channel activity is responsible for vacuole formation, we determined the effect of several anion channel inhibitors on the channel formed in planar lipid bilayers by cleaved and activated VCC (Table 1 and Fig. 1). The electrophysiological properties of the VCC toxin were found to be essentially the same as those reported previously (Krasilnikov *et al*., 1992; Menzl *et al*., 1996). Different to VacA (Tombola *et al*., 1999b), DIDS and SITS were found to be the most active compounds on the VCC channel. DIDS and SITS are two well-characterized inhibitors of the chloride/bicarbonate anion transporter of the red cell membrane (Simchowitz, 1988).

As shown in Fig. 1, DIDS induced a fast block with transitions to either an essentially non-conducting state or to a state with approximately 60% of the maximal conductance (Fig. 1B and G), with an IC_{50} of 76 μM at V = −50 mV (as measured from titrations of multi-channel currents). The block was strongly voltage-dependent as at positive applied potentials DIDS was completely ineffective. SITS behaved analogously, but had a higher IC_{50} (Table 1). IAA-94 was less effective on a molar basis, but produced longer (on average) blockages, and it also induced some rapid events at positive potentials. NPPB, the most potent inhibitor of VacA (IC_{50} 24 μM), only weakly inhibited VCC ($IC_{50} > 1$ mM). It induced mainly fast events, with occasionally longer ones, at negative potentials, and some flickering blocks at positive potentials. Both IAA-94 and NPPB induced partial as well as complete blockages, and the partially blocked state(s) had a similar conductance(s) in all cases. The other compounds tested (see Table 1) had little or no activity on the channel in lipid bilayers.

The fast block shown in Fig. 1 appeared immediately after the addition of the inhibitor. It was reversible, as upon perfusion of the chamber with fresh medium, the frequency of the blockages decreased substantially, due to wash-out of the blocker(s) (Fig. 2A and B). In the experiment shown in Fig. 2A, a cocktail of the inhibitors, listed

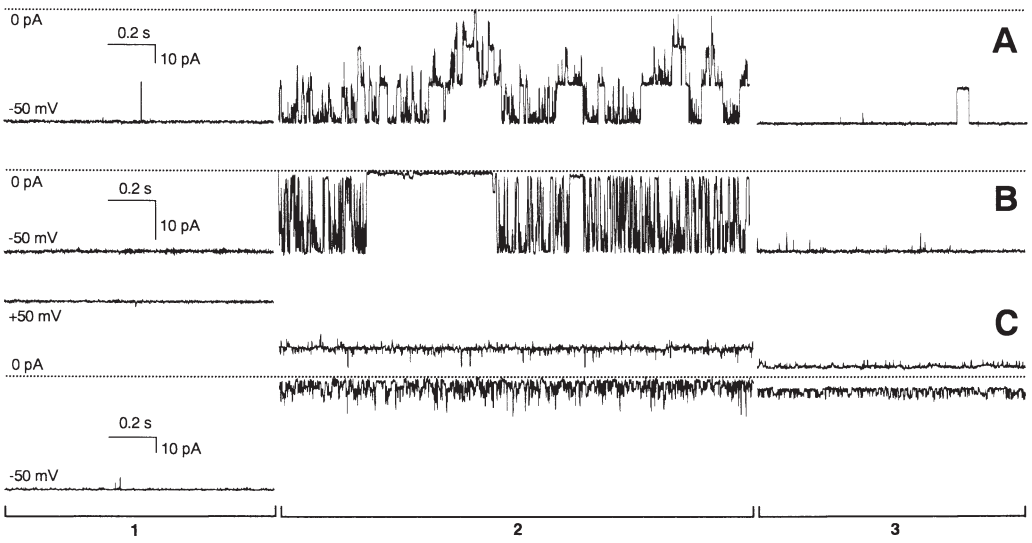

Fig. 2. Reversibility of the VCC channel inhibition by anion channel inhibitors**.** Three segments (1–3) of current recordings from three different experiments (A–C) are shown. A and B illustrate reversible block, whereas (C) shows an example of irreversible inhibition. Segment 1: with no addition of inhibitors the channel is open and the trace shows current flow. Segment 2: in the presence of inhibitors in the *cis* chamber, events of channel block shift the trace upward towards zero current. Segment 3: perfusion of the *cis* chamber removes non-covalently bound inhibitors and restores initial current flow. During the recording of segment 2, the following inhibitors were present: A, 100 μM each of IAA-94, NPPB, niflumic acid, flufenamic acid, NPA; B, 50 μM DIDS; C, 200 μM DIDS. Three channels were initially open in A, one in B and four in C. In segment 2 of panel C three out of the four channels were already irreversibly inhibited. Filtering and digitizing as in Fig. 1.

in the figure legend, was used when the reversibility of their inhibition had to be tested. From such experiments, it can be concluded that all the inhibitors contained in the cocktail act reversibly. In the case of DIDS and SITS, the inhibition suddenly changed, after a period of the order of minutes, roughly inversely proportional to the concentration of inhibitor, into a different and irreversible type of inhibition, as illustrated in Fig. 2C. After this transition, the channel adopted a very low basal conductance, with superimposed fast spikes of low amplitude (Fig. 2C, part 3). In contrast to the reversible block, this permanent inhibition could not be eliminated by washing, and was not voltage-sensitive. If incubation with the inhibitor was continued, even the small-amplitude openings eventually disappeared (not shown). This is consistent with a covalent binding of DIDS and SITS to VCC. The results presented in Figs 1 and 2 show at least two discrete blocked states, presumably corresponding to the presence of at least two different inhibitor binding sites within the pore. Whether these sites are the same for the different inhibitors, and whether the nearly complete blocking events are determined by the binding of two molecules, or by only one at the appropriate site, remains to be determined.

The planar bilayer experiments indicate that VCC always inserts into membranes in one of the two possible orientations. This statement is based on the asymmetry of the I/V curves determined in both multi- and single-channel experiments as well as on the strict voltage dependence of the block itself. The toxin-containing chamber (*cis*) thus corresponds to the extracellular medium, and the normal membrane potential of most cell types is close to the value used in the experiments reported here (+50 mV).

These investigations using planar lipid bilayers showed that the VCC anion channel is inhibited by general inhibitors of anion channels with the following order of potency: DIDS > SITS > IAA-94 > NPPB >> niflumic acid ≈ flufenamic acid > NPA. This scale is different from the one determined for the VacA anion channel (Tombola *et al*., 1999b), indicating a different structural organisation of the two channels. The main differences emerged before were the higher conductance and the lower selectivity of the VCC channel with respect to the VacA channel (Menzl *et al*., 1996; Tombola *et al*., 1999a).

Different cell lines exhibit a different sensitivity to VCC

The data described above allowed us to test the possibility that the VCC anion channel is required for the formation of vacuoles in VCC exposed cells. If this is the case, the VCC channel inhibitors must inhibit vacuolation with an order of potency similar to the one displayed in planar lipid bilayers, as previously established with VacA (Tombola *et al*., 1999b). In order to proceed with these experiments, the sensitivity of different cell lines to vacuolation induced by VCC was established. The vacuolating activity of VCC on Vero cells and HeLa cells in culture was recently determined (Coelho *et al*., 2000; Figueroa-Arredondo *et al*., 2001). Here, we extended this analysis to BHK, MDCK-I and A431 cells, which were previously shown to be highly sensitive to the *H. pylori* cytotoxin VacA (de Bernard *et al*., 1998). The five cell lines were challenged with increasing VCC concentrations, in the range between 8 pM and 160 pM. Table 2 shows that VCC-induced vacuolation in Vero, HeLa and A431 cells, but not in MDCK-I and BHK cells, at concentrations between 25 pM and 40 pM, indicating that this toxin is much more toxic than VacA, which is effective at concentrations >5 nM. VCC-induced vacuolation did not revert and cells died after 24 h, with the exception of Vero cells which developed very large vacuoles. All cell lines tested here died by osmotic lysis at VCC concentrations >60 pM. At variance, VacA treated cells do not die and slowly recover from vacuolation upon toxin removal from the medium (our unpublished observations).

Table 2. Sensitivity of different cell lines to VCC.*

Cell line	Vacuolation (25–40 pM VCC)	Lysis (>60 pM VCC)
Vero	++	+
HeLa	+	+
MDCK I	–	+
BHK	–	+
A431	+	+

*Cells were incubated 24 h with increasing concentrations of VCC. Vacuolation and lysis of the cells were determined in the two ranges of concentrations indicated. The extent of vacuolation was estimated by measuring the amount of neutral red uptake.
++, Strong vacuolation; +, weak vacuolation; –, undetectable vacuolation.

In addition, we investigated the effects of VCC on polarized epithelial monolayers formed by T84 and MDCK-I cell lines, a system more closely related to the *in vivo* situation. The Trans-epithelial Electric Resistance (TER) of monolayers is lowered by VacA added in the apical compartment with no formation of macroscopic vacuoles (Papini *et al*., 1998). Neither vacuolation nor rapid decrease of TER were observed on monolayers of T84 and MDCK-I treated with increasing concentrations of VCC (between 40 pM and 400 pM). However, a progressive damage of the epithelium was evident at high concentrations of the toxin (>150 pM), which caused an extensive cell lysis (not shown).

Inhibitors of the VCC anion channel prevent Vero cells vacuolation and death

Based on this preliminary information, Vero cells were chosen for the assay of anion channel inhibitors on VCC-

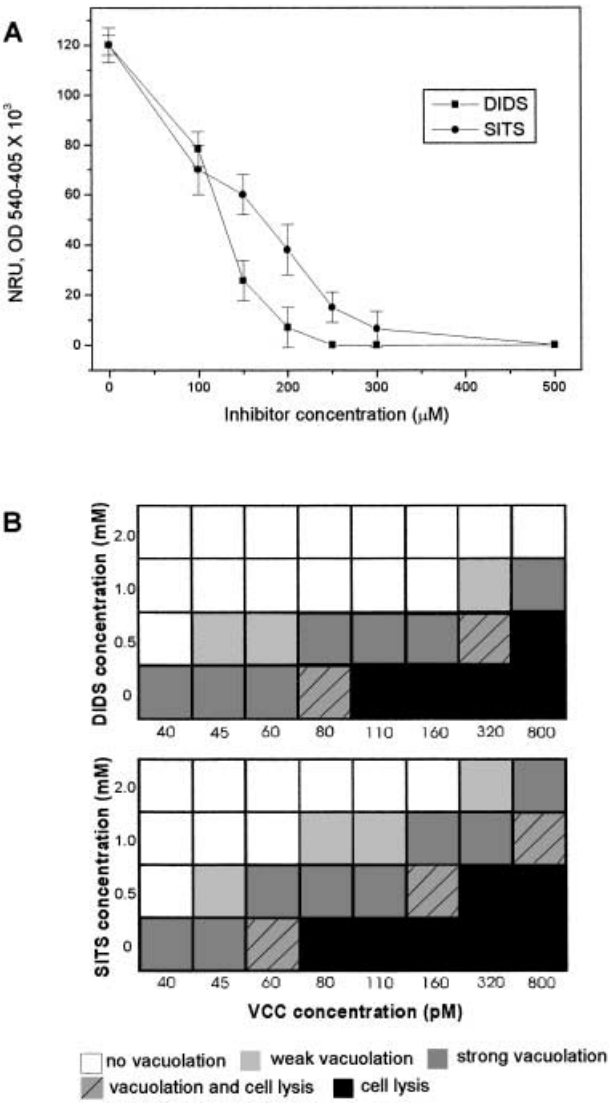

Fig. 3. DIDS and SITS prevent vacuolation and death of VERO cells.
A. Cells were incubated with VCC (40 pM) in the presence of the indicated concentrations of inhibitors (DIDS (squares) and SITS (circles)). After 18 h, the uptake of neutral red was determined. Values are the means of three experiments and bars represent ±SD values.
B. VERO cells were incubated with increasing amounts of VCC (40–800 pM) at different concentrations of inhibitors DIDS and SITS (0–2 mM). The extents of vacuolation and cell death were evaluated by visual inspection after 24 h of toxin intoxication.

induced vacuolation. As documented in Table 1, only SITS and DIDS were found to be effective in preventing vacuolation of VCC-treated Vero cells. Even at concentrations that began to show general cell toxicity effects, no inhibitory activity on vacuolation was detectable with the other channel inhibitors.

Figure 3A reports the dose dependence of DIDS and SITS inhibition of cell vacuolation induced by VCC and provides values of ID_{50} around 150 μM. These inhibitors were also tested for their effect in preventing cell death after 24 h of incubation with the toxin. As depicted in Fig. 3B, both inhibitors were capable of reducing cell death (detected by Trypan blue exclusion) in a dose-dependent way, with DIDS showing a higher activity. DIDS and SITS similarly protected T84 and MDCK-1 cells from VCC-induced death. These results clearly indicate that the anion channel activity of VCC is the molecular lesion of the cell which eventually results in the formation of vacuoles and cell death.

Ammonium chloride increases the extent of VCC-induced vacuolation

VacA-induced vacuoles are acidic and accumulate the weak base dye neutral red (Cover *et al*., 1991; Papini *et al*., 1993) and both the kinetics and the extent of vacuolation are strongly stimulated by the presence of ammonium chloride in the medium. Figure 4 shows a time course analysis of the effect of 5 mM ammonium chloride on the vacuolation induced by 40 pM VCC on Vero cells. Similarly to what was found with VacA, the presence of ammonium ions in the medium containing VCC caused an earlier and more extensive vacuolation and the volume of vacuoles was larger. The difference induced by ammonium ions disappeared after 22 h of incubation. Therefore, at variance from VacA, in the case of VCC-treated cells the kinetics of vacuolation, but not its extent, was influenced by ammonium chloride.

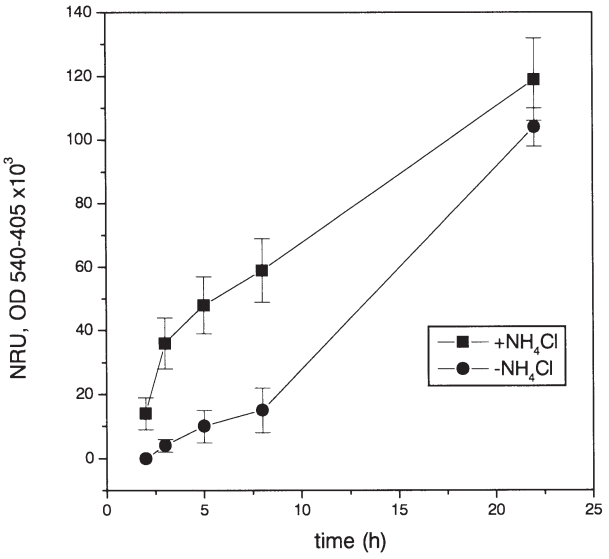

Fig. 4. Stimulating effect of NH_4Cl on VCC-induced cell vacuolation. VERO cells, were incubated with 40 pM VCC in the presence (squares) or absence (circles) of 5 mM NH_4Cl in the culture medium. At the indicated times the vacuolation extent was assayed by measuring neutral red uptake. Data are average of four independent assays run in triplicates and bars represent ±SD values.

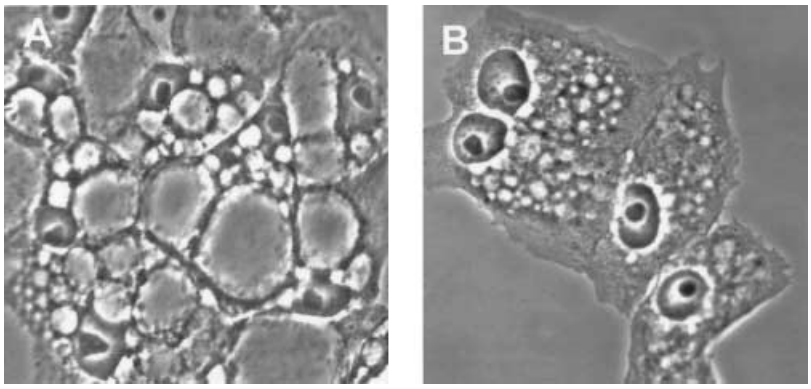

Fig. 5. Vacuolation of VERO cells induced by VCC in the presence (B) or absence (A) of bafilomycin A1. Cells were treated for 30 min with or without bafilomycin A1 (100 nM), and then incubated for 36 h with VCC 40 pM.

Effect of bafilomycin A1 on cell vacuolation induced by VCC

Bafilomycin A1 is a specific inhibitor of the vacuolar ATPase proton pump (v-ATPase), which is responsible for the acidification of intracellular acidic compartments (Nelson *et al*., 2000). This drug completely prevents and reverts VacA-induced vacuoles (Cover *et al*., 1993; Papini *et al*., 1993), but was reported to have no effect on the development of vacuoles caused by VCC (Coelho *et al*., 2000). In addition, bafilomycin A1 was found here to be unable to revert the vacuolation process in VCC-treated Vero cells, but it suppressed the stimulatory effect of NH_4Cl (not shown). Furthermore, in the presence of bafilomycin A1, VCC-treated Vero cells did not develop the characteristic large vacuoles, as shown in Fig. 5. This result suggests that the v-ATPase is required for the formation of the large vacuoles, probably because they originate from extensive membrane fusion events involving endosomal compartments. Indeed, the presence of an active working v-ATPase and/or the acidity of the endosomal compartments are necessary to maintain an efficient endosomal traffic (Clague *et al*., 1994). Moreover, the membrane domain of the v-ATPase was recently reported to be involved in membrane fusion events within the cell (Peters *et al*., 2001).

VCC-induced vacuoles originate from different intracellular compartments

To shed light on the organelle/s from which vacuoles derive, vacuolated cells were labelled with cell marker specific antibodies. Many but not all the vacuole membranes were labelled with the antiv-ATPase antibody (Fig. 6B). Moreover, large vacuoles presented an internal labelling that might correspond to the internal membranous vesicle observed previously (Figueroa-Arredondo *et al*., 2001). Figure 6D and F show that vacuolar membranes are enriched in rab7, the small GTPase which regulates the late stages of endosomal trafficking (Chavrier *et al*., 1990; Meresse *et al*., 1995) and in LAMP-1, a lysosome-associated membrane glycoprotein (Rohrer *et al*., 1996). The intensity of labelling of vacuoles with the fluid phase marker Lucifer yellow (Fig. 6H) is variable and independent from their size. As this dye traces the endocytic route of membrane trafficking, such pattern of labelling is in keeping with the possibility that, in addition to endo/lysosomal compartments, other cell organelles contribute to the biogenesis of VCC-induced vacuoles. This is further supported by the finding shown in Fig. 6L that some vacuoles contain elements of the Trans Golgi Compartment (TGN), as they are labelled with the anti TGN46 antibody, which does not label VacA-induced vacuoles. On the other hand, vacuoles are not labelled with antibodies specific for the transferrin receptor and for calreticulin, markers of early endosomes and of the endoplasmic reticulum, respectively (data not shown). Hence VCC-induced vacuoles include endo/lysosomal and TGN membranes, but do not include early endosomal or endoplasmic reticulum (ER) membranes.

In the case of VacA, it was shown that a functional rab7 was essential for vacuolation to occur as inactive rab7 molecules acted as dominant negative (Papini *et al*., 1997). This is not the case of VCC. In fact, Vero cells expressing the inactive mutant of rab7 (rab7 T22N) do vacuolate in the presence of VCC (data not shown). Taken together, these experiments indicate that VCC-induced vacuoles originate from more than one type of acidic compartment, in agreement with the finding that different VCC-induced vacuoles within the same cell do exhibit a different acidity (Figueroa-Arredondo *et al*., 2001).

VCC is internalized and localizes on vacuolar membrane

VacA is internalized by cells in culture (Garner and Cover, 1996; Szabo *et al*., 1999; Ricci *et al*., 2000). Similar data were not available for VCC. Here, we have studied the distribution of VCC with specific antibodies after either continuous incubation of Vero cells with the toxin at 37°C or after incubation with 800 pM VCC at 4°C, washing and

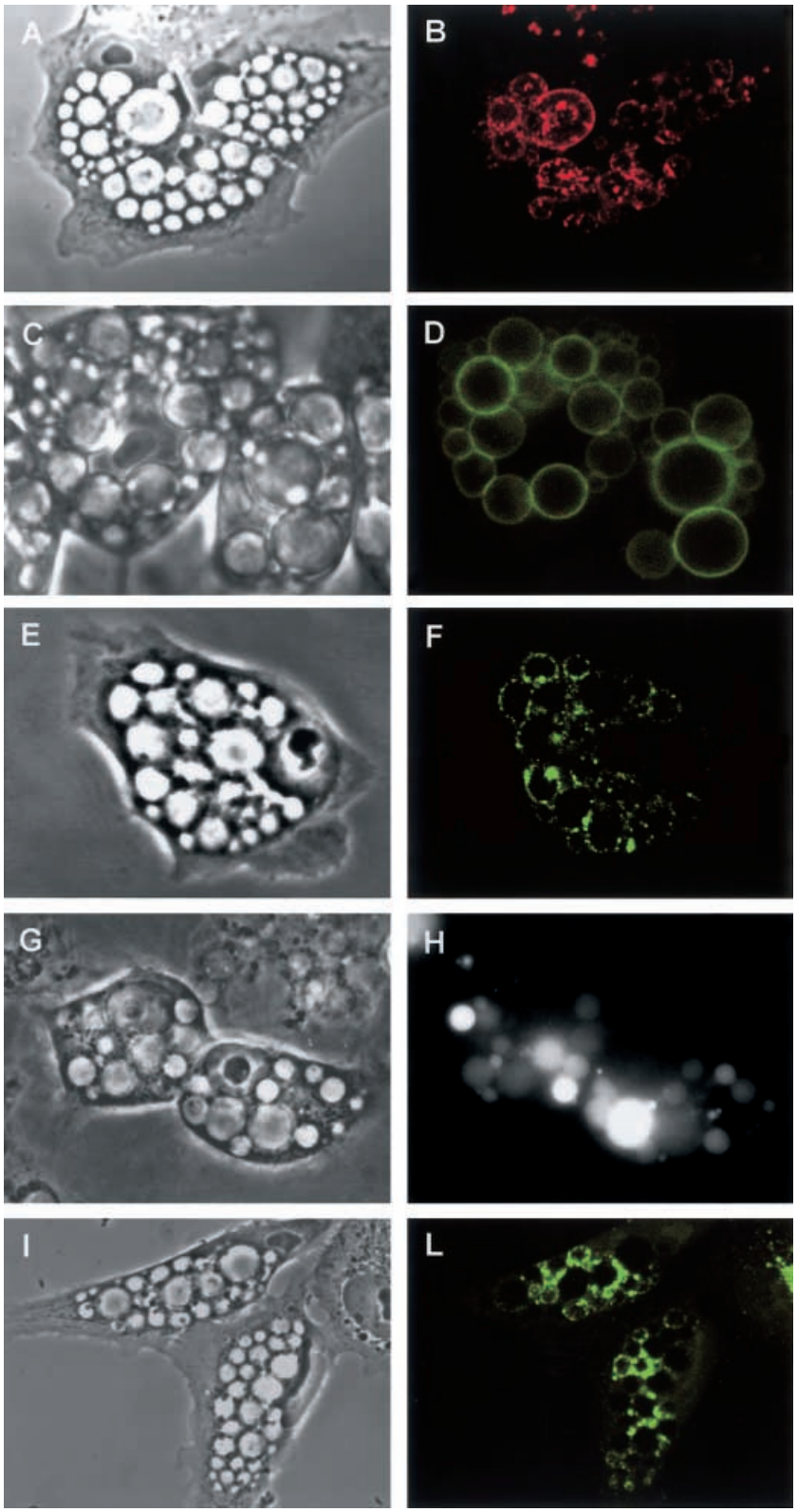

Fig. 6. Immunofluorescence localization of v-ATPase, rab7, LAMP1, Lucifer yellow and TGN46 in VCC vacuolated VERO cells. (B, F, L) VERO cells were treated with VCC, processed for immunofluorescence and than analysed with a confocal microscope. Cells were labelled with mouse monoclonal anti-v-ATPase antibody (B), rat anti-LAMP-1 antibody (F) and sheep anti-TGN46 antibody (L). Secondary anti-mouse IgG was Texas red conjugated; secondary antibodies anti-sheep and anti-rat IgG were FITC-conjugated. (D) VERO cells, transfected with pGEM7Zf-GFP-rab7, were treated with VCC, fixed with paraformaldehyde, washed with PBS and then analysed with a confocal microscope, using a FITC filter. (H) Localization of Lucifer Yellow in vacuolated VERO cells following incubation with VCC 40 pM and Lucifer yellow 500 $\mu g\,ml^{-1}$ for 8 h. Cells were washed with ice-cold PBS and then immediately observed with a Zeiss Axioplan fluorescence microscope. (A, C, E, G, I) Corresponding phase-contrast pictures of the same cells.

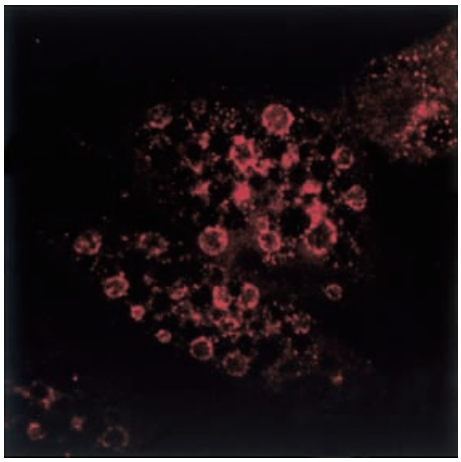

Fig. 7. Intracellular distribution of VCC in toxin-treated Vero cells. Vero cells, pre-cooled at 4°C, were treated with VCC 800 pM for 2 h at 4°C, washed with cold PBS and then heated at 37°C. After 4 h of intoxication, cells were fixed and processed for immunofluorescence. Cells were labelled with rabbit anti-VCC antibody. Secondary antibody, anti-rabbit IgG, was Texas red conjugated. The image was acquired with a confocal microscope.

incubation at 37°C. Under both conditions, VCC entered the cells, and as soon as vacuoles were detectable it was found associated with their membranes. This membrane localization becomes particularly evident when vacuoles attained a significant size (Fig. 7).

Discussion

The formation of vacuoles inside the cell, i.e. translucid and membrane-encased intracellular formations, is a widespread phenomenon in cell pathology, and may be caused by a variety of molecular lesions (Virchow, 1859; Majno and Joris, 1996). Recently, some bacterial protein toxins were shown to induce the formation of vacuoles in cells in culture and in tissues *in vivo* (Cover and Blaser, 1992; Telford *et al*., 1994; Abrami *et al*., 1998; Coelho *et al*., 2000; Mitra *et al*., 2000). Such purified molecules that exert a specific molecular lesion to the cell are specific tools for the study of the cell structure and physiology, as other bacterial toxins do (Rappuoli and Montecucco, 1997; Schiavo and van der Goot, 2001).

VCC forms anion selective channels in planar lipid bilayers (Krasilnikov *et al*., 1992; Menzl *et al*., 1996), similarly to VacA (Iwamoto *et al*., 1999; Tombola *et al*., 1999a). Such VacA activity was shown to be at the basis of the VacA-induced vacuolation of late endosomal compartment by using anion channel inhibitors and by manipulating the intracellular Cl^- content (Tombola *et al*., 1999b; Montecucco and Rappuoli, 2001; Morbiato *et al*., 2001).

In the present work, we have identified DIDS and SITS as inhibitors of the VCC anion channel activity. Besides a voltage-dependent block, these two compounds produced a voltage-independent, irreversible inhibition, presumably due to covalent modification of the protein. The other compounds tested did not cause any significant inhibition at positive (i.e. physiological) potentials.

DIDS and SITS also largely prevented the VCC intoxication of Vero cells, selected here for their particular reactivity to this toxin, which results in vacuolation and death. Compounds that failed to inhibit VCC channels also failed to protect the cells. This correlation of *in vitro* and *in vivo* inhibitory activities is a strong indication that the VCC anion channel activity is responsible for vacuole formation and for cell death.

We have attempted to identify the intracellular compartments from which vacuoles originate. VCC-induced vacuoles were previously shown to be acidic (Coelho *et al*., 2000) and accordingly most of them were found here to contain v-ATPase, rab7, LAMP-1 and TGN46, which are markers of acidic compartments (endosomes, lysosomes and TGN). In addition, ammonium chloride was found to increase the rate of vacuolation, an effect abolished by bafilomycin A1. This drug, however, did not prevent VCC-induced vacuolation. Thus, there are several differences from VacA, which does not cause cell death and induces the selective vacuolation of late endosomal/lysosomal compartments in a way completely prevented and reversed by bafilomycin A1.

On the basis of what is known about the mode of action of VacA, and of the present findings, we would like to propose the following scenario for the cellular intoxication of cells by VCC (Fig. 8). This toxin inserts in the plasma membrane and induces an anion current larger than the one induced by VacA on a 'per channel' basis. At high toxin doses, this alteration is of such a magnitude as to cause cell death, an end result not attained in the presence of VacA. However, at lower concentrations, the change in cell permeability induced by VCC is compatible with cell life and the toxin is then internalized, as VacA does (Garner and Cover, 1996; Szabo *et al*., 1999; Ricci *et al*., 2000). VCC may follow the same intracellular route: plasma membrane-early endosomes-late endosomes-lysosomes. However, the finding that TGN membranes are included in VCC vacuoles leaves open the possibility that it may also, at least in part, follow the route of another protein toxin released by *V. cholerae*, the cholera toxin, which reaches the TGN from the early endosomes (Majoul *et al*., 1996; Sandvig *et al*., 1997; Lencer *et al*., 1999; Falnes and Sandvig, 2000). As a consequence of these intracellular trafficking events, VCC and VacA anion channels become associated with intracellular compartments characterized by the presence of the v-ATPase, which pumps protons inside the organelle thus rendering their lumen acidic. This pump is electrogenic, and its activity is accompanied by the building up of a proton gradient which progressively inhibits further pumping activity

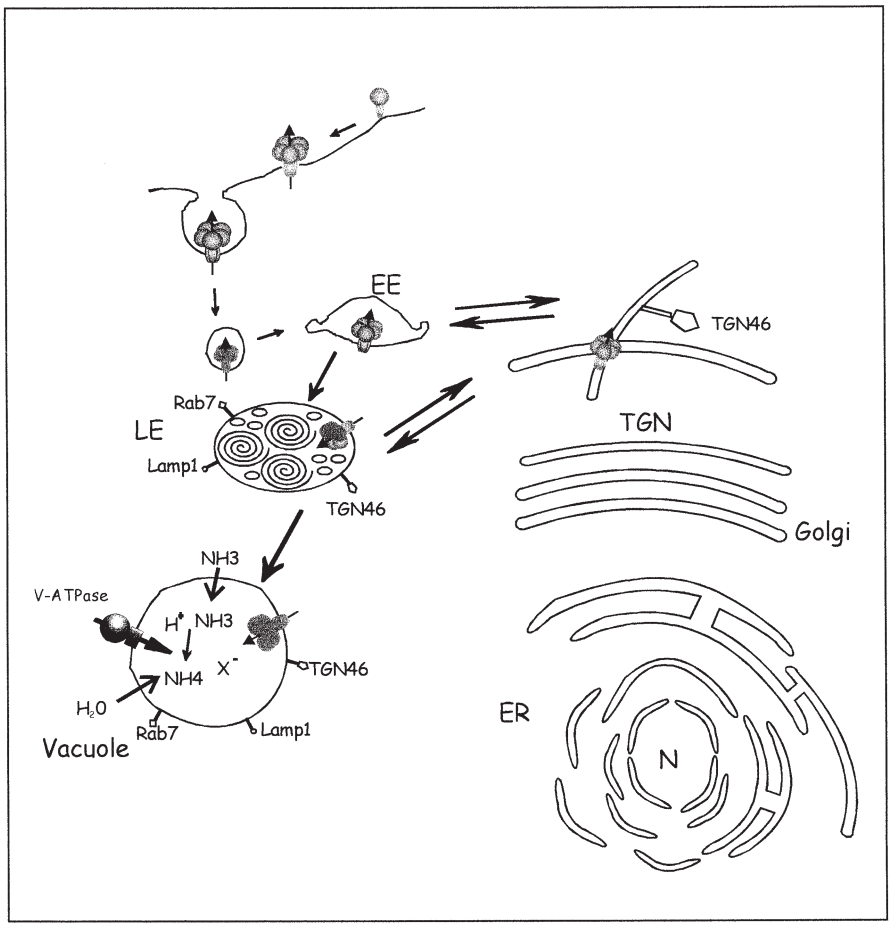

Fig. 8. A model for the mechanism of cell intoxication of VCC. The toxin binds and inserts into the plasma membrane of cells forming an ion channel larger and less specific for anions than the one induced by VacA. VCC is suggested to be slowly removed from the plasma membrane by endocytosis, similarly to VacA, to reach late endosomal compartments. The finding that TGN membranes are included in VCC-induced vacuoles suggest that it may, in addition, follow the route of cholera toxin which reaches the TGN from early endosomes. Hence, VCC anion channels become associated with intracellular compartments endowed with the electrogenic v-ATPase proton pump, whose activity generates a proton gradient. The presence of a large conductance channel as that formed by VCC is expected to promote the activity of the v-ATPase, therefore incrementing the capability of the organelle of accumulating membrane permeant weak bases. This would explain the increased rate of vacuolation caused by ammonium ions in the medium. The increased concentration of osmolites in the organelle lumen would cause their swelling with formation of the large translucid vacuoles, shown in Fig. 5.

(Nelson *et al*., 2000). The acidity of the lumen appears to be controlled by an endogenous chloride channel, which is present in different isoforms in different cell membranes (Piwon *et al*., 2000; Schwake *et al*., 2001; Vandewalle *et al*., 2001). The presence on the membrane of the toxin anion channel is expected to promote the activity of the v-ATPase, therefore incrementing the capability of the organelle of accumulating membrane-permeant weak bases. This would explain the increased rate of vacuolation caused by ammonium ions in the medium. The accumulation of molecules in the organelle lumen would cause their swelling into the translucid vacuoles visible in Fig. 5. VCC is expected to be more active than VacA based on its larger conductance. Moreover it is less ion selective than VacA, and these differences could account for the different properties of the vacuoles induced by the two toxins. Several aspect of the scenario depicted in Fig. 8 remain to be investigated in details. However, we believe that it provides a working model useful to design novel experiments aimed at the clarification of the cellular mechanism of intoxication of VCC.

Experimental procedures

Reagents

Vibrio cholerae cytolysin (VCC) was purified from culture supernatants of *V. cholerae* O1 El Tor 8731 (Hall and Drasar, 1990) by ethanol precipitation (final concentration, 40%), preparative isoelectric focusing in a sucrose density gradient, and hydroxyapatite chromatography and the cleaved and active form of VCC was obtained (Zitzer *et al*., 1997b; 1999). Cell culture media, neutral red and Lucifer yellow were from Sigma. Bafilomycin

A1 was a kind gift of Professor K. H. Altendorf (University of Osnabruck, Germany). Fetal calf serum (FCS) was purchased from PAA Laboratories. DOTAP and bovine serum albumin (BSA) were from Roche. Cellulose Transwell porous filters were purchased from Corning. Restriction enzymes were from Promega. Recombinant vaccinia virus vT7 (Fuerst *et al.*, 1986) was a gift from M. Zerial (Max Planck Institute, Dresden). R(+)-2-((2-cyclopentenyl-6,7-dichloro-2,3-dihydro-2-methyl-1-oxo-1H-inden-5-yl)oxy)acetic acid (IAA-94) and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) were purchased from Research Biochemicals International (Natick, MA, USA). *N*-phenylanthranilic acid (NPA), 2-[3-(trifluoromethyl) anilino]benzoic acid (flufenamic acid), 2-[3-(trifluoromethyl) anilino]nicotinic acid (niflumic acid), acetylsalicylic acid, 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (disodium salt) (DIDS), 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (disodium salt) (SITS) and N-p-[2-(5-chloro-2-methoxybenzamido)ethyl]benzene-sulphonyl-*N*′-cyclohexylurea (glybenclamide) were from Sigma. All inhibitors were used as 25, 50 or 100 mM stock solutions in DMSO, except for DIDS and SITS, which were dissolved in water, and acetylsalicylic acid, in ethanol. All other reagents used were of the highest purity available.

Antibodies

Monoclonal antibody anti-c-Myc was purchased by Roche. Mouse monoclonal antibody against the regulatory subunit (116 KDa) of human v-ATPase, termed Osw2 (Sato and Toyama, 1994) was a kind gift from Dr Sato. Affinity-purified rabbit polyclonal anti-transferrin receptor antibody, sheep polyclonal anti-TGN46 antibody, rat polyclonal anti-LAMP-1 antibody and rabbit polyclonal anti-calreticulin antibody were kind gifts of M. Zerial (Max Planck Institute, Dresden), A. Luini (Department of Cell Biology and Oncology, Chieti), G. Griffith (Max Planck Institute, Dresden) and R. Betto (Department of Biomedical Science, Padova) respectively. Texas red-labelled goat anti-rabbit and anti-mouse antibodies were purchased from DAKO. FITC-labelled rabbit anti-sheep antibody was from Calbiochem.

Cells

VERO, MDCK I, HeLa, BHK, A431 and T84 were cultured in plastic flasks in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS under 5% CO_2 at 37°C. Twenty-four hours before experiments cells were released with Trypsin/EDTA and seeded in 96- or 24-well titration plates in their culture medium at a density of 4×10^4 per cm^2. To form monolayers the suspended cells were seeded on cellulose Transwell porous filters (pore diameter 0.4 μm) at the density of 0.75×10^6 per cm^2. The medium of the lower and upper filter chambers were changed every 24 h, and the formation of a stable monolayer was assessed by monitoring the TER. The TER was measured with a Millipore apparatus as already described (Papini *et al.*, 1998). Within 7–14 days from seeding on filters MDCK I and T84 formed monolayers with TER values about 12 000 and 2 900 Ω per cm^2 respectively.

Cell vacuolation

Cells were incubated with VCC at the indicated concentrations in DMEM 10% FCS. When necessary, 5 mM NH_4Cl was added. At different time periods, cells were inspected for vacuolation under the optical microscope. To quantify the extent of vacuolation, cells were incubated for 8 min at RT with freshly prepared 0.05% neutral red in PBS containing 0.3% BSA and washed three times with the same buffer. After addition of 70% ethanol in water containing 0.37% HCl, absorbance was measured with a Packard Spectra Count™ at 534 nm with subtraction of adsorbance at 405 nm.

When needed, cells were preincubated for 1 h with bafilomycin A1 0.1 μM before the addition of VCC; drug was maintained during the intoxication. The effects of bafilomycin A1 on vacuolation was determined as above.

The experiments carried out with the specific chloride channel inhibitors, were performed co-incubating the toxin with the drugs at the indicated concentrations.

Immunocytochemistry and confocal laser scanning microscopy

VERO cells, grown on glass coverslips, were treated with VCC, washed twice with PBS, and then fixed with 3% (w/v) paraformaldehyde, 0.025% (v/v) glutaraldehyde in PBS for 15 min They were permeablized with pre-cooled methanol (–20°C) for 10 s and incubated with 0.1% $NaBH_4$ (w/v) in PBS for 10 min. After washing the cells three times with PBS, the cells were incubated for 1 h with the primary antibody. After several washes with PBS 1% BSA, binding was visualized with Texas red-conjugated anti-rabbit or anti-mouse IgG or FITC-conjugated anti-sheep IgG. Samples were mounted on 90% (v/v) glycerol, 0.3% (w/v) n-propylgallate in PBS. A MRC-1024 laser scanning confocal imaging system (Bio-Rad) was used to detect the fluorescence signals.

Plasmids

Vector pGEM1Myc3 was a generous gift of H. Stenmark (Institute for Cancer Research, Oslo); pGEM plasmid containing the gene encoding for rab7 T22N was from Dr C. Bucci (University of Naples, Italy). Plasmid pGEM7Zf-GFP-Rab7 was obtained by fusing GFP C-terminally to Rab7.

Transfection of cells

Cells were washed once with internalization medium (IM: DMEM, 20 mM HEPES, pH 7.2), incubated in IM containing recombinant vaccinia virus vT7 (Fuerst *et al.*, 1986) expressing the T7 polymerase for 30 min at room temperature and transfected with DMEM containing 3.7 g l^{-1} $NaHCO_3$, 10 mM Na-HEPES, 10 mM hydroxyurea, pH 7.2, DNA (9.2 ng ml^{-1}) and DOTAP (27.4 ng ml^{-1}) for 2 h at 37°C (Stenmark *et al.*, 1995). Cells were washed three times and incubated for 4 h with DMEM, 3.7 g l^{-1} $NaHCO_3$, 10% FCS, 10 mM hydroxyurea, pH 7.2, and further incubated in the same medium with 40 pM VCC for 5 h. Transfection efficiency and staining of the expressed protein were determined by immunocytochemical assay as described above; expressing chimera GFP-rab7 cells were directly analysed by confocal microscope, using a FITC filter.

Planar lipid bilayer experiments

Experiments were performed essentially as previously described (Tombola *et al*., 1999a,b). Diphytanoylphosphatidylcholine (DPhPC) (Avanti Polar Lipids, Alabaster, AL, USA) plus 20% (w/w) cholesterol (Sigma) was used as membrane lipid. The medium was 1 M NaCl, 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 10 mM HEPES/Na, pH 7.2, in both chambers, except that for selectivity determinations the NaCl concentration was lowered to 100 mM on one side of the membrane. In some experiments, the medium contained 0.1 mg ml^{-1} BSA (Sigma). The voltage reported is that of the chamber into which the toxin was added (*cis*), while the *trans* electrode was grounded. VCC was added at the concentration of 0.5–2.5 nM and experiments were performed after incorporation of either one or a few channels. Incorporation was stopped by perfusing the 3 ml chamber with 20 ml of fresh medium. Inhibitors were added at the indicated concentrations to the *cis* chamber. In reversion-of-inhibition experiments the compartment was perfused as above.

Acknowledgements

We gratefully acknowledge the financial support of CNR, of Progetto Finalizzato Biotecnologie (97.01168.PF 49) and of MURST-COFIN Project on 'Meccanismi e segnali nel trasporto cellulare di proteine'. A.Z. was supported by Deutsche Forschungsgemeinschaft (SFB 311) and Dr A. Coelho was partially supported by a UNESCO travelling grant.

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