Effect of the chaotropic anions thiocyanate and perchlorate on the entry of ricin into Vero cells

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The effect of different anions on the sensitivity of Vero cells to ricin was investigated. The cells were equally sensitive to ricin when NaCl was replaced by NaBr, NaI, Na₂SO₄ or with iso-osmotic concentrations of mannitol. In contrast, NaSCN and NaClO₄ strongly protected against ricin at pH 7.2, but not at pH 7.6. The possibility that the protective effect is due to the ability of chaotropic anions to decrease the pH close to the membrane is discussed.

Ricin is one of several structurally related toxins that inhibit protein synthesis in cells. These toxins consist of two polypeptide chains connected by a disulphide bond. One chain binds to cell-surface receptors while the other chain enters the cytosol and inhibits protein synthesis enzymically. There is now good evidence that all of these toxins enter from intracellular compartments (for review, see Olsnes & Sandvig, 1983). However, the toxins appear to enter the cytosol from different vesicular compartments and also to differ with respect to the mechanisms by which they are transferred across the limiting membrane. Studies with compounds and conditions that interfere with the entry have proved to be valuable to elucidate the entry mechanisms. We have earlier found that the ionic conditions in the medium surrounding the cells are important for the entry process (Sandvig & Olsnes, 1982, 1984; Olsnes & Sandvig, 1983). Thus ricin and abrin appear to require calcium transport across the membrane in order to enter the cytosol, while there is no specific requirement for sodium. Since entry of diphtheria toxin at low pH seems to require an anion flux across the membrane, we decided to investigate if anions are of importance for ricin entry as well. We here report studies on the effect of various anions on ricin entry.

Materials and methods

Ricin and abrin were purified as earlier described (Olsnes & Pihl, 1973*a,b*). ¹²⁵I-labelled toxin was prepared by the Iodogen method (Fraker & Speck, 1978). Binding of ¹²⁵I-ricin to Vero cells and endocytosis of ¹²⁵I-ricin were measured as described earlier (Sandvig & Olsnes, 1979). Vero

cells, mouse L cells and HeLa cells were maintained in minimum essential medium containing 10% (v/v) foetal-calf serum (Gibco, Glasgow, Scotland, U.K.). The sensitivity of cells to toxin was tested by measuring the ability of the toxins to inhibit protein synthesis (Sandvig & Olsnes, 1979, 1984). The difference between duplicates was less than 5% of the average value.

Results and discussion

To study if Cl⁻ is involved in the entry of ricin into cells, Vero cells were incubated in a Hepes buffer (pH 7.2) containing the sodium salt of different anions. Ricin was then added and, after 3 h incubation, the rate of protein synthesis was measured. As shown in Fig. 1, the presence of Cl⁻ is not essential for ricin entry, which occurred equally well in the presence of SO₄²⁻, Br⁻ and I⁻ as in the presence of Cl⁻. Furthermore, ricin was equally toxic when the concentration of anions was strongly reduced by using mannitol instead of salt to provide iso-osmotic conditions. On the other hand, the presence of the chaotropic anions SCN⁻ and ClO₄⁻ strongly protected the cells against ricin at pH 7.2 (Fig. 1). Similar results were obtained with mouse L cells and HeLa cells (results not shown).

High concentrations of SCN⁻ were needed to afford protection. Thus a mixture of 70 mM-Cl⁻ and 70 mM-SCN⁻ did not give any protection (results not shown). The sensitivity of cells to ricin was rapidly restored when cells that had been incubated in the presence of SCN⁻ were transferred to normal medium.

Control experiments showed that incubation of

Fig. 1. Effect of different anions on the sensitivity of Vero cells to ricin

Vero cells growing in 24-well disposable trays were incubated for 1 h at 37°C in a buffer containing 20 mM-Hepes, pH 7.2, 1 mM- $\text{Ca}(\text{OH})_2$, 5 mM-glucose and 0.14 M of the salts indicated below. Then increasing concentrations of ricin were added and, after 3 h further incubation at 37°C, protein synthesis was measured as described in the Materials and methods section. The salts in the buffer were: $\times$, NaCl; $\blacktriangle$, NaBr; $\square$, Na_2SO_4 ; $\blacksquare$, NaI; $\triangle$, NaClO_4 ; $\circ$, NaSCN.

ricin in buffers containing NaSCN and NaClO_4 did not inactivate the toxin. Furthermore, as shown in Fig. 2, the protection increased when the cells were incubated with SCN^- before ricin was added, indicating that the protection obtained is due to an effect of these ions on the cells.

Since the intoxication of cells by ricin involves binding of toxin to cell surface receptors and endocytosis of the toxin molecules, we tested if either one of these processes was affected by SCN^- . After incubation of Vero cells in the absence and presence of SCN^- for 1 h, binding was tested in two different ways. In one case we measured binding of ^{125}I -ricin in the absence and presence of SCN^- as described in the Materials and methods section. We also measured toxin binding in a functional assay where we estimated the amount of toxin bound from its subsequent ability to intoxicate cells. In this assay we added unlabelled ricin to Vero cells in the absence or presence of SCN^- , incubated the cells for 30 min to

Fig. 2. Effect of preincubation time with NaSCN on the sensitivity of Vero cells to ricin

Vero cells growing in 24-well disposable trays were incubated for 1 h at 37°C in a buffer containing 20 mM-Hepes, pH 7.2, 1 mM- $\text{Ca}(\text{OH})_2$, 5 mM-glucose and 0.14 M of the salts indicated below for increasing periods of time before ricin was added. After 3 h incubation at 37°C in the presence of toxin, protein synthesis was measured as described in the Materials and methods section. The additions were: $\times$, 0.14 mM-NaCl; $\circ$, 0.14 mM-NaSCN added immediately before ricin; $\triangle$, 0.14 mM-NaSCN added 15 min before ricin; $\square$, 0.14 mM-NaSCN added 30 min before ricin.

allow binding to occur, washed the cells to remove unbound toxin, and then transferred them to normal medium. Finally, we measured the rate of protein synthesis in the cells. Both methods indicated that binding of ricin to the cell surface was unaffected by SCN^- . Also endocytosis of ^{125}I -ricin, measured as the amount of cell-associated ricin that could not be removed with lactose (Sandvig & Olsnes, 1979), was not reduced by the presence of the chaotropic anions (results not shown). Therefore, SCN^- apparently inhibits a late step in the entry of ricin, possibly the transfer of ricin across the limiting membrane of an intracellular compartment. All the experiments described in this paper were also carried out with the toxic lectin abrin, which has similar requirements to ricin for entry (Sandvig & Olsnes, 1982). The results obtained with abrin were similar to those obtained with ricin (results not shown).

Chaotropic anions like SCN^- and ClO_4^- are known to lower the interfacial pH of charged lipid monolayers (Fromherz & Masters, 1974; McLaughlin *et al.*, 1975). We have earlier presented evidence that ricin and abrin, in contrast with diphtheria

Fig. 3. Effect of pH on the ability of SCN^- to protect Vero cells against ricin

Vero cells growing in 24-well disposable trays were incubated for 1 h at 37°C in buffers containing 20 mM-Hepes, pH 7.2 or pH 7.6, 1 mM- $\text{Ca}(\text{OH})_2$, 5 mM-glucose and 0.14 M of the salts indicated below. Then increasing concentrations of ricin were added and, after 3 h incubation at 37°C, protein synthesis was measured as described in the Materials and methods section. The additions were: ○, 0.14 mM- NaCl , pH 7.2; ●, 0.14 M- NaSCN , pH 7.2; □, 0.14 M- NaCl , pH 7.6; ■, 0.14 M- NaSCN , pH 7.6.

toxin and modeccin, do not require low pH at any stage of their entry route. On the contrary, compounds known to increase the pH of intracellular compartments increase the sensitivity of cells to these toxins, and low pH in the medium protects

cells against abrin and ricin (Sandvig & Olsnes, 1982; Olsnes & Sandvig, 1983). We therefore tested if the protective effect of SCN^- could be overcome by increasing the pH of the buffer. As shown in Fig. 3, this was indeed the case. When the pH in the buffer was increased from 7.2 to 7.6, the protective effect of SCN^- was completely abolished. If the protection afforded by the chaotropic anions is indeed due to their ability to reduce the pH close to the membrane below that in the bulk solution, it is likely that even a slightly increased pH in the medium may be sufficient to raise the pH close to the membrane to a value that permits toxin entry.

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