PERMEABILIZATION OF MAMMALIAN CELLS TO PROTEINS BY THE IONOPHORE NIGERICIN

Miguel A. ALONSO and Luis CARRASCO
Department of Microbiology, Centro de Biología Molecular, Universidad Autónoma, Madrid-34, Spain

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1. Introduction

Little is known about the mechanisms by which macromolecules cross the membrane of cells [1]. There are a number of examples that illustrate the passage of intact proteins into the interior of cells. In some instances internalization involves binding to a cellular receptor followed by the formation of a coated pit in such a way that the protein appears in a coated vesicle. The macromolecule still has to cross the membrane of the coated vesicle to enter the cytoplasm of the cell. A number of protein toxins are composed of two subunits; one of these subunits, the haptomer, is involved in the attachment of the toxin to the cell surface and promotes, by an unknown mechanism, the passage of the other subunit, or effectomer, into the cell [2,3]. Abrin, ricin and several other analogous plant toxins are specific inhibitors of protein synthesis in eukaryotic cells; in these cases the effectomer has the property of enzymatically degrading the ribosome [4].

We have observed that the effectomer of some of these toxins was able to cross the cell membrane and reach the cellular cytoplasm when animal viruses absorbed to the cells [5]. This result suggests that entry of the virus into the cell increases the permeability of the cell membrane and allows the passage of these macromolecules into the cytoplasm. We have also reported that some ionophores permeabilize the cell to low molecular mass (M_r) compounds [7]. It was of interest therefore to determine whether the treatment of cells with some ionophores renders the cell permeable to macromolecules. The results are presented here.

2. Materials and methods

2.1. *Cell lines*

Human HeLa cells, mouse L929 and 3T3 cells, hamster BHK cells and monkey Vero cells were grown and propagated in Dulbecco's modified Eagle's medium supplemented with 10% calf serum.

2.2. *Assay of protein synthesis*

Cells were grown in 24 well plates containing 1 ml Dulbecco modified Eagle's medium supplemented with 10% newborn calf serum. When the cell monolayer was confluent, the medium was removed and replaced by 0.5 ml methionine-free Eagle's medium supplemented with 1% newborn calf serum and containing the indicated concentrations of nigericin and alpha-sarcin. Labelling of newly synthesised proteins was carried out by incubating the cell cultures with 0.12 μCi [^{35}S]methionine (1375 Ci/mmol) from 4–5 h after the addition of the inhibitors or from zero time. Then, the medium was removed and the cell monolayer washed with phosphate buffer and then 1 ml 5% trichloroacetic acid was added. The precipitated cell monolayer was then washed 3 times with ethanol and dried under an infrared lamp. 250 μl 0.1 N NaOH with 1% sodium dodecyl sulphate were added and the mixture was kept for 15 min at room temperature. 100 μl were withdrawn and counted in a liquid scintillation spectrometer.

2.3. *Source of inhibitors*

Alpha-sarcin, mitogillin and restrictocin were a generous gift from D. M. Shuurmans (Department of Public Health; Lansing MI) and J. E. Davies (University of Wisconsin, Madison WI); ricin A chain and abrin A chain were gifts from S. Olnes (Norsk Hydro's Institut

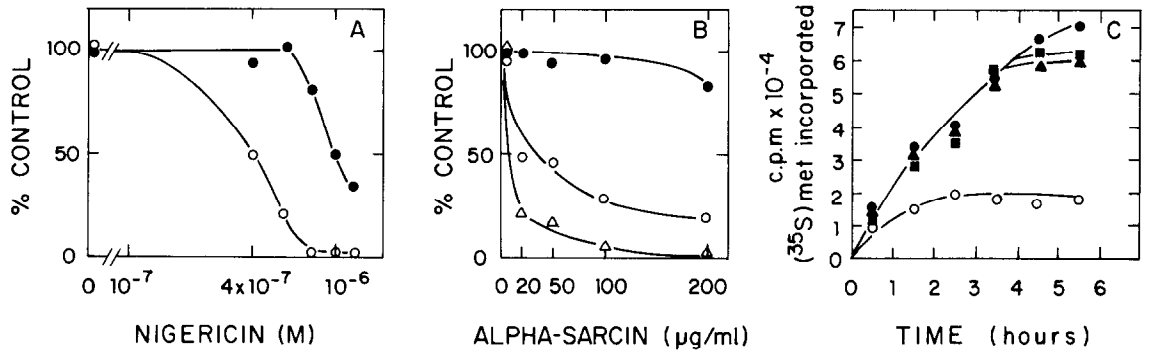

Fig.1. Effect of alpha-sarcin and nigericin on protein synthesis in HeLa cells. Human HeLa cells were grown as described and pulsed with [^{35}S]methionine from 4–5 h after the addition of the inhibitors (A,B) or from zero time (C). Protein synthesis was estimated as in section 2. (A) (•) Nigericin; (○) nigericin + 20 μg alpha-sarcin/ml. (B) (•) Alpha-sarcin; (○) alpha-sarcin + 4 × 10^{-7} M nigericin; (△) alpha-sarcin + 6 × 10^{-7} M nigericin. (C) (•) No additions; (▲) 6 × 10^{-7} M nigericin; (■) 100 μg alpha-sarcin/ml; (○) 6 × 10^{-7} M nigericin + 100 μg alpha-sarcin/ml.

for Kreftforskning; Oslo). Nigericin was from Lilly Laboratories.

3. Results and discussion

The effectomer moiety of some toxins blocks protein synthesis in cell-free systems by enzymatically degrading the ribosome [2,4]. Cultured cells are impermeable to this effectomer molecule; however, it is able to cross the cell membrane and reach the cellular cytoplasm when viruses infect animal cells [5]. To test if ionophores are also able to permeabilize animal cells to macromolecules, human HeLa cells were incubated both with the ionophore nigericin and the toxin alpha-sarcin. The latter is a protein of 16 800 M_r and is produced by *Aspergillus giganteus*. Alpha-sarcin inhibits translation in cell-free systems from eukaryotic cells, but not in intact cells, presumably because it is unable to cross the membrane of intact cells and reach the ribosomes [5]. The results shown in fig.1 indicate that the presence of nigericin in the culture medium allowed the entry of alpha-sarcin into HeLa cells. The finding that protein synthesis was inhibited by alpha-sarcin indicates that the toxin reaches the ribosomes in an active form, although we do not know whether it is partially degraded or not. In order to test whether the increased permeability induced by the presence of nigericin is specific we tested for the entry of other toxin moieties that do not cross the membrane by themselves. Table 1 indicates that in all cases the presence of nigericin promoted the inhibition of protein synthesis by these proteins.

Fig.2 shows the permeabilization to alpha-sarcin by nigericin in different cell lines. In all instances nigericin permeabilized the cells to this macromolecule although to different extents. Mouse and hamster cells were permeabilized at $<10^{-8}$ M nigericin, whereas

Table 1
Effect of different toxins on protein synthesis in nigericin-treated HeLa cells

Toxin	Concentration (μg/ml)	Minus nigericin		Plus 6 × 10^{-7} M nigericin	
		cpm	%Control	cpm	%Control
Control	–	32 174	100	27 235	100
Alpha-sarcin	100	30 010	95	1002	3
Mitogillin	100	30 693	94	906	3
Restrictocin	100	30 174	92	2097	6
Ricin A chain	9	28 337	87	7841	24
Abrin A chain	9	28 101	87	9335	29

Protein synthesis was measured as described in fig.1(A,B)

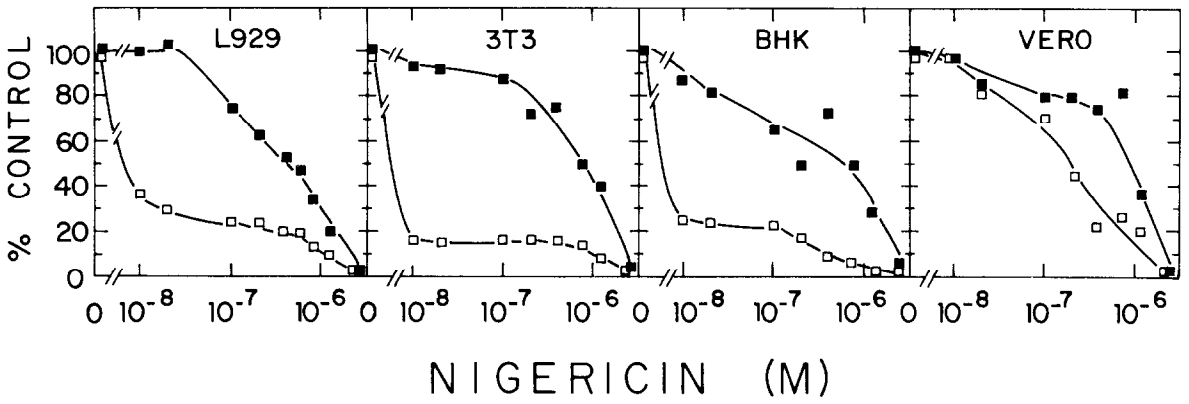

Fig.2. Effect of alpha-sarcin and nigericin on protein synthesis in a variety of mammalian cell lines. The experimental conditions were as in fig.1 (A,B): (■) control; (□) + 20 μg alpha-sarcin/ml.

human and monkey cells needed higher concentrations of the ionophore to allow the passage of alpha-sarcin. This permeabilization method is reversible. The treatment of human HeLa cells with 8×10^{-7} M nigericin for 6 h and subsequent washing of the ionophore has no subsequent effect on cell growth (not shown).

These findings, in addition to revealing an unsuspected action of nigericin on the membrane, provide an alternative and very easy method for introducing proteins into cells. It is difficult to quantitate the efficiency of this permeabilization method compared with the microinjection or the liposome techniques. A possible disadvantage of the nigericin method is the side effects that nigericin by itself could have on cell metabolism. However, the concentrations of nigericin necessary to permeabilize the cell are low and it can be removed from the medium after the protein has been introduced into the cell. Alternatively UV-inactivated virus can also be used for permeabilization with no major consequences for cell metabolism (as in [5,6]).

Our findings also raise questions about the mechanism by which nigericin promotes the entry of toxins into cells. We had observed that a decrease in membrane potential was sufficient to permeabilize human cells to low M_r compounds [7]. However, this mechanism does not seem to operate in this instance because in a medium with high $[K^+]$ and low $[Na^+]$ the toxins did not cross the membrane. By contrast, the presence of other membrane-active compounds like valinomycin also promotes to some extent the inhibition of protein synthesis by alpha-sarcin.

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