MECHANISM OF ACTION OF CESALIN, AN ANTITUMOR PROTEIN*

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SUMMARY

A protein, cesalin, isolated from Caesalpinia gilliesii is cytotoxic to KB cells in tissue culture. It has been shown to bind to the plasma membrane of this cell line and to inhibit Na^+,K^+-ATPase (ATP phosphohydrolase EC 3.6.1.3). Similar studies with HTC cells show no cytotoxicity or inhibition of plasma membrane Na^+,K^+-ATPase. The Na^+,K^+-ATPase of human erythrocytes and rat brain and kidney tissues are not inhibited. 5'-Nucleotidase and Mg^{++}-ATPase are not inhibited by cesalin in any cells tested.

INTRODUCTION

It is well known that the interaction of some proteins with cell surface membranes may alter intracellular processes. Numerous studies have shown that peptide hormones bind to specific receptors on the exterior surface of the plasma membrane (1). The enterotoxin secreted by Vibrio cholerae interacts with eucaryotic cell membranes and stimulates adenylate cyclase (2). The toxin of Corynebacterium diphtheriae binds to specific ganglioside receptors on the membrane surface and, after penetration into the cell, inhibits protein synthesis by enzymatic modification of elongation factor 2 (3). Varied effects of lectin interaction have been observed, some of which concern transport processes. Phytohemagglutinin stimulates transport of uridine into lymphocytes (4) while treatment of chick embryo fibroblasts with concanavalin A or Robinia pseudoacacia lectin inhibits incorporation of thymidine into DNA without inhibiting transport into the cells (5).

We report a protein, cesalin, purified from the seeds of Caesalpinia gilliesii, which possesses antitumor activity. It appears to act in tissue

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culture by directly altering transport functions. Cesalin has been shown to bind to plasma membranes and to affect the Na^+,K^+-ATPase of cells for which it is cytotoxic.

MATERIALS AND METHODS

KB (human epidermoid carcinoma derived) cells are routinely subcultured by seeding at 4 x 10^4 cells/cm^2 in Falcon monolayer flasks in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal calf serum and 100 units/ml penicillin and 100 µg/ml streptomycin. HTC (rat hepatoma derived) cells (6) are seeded at 2-2.5 x 10^5 cells/ml in spinner flasks with Swim's S-77 medium supplemented with cystine, glutamine, penicillin, streptomycin, 5% fetal calf serum and 5% calf serum. All media and supplements were purchased from Grand Island Biological Company. Ouabain was purchased from Sigma Chemical Co. FITC-conjugated IgG from goat anti-rabbit IgG serum was purchased from Miles Laboratories.

Protein concentrations of solutions were determined by the method of Lowry (7) using crystalline bovine serum albumin as standard. Due to the presence of substances interfering with the Lowry method, total KB cell protein was determined by the microbiuret method of Goa (8).

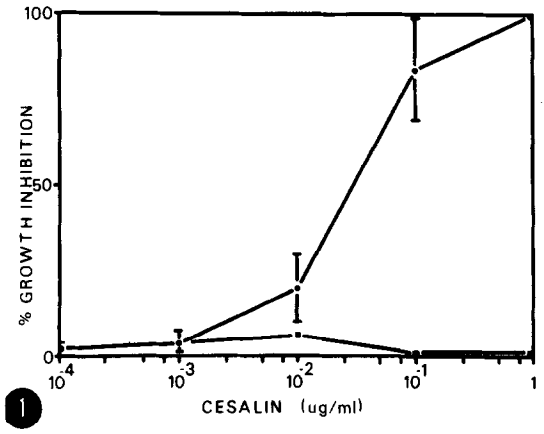
5'-Nucleotidase was assayed by the method of Michell and Hawthorne (9) and released phosphate measured by the method of Lowry (10). Phosphate released during ATPase assays was determined by the method of Marsh (11) due to the instability of ATP in the system of Lowry.

Growth inhibition was determined by adding cesalin at various concentrations in phosphate buffered saline (PBS) (10 mM Na phosphate, 0.14 M NaCl, 4 mM KCl, pH 7.5) to KB monolayer cultures according to the procedure of Smith, et al. (12) or to HTC spinner cultures. Growth of cesalin-treated cultures and PBS-treated controls was monitored by hemocytometer counting of HTC cells or, for KB cells, determination of total cell protein after 4 days growth.

Cesalin was prepared from the meal of Caesalpinia seeds (13). Na^+,K^+ ATPase was partially purified by preparation of a plasma membrane-enriched fraction from KB and HTC cells using the method of Butters and Hughes (14). Plasma membrane-enriched microsomes from rat brain and kidney were prepared according to the method described by Dorling and LePage (15) for rat liver plasma membranes. Na^+,K^+-ATPase was determined as ouabain-sensitive activity by incubation of the enzyme in buffer containing 25 mM HEPES, 3 mM $MgCl_2$, 0.13 M NaCl, 20 mM KCl, 3 mM ATP (final concentrations) in the presence and absence of 10^{-3} M ouabain (16). The incubation was for 20 min. at 37° with enzyme concentrations at which the reaction rate was linear for at least 30 min. Reactions were started by the addition of ATP.

RESULTS AND DISCUSSION

The growth response of HTC and KB cells to various levels of cesalin are shown in Figure 1. The concentration that inhibits growth of KB cells by 50% (ED_{50}) is 2.0 - 5.0 x 10^{-2} µg/ml. Growth of HTC cells was unaffected by concentrations up to 11 µg/ml. Cesalin-treated KB cells appear by phase contrast



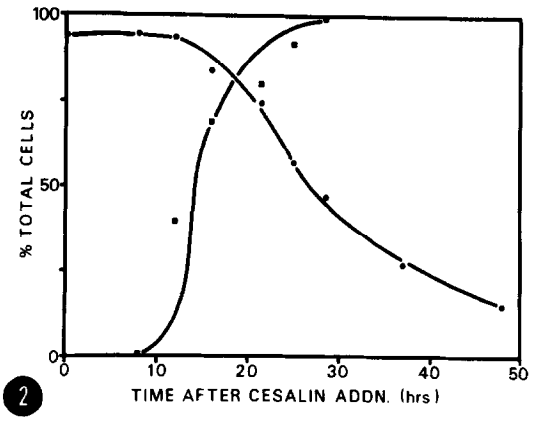


Figure 1 Inhibition of growth of KB and HTC cells by cesalin; for experimental procedure see Materials and Methods. ●—● KB cells ■—■ HTC cells.

Figure 2 Effect of cesalin (1mg/ml) on KB cells; ■—■ percentage of cells that have spontaneously released from substratum; ●—● percentage of cells that exclude trypan blue.

microscopic examination to be morphologically normal for 1-10 hrs. After 12-16 hours they begin to detach from the substratum and by 24 hours they have all released. The treated cells show no reduction in their ability to exclude trypan blue dye until 14-16 hours after cesalin addition. After 24 hours 50% of the cells still exclude dye. The time course of these events is shown in Figure 2. Cells which are harvested from the surface of the flask with trypsin (0.25% in Earle's balanced salts, 5 min., 37° C) or EDTA (0.5 mM in PBS, 7 min., 37° C) during the first 6 hours of cesalin treatment will reattach themselves to a new culture flask surface. This property was lost after 7 hours of cesalin treatment. However, cesalin-treated cells that have reattached in the absence of further cesalin exposure will begin to detach 12-16 hours after the initial cesalin addition and begin to die just as do the cells left in continuous contact with cesalin. Cells treated with cesalin for as little as 30 minutes, the shortest time tested, could not be rescued from eventual death by either trypsin or EDTA treatment (see above) or multiple washings with Earle's balanced salts solution or complete culture medium.

Assay of the membrane phosphatases showed that cesalin inhibits ouabain-

Table 1

Inhibition of Na^+,K^+-ATPase
By Cesalin

Cesalin Added (ug)	Na^+,K^+-ATPase Activity (umoles Pi/mg·hr)[1]	% Inhibition
0	4.98 ± 0.96[2]	–
1	3.54 ± 0.83	29
10	3.09 ± 0.69	38
100	3.53 ± 1.0	29

[1] Na^+,K^+-ATPase activity determined as that ATPase activity inhibited by 10^{-3} M ouabain. Enzyme source is plasma membrane of KB cells purified 6X by density gradient centrifugation.

[2] S.E.M.

sensitive Na^+,K^+-ATPase but not 5'-nucleotidase or Mg^{++}-ATPase in KB plasma membranes. The degree of inhibition of Na^+,K^+-ATPase by various levels of cesalin is shown in Table 1. The total ATPase activity inhibited by cesalin was never greater than that inhibited by 10^{-3} M ouabain, which inhibition could not be increased further by adding cesalin. Na^+,K^+-ATPase from HTC cells, whose growth is unaffected by cesalin, is not inhibited at all under these conditions. Similarly, Na^+,K^+-ATPases from human erythrocytes and rat brain and kidney were not inhibited.

Studies with [125I] cesalin indicated that it binds to the plasma membrane of KB cells and not to HTC cell plasma membranes. Corroborative evidence that cesalin acts at the cell surface comes from indirect fluorescent antibody

staining of cesalin-treated KB cells. Cells were incubated 60 min with cesalin, washed and incubated with rabbit antiserum against cesalin. The cells were again washed extensively and incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit γ-globulin. Intense membrane fluorescence was observed on cesalin-treated cells with minimal or no non-specific staining of buffer-treated controls or controls without cesalin-specific antiserum. It follows that cesalin was present on the cell membrane after 1 hour, by which time cytotoxic action is known to be irreversible.

Inhibition of Na^+,K^+-ATPase would be expected to result in dysfunction of ion transport and ion-coupled transport of other metabolites. Cesalin treatment of KB cells has been shown in preliminary studies to alter glucose and nucleoside transport. Similar changes in the transport of these materials can be produced by the addition of ouabain. While uptake of thymidine and uridine by cesalin-treated KB cells is significantly reduced, rupturing the cell membrane shows that the cells' ability to incorporate nucleosides into macromolecular products is nearly unaffected. This supports the proposal that cesalin interacts with the plasma membrane without inhibiting nucleic acid synthesizing enzymes.

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