Novel Blockade of Cell Surface Expression of Virus Glycoproteins by Leucinostatin A

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The nonapeptide leucinostatin A (LSA) inhibited syncytium formation without profoundly affecting HN glycoprotein synthesis in Newcastle disease virus (NDV)-infected BHK cells. At similar doses of LSA, cytopathic effect and infectious virus production were suppressed in vesicular stomatitis virus (VSV)-infected BHK cells. Blockade by LSA of cell surface expression of NDV-HN and VSV-G glycoproteins was demonstrated, accompanied by intracellular accumulation of these virus glycoproteins. LSA acts as an inhibitor of mitochondrial F-type H^+-translocating ATPase, a key enzyme in the generation of ATP, but its action against cell surface expression of virus glycoproteins was independent of the depletion of intracellular ATP. LSA also acts as an ionophore, but its action on intoxication by ricin and diphtheria toxin was different from that of monensin. This novel action of LSA is expected to be useful in investigation of the mechanism of intracellular trafficking of proteins.

Glycoproteins play a pivotal role in diverse aspects of cell physiology. Glycoproteins are synthesized on the rough endoplasmic reticulum with concomitant glycosylation, and are translocated intracellularly through the Golgi apparatus *en route* to their respective destinations such as the cell surface, extracellular milieu, lysosomes, and other intracellular organelles. This trafficking is achieved by repeated cycles of budding and fusion of transport vesicles[1]. Much effort has been devoted to unraveling the mechanism of intracellular trafficking. Inhibitors of trafficking are powerful tools in the study on intracellular trafficking, as shown by our discovery of the novel action of brefeldin A (BFA)[2] and subsequent advances in its application[3]. However, only a very limited number of compounds have been shown to affect intracellular trafficking processes. In the search for inhibitors of intracellular trafficking of glycoproteins, the present study showed that leucinostatin A (LSA) blocked cell surface expression of virus glycoprotein without profoundly affecting its synthesis. LSA, a nonapeptide antibiotic[4,5], acts as an uncoupler on mitochondria[6] and inhibits oxidative phosphorylation and mitochondrial F-type H^+-translocating ATPase (F-ATPase)[7]. Recently, LSA was demonstrated to act as a weak ionophore[8]. Here, we investigated the action of LSA on intracellular trafficking in relation to these reported characteristics.

Materials and Methods

Reagents and Viruses

LSA was isolated from a culture of an unidentified fungus and identified spectrometrically (fast-atom bombardment mass spectrometry and ^1H and ^{13}C NMR spectroscopies). Newcastle disease virus (NDV, the Miyadera strain) and vesicular stomatitis virus (VSV, New Jersey serotype) were obtained from the National Institute of Animal Health of Japan, and cultured as described previously[9]. Unless otherwise specified, all chemicals used in the present study were obtained from Sigma (St. Louis, MO), and were of analytical grade.

Syncytium Formation and Cytopathic Effect

Monolayer cultures of baby hamster kidney (BHK) cells in 96-well microtitre plates were infected with NDV or VSV, and syncytium formation (SF) and cytopathic effects (CPE) in NDV- and VSV-infected cells, respectively, were observed under an optical microscope[9]. Infectious virus production was quantified and expressed as cytopathic units (CPU): the medium fraction of VSV-infected BHK cells in each well was serially diluted 2-fold and added to BHK cells in 96-well microtitre plates, and CPU was expressed as the maximum number of dilutions tolerated to cause CPE.

Hemagglutination and Hemadsorption

Synthesis of NDV-HN glycoprotein was quantified by determining hemagglutination units (HAU) in whole lysates of NDV-infected cells[9]. NDV-HN expressed on the cell surface was quantified by hemadsorption (HAD) as described previously[10]. Briefly, to NDV-infected BHK cells in 6-well plates was added a 2-ml suspension of 1% (v/v) chicken red blood cells. The cells were maintained at 4°C for 30 minutes with occasional stirring. Unadsorbed red blood cells were removed and adsorbed red blood cells were quantified by measuring absorption at 550 nm.

Fluorescence-activated Cell Sorting (FACS)

Suspension cultures of FM3A cells in Dulbecco-modified EAGLE's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% calf serum were infected with VSV at an input multiplicity of infection of about 5 plaque-forming units/cell and incubated for 1 hour at 37°C. Unadsorbed viruses were removed by centrifugation in a bench-top centrifuge. Infected cells were suspended in medium with or without inhibitors, incubated at 37°C for a further 7 hours, collected by centrifugation, and fixed with 0.3% paraformaldehyde. The fixed cells were treated with anti-VSV-G antibody and stained with fluorescein isothiocyanate-labeled anti-rabbit IgG antibody as described previously[9]. The stained cells were analyzed with a FACSort (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Immunofluorescence Microscopy

BHK cells on glass coverslips were infected with VSV, and treated with drugs as specified in the figure legend. The procedure for the immunofluorescence staining of cell surface and intracellular VSV-G glycoprotein was the same as reported previously[9]. One set of cells was processed for immunostaining after fixation to stain cell surface VSV-G, and another after treatment with methanol following fixation to stain intracellular VSV-G.

Vital Stain of the Golgi Apparatus with *N*-{6-[7-Nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproyl}-sphingosine

BHK cells grown on glass coverslips were treated with drugs as specified in the figure legend. *N*-{6-[7-Nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]caproyl}-sphingosine (C_6-NBD-ceramide; Molecular Probe, Eugene, OR) was then added to stain the Golgi apparatus. Uptake and back-exchange of C_6-NBD-ceramide were done according to the reported method[11], and Golgi stain was observed under a fluorescence microscope.

Preparation and Assay of ATPases

Mitochondria and vacuoles were prepared from protoplasts of the yeast *Saccharomyces cerevisiae*[12,13], and F-ATPase and vacuole-type H^+-translocating ATPase (V-ATPase) activities were determined by quantitating liberated inorganic phosphate as described previously[9].

Intracellular ATP Content

Intracellular ATP content was determined using virus-infected cells to correlate directly the inhibition of SF and CPE with ATP content. Virus-infected BHK cells in microtitre plates were disrupted by 5 cycles of sonication with a Sonifier at the minimum output and 50% duty cycle, and ATP therein was quantified with a luciferin-luciferase kit (Kikkoman Co., Chiba, Japan) according to the manufacturer's recommendation. Sonication and quantitation in each well was done one well at a time, and cycle durations were kept as equal as possible to avoid error caused by degradation of ATP after cell disruption.

Intoxication by Ricin and Diphtheria Toxin

BHK cells in microtitre plates were pretreated with designated concentrations of LSA, BFA or monensin for 5 hours. Ricin or diphtheria toxin was then added, and incubation was continued for a further 1 hour. Thereafter, protein synthesis was followed by quantitating incorporation of [^{35}S]methionine (1 μCi/ml) into 10% trichloroacetic acid-insoluble fractions[14].

Methylorange Transfer

Ionophore activity of LSA was determined by a previously reported method[15]. Briefly, 200 μl of 2 mg/ml methylorange was added to 2 ml of phosphate buffer (0.1 M, pH 6.0) containing a designated concentration of LSA. The mixture was held at room temperature for 30 minutes and then extracted with 2 ml of *n*-butanol, and absorption at 430 nm was measured to quantitate methylorange transferred from the aqueous to the butanol phase.

Results

LSA Blocks SF, CPE and Infectious Virus Production without Profoundly Affecting Virus Glycoprotein Synthesis

Confluent monolayer cultures of BHK cells in microtitre plates were infected with NDV or VSV. At 1 hour after infection, designated concentrations of LSA were added, and incubation was continued for a further 18 hours as described in Materials and Methods. LSA suppressed SF, with a MIC value of 20 nM (Table 1). At a similar drug concentration range, CPE and infectious virus production in VSV-infected cells were profoundly inhibited. LSA partly inhibited NDV-HN glycoprotein synthesis as quantitated by hemagglutination at doses at which SF was profoundly suppressed, but no remarkable inhibition was demonstrated at any concentration up to 20 μM, 1000 times greater than the MIC. Synthesis of both NDV and VSV glycoproteins depends on cellular

Table 1. Effect of LSA on virus-infected cells.

Concentration (μM)		20	10	5	2.5	1.25	0.63	0.31	0.16	0.08	0.04	0.02	0.01	0.005
VSV	CPE	−	−	−	−	−	−	−	−	−	−	−	++	++
	CPU	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	3.1	3.1	12.5	37.5	50
NDV	SF	−	−	−	−	−	−	−	−	−	−	−	+	++
	%HAU	82	82	82	82	82	82	82	82	82	82	82	100	100

Confluent monolayer cultures of BHK cells in 96-well microtitre plates were infected with VSV or NDV. LSA was added at 1 hour after infection and the cells were incubated for a further 18 hours. CPE, CPU, SF and HAU were determined as described in Materials and Methods. The degrees of CPE and SF were expressed as follows: -, none; +, moderate; ++, severe.

Table 2. Reversibility of the action of LSA on VSV multiplication.

Concentration (μM)		20	10	5	2.5	1.3	0.63	0.31	0.16	0.08	0.04	0.02	0.01	0.005
LSA	Present throughout	−	−	−	−	−	−	−	−	−	−	−	++	++
	Removed after 1 hour	−	−	−	−	−	−	−	−	−	−	−	++	++
	2 hours	−	−	−	−	−	−	−	−	−	−	−	++	++
	4 hours	−	−	−	−	−	−	−	−	−	−	−	++	++
BFA	Present throughout	−	−	−	−	−	−	+	++	++	++	++	++	++
	Removed after 4 hours	++	++	++	++	++	++	++	++	++	++	++	++	++

Confluent monolayer cultures of BHK cells in 96-well microtitre plates were infected with VSV, and LSA or BFA was added at 1 hour after infection. Cells were washed with fresh medium three times at the indicated times. The degree of CPE was determined at 14 hours after the infection, as described in Table 1.

machineries, and it might therefore be said that LSA inhibits CPE and infectious virus production in VSV-infected cells even though VSV-G was not quantified in the present experiments. This was supported by the observation of intracellular accumulation of VSV-G in the presence of LSA (see below). Virus glycoprotein synthesis under the present experimental conditions reflects virus mRNA synthesis followed by synthesis and glycosylation of virus proteins because LSA was added at an early stage of virus multiplication. The results shown in Table 1 therefore suggest that LSA inhibits SF and infectious virus production without profoundly affecting synthesis of virus-coded macromolecules.

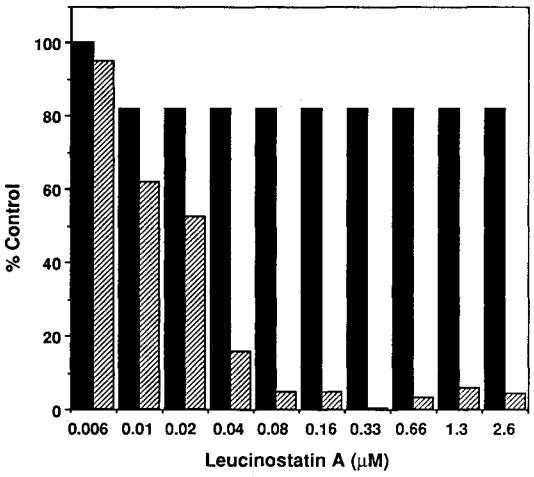
LSA is an inhibitor of mitochondrial F-type H^+-translocating ATPase. It has been shown to bind to F-ATPase, and the binding site has been suggested to be located in or near the F_0 sector of F-ATPase[7]. In this connection, we examined the reversibility of the action of LSA against CPE. LSA was added to BHK cells at 1 hour of VSV infection and then washed out after 1, 2 or 4 hours of treatment. CPE was observed at 14 hours after infection. As shown in Table 2, CPE in BHK cells treated with LSA for 1 hour was inhibited to the same degree as in those treated with LSA throughout the experiment. BFA was used as a control drug to ensure that the washing procedure employed in this experiment was sufficient to remove drug. The inhibitory action of BFA was reversed even after 4 hours of treatment as determined at the same time. This poor reversibility of the action of LSA against VSV multiplication was in a good accordance with its action against F-ATPase, but it remains for further studies to show whether the blockade of infectious virus production was caused by the inhibition of F-ATPase.

Effect of LSA on Cell Surface Expression of Virus Glycoproteins

SF in NDV-infected cells takes place after the causative virus glycoprotein, F glycoprotein, is expressed on the cell surface. There are three possible explanations for the suppression of SF by LSA without inhibition of glycoprotein synthesis: (1) blockade of cell surface expression of virus glycoprotein; (2) production of virus glycoprotein nonfunctional in SF; or (3) blockade of the SF process itself. To determine which of these causes the blockade of SF without profoundly affecting glycoprotein synthesis, we next investigated the effect of LSA on cell surface expression of virus glycoproteins. BHK cells in 6-well plates were infected with NDV and incubated at 37°C for 14 hours in the presence of LSA at concentrations indicated in Fig. 1, and the total and cell surface-expressed NDV-HN glycoprotein were quan-

tified by hemagglutination and hemadsorption activities, respectively. To quantify the total amount of NDV-HN synthesized, whole NDV-infected cultures were disrupted by brief sonication, and chicken red blood cells were added to determine hemagglutination activity in the lysates. Again, no profound decrease in HAU was observed at any concentration tested (Fig. 1, ■).

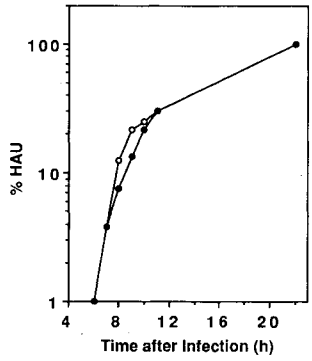
Fig. 1. Blockade of cell surface expression of NDV-HN glycoprotein by LSA without profound action on its synthesis.



Monolayer cultures of BHK cells in 6-well or microtitre plates were infected with NDV, and LSA of indicated concentrations was added to the cultures at hours after the infection. % HAU (■) and % HAD (▨) was determined at 14 hours of infection. Synthesis of NDV-HN protein was quantified by determining HAU in whole lysates of infected cultures in microtitre plates, and its cell surface expression was quantified by measuring the amounts of chicken red blood cells adsorbed to intact, infected cells in 6-well plates. Results are expressed as a % of control value.

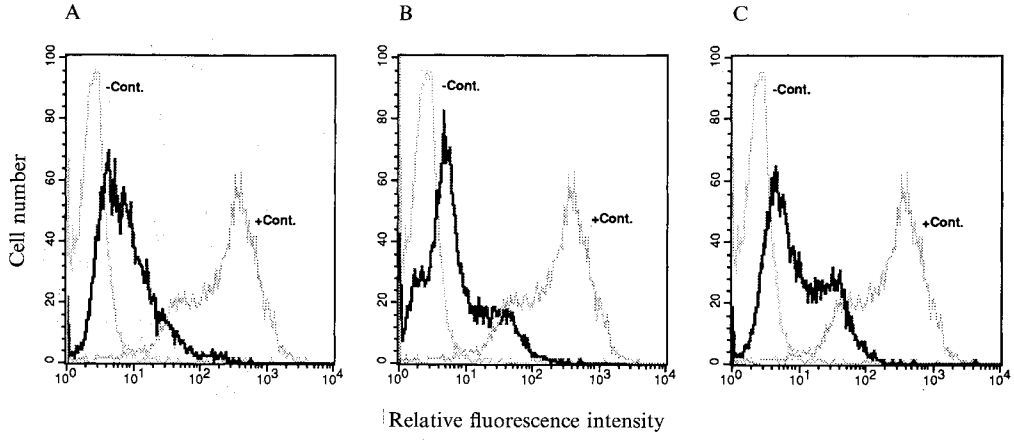
However, the binding of extracellularly added chicken red blood cells to the surface of intact NDV-infected cells, as expressed as % HAD, decreased depending on the concentration of LSA (Fig. 1, ▨), indicating that LSA blocks dose-dependently the cell surface expression of NDV-HN glycoprotein. Taken together, these results indicate LSA blocks cell surface expression of NDV-HN without profoundly affecting its synthesis. One further possibility remained for the decrease in cell surface-expressed NDV-HN, namely that delayed synthesis of NDV-HN was followed by the apparent suppression of its cell surface expression. This possibility was ruled out by the observation shown in Fig. 2. The kinetics of NDV-HN synthesis in LSA-treated cells was delayed

Fig. 2. Effect of LSA on the kinetics of NDV-HN synthesis.



Monolayer cultures of BHK cells infected with NDV were incubated in the presence (●) or absence (○) of 0.16 μM LSA. At indicated times after infection, cells were disrupted by sonication and total HAU were determined as described in Material and Methods. The HAU of control at 22 hours is set at 100%.

Fig. 3. Blockade of cell surface expression of VSV-G protein as determined by FACS.

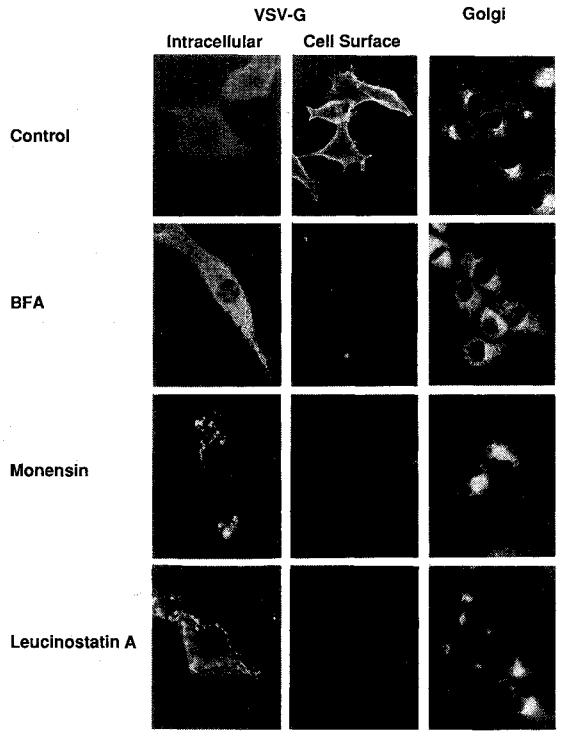


Suspension cultures of FM3A cells were infected with VSV, and unadsorbed virus was removed after 1 hour of adsorption. The infected cells were then suspended in the medium without or with LSA (0.13 μM), BFA (2 μg/ml), monensin (5 μg/ml) or cycloheximide (10 μg/ml), and incubated for a further 8 hours at 37°C. The cells were collected, fixed with paraformaldehyde, treated with anti-VSV-G antibody, stained with FITC-conjugated anti-rabbit IgG antibody, and then analyzed by FACS as described in Materials and Methods. The results with the false- or cycloheximide-treated cells are indicated as +control and −control, respectively, and superimposed on the results of cells treated with BFA (A), monensin (B) or LSA (C).

only slightly compared with that of the control, and this delay is not sufficient to explain the nearly complete suppression of cell surface expression of NDV-HN glycoprotein shown in Fig. 1.

Blockade of cell surface expression of virus glycoprotein was also demonstrated in the case of VSV-G glycoprotein by FACS analyses (Fig. 3). FM3A cells were utilized because their growth in suspension facilitates the use of FACSort. BFA and monensin were used as control drugs in this study. BFA is a potent inhibitor of intracellular trafficking[2], and most of the cells were counted between 4 and 10 relative fluorescence intensity as shown in Fig. 3A. Monensin is another inhibitor of trafficking, but its action was not as remarkable as that of BFA and a minor portion of VSV-G protein was translocated to the cell surface as indicated by the presence of weakly fluorescence-labeled cells demonstrated as a shoulder at a relative fluorescence intensity of 10 to 60 (Fig. 3B). Most of the cells treated with LSA were sorted at a relative fluorescence intensity similar to that of BFA-treated cells, but, as in the case of monensin, a small peak of weakly stained cells was observed (Fig. 3C). This blockade of cell surface expression of VSV-G was not caused by inhibition of VSV-G protein synthesis, because intracellular accumulation of VSV-G protein was demonstrated by immunofluorescence microscopy (Fig. 4). Without methanol treatment, which facilitates internalization of antibodies into the cell, only VSV-G expressed on the cell surface is stained. This was demonstrated by the observations shown in Fig. 4: the cell surface of control cells was stained strongly whereas that of cells treated with inhibitors of intracellular trafficking, BFA and monensin were not. On the contrary, prominent intracellular staining was demonstrated with BFA- or monensin-treated cells. A strong intracellular, but not cell surface, stain was demonstrated with LSA-treated cells, indicating the blockade of cell surface expression and a concomitant intracellular accumulation of VSV-G glycoprotein in LSA-treated cells. The localization of the clouds of stain in LSA-treated cells was different from that in cells treated with BFA or monensin, which block intracellular trafficking from the endoplasmic reticulum to the Golgi[2,3] or in the Golgi cisternae[16], respectively. The Golgi complex is induced to fragment by monensin, but it remains localized to the perinuclear region[16]. In BFA-treated cells, the Golgi components are redistributed to the endoplasmic reticulum or intermediate compartments[3]. These effects of monensin and BFA were reproduced in BHK cells when cells were treated with the drugs and then stained with C_6-NBD-ceramide (Fig. 4). The Golgi stain in LSA-treated cells was apparently similar to that in the control, suggesting that the cytoplasmically dispersed, punctuate immunofluorescence stain in LSA-treated cells is not caused by fragmentation of the Golgi apparatus. However, the determination of the site(s) of intracellular accumulation of VSV-G awaits further study. Taken together, these results indicate that LSA blocks cell surface expression of both NDV and VSV glycoproteins, and that the 2nd and 3rd possibilities mentioned above are, not exclusively, ruled out.

Fig. 4. Immunofluorescence microsopy of cell surface and intracellular VSV-G and vital stain of the Golgi apparatus.



BHK cells on glass coverslips were infected with VSV, and BFA (2 μg/ml), monensin (5 μg/ml) or LSA (0.13 μM) was added at 1 hour of infection and present throughout the experiment before fixation with paraformaldehyde. The infected cells were added with cycloheximide to become 10 μg/ml at 8 hours after the infection, and were incubated for a further 2 hours to chase the intracellular VSV-G. Thereafter, cells were fixed and processed for immunofluorescence microscopy. Cell surface VSV-G was stained without treatment with methanol after fixation, and intracellular VSV-G with treatment. BHK cells on glass coverslips were treated for 3 hours with drugs of the same concentration as in the immunofluorescence stain which were then added with C_6-NBD-ceramide to a concentration of 2 μM. Uptake and back-exchange of C_6-NBD-ceramide were measured according to the reported methods[11], and photographs were taken using a fluorescence microscopy.

Effect of LSA on F- and V-ATPases Compared with Its Action on Cell Surface Expression of Virus Glycoprotein

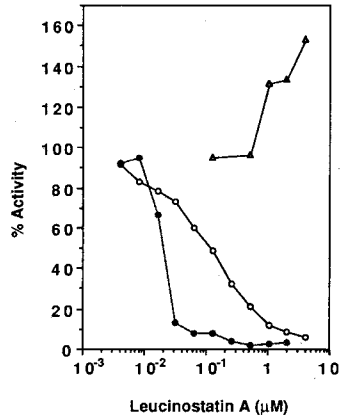
LSA is an uncoupler of oxidative phosphorylation and inhibits mitochondrial F-ATPase[7,8]. In this respect, oligomycin and carbonylcyanide *m*-chlorophenylhydrazone demonstrated a similar but less profound effect on SF formation to that of LSA (data not shown). LSA inhibited mitochondrial F-ATPase dose-dependently, with a 50% inhibitory concentration of *ca.* 0.1 μM (Fig. 5, ○). This value is similar to that reported for mitochondrial ATPase of various origins, including rat liver[7], suggesting that LSA inhibits F-ATPase similarly independent of source, and that the same could be said for F-ATPase in BHK cells. A direct comparison is impossible because the assay systems were different, but a relatively good correlation was observed between F-ATPase (Fig. 5, ○) and HAD (Fig. 5, ●) inhibition, and HAD was strongly suppressed at LSA concentrations enough to inhibit F-ATPase by more than 30%. Vacuolar-type H^+-translocating ATPase (V-ATPase) was not inhibited by LSA, but was rather stimulated at LSA concentrations above 1 μM (Fig. 5, △).

F-ATPase is involved in the synthesis of ATP during the terminal steps of oxidative phosphorylation. Because many stages of protein trafficking require energy in the form of ATP[17], it is possible that the inhibition of cell surface expression of virus glycoprotein follows the depletion of intracellular ATP. To address this question, intracellular ATP was quantified fluorometrically. ATP content in NDV-infected cells was nearly constant (200~220 pmol/well) at LSA concentrations between 0.004 and 2 μM (Fig. 6). ATP content in the control NDV-infected cells was *ca.* 187 pmol/well, and this value was slightly less than that in cells treated with LSA at concentrations at which SF was effectively suppressed. This decrease in the control cells could be caused partly by damage of cells following virus multiplication. Note, however, that ATP content decreased after more than 13 hours of treatment to less than 10% of the false-treated control in two of seven repeated experiments (data not shown). We can not explain this discrepancy. However, LSA inhibited SF and CPE without severely suppressing glycoprotein synthesis irrespective of whether intracellular ATP content was affected or not, indicating that the action of LSA is independent of the intracellular amount of ATP.

LSA Action as an Ionophore and Its Effect on Intoxication by Diphtheria Toxin and Ricin

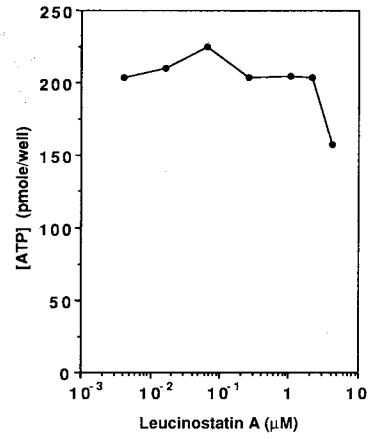
CSERMELY *et al.* reported that LSA acts as a weak ionophore and facilitates the transport of mono- and divalent cations through the plasma membrane of T lymphocytes[8]. Ionophores such as monensin inhibit intracellular protein trafficking[16]. Recently, helioferins, antifungal lipopeptides having structures similar to LSA, were demonstrated to mediate anion transfer from aqueous to toluene phases[15]. The same was demonstrated with LSA: 1 mol of LSA transferred about 0.6 mol of methylorange, an hydrophilic anionic dye, to the

Fig. 5. Relatively good accordance is seen between the inhibition of cell surface expression of NDV-HN and the inhibition of F-ATPase.



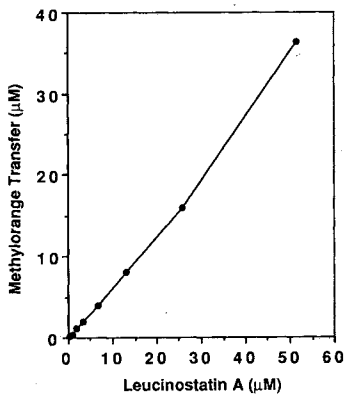
Effect of LSA on F- (○) and V-ATPases (△) was determined by quantifying liberated Pi as described in Materials and Methods, and its dose-dependency was compared with that of the inhibition of cell surface expression of NDV-HN (●) determined by quantifying HAD as described in the legend to Fig. 1.

Fig. 6. Intracellular ATP content in LSA-treated, NDV-infected BHK cells.



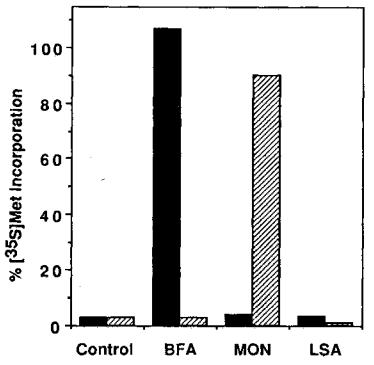
NDV-infected BHK cells were incubated in the presence of LSA at concentrations indicated in the figure, and ATP contents were determined by the luciferin-luciferase methods as described in Materials and Methods. Note that ATP content decreased in some cases on repeated experiments as described in the text.

Fig. 7. Anion transfer activity of LSA. LSA was mixed with methylorange solution in water to the final concentrations indicated in the figure.



The mixtures were extracted with *n*-butanol and the amounts of methylorange transferred to the butanol layer were quantified by measuring absorption at 430 nm.

Fig. 8. LSA does not detoxify the action of ricin and diphtheria toxin.



Monolayer cultures of BHK cells in microtitre plates were treated for 5 hours with 0.13 μM LSA. Fresh MEM was then added with or without ricin (■) or diphtheria toxin (▨). At 1 hour after the addition of the toxins, the cells were labeled with [^{35}S]methionine for 1 hour. Incorporation of [^{35}S]methionine into acid-insoluble products was measured as described in Materials and Methods. Cells were treated with BFA (5 μg/ml) or monensin (MON, 10 μg/ml) for comparison as in the case of LSA. % Incorporation = (counts in cells treated with both drug and toxin − background)/(counts in cells treated with drug − background) × 100.

toluene phase (Fig. 7). Taken together, the results demonstrate that LSA is an ionophore having affinity for both cations and anions. Unlike monensin, LSA did not perturb the accumulation of acridine orange in acidic intracellular organelles (data not shown). Ricin and diphtheria toxin added outside of cells are endocytosed to the trans Golgi network and endosomes and/or lysosomes, respectively, before exerting their toxic effect[18,19]. Drugs which perturb the H^+ gradient in intracellular acidic organelles such as ionophores and V-ATPase inhibitors prevent the intoxication by diphtheria toxin but not ricin[20], while a reverse effect is seen for BFA, which affects Golgi functions and trafficking between the endoplasmic reticulum and the Golgi complex[14,21]. These effects of BFA and monensin were reproduced in BHK cells as shown in Fig. 8. LSA did not detoxify the actions of either diphtheria toxin or ricin (Fig. 8), indicating again that the actions of LSA and monensin are different even though they are both ionophores having affinity for cations.

Discussion

The present study has demonstrated for the first time that LSA blocks cell surface expression of virus glycoproteins without profoundly affecting their synthesis. LSA acts as an uncoupler on mitochondria[6] and inhibits oxidative phosphorylation and mitochondrial F-ATPase[7]. It also acts as a weak ionophore, having affinity for cations and an anion (ref. 8 and this study). The action of LSA on intracellular trafficking of virus glycoproteins is discussed in the following in relation to these reported characteristics of LSA.

Many stages of intracellular translocation of proteins require energy in the form of ATP[17]. F-ATPase, also called ATP synthase, is a key enzyme in the regeneration of ATP from ADP. The effect of LSA on intracellular ATP content varied on repeated experiments, and we have no answer for this fluctuation. Experiments on the time course of the effect of LSA on ATP content demonstrated that the decline in ATP content, when it was seen, occurred after 13 hours of treatment at the least (data not shown). However, suppression of SF and CPE was constantly observed and the MIC value did not change on more than ten repeated experiments irrespective of its effect on ATP content. These results suggest that the cause of the blockade of cell surface expression of virus glycoproteins resides elsewhere than the effect of LSA on the intracellular content of ATP.

Energized membrane-bound organelles participate in many stages of intracellular trafficking of proteins. Ionophores and V-ATPase inhibitors such as monensin and folimycin (concanamycin A) perturb the trafficking processes[9,16]. LSA is a potent inhibitor of F-ATPase, but, as demonstrated in this paper, has no inhibitory action on V-ATPase. LSA acts also as an ionophore (ref. 8 and this study). The action of LSA did not affect acidification of intracellular organelles and intoxication by ricin and diphtheria toxin. This action of LSA was different from that of monensin, an ionophore well known to affect intracellular trafficking and acidification of intracellular organelles and to detoxify the action of diphtheria toxin but not of ricin. In addition, our unpublished results indicate that many ionophores affect

glycoprotein synthesis *per se*, but their effect on cell surface expression of glycoprotein as judged by the suppression of SF could not be distinguished from their action on glycoprotein synthesis under the assay method employed in this study.

Finally, it should be noted that blockade of cell surface expression of virus glycoproteins was observed with venturicidin A, another F-ATPase inhibitor structurally unrelated to LSA (unpublished observation). It therefore awaits further study to demonstrate whether the blockade of cell surface expression of glycoproteins is a result of the inhibition of F-ATPase or is caused by a hitherto unknown effect(s) of these drugs. Results of immunofluorescence microscopy demonstrate that the site(s) of intracellular accumulation of VSV-G glycoprotein in LSA-treated cells is apparently different from that in BFA- and monensin-treated cells, and suggest that LSA may have a novel site of action in the trafficking pathway of glycoproteins. Elucidation of the mechanism of the blockade of protein trafficking by LSA remains to be done, but we expect that the novel action of LSA will be useful in investigation of the mechanism of intracellular trafficking of glycoproteins.

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References

1) ROTHMAN, J. E.: Mechanisms of intracellular protein transport. Nature 372: 55~63, 1994
2) TAKATSUKI, A. & G. TAMURA: Brefeldin A, a specific inhibitor of intracellular translocation of vesicular stomatitis virus G protein: intracellular accumulation of high-mannose type G protein and inhibition of its cell surface expression. Agric. Biol. Chem. 49: 899~902, 1985
3) KLAUSNER, R. D.; J. G. DONALDSON & J. LIPPINCOTT-SCHWARTZ: Brefeldin A: Insights into the control of membrane traffic and organelle structure. J. Cell Biol. 116: 1071~1080, 1992
4) FUKUSHIMA, K.; T. ARAI, Y. MORI, M. TUBOI & M. SUZUKI: Studies on peptide antibiotics leucinostatins. II. The structure of leucinostatins A and B. J. Antibiotics 36: 1613~1630, 1983
5) CERRINI, S.; D. LAMBA, A. SCATTURIN, C. ROSSI & G. UGHETTO: The crystal and molecular structure of the α-helical nonapeptide antibiotic leucinostatin A. Biopolymers 28: 409~420, 1989
6) FUKUSHIMA, K.; T. ARAI, Y. MORI, M. TSUBOI & M. SUZUKI: Studies on peptide antibiotics, leucinostatins. I. Separation, physico-chemical properties and biological activities of leucinostatins A and B. J. Antibiotics 36: 1606~1612, 1983
7) SHIMA, A.; K. FUKUSHIMA, T. ARAI & H. TERADA: Dual inhibitory effects of the peptide antibiotics leucinostatins on oxidative phosphorylation in mitochondria. Cell Struct. Funct. 15: 53~58, 1990
8) CSERMELY, P.; L. RADICS, C. ROSSI, M. SZAMEL, M. RICCI, K. MIHÁLY & J. SOMOGYI: The nonapeptide leucinostatin A acts as a weak ionophore and as an immunosuppressant on T lymphocytes. Biochim. Biophys. Acta 1221: 125~132, 1994
9) MUROI, M.; N. SHIRAGAMI, K. NAGAO, M. YAMASAKI & A. TAKATSUKI: Folimycin (concanamycin A), a specific inhibitor of V-ATPase, blocks intracellular translocation of the glycoprotein of vesicular stomatitis virus before arrival to the Golgi apparatus. Cell Struct. Funct. 18: 139~149, 1993
10) MUROI, M.; N. SHIRAGAMI & A. TAKATSUKI: Destruxin B, a specific and readily reversible inhibitor of vacuolar-type H^+-translocating ATPase. Biochem. Biophys. Res. Commun. 205: 1358~1365, 1994
11) LIPSKY, N. G. & R. E. PAGANO: A vital stain for the Golgi apparatus. Science 228: 745~747, 1985
12) LLOYD, D.: General methodology for isolation and characterization of mitochondria from microorganisms. Method Enzymol. 55: 135~144, 1979
13) UCHIDA, E.; Y. OHSUMI & Y. ANRAKU: Purification of yeast vacuolar membrane H^+-ATPase and enzymological discrimination of three ATP-driven proton pumps in Saccharomyces cerevisiae. Methods Enzymol. 157: 544~562, 1988
14) YOSHIDA, T.; C. CHEN, M. ZHANG & H. C. WU: Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxicity of ricin, modeccin, and *Peudomonas* toxin. Exp. Cell Res. 192: 389~395, 1991
15) GRÄFE, U.; W. IHN, M. RITZAU, W. SCHADE, C. STENGEL, B. SCHLEGEL, W. F. FLECK, W. KÜNKEL, A. HÄRTL & W. GUTSCHE: Helioferins; novel antifungal lipopeptides from *Mycogone rosea*: screening, isolation, structures and biological properties. J. Antibiotics 48: 126~133, 1995
16) MOLLENHAUER, H. H.; D. J. MORRÉ & L. D. ROWE: Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. Biochimi. Biophys. Acta 1031: 225~246, 1990
17) SCHMID, S. L. & H. DAMKE: Coated vesicles: a diversity of form and function. FASEB J. 9: 1445-1453, 1995
18) SIMPSON, J. C.; C. DASCHER, L. M. ROBERTS, J. M. LORD & W. E. BALCH: Ricin cytotoxicity is sensitive to recycling between the endoplasmic reticulum and the Golgi complex. J. Biol. Chem. 270: 20078~20083, 1995
19) SANDVIG, K. & B. VAN DEURS: Endocytosis and intracellular sorting of ricin and Shiga toxin. FEBS Lett. 346: 99~102, 1994
20) YOSHIDA, T.; C. CHEN, M. ZHANG & H. WU: Increased cytotoxicity of ricin in a putative Golgi-defective mutant of Chinese hamster ovary cell. Exp. Cell Res. 190: 11~16, 1990
21) SANDVIG, K.; K. PRYDZ, S. H. HANSEN & B. VAN DEURS: Ricin transport in brefeldin A-treated cells: correlation between Golgi structure and toxic effect. J. Cell Biol. 115: 971~981, 1991