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# Inhibition of proteolytic cleavage of the hemagglutinin of influenza virus by the calcium-specific ionophore A23187

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**At calcium-specific ionophore A23187 concentrations of ~0.25 μM [which still allow assembly and release of fowl plague virus (FPV) particles] post-translational proteolytic cleavage of the viral hemagglutinin precursor HA into the fragments $HA_1$ and $HA_2$ is inhibited. The resulting virus particles with uncleaved hemagglutinin, that cannot be obtained under normal conditions, provide a suitable substrate for *in vitro* assays of the protease sensitivity of the FPV hemagglutinin. Proteolytic activation is accomplished with trypsin. Treatment with cathepsin B at low pH yields aberrant cleavage products suggesting that the cellular cleavage enzyme is not of lysosomal origin. A protease that cleaves the FPV hemagglutinin in the correct place can be detected in lysates of MDBK cells. This enzyme is calcium dependent and has a neutral pH optimum.**

*Key words:* influenza virus/hemagglutinin/proteolytic activation/proteases/ionophores

## Introduction

The hemagglutinin of influenza virus is translated as an integral membrane glycoprotein at the rough endoplasmic reticulum (ER) and is then transported to the cell surface. During transport the precursor HA undergoes post-translational proteolytic cleavage into the fragments $HA_1$ and $HA_2$. Cleavage which is a pre-condition for the fusion capacity of the hemagglutinin (Huang *et al.*, 1980, 1981; Lenard and Miller, 1981; Maeda and Ohnishi, 1980; White *et al.*, 1981) and, thus, for virus infectivity (Klenk *et al.*, 1975; Lazarowitz and Choppin, 1975) involves the sequential action of a trypsin-like endoprotease and carboxypeptidase N which are both of host origin (Lazarowitz *et al.*, 1973; Garten *et al.*, 1981; Garten and Klenk, 1983). There is increasing evidence that structural differences at the cleavage site are important for the spread of infection and for pathogenicity (Bosch *et al.*, 1979, 1981). In most hemagglutinins, $HA_1$ and $HA_2$ are linked by a single arginine. Many cells lack a suitable endoprotease to activate these hemagglutinins. Thus, virions with uncleaved HA are obtained that can be activated *in vitro*. However, with other virus strains, notably with fowl plague virus (FPV), there is an intervening sequence of several basic amino acids (Porter *et al.*, 1979). This type of hemagglutinin is activated by a protease present in all cells. Thus, virions with uncleaved HA are not available for *in vitro* studies (Klenk *et al.*, 1975).

Ionophores have proved to be useful tools for studying glycoprotein processing. The monovalent carboxylic ionophore monensin has probably found the widest application. It is assumed that by blocking the intracellular transport, presumably somewhere within the Golgi apparatus, this compound interferes with the biosynthesis of cellular membrane and secretory proteins (Ledger *et al.*, 1980; Tartakoff and Vassalli, 1977, 1978) and of the glycoproteins of several viruses, such as vesicular stomatitis virus, alphaviruses and coronaviruses (Johnson and Schlesinger, 1980; Straus and Lodish, 1980; Kääriäinen *et al.*, 1980; Niemann *et al.*, 1982). However, with influenza virus, monensin did not inhibit glycoprotein processing and virus assembly (Alonso and Compans, 1981).

We have analyzed the effects of the calcium-specific ionophore A23187 on the biosynthesis of the hemagglutinin of FPV. We have found that A23187 specifically inhibits cleavage of this hemagglutinin without interfering with particle formation. Thus, virions are available which can be used for cleavage studies of the FPV hemagglutinin under *in vitro* conditions.

## Results

### *Replication of FPV in the presence of ionophore A23187*

Figure 1 shows the effect of ionophore A23187 on the growth of FPV (Dobson strain) in BHK21-F cells. There is a dose-dependent decrease in virus production, but up to a concentration of 0.25 μM substantial amounts of virus particles are still released into the medium. The Rostock strain of FPV, which grows to lower titres in BHK cells (Lohmeyer *et al.*, 1979), shows essentially the same sensitivity to the ionophore. Virus-infected cells exhibit hemadsorption in the presence of the inhibitor, and, particularly with respect to the surface spikes, virus particles are morphologically indistinguishable from control virions (data not shown).

We have analyzed the effect of A23187 on the biosynthesis of the viral glycoproteins. Figure 2 shows a pulse-chase exper-

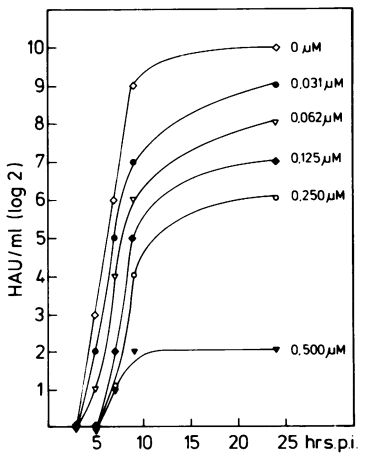


**Fig. 1.** Growth of FPV (Dobson strain) in the presence of ionophore A23187. Monolayers of BHK cells were infected at a multiplicity of 50 p.f.u./cell. Cultures were maintained in calcium-free REM containing A23187 at the concentrations indicated. At appropriate times after infection virus released into the culture medium was determined by hemagglutination test.

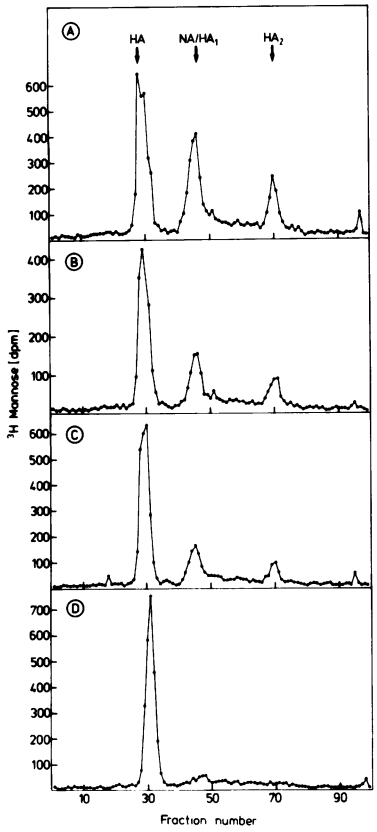


**Fig. 2.** Biosynthesis of the hemagglutinin of FPV (Rostock strain) in the presence of ionophore A23187. Monolayers of BHK cells were infected at a multiplicity of 50 p.f.u./cell. Cultures were maintained in calcium-free REM containing no **(A)**, 0.062 μM **(B)**, 0.125 μM **(C)**, and 0.250 μM **(D)**, A23187. After 4 h the cultures were pulse-labeled for 2 h with [2-$^3$H]-mannose. Cell lysates were analyzed on polyacrylamide column gels. The positions of the hemagglutinin precursor (HA) and its cleavage products ($HA_1$ and $HA_2$) and of the neuraminidase (NA) are indicated.

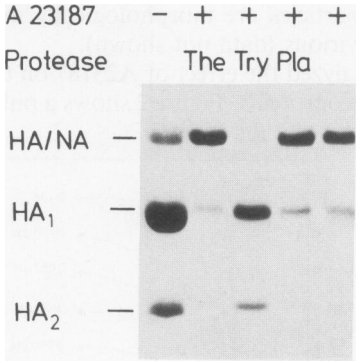


**Fig. 3.** Proteolytic cleavage of the hemagglutinin of FPV (Dobson strain) by thermolysin, trypsin and plasmin. Virus was grown in BHK cells in the absence **(lane 1)** or presence **(lanes 2–5)** of 0.250 μM A23187. Purified virions (~200 HAU) were suspended in 25 μl PBS and incubated *in vitro* for 20 min at 37°C with thermolysin (The), trypsin (Try), and plasmin (Pla). Each enzyme was present at a concentration of 10 μg/ml. The samples shown in **lanes 1** and **5** were not treated with proteases. The virus preparations were then analyzed by electrophoresis on a slab gel.

iment in which infected cells were labeled with tritiated mannose. A23187 interferes with proteolytic cleavage of the hemagglutinin as indicated by the dose-dependent decrease of the cleavage fragments $HA_1$ and $HA_2$. Synthesis of the uncleaved precursor HA is not affected, and predominantly

**Table I.** Activation of the hemolytic capacity of the FPV hemagglutinin by trypsin treatment

| Virus | Trypsin | Hemolysis (OD units) |
|---|---|---|
| FPV-A23187 | – | 0.086 |
| FPV-A23187 | + | 0.384 |
| FPV | – | 0.315 |

FPV (Rostock strain) was grown in BHK cells with (FPV-A23187) or without (FPV) A23187 (0.250 μM) present in the medium. Virus was pelleted from the medium by ultracentrifugation, and an aliquot of FPV-A23187 was incubated with trypsin (10 μg/ml) for 15 min at 37°C. The virus samples were then purified by equilibrium centrifugation on potassium tartrate gradients. Hemolysis was carried out at pH 5.6 using 16 HAU of purified virus.

in this form the hemagglutinin is also incorporated into virus particles (Figures 3, 4 and 5). HA synthesized in the presence of A23187 can also be labeled with [1-$^3$H]galactose (data not shown), indicating that processing of the carbohydrate side chains to hybrid or complex type oligosaccharides is possible under these conditions.

### *Proteolytic activation of the FPV hemagglutinin by defined proteases*

Virus particles containing uncleaved HA after growth in the presence of A23187 were incubated with trypsin. Figure 3 demonstrates that the FPV hemagglutinin is readily cleaved by this enzyme as had to be expected from the structure of the cleavage site (Porter *et al.*, 1979; Garten *et al.*, 1982). Other proteases have also been tested. These include plasmin which cleaves the hemagglutinin of strain A/WSN/34 (H1N1) (Lazarowitz *et al.*, 1973), thermolysin which cleaves the hemagglutinin of strain A/chick/Germany/N/49 (H10N7) (Garten *et al.*, 1981) and chymotrypsin (data not shown) which cleaves the hemagglutinin of both of these strains. The FPV hemagglutinin is susceptible to none of these enzymes.

Correct cleavage of the hemagglutinin by trypsin should be accompanied by an activation of the fusion capacity. We have therefore analyzed FPV grown in the presence of A23187 for its capacity to induce hemolysis at low pH. Table I demonstrates that such virus requires trypsin cleavage to express hemolytic activity comparable with that of control virus grown in the absence of A23187. Thus, proteolytic cleavage is clearly a pre-condition for the fusion activity of the FPV hemagglutinin, too.

Since the FPV hemagglutinin is cleaved in a large variety of different tissues, trypsin, a protease of the digestive tract, can be ruled out as the enzyme responsible for cleavage *in vivo*. Search for the natural enzyme should therefore focus on the so-called tissue proteinases. Among these, cathepsin B is very active in splitting the peptide bond on the carboxyl side of a pair of basic amino acid residues and has therefore been suspected of being the natural activator of prohormones and zymogens that require cleavage of such peptide bonds for activation (Ansorge *et al.*, 1977). Because of its similar cleavage site we have exposed uncleaved FPV hemagglutinin to a preparation of purified cathepsin B1 (Barrett, 1973). At pH 7.2 this enzyme does not cleave HA (data not shown). Since cathepsin B is a lysosomal enzyme, incubation was also carried out at pH 5.4. Under these conditions HA is readily cleaved, but the cleavage products are not identical with authentic $HA_1$ and $HA_2$ (Figure 4). The largest cleavage fragment migrates slightly faster than $HA_1$, a second fragment migrates behind $HA_2$, and a third fragment co-migrates with

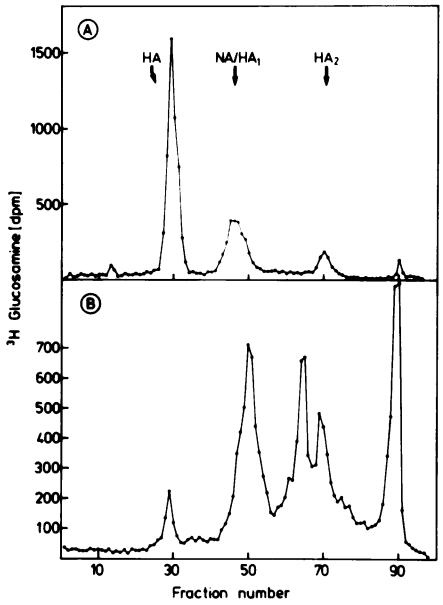


**Fig. 4.** Proteolytic cleavage of the hemagglutinin of FPV (Rostock strain) by cathepsin B1. Virus was grown in BHK cells in the presence of 0.250 μM A23187. Purified virions (~200 HAU) were suspended in 25 μl 0.1 M acetate buffer (pH 5.4) and incubated for 20 min at 33°C without **(A)** or with cathepsin B1 (10 μg/ml) **(B)**. The samples were analyzed on polyacrylamide column gels.

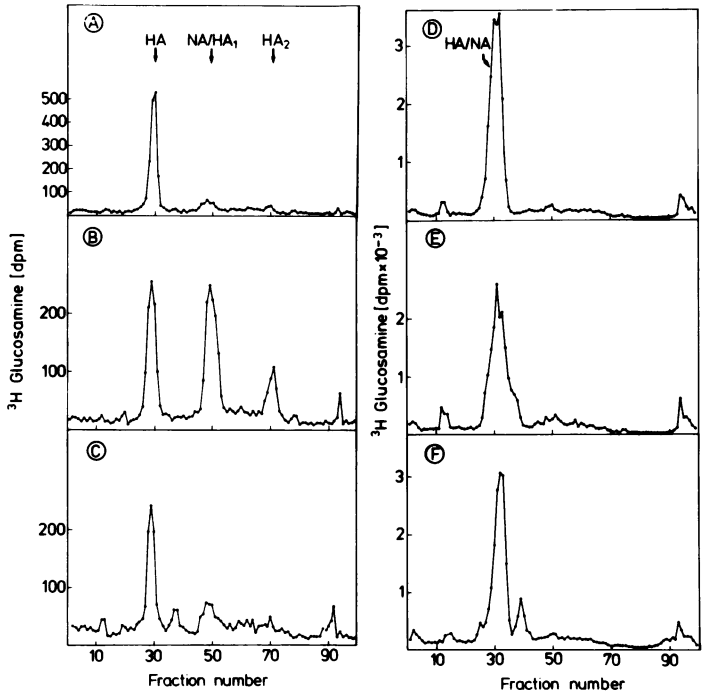


**Fig. 5.** Proteolytic cleavage of the hemagglutinin of FPV (Rostock strain) and of virus N by cell lysates. FPV was grown in BHK cells in the presence of 0.250 μM A23187. Purified virions (~200 HAU) were incubated without **(A)** or with lysates of MDBK cells as described in Materials and methods. Incubation with lysates was carried out at pH 7.2 **(B)** or at pH 5.4 **(C)**. Purified particles of virus N that had been grown in CE cells in the absence of A23187 were also incubated without **(D)** or with cell lysates at pH 7.2 **(E)** or pH 5.4 **(F)**.

$HA_2$. Thus, it is not likely that cathepsin B acts as the natural activator of the influenza hemagglutinin. It is worth noting that the results shown in Figure 4B resemble an observation

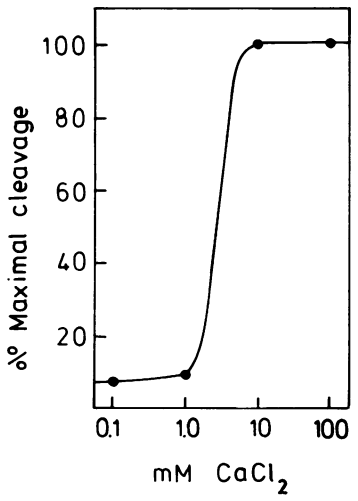


**Fig. 6.** Effect of calcium on proteolytic cleavage of the hemagglutinin of FPV (Dobson strain) by cell lysates. Purified virus that had been grown in BHK cells in the presence of 0.250 μM A23187 was incubated with cell lysates of MDBK cells at pH 7.2 as described in Materials and methods, except that the calcium chloride concentrations were varied as indicated. The virus samples were then analyzed on polyacrylamide column gels and the extent of hemagglutinin cleavage was determined at the various calcium concentrations.

made by Skehel *et al.* (1982) who found that, after exposure to low pH, $HA_1$ was converted by trypsin into several smaller fragments, whereas $HA_2$ was not altered.

### *Proteolytic activation of the FPV hemagglutinin by cell lysates*

Fowl plague virions with uncleaved HA can also be used as a substrate to analyze the proteolytic activity present in cells under *in vitro* conditions (Figure 5A–C). Virus has been exposed to lysates of uninfected MDBK cells. The authentic cleavage products could be detected when incubation was carried out at neutral pH (Figure 5B), but not at pH 5.4 (Figure 5C). This finding also argues against the involvement of lysosomal enzymes in the activation process. In a control experiment (Figure 5D–F), lysates of MDBK cells were inactive when analyzed on strain A/chick/Germany/N/49 (H10N7) which has a hemagglutinin with a single arginine at the cleavage site. Thus, in the *in vitro* system the protease that specifically cleaves the FPV hemagglutinin is measured. It should be mentioned that cleavage could not be accomplished when cell lysates of chick embryo cells or of BHK21-F cells were used (data not shown). At present, we have no clear explanation for this observation. Conceivably, protease inhibitors could be involved, which might be present in different amounts in these cell cultures.

In our experiments A23187 was administered in calcium-free medium. The cells should therefore be calcium depleted. The observation that cleavage of the FPV hemagglutinin is inhibited in such cells suggests that the cleavage enzyme is calcium dependent. We have therefore carried out the *in vitro* cleavage by the cell lysate with variable amounts of calcium added to the incubation mixture. Figure 6 shows that cleavage requires a calcium concentration of 10 mM.

## Discussion

We have investigated the effects of the calcium ionophore A23187 on the biosynthesis of FPV hemagglutinin. Our data show that proteolytic cleavage of the hemagglutinin is in-

hibited at an ionophore concentration of ~0.25 μM, which still allows viral protein synthesis, intracellular transport of the hemagglutinin, processing of the carbohydrate side chains and formation of virus particles. The specific effect of A23187 on proteolytic cleavage of the FPV hemagglutinin contrasts with observations made with Sindbis virus where the precursor glycoprotein PE2 remains uncleaved, because A23187 interferes in that system primarily with intracellular transport and therefore prevents the access of the glycoprotein to the cellular organelle in which cleavage takes place (Johnson and Schlesinger, 1980).

Since growth in the presence of A23187 yields fowl plague virions that contain uncleaved hemagglutinin, it is now possible to analyze proteolytic cleavage of this hemagglutinin with defined proteases *in vitro*, as has been done before with those hemagglutinins that are incorporated into virions in uncleaved form because the host cells lack a suitable cleavage enzyme. The FPV hemagglutinin is cleaved by trypsin, but it was interesting to see that this could not be accomplished by any of the other proteases analyzed, although each one of these has been found to cleave other hemagglutinins. This observation is compatible with the view that the accessibility of the cleavage site to proteases varies with different hemagglutinins. As has been pointed out above, when looking for the neutral cleavage enzyme, one should concentrate on tissue proteases. We have followed two routes in this search. Firstly, we thought that a lysosomal enzyme might be a suitable candidate. Although it is unlikely that the hemagglutinin passes through lysosomes on its way to the cell surface, it is conceivable that the hemagglutinin and lysosomal enzymes encounter each other en route from their common site of synthesis in the rough ER to their different destination sites. However, when a purified preparation of the lysosomal enzyme cathepsin B was employed, the hemagglutinin cleavage products differed from authentic $HA_1$ and $HA_2$. In the second approach, lysates of uninfected cells were assayed for their capacity to cleave HA *in vitro*. Using this system, correct cleavage fragments were obtained when the reaction was carried out at neutral pH. Another requirement was a relatively high calcium concentration. This finding is compatible with the view that when administered in the calcium-free medium as has been done in this study, A23187 inactivates the cleavage enzyme by calcium depletion of the host cell. It is interesting to note that a protease from amnion fluid that cleaves the F protein of Sendai virus also requires calcium ions (Appleyard and Davis, 1983). Our observations taken together indicate that the cellular enzyme that activates the FPV hemagglutinin is calcium dependent and has an optimal activity at neutral pH. It is neither cathepsin B nor another lysosomal enzyme. Its exact nature and intracellular location remain to be elucidated. However, it is reasonable to assume that it is a component of the Golgi apparatus or of membrane vesicles mediating transport between Golgi and plasma membrane, since proteolytic cleavage occurs late in transport, but before arrival of the hemagglutinin at the cell surface (Klenk *et al.*, 1974; Matlin and Simons, 1983).

The hemolytic activity of the FPV hemagglutinin depends on cleavage. Although proteolytic activation of the fusion capacity has not been definitely shown before with FPV, this observation does not come as a surprise with the large body of information on other influenza hemagglutinins available. It should be pointed out, however, that there are numerous other viral glycoproteins, notably those of retroviruses, that undergo proteolytic processing at FPV hemagglutinin-like cleavage sites containing at least two basic amino acids (Oroszlan *et al.*, 1980; Hunter *et al.*, 1983; Koch *et al.*, 1983; Wünsch *et al.*, 1983; Adachi *et al.*, 1984). These glycoproteins also appear to be responsible for initiation of infection, and it is conceivable that this function, particularly fusion capacity, requires proteolytic activation, too. However, definite proof for these assumptions has not been obtained yet, because for their metabolic instability the uncleaved precursor glycoproteins are not available for proteolytic activation *in vitro*. Inhibition of *in vivo* cleavage with A23187 may provide a suitable system to overcome this problem.

## Materials and methods

*Viruses and cells*

The influenza strains A/FPV/Rostock/34 (H7N1), A/FPV/Dutch/27 (H7N7) (Dobson variant adapted to growth in BHK cells), and A/chick/Germany/N/49 (H10N7) were used. Seed stocks were propagated in the allantoic cavity of 11-day-old embryonated eggs. Virus growth was analyzed in confluent monolayer cultures of BHK21-F cells (Holmes and Choppin, 1966) which were infected with 50 plaque-forming units (p.f.u.) per cell. In some experiments MDBK cells and primary cultures of chick embryo (CE) cells were used. After 45 min adsorption, the virus inoculum was removed and replaced with reinforced Eagle's medium (REM) (Bablanian *et al.*, 1966). When virus was grown in the presence of ionophore A23187, REM lacking calcium chloride was used.

Plaque assays and hemagglutinin titrations were carried out according to standard procedures. Hemadsorption was analyzed on BHK monolayers (5 cm diameter) 8 h after infection. After removal of the incubation medium the cells were rinsed with phosphate-buffered saline (PBS) and incubated with 2 ml of a 0.5% suspension of chicken erythrocytes in PBS for 15 min at 20°C. After five washings with PBS the monolayers were inspected in the microscope.

To analyze hemolytic activity, preparations of purified virus containing ~16 hemagglutinating units (HAU) on 0.5 ml saline were added to 0.5 ml ammonium acetate buffer (0.5 M, pH 5.6) and 1.5 ml of a 1% suspension of human erythrocytes in saline. After incubation for 15 min at 37°C the samples were subjected to low speed centrifugation, and released hemoglobin was measured in the supernatant by spectrophotometry at 546 nm (Huang *et al.*, 1981).

*Purification of virus particles and analysis of viral glycoproteins*

To obtain radioactive particles $^3$H-labeled sugars were added to the growth medium at a concentration of 2 μCi/ml. In a typical experiment, virus labeled with a specific sugar was obtained from 80 Petri dishes (14 cm diameter). 16 h after infection virus particles were purified from the medium by centrifugation on potassium tartrate gradients (15–35%) as described previously (Compans *et al.*, 1970).

To analyze intracellular synthesis of viral glycoproteins, monolayers of BHK cells on 5 cm Petri dishes were incubated with 2 ml REM. 4 h after infection the cells were labeled by adding radioactive sugars at a concentration of 50 μCi/ml for the times indicated. After the labeling period the cells were lysed, and viral proteins were analyzed by polyacrylamide gel electrophoresis.

For gel electrophoresis, samples were boiled for 2 min with SDS (2%) and mercaptoethanol (2%). Cylindrical gels (Schwarz and Klenk, 1974) or slab gels (Laemmli, 1970) containing 10% acrylamide and 0.27% bisacrylamide were used. Cylindrical gels were sliced and radioactivity was determined by liquid scintillation counting. Slab gels were analyzed by fluorography (Bonner and Laskey, 1974).

*Incubation of virus with cell lysates*

A monolayer culture of MDBK cells was scraped with a rubber policeman from the Petri dish (5 cm diameter) in 1 ml PBS or in 1 ml acetate buffer (pH 5.4), each containing 10 mM $CaCl_2$ and 2% octylglucoside. The cells were sonicated with a Branson sonifier for 3 s. The virus sample (~200 HAU in 25 μl distilled water) was incubated with 50 μl of the cell lysate for 60 min at 37°C.

*Enzymes and chemicals*

Ionophore A23187 was purchased from Calbiochem, Giessen, FRG. It was kept as a 1 mM stock solution in ethanol (reagent grade). Trypsin, TPCK-treated, was obtained from Serva, Heidelberg, FRG. Chymotrypsin A4 and thermolysin were purchased from Boehringer, Mannheim, FRG. Plasmin from porcine blood was from Sigma, München, FRG. Cathepsin B1 purified

from human liver (Barrett, 1973) was a gift of Dr A.J.Barrett, Strangeways Research Laboratory, Cambridge, UK. n-Octylglucoside was synthesized as described previously (Kohama *et al.*, 1981). Reagents for polyacrylamide gel electrophoresis were from Bio-Rad, München, FRG. D-[6-$^3$H]glucosamine-HCl (10 Ci/mmol) and D-[2-$^3$H]mannose (2 Ci/mmol) were from Amersham, Buchler, Braunschweig, FRG.

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