Intracellular processing and transport of influenza-virus envelope proteins in Madin–Darby canine kidney cells

Effects of the carboxylic ionophores monensin and nigericin

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We have investigated the effects of the carboxylic ionophores monensin and nigericin on the intracellular processing and transport of the influenza-virus envelope proteins haemagglutinin and neuraminidase in Madin–Darby-canine-kidney-cell monolayers. In the presence of either ionophore, haemagglutinin acquires resistance to the enzyme endoglycosidase H more slowly than it does in untreated cells. In addition, the ionophores cause a block in oligosaccharide-processing events that are believed to occur normally in the *trans* elements of the Golgi complex. This block is not overcome even at long chase times. Finally, the ionophores cause a substantial slowing of the delivery of both haemagglutinin and neuraminidase to the plasma membrane. We conclude that the ionophores cause delays in the intracellular transport of these proteins both early and late in the pathway, that is, before and after passage through the *trans*-Golgi, and perturb the processing functions of this compartment. The possible significance of these observations with regard to the intracellular transport of newly synthesized plasma-membrane proteins in epithelial cells is discussed.

INTRODUCTION

In ion-transporting epithelia, functional asymmetry depends on the differential localization of proteins in the two domains of the plasma membrane: apical and basolateral. For example, in the epithelial-cell line Madin–Darby canine kidney (MDCK), the enzyme aminopeptidase is found only in the apical surface, whereas ($\text{Na}^+ + \text{K}^+$)-dependent ATPase is found only in the basolateral surface (Louvard, 1980). Investigations of the mechanisms underlying this asymmetric targeting of plasma-membrane proteins have used epithelial-cell lines infected with enveloped viruses such as influenza and vesicular-stomatitis virus (VSV). These viruses bud asymmetrically from epithelial monolayers [influenza virus apically and VSV basolaterally (Rodriguez-Boulan & Sabatini, 1978)] and the envelope proteins (influenza-virus haemagglutinin and neuraminidase, and VSV glycoprotein G) are inserted exclusively into the surface from which the particular virus will bud (Rodriguez-Boulan & Pendergast, 1980).

Carboxylic ionophores, such as monensin and nigericin, interfere with the intracellular processing of proteins and have been used widely to characterize the transport pathways of newly synthesized proteins (Griffiths *et al.*, 1983; Tartakoff, 1983). Typically, monensin causes a dramatic swelling of the normally compressed cisternae of the Golgi complex and a concomitant block in the intracellular transport of membrane proteins within this organelle. In MDCK-cell monolayers, monensin has a particularly interesting effect, apparently blocking the

transport of VSV G (in the Golgi complex), but not that of influenza-virus haemagglutinin (Alonso-Caplen & Compans, 1983; Edwardson, 1984; Rodriguez-Boulan *et al.*, 1984). Reports differ as to whether the transport of haemagglutinin is completely unaffected (Alonso-Caplen & Compans, 1983) or delayed (Edwardson, 1984; Rodriguez-Boulan *et al.*, 1984). This ability of monensin to discriminate between haemagglutinin and VSV G has been taken to indicate that the pathways taken by the two proteins diverge at a stage preceding the point at which transport of VSV G is blocked (Alonso-Caplen & Compans, 1983). However, more recent evidence (Rindler *et al.*, 1984; Fuller *et al.*, 1985) suggests that these two groups of proteins are segregated as they exit the Golgi complex. Furthermore, it has been shown recently that VSV G can recycle between the plasma membrane and endosomes, whereas haemagglutinin cannot (Gottlieb *et al.*, 1986), which raises the possibility that VSV G reaches the plasma membrane in the presence of monensin but does not accumulate there because it is trapped in endosomes as a consequence of the action of the drug on this organelle (Marsh *et al.*, 1982).

Despite the widespread use of carboxylic ionophores, their precise target compartment(s) and mechanism of action on the exocytic route remain unclear. It has been demonstrated (Anderson & Pathak, 1985) that a compartment on the *trans* side of the Golgi complex, which has been termed the '*trans*-Golgi network' (Griffiths & Simons, 1986) is mildly acidic. Since the ionophores are known to facilitate the electroneutral exchange of Na^+ ,

Abbreviations used: BHK, baby-hamster kidney; BSA, bovine serum albumin; EMEM, Eagle's minimum essential medium; GMEM, Glasgow's modified Eagle's medium; MDCK, Madin–Darby canine kidney; PBS, phosphate-buffered saline (for composition, see the text); SFV, Semliki-Forest virus; Tos-Phe-CH₂Cl, tosylphenylalanylchloromethane ('TPCK'); VSV, vesicular-stomatitis virus; VSV G, VSV glycoprotein G; endo H, endoglycosidase H.

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K⁺ and H⁺ across lipid bilayers, and hence to cause the dissipation of trans-bilayer concentration gradients of these ions (Pressman, 1976), it is possible that the ionophores are having an effect here analogous to that in the endosome (Marsh *et al.*, 1982), that is, dispersing a trans-membrane pH gradient and disrupting compartmental functions that depend on an acidic environment. Such a mechanism of action has been proposed for weak bases, such as NH₄Cl, that also interfere with the exocytic transport of plasma-membrane proteins (Matlin, 1986). However, monensin typically blocks the transport of proteins from the *medial*- to the *trans*-Golgi (Griffiths *et al.*, 1983) and causes the vacuolation of the entire Golgi complex, effects which indicate that it does not specifically perturb the function of the *trans*-Golgi network. Of possible relevance to the mechanism of action of the ionophores is the observation (Alonso-Caplen & Compans, 1983) that nigericin (a K⁺-selective ionophore), at a concentration of 1 µM, decreases the yield of influenza virus from infected MDCK cells by about 10-fold, whereas monensin (an Na⁺-selective ionophore) at the same concentration has no effect on virus yield. This result emphasizes the fact that the effects of the ionophores on compartmental concentrations of Na⁺ and K⁺ cannot be discounted.

In an attempt to resolve some of the complexities of ionophore treatment of epithelial cells, we have examined the effects of monensin and nigericin on the intracellular processing and transport of the two influenza-virus envelope proteins, haemagglutinin and neuraminidase, in MDCK-cell monolayers. Specifically, we set out to determine whether these two ionophores do indeed cause a delay in the delivery of the proteins to the cell surface, what stages of the transport pathway were affected, whether both envelope proteins were affected similarly and whether the two ionophores produced the same effects, despite their different ion selectivities.

MATERIALS AND METHODS

Cells and viruses

MDCK cells were grown as monolayers in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) newborn-calf serum, non-essential amino acids and gentamicin (160 µg/ml). Baby-hamster kidney (BHK-21) cells were grown in Glasgow's minimum essential medium (GMEM) supplemented with 10% newborn-calf serum, 10% tryptose/phosphate broth and gentamicin (160 µg/ml). Cells were grown at 37 °C in an atmosphere of CO₂/air (1:19). All cells, media and supplements were supplied by Flow Laboratories, Rickmansworth, Herts., U.K.

The WSN (H1N1) strain of influenza virus, initially obtained from Dr. S. C. Inglis (Department of Pathology, University of Cambridge, Cambridge, U.K.) was grown in fertile hens' eggs, purified by centrifugation on a continuous sucrose density gradient [15–60% (w/v) in 50 mM-Tris/HCl (pH 7.4)/100 mM-NaCl] for 2 h at 100 000 g and assayed by plaquing on MDCK-cell monolayers. Stocks of virus were stored in liquid N₂.

Semliki-Forest virus (SFV), initially obtained from Dr. T. R. Hesketh (Department of Biochemistry, University of Cambridge, Cambridge, U.K.) was grown on BHK-21 cells. Cells (10⁸ at 50% confluence) were washed twice with EMEM containing 0.2% (w/v)

bovine serum albumin (BSA; Sigma, Poole, Dorset) and 20 mM-Hepes, pH 6.8, and infected with > 10 plaque-forming units of SFV/cell in the same medium for 1 h at 37 °C. The medium was then replaced with 50 ml of complete GMEM. After 15 h at 37 °C the medium was harvested and centrifuged at 5000 g for 30 min to sediment cell debris. SFV was sedimented by centrifugation at 60 000 g for 2.5 h and purified by centrifugation for 2 h at 100 000 g on a gradient consisting of 10–20% (w/v) sucrose in 50 mM-Tris/HCl (pH 7.4)/100 mM-NaCl above 25–50% (w/v) sucrose in the same buffer. Stocks of virus were stored in liquid N₂.

Infection

MDCK cells were grown on 3 cm-diameter culture dishes until just confluent. Monolayers were washed twice with 2 ml of EMEM containing 0.2% (w/v) BSA and infected with > 10 plaque-forming units of influenza virus in the same medium for 1 h at 37 °C. The medium was then replaced with EMEM containing 2% newborn-calf serum. Monensin or nigericin (Calbiochem-Behring, Bishops Cleeve, Herts., U.K.) was present at 1 µM where appropriate.

Labelling with [³⁵S]methionine

MDCK-cell monolayers, 5 h post-infection, were washed twice with 1 ml of EMEM lacking methionine and then labelled for 10 min by exposure to 0.5 ml of the same medium containing 60 µCi of [³⁵S]methionine (Amersham International, Amersham, Bucks, U.K.)/ml. Further labelling was prevented, and incubation continued, by addition of 2 ml of EMEM containing 10 times the normal level of unlabelled methionine (1.5 mg/ml). To terminate the incubation, monolayers were washed twice with ice-cold phosphate-buffered saline (PBS), containing 137 mM-NaCl, 2.7 mM-KCl, 8.1 mM-Na₂PO₄ and 1.5 mM-KH₂PO₄, pH 7.4. Cells were disrupted and cellular DNA was digested by addition of 10 µl of 2% (w/v) SDS in 50 mM-Tris/HCl, pH 8.8, followed by 2 µl of DNAase I (10 mg/ml; Sigma Type IV). Cells were scraped from the dishes with a rubber policeman.

Treatment with endoglycosidase H (endo H)

A sample (10 µl) of cell lysate was taken, 10 µl of 2% (w/v) SDS was added and the mixture boiled for 2 min. After cooling, 200 µl of 100 mM-sodium citrate, pH 5.5, containing 2 munits of endoglycosidase H (Boehringer Mannheim, Mannheim, Germany) and the proteinase inhibitors (all from Sigma) phenylmethanesulphonyl fluoride (1 mM), antipain (1 µg/ml), benzamidine (17.5 µg/ml), pepstatin (1 µg/ml) and aprotinin (10 µg/ml) were added and the sample was incubated at 37 °C for 15 h. Protein was precipitated by addition of 200 µl of ice-cold acetone and resuspended in 50 µl of gel-electrophoresis sample buffer (see below).

In experiments involving endo H, control cell lysates were processed for gel electrophoresis immediately after harvesting. It was shown in a preliminary experiment that incubation of these lysates at 37 °C for 15 h in sodium citrate buffer containing proteinase inhibitors had no effect on either the mobilities or the recoveries of the viral proteins. Hence, any loss of material occurring during incubation with endo H can be ascribed to the action of contaminating enzymes.

Fig. 1. Polarization of an MDCK-cell monolayer

An MDCK-cell monolayer growing on a nitrocellulose filter (0.22 μm pore size; Millipore, Harrow, Middx., U.K.) was infected with influenza virus. At 7 h post-infection the monolayer was fixed and processed for electron microscopy. The arrowhead shows a budding virion. The bar represents 1 μm .

Assay for delivery of haemagglutinin to the plasma membrane

To assay delivery of haemagglutinin to the plasma membrane, [^{35}S]methionine-labelled monolayers were washed twice with 2 ml of ice-cold PBS and treated with 1 ml of 1 mg of Tos-Phe-CH₂Cl-treated trypsin (Cooper Biomedical, Bury St. Edmunds, Suffolk, U.K.)/ml in ice-cold PBS for 15 min, with gentle rocking. Monolayers were then exposed to 1 ml of soybean trypsin inhibitor (1 mg/ml) in ice-cold PBS for 15 min and washed twice with 2 ml of ice-cold PBS. Cells were harvested and processed for gel electrophoresis as described above.

SDS/polyacrylamide-gel electrophoresis

Samples of cell lysate were prepared for gel electrophoresis by addition of 3 vol. of sample buffer containing 200 mM-Tris/HCl, pH 6.8, 2% (w/v) SDS, 500 mM-sucrose, 20 mM-EDTA, 5% (v/v) 2-mercaptoethanol and 0.02% (w/v) Bromophenol Blue. Samples were boiled for 2 min and cooled to room temperature before application to a 1 mm-thick 15%-(w/v)-polyacrylamide gel in 0.1% (w/v) SDS with a 3% stacking gel. The gel running buffer was Tris/glycine, pH 8.3, containing 0.1% (w/v) SDS. After running, the gel was fixed for 15 min in 10% (w/v) trichloroacetic acid and then immersed for 30 min in AMPLIFY (Amersham). The gel was dried at 80 °C under reduced pressure and exposed to X-ray film (Kodak) at –70 °C, usually for 1 week. Fluorographs were scanned with a Joyce–Loebl Mark III densitometer scanner.

Preparation of [^3H]SFV

Preparation of [^3H]SFV was as described for SFV, except that the post-infection maintenance medium contained 5 mCi of [^3H]N-acetyl-D-mannosamine (30 Ci/mmol; New England Nuclear, Stevenage, Herts., U.K.). The envelope glycoproteins of shed virions consequently contained [^3H]sialic acid (Monaco & Robbins, 1973).

Assay for delivery of neuraminidase to the plasma membrane

At various times after infection, monolayers were washed twice with 2 ml of ice-cold PBS containing 0.9 mM-CaCl₂ and 0.5 mM-MgCl₂, then exposed to [^3H]SFV (approx. 20 000 d.p.m.) in 0.5 ml of ice-cold PBS containing BSA (2 mg/ml) for 30 min, with gentle rocking. Protein was precipitated by addition of 10% (w/v) trichloroacetic acid, followed by incubation on ice for 30 min. Protein was pelleted by centrifugation for 2 min in an Eppendorf Microfuge. Supernatants were removed and radioactivity was counted by liquid-scintillation spectroscopy, using 0.6% (w/v) butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole]/70% (v/v) toluene/30% (v/v) Triton X-100 as scintillant.

Electron microscopy

Influenza-virus-infected monolayers on Millipore filters were fixed for 6 h at 4 °C with 2% (v/v) glutaraldehyde, 2% (w/v) paraformaldehyde in 0.1 M-Pipes buffer, pH 7.2, containing 0.1 M-sucrose. Samples were post-fixed with 1% (w/v) osmic acid, dehydrated, stained with uranyl acetate and lead citrate and embedded in Spurr resin. Thin sections (60–80 nm) were cut on a Huxley Mark II ultramicrotome, mounted on 400-mesh copper grids and examined in a Philips 300 electron microscope.

RESULTS

Polarization of MDCK-cell monolayers

An electron micrograph of a section through an MDCK cell growing in a monolayer on a nitrocellulose filter is shown in Fig. 1. The monolayer was infected with influenza virus and processed 7 h post-infection. Influenza virions are seen budding exclusively from the apical surface of the cell, a result which demonstrates the polarization of the monolayer.

Fig. 2. Intracellular processing of haemagglutinin

(a) Endo H digestion of haemagglutinin in control monolayers. Influenza-virus-infected MDCK cell monolayers, 5 h post-infection, were pulse-labelled for 10 min with [35 S]methionine and then chased in the presence of excess unlabelled methionine for the indicated times. Cells were then disrupted and scraped from the dishes. Samples of cell lysate were processed for SDS/polyacrylamide-gel electrophoresis, either immediately or after digestion with endo H for 15 h at 37 °C. The gel was dried and fluorographed. Bands shown are: HAR, haemagglutinin resistant to endo H; HAS, haemagglutinin sensitive to endo H; HAp, product of digestion of HAS by endo H; NP/NA, nucleoprotein/neuraminidase; M, matrix protein; NS, non-structural protein. (b) Comparison of HAR forms produced in untreated and ionophore-treated cells. Labelling and processing was as in (a). Chase time was 1 h. Monolayers were either untreated (lane 1) or treated from 4 h post-infection with either 1 μ M-monensin (lane 2) or 1 μ M-nigericin (lane 3). Only the region of the fluorograph containing HA is shown. NP/NA is included as a reference band. (c) Endo H digestion of haemagglutinin in monensin-treated monolayers. As for (a), except that monolayers were treated from 4 h post-infection with 1 μ M-monensin. (d) Endo H digestion of haemagglutinin in nigericin-treated monolayers. As for (a), except that monolayers were treated from 4 h post-infection with 1 μ M-nigericin. (e) Time course of acquisition by haemagglutinin of resistance to endo H, as determined by densitometric scanning of the fluorographs shown in (a), (c) and (d). Values for the percentage of haemagglutinin resistant to endo H were calculated from the densities of the HAR and HAS bands in the 'minus endo H' lanes. ○, Control; ●, + monensin; ■, + nigericin.

Intracellular processing of haemagglutinin

It has been shown previously that haemagglutinin acquires high-mannose oligosaccharides as it is being synthesized in the rough endoplasmic reticulum and that these oligosaccharides are processed in the Golgi complex by the removal of glucose and mannose residues and the addition of *N*-acetylglucosamine, galactose and fucose (Nakamura & Compans, 1979). Mannose trimming results in the acquisition of resistance to the enzyme endoglycosidase H (endo H), and the addition of terminal sugars is seen as an increase in molecular mass (Edwardson, 1984). Fig. 2(a) shows the time course of the development by haemagglutinin of resistance to endo H in a control monolayer. Careful inspection of the

fluorograph reveals that even the fully processed form of haemagglutinin seen at long chase times is partially sensitive to endo H. This result was expected, since it has been shown that the mature form of haemagglutinin of the WSN strain of virus has six oligosaccharide moieties, of which four are resistant to endo H, one is sensitive and one partially sensitive (Nakamura *et al.*, 1980). Densitometric scanning of the fluorograph (Fig. 2e) revealed that the half-time for conversion of haemagglutinin into its Golgi-processed form is 18 min (10 min pulse plus 8 min chase).

In the presence of either monensin or nigericin, the Golgi processing of haemagglutinin was interrupted, resulting in the production of a new form of haemagglutinin with a molecular mass lower than that of the

Fig. 3. Delivery of haemagglutinin to the plasma membrane

(a) Control. Influenza-virus-infected MDCK-cell monolayers, 5 h post-infection, were pulse-labelled for 10 min with [35 S]methionine and chased in the presence of excess unlabelled methionine for the indicated times. Monolayers were then treated on ice with Tos-Phe- CH_2Cl -treated trypsin (0.5 mg/ml) in PBS for 15 min. Samples of cell lysates were electrophoresed on an SDS/polyacrylamide gel, and the gel was dried and fluorographed. (b) Effect of monensin. As for (a), except that monolayers were treated from 4 h post-infection with 1 μM -monensin. Only the region of the fluorograph containing HA is shown. NP/NA is included as a reference band. (c) Effect of nigericin. As for (a), except that monolayers were treated from 4 h post-infection with 1 μM -nigericin. (d) Acces-

normal fully processed form (Fig. 2b). This new form was found not to be completely sensitive to endo H (Figs. 2c and 2d), although the decrease in molecular mass produced by endo H treatment was greater than that seen for the normal form. This result indicates that some of the oligosaccharides had been fully trimmed to three mannose residues and further processed by the (incomplete) addition of the terminal sugars, *N*-acetylglucosamine, galactose and fucose, but that at least one oligosaccharide that is normally processed to endo H resistance had not undergone complete mannose trimming. The half-times for conversion of haemagglutinin to the new 'endo H-resistant' form were 35 min and 27 min in the presence of monensin and nigericin respectively (Fig. 2e). Hence, the ionophores have effects on mannose trimming, on the addition of terminal sugars and on the rate of delivery of haemagglutinin into the compartment in which these processing events are believed to occur, that is, the *trans*-Golgi (Griffiths *et al.*, 1983).

Unfortunately, neuraminidase is not detectable on these gels, because it co-migrates with nucleoprotein. Hence, it was not possible to examine the effects of the ionophores on its processing.

Delivery of haemagglutinin to the plasma membrane

The delivery of haemagglutinin to the apical surface of MDCK-cell monolayers was assayed by measuring its sensitivity to exogenously applied trypsin (Matlin & Simons, 1983; Edwardson, 1984). Digestions were carried out on ice in order to block all further transport of haemagglutinin. It was found that, at this temperature, a 15 min treatment with trypsin (0.5 mg/ml) resulted in a complete digestion of haemagglutinin at long chase times. 'Intracellular' viral proteins were protected from proteolysis at all times.

Infected cells were pulse-labelled with [35 S]methionine for 10 min and chased for various times in the presence of excess unlabelled methionine. Duplicate monolayers were cooled on ice and one monolayer of each pair was treated with trypsin. The percentage of radiolabelled haemagglutinin at the cell surface was determined from the percentage removal of the haemagglutinin band in the presence of trypsin (Edwardson, 1984). This procedure was used in preference to measuring the appearance of the fragment HA₂ (Matlin & Simons, 1983), because we were unable to detect this band reproducibly above the background labelling of the gel (compare, for example, lanes 4 and 6 in Fig. 3a).

In control monolayers, the half-time for the appearance of haemagglutinin at the cell surface was 65 min (Figs. 3a and 3d), in close agreement with the result obtained by both Matlin & Simons (1983) and Edwardson (1984). In the presence of monensin (Figs. 3b and 3d) and nigericin (Figs. 3c and 3d), the corresponding half-times were 115 min and 130 min respectively. Hence both ionophores cause a delay in the transport of haemagglutinin to the cell surface. This delay is considerably greater than the delay in acquisition of resistance to endo H, which indicates that the ionophores cause an additional slowing of the transport of haemagglutinin between the Golgi complex and the plasma membrane.

sibility of haemagglutinin to trypsin, as determined by densitometric scanning of the fluorographs shown in (a), (b) and (c). ○, Control; ●, + monensin; ■, + nigericin.

Fig. 4. Dependence of release of free [³H]sialic acid on incubation time and virus titre

(a) Influenza-virus-infected MDCK-cell monolayers at 4.5 h post-infection were cooled on ice and then exposed to SFV with glycoproteins bearing [³H]sialic acid (approx. 20000 d.p.m.) in 0.5 ml of ice-cold PBS, containing 2 mg of BSA/ml, for various times. Protein was precipitated by addition of 10% (w/v) trichloroacetic acid. Release of free [³H]sialic acid into the trichloroacetic acid supernatant was determined and expressed as a percentage of the total radioactivity added. Values are means $\pm$ S.E.M. ($n = 3$). Error bars are not shown where they lie within the dimensions of the symbol. (b) Release of free [³H]sialic acid from [³H]SFV produced by various titres of influenza virus over a 30 min incubation period was determined as in (a). Values are means $\pm$ S.E.M. ($n = 3$). Error bars are not shown where they lie within the dimensions of the symbol.

Fig. 5. Delivery of neuraminidase to the plasma membrane

(a) Effect of monensin. Influenza-virus-infected MDCK-cell monolayers, either control (○) or treated with 1 μ M-monensin from 1 h post-infection (●), were cooled on ice at various times, and surface neuraminidase activities were determined as in Fig. 4(a) (incubation period 30 min). Values are means $\pm$ S.E.M. ($n = 3$). Error bars are not shown where they lie within the dimensions of the symbol. (b) Effect of nigericin. As for (a), except that monolayers were either control (○) or treated with 1 μ M-nigericin from 1 h post-infection (■).

Delivery of neuraminidase to the plasma membrane

To measure the kinetics of the delivery of neuraminidase to the cell surface, a novel assay was developed that exploits the enzyme activity of this protein. At

various times after infection, MDCK-cell monolayers were cooled on ice and the amount of neuraminidase activity present at the cell surface was measured through the cleavage of free [³H]sialic acid from the envelope proteins of [³H]sialic acid-labelled SFV, added as a

Fig. 6. Viral budding

Electron micrographs of sections of the apical surface of influenza-virus-infected MDCK-cell monolayers at 7 h post-infection. (a) Control; (b) + monensin ($1\ \mu\text{M}$) from 1 h post-infection; (c) + nigericin ($1\ \mu\text{M}$) from 1 h post-infection. Arrowheads indicate budding virions. The bar represents $1\ \mu\text{m}$.

suspension above the monolayers. It was established in preliminary experiments that the release of free [^3H]sialic acid obtained was linear over the 30 min incubation period used, and approximately linear over the appropriate range of neuraminidase activities (Fig. 4). Release is therefore proportional to the amount of enzyme present at the cell surface. Release was measured at hourly intervals from the time of infection to 7 h post-infection, in the absence and presence of the carboxylic ionophores (Fig. 5). In the presence of each ionophore, the curve relating the surface delivery of neuraminidase to time post-infection was shifted in an approximately parallel manner to a later time. The extent of the shift was about 45 min for both monensin and nigericin. In both experiments illustrated, surface neuraminidase was detected at 1 h post-infection. This activity was associated with virions that remain bound to the cell surface at the end of the 1 h infection period. After incubation for a further 1 h, these virions are internalized, and enzyme activity at the cell surface falls almost to zero.

Viral budding

Fig. 6 shows electron micrographs of sections of the apical surfaces of monolayers taken 7 h post-infection. It is apparent that viral budding is occurring not only in the control monolayer (Fig. 6a), but also in monensin-treated (Fig. 6b) and nigericin-treated (Fig. 6c) monolayers. No budding from the basolateral surface was seen, either in control or ionophore-treated monolayers (results not shown). Hence, the ionophores apparently do not cause any mis-sorting of the viral envelope proteins.

DISCUSSION

We have shown that the two carboxylic ionophores monensin and nigericin cause a delay in the production of the Golgi-processed, endo H-resistant, form of haemagglutinin and a substantially greater delay in the transport of both haemagglutinin and neuraminidase to the plasma membrane. These results indicate strongly that the ionophores do not exert a single well-defined effect on the exocytic route, but affect transport both into and out of the *trans* elements of the Golgi complex.

The observed delay in the delivery of the envelope proteins to the cell surface confirms and extends the results of earlier studies (Edwardson, 1984; Rodriguez-Boulán *et al.*, 1984), but conflicts with the finding of Alonso-Caplen & Compans (1983) that monensin did not affect the rate of transport of haemagglutinin to the cell surface. The reason for this discrepancy is not clear to us. One would certainly expect that the method used by this last group (indirect immunofluorescence coupled to fluorescence-activated cell sorting) would be sufficiently sensitive to detect a difference in half-time of delivery of about 1 h.

In addition to their effects on the kinetics of the intracellular transport of haemagglutinin and neuraminidase, both monensin and nigericin were shown to interrupt oligosaccharide-processing events that are believed to occur normally in the *trans* elements of the Golgi complex (Griffiths *et al.*, 1983). It is also clear that it is the incompletely glycosylated form of haemagglutinin that is delivered to the cell surface in presence of the ionophores. There are two possible interpretations of these observations. Either haemagglutinin is able to by-pass the *trans*-Golgi complex and so escape an ionophore-induced block of transport, or else it passes through this organelle as normal, but is not completely processed (perhaps because the relevant enzymes no longer recognize haemagglutinin as a substrate). It is impossible, with the available information, to distinguish between these possibilities. However, if a by-pass is occurring, it is clear that this does not represent the major route normally followed by haemagglutinin, since in untreated cells this protein passes completely through the Golgi complex, into the *trans*-Golgi network and thence to the plasma membrane (Griffiths & Simons, 1986).

Like the carboxylic ionophores, the 'acidotropic' base NH_4Cl has been shown to delay the transport of haemagglutinin between the *trans*-Golgi and the plasma membrane (Matlin, 1986). It was suggested that this effect of NH_4Cl was a consequence of its ability to buffer the intracisternal pH of the normally acidic *trans* elements of the Golgi complex, and it is possible that the effects of

the ionophores late in the transport pathway also are brought about through a perturbation of intracisternal pH. Unlike the ionophores, however, NH_4Cl does not significantly affect either the rate of acquisition by haemagglutinin of resistance to endo H or the pattern of its terminal glycosylation. Hence, the ionophores must have effects on the functioning of the Golgi complex that are not shared by NH_4Cl . Perhaps changes in the ionic composition of the cytosol are important here, as suggested by Tartakoff (1983). It is clear from the present study, however, that the different ion selectivities of monensin and nigericin do not cause significant differences in their effects on intracellular transport and processing.

The most common effect of monensin on the intracellular transport of membrane proteins is to produce a complete block within the Golgi complex (Tartakoff, 1983). Hence, the delay seen with haemagglutinin and neuraminidase is anomalous, although not unique [for example, the mouse macrophage Fc receptor reaches the plasma membrane in monensin-treated cells, but is not galactosylated (Green *et al.*, 1985)]. Interestingly, the delaying effect of monensin is seen also on the export of endogenous secretory proteins from the apical surface of MDCK-cell monolayers (C. Y. Nadin, P. U. Daniels & J. M. Edwardson, unpublished work). It is possible, therefore, that this effect may reflect a general feature of transport to the apical surface of these cells. The relevance of these observations to the biogenesis of polarity in epithelia is still unclear. Perhaps the crucial question is whether, in monensin-treated MDCK cells, the transport of VSV G is truly blocked in the Golgi complex or whether, instead, it is trapped in endosomes after passing through the plasma membrane. If the latter effect is the real one, then the actions of the ionophores on this cell line would be unconnected with the issue of polarity.

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