INFLUENZA VIRUS M2 PROTEIN AND HAEMAGGLUTININ CONFORMATION CHANGES DURING INTRACELLULAR TRANSPORT

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Summary. – The influenza virus M2 protein has an ion channel activity that permits ions to enter the virion during its uncoating and also modulates pH of intracellular compartments. M2 protein is a homotetramer consisting of either a pair of disulfide-linked dimers or a disulfide-linked tetramer. The M2 trans-membrane domain peptide adopts an alfa helical secondary structure. In polarized cells, M2 protein is expressed at the apical cell surface. The amantadine-induced, M2-mediated conversion of influenza A virus haemagglutinin (HA) to the low pH conformation occurs in an acidic trans-Golgi compartment. The M2 protein ion channel activity can affect the conformation of cleaved HA during intracellular transport.The equine influenza virus 1 HA expressed from cDNA does not require coexpression of a functional M2 protein to maintain HA in its native conformation.

Key words: influenza A virus; M2 protein; haemagglutinin

Introduction

The structural proteins and genomic nucleic acids of an influenza virus particle are synthesized in different compartments of the infected cell. Haemagglutinin (HA) is the major membrane glycoprotein of influenza virus. It is a homotrimer and each of the identical subunits consists of two disulfide-linked glycopolypeptides, HA1 and HA2 (Wiley and Skehel, 1987). M2 protein is a minor component of the envelope of influenza A viruses (Zebedee and Lamb, 1988) which participates in two stages of virus replication, uncoating of infecting virus particles and maturation of the HA glycoprotein (Sugrue *et al.*, 1990a). M2 protein is encoded by a spliced mRNA derived from genome RNA segment 7; it is located in membrane with 23 N-terminal extracellular residues, 19 residues are located in the transmembrane domain, and 54 C-terminal residues represent a cytoplasmic domain (Lamb and Choppin, 1983; Lamb *et al.*, 1985). M2 protein forms a homotetramer consisting of either a pair of disulfide-linked dimers or a disulfide-linked tetramer (Sugrue and Hay, 1991).

Amantadine and rimantadine hydrochloride are specific inhibitors of replication of influenza A viruses (Dolin *et al.*, 1982). The characterization of drug-resistant mutants has identified M2 protein as the target of drug action (Hay *et al.*, 1985; Belshe *et al.*, 1988).

The aim of the present review is to summarize the structure and function of M2 protein and ultrastructural analysis of its effect on conformational changes of HA durig its exocytosis in infected cells.

Structural phenomena of M2 protein in influenza A viruses

The data presented by Sugrue and Hay (1991) indicate that in the plasma membrane of infected cells M2 protein of influenza A viruses exists as a homotetramer made up of two disulfide-linked dimers held together by non-covalent interactions. The M2 monomer has M_r of 15 K and is 97 residues long. M2 is an integral membrane protein with a single transbilayer domain (Lamb *et al.*, 1985). The C-terminal domain is palmitoylated (Sugrue *et al.*, 1990b), and *in situ*, M2 is a tetramer stabilized by inter-subunit disulfide bridges be-

Abbreviations: DNP = 2,4-dinitrophenol; FITC = fluorescein isothiocyanate; FPV = fowl plague virus; HA = haemagglutinin; MoAb = monoclonal antibody; TRITC = tetramethyl rhodamine isothiocyanate

tween the N-terminal domains (Sugrue and Hay, 1991; Holsinger and Lamb, 1991; Sansom and Kerr, 1993).

Sansom and Kerr (1993) presented a molecular modelling study of M2 protein ion channel. In this molecular model M2 channel is a bundle of four parallel M2 trans-membrane helices which surrounds a central ion-permeable pore. The helices are tilted so that the N-terminal mouth of the pore is wider than the C-terminal one. The channel is lined by valine 27, serine 31 and isoleucine 42. Aspartic acids 24 and 44 are located at opposite mouths of the pore, which is narrowest in the vicinity of isoleucine 42.

The computational techniques developed for channel-forming peptides (Sansom *et al.*, 1991) and channel and transport proteins (Sansom, 1992a,b) led to two postulates underlying the construction of M2 protein ion channel. The first postulate is that the trans-bilayer domain of M2 protein is helical. The M2 sequence exhibits features characteristic of a trans-bilayer helix, namely a hydrophobic domain. The trans-bilayer domain is alfa-helical, and thus M2 is a class III membrane protein. The second postulate is that the M2 helix is the region which forms the trans-bilayer pore and is amphipathic. The amphipathic helices are a key element of the helix bundle motif model of ion channel.

The M2 channel has been modelled as a bundle of four approximately parallel helices. Holsinger and Lamb (1991) and Sugrue and Hay (1991) experimentally evidenced the tetrameric nature of M2. The constructed model was subjected to energy minimization in order to remove any stereochemical conflicts. Thus, with the backbone atoms fixed and only the side-chain atoms free to move, the monomer was subjected to 100 cycles of steepest descents minimization followed by 250 cycles of adopted-basis Newton-Raphson minimization. The main-chain conformation of the resultant structure remained alfa-helical (Sansom and Kerr, 1993).

In order to test the hypothesis that M2 protein can in fact act as a proton chanel, Duff and Ashley (1992) applyed a synthetic 25-residue peptide corresponding to the predicted trans-membrane sequence common to several strains of influenza A. This 25-residue peptide was incorporated into voltage-clamped planar lipid bilayers. They observed single proton-selective ion channels with a conductance of ~ 10 pS at a pH of 2.3, consistent with the association of several monomers around a central water-filled pore. The channels were reversibly blocked by the anti-influenza drug amantadine.

Influenza A virus M2 ion channel protein - a function analysis

A function analysis of the influenza A virus M2 ion channel protein was performed on various influenza A virus strains and on various virus-infected tissue culture cells.

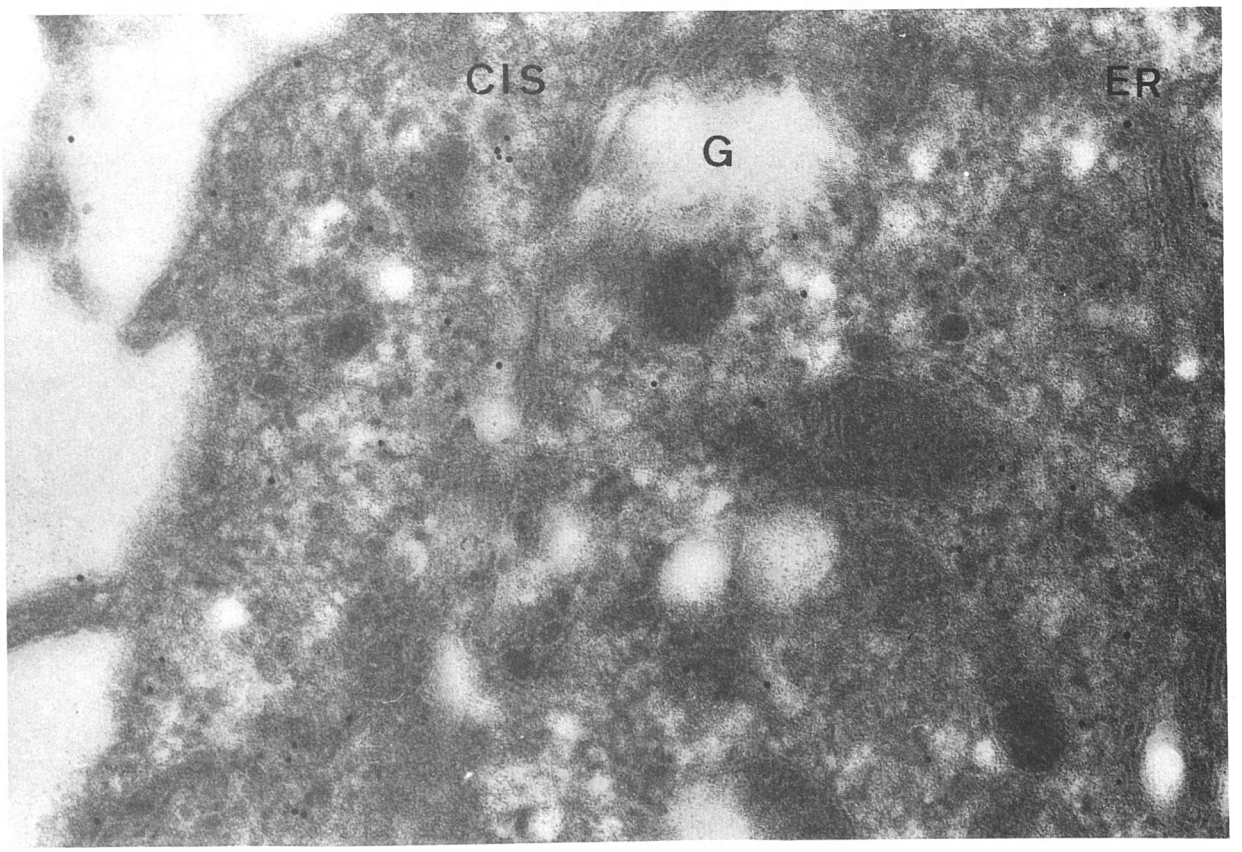
M2 protein of human influenza virus A/Udorn/72 and mutants containing changes on one face of the alfa-helix of M2 trans-membrane domain were expressed in oocytes of *Xenopus laevis*. The membrane currents of oocytes expressing mutant M2 ion channels were measured at both normal and low pH. The amantadine-resistant mutant containing a change of alanine at residue 30 to threonine was found to have a significantly attenuated low pH-activation response. The specific activity of the channel current of the amantadine-resistant mutants was studied by measuring the membrane current of individual oocytes followed by quantification of the amount of M2 protein expressed in single oocytes by the immunoblot analysis. The measurements indicated that changing residues on this face of the alfa-helix of the M2 trans-membrane domain altered properties of the M2 ion channel. Some changes of the M2 proteins in the transmembrane domain were found to be modified by addition of an N-linked carbohydrate chain at an asparagine residue that is membrane-proximal and which is not modified in the wild-type M2 protein. These N-linked carbohydrate chains were further modified by addition of polylactosaminoglycan. A glycosylated M2 mutant protein (M2+V, A30T) exhibited an ion channel activity with a voltage-activated, time-dependent kinetic component. A prevention of carbohydrate addition did not affect the altered channel activity.

The ability of M2 protein to tolerate deletions in the transmembrane domain was studied by expressing 3 mutants (del 29-31, del 28-31, and del 27-31) containing deletions of three, 4, and 5 amino acids in the transmembrane domain, respectively. No ion channel activity was detected by expression of M2 del 29-31 and del 27-31, whereas expression of M2 del 28-31 resulted in an ion channel activity that was activated by hyperpolarization (and not low pH) and was resistant to amantadine block. The examination of the oligomeric form of M2 del 28-31 indicated that the oligomer was different from the wild-type M2, and the data were consistent with M2 del 28-31 forming a pentamer (Holsinger *et al.*, 1994).

The influenza virus A/Weybridge (H7N7) M2 protein was expressed from a recombinant baculovirus in *Spodoptera frugiperda* Sf9 cells, purified and reconstituted into artificial membrane vesicles. The specific inhibitor amantadine overcame the toxic activity of the protein and boosted the rate of M2 synthesis, allowing yields of about 1 mg of purified M2 protein per g of Sf9 cells. M2 protein expressed in this system was phosphorylated and palmitoylated, and displayed properties similar to those of the authentic virus protein. The purified wild-type M2 protein and an amantadine-resistant mutant M2 with a deletion in the trans-membrane domain (amino acids 28 to 31) were incorporated into lipid vesicles, which were loaded with the fluorescent pH indicator pyranine. On imposition of an ionic gradient, M2 caused

a decrese in intravesicular pH, which was susceptible to inhibition by 0.1 to 1 μmol/l rimantadine or N-ethyl-rimantadine. M2 with a deletion of amino acids behaved similarly but exhibited the expected drug resistance. These results indicate that the isolated M2 functions as an ion channel and demonstrates *in vitro* a M2-mediated proton translocation (Schroeder *et al.*, 1994).

The *Xenopus oocyte* expression system is widely used for the expression and study of ion channels. Data presented by Giffin *et al.* (1995) characterize the electrophysiological properties of the M2 channel and indicate that the channel is activated as pH is lowered. In addition, the cellular assay also indicates that the pH induced cell „death" directly correlates with the pH activation properties of the M2 channel. Authors believe that the oocyte „death" in the assay is likely due to the continual influx of sodium cations into the cell. Increasing the sodium concentration from 96 to 150 mmol/l decreases the time course of the „death". The ability of amantadine/rimantadine to prevent the cell „death" correlates with the ability of amantadine to block the channel. Enami *et al.* (1990) and Enami and Palese (1991) generated influenza A viruses that contained the influenza virus genes derived from cDNA clones. Using this technique, influenza A viruses with mutations in the coding and non-coding regions of the PB2, HA, neuraminidase and NS genes (Subbarao *et al.*, 1993; Li *et al.*, 1992, 1993; Castrucci *et al.*, 1992; Enami *et al.*, 1991) allowed to analyze the roles of structural features of viral proteins in the replication. This reverse genetics system used Castrucci and Kawaoka (1995) for generation of an influenza A virus mutant containing a deletion of the C-terminal residue of M2 protein with amantadine resistance as a selection criterion. Transfection with an artificial M ribonucleoprotein complex of influenza virus A/Puerto Rico/8/34 (H1N1), a naturally occuring amantadine-resistant virus, and superinfection with amantadine-sensitive influenza virus A/equine/Miami/1/63 (H3N8), followed by cultivation in the presence of the drug, led to the generation of a transfectant virus with the A/Puerto Rico/8/34 (H1N1) M gene. The generated virus contained a deletion in the M gene product, M2 protein. Viruses lacking the C-terminal glutamine of M2, but not those lacking 5 or 10 C-terminal residues, were rescued in the presence of amantadine. This result indicates that the C-terminal residues of the M2 protein play an importamnt role in influenza virus replication and



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Immunogold localization of HA in FPV-infected MDCK cell
LR Gold embedding used. MoAb HC2 recognizes a peripheral domain of HA1 (site A) and reacts with most forms of both the HA0 precursor and cleaved HA1/HA2 molecules. G - Golgi stack, CIS - cis part of Golgi stack, ER - endoplasmic reticulum.

pathogenesis. The influenza viruses with either 5 or 10 amino acids deleted from the C-terminus of M2 protein damaged virus replication either fully or to such a degree that their rescue was inhibited in the reverse genetics system. The reason for the failure to generate the COOH-5 and COOH-10 mutants is unknown. According to Castrucci and Kawaoka (1995), (a) specific signals required for interaction of the M2 C-terminus with other viral proteins were obliterated by the mutations or (b) such deletions may have resulted in misfolding of the proteins and inhibition of their transport to the cell surface.

M2 protein and influenza A virus HA during intracellular transport

Amantadine treatment of cells infected with H7 strains of influenza A viruses causes an M2 protein-mediated conversion of HA from its native to low pH conformation (Čiampor *et al.*, 1992a,b). Studies of immunofluorescence with confocal laser imaging system and electron microscopic observations showed that the structural alteration and hence drug action occur shortly after HA exits from the Golgi complex during its passage through the trans-Golgi region. Using the anti-DNP pH probe it became evident that virus infection causes increased acidity of the trans Golgi region and that vesicles containing low pH HA in amantadine-treated virus-infected cells are particularly acidic. These results therefore indicate that the alteration in HA is the direct consequence of exposure to an adverse low pH and provide further support for the conclusion that M2 protein, the target of the amantadine action, is involved in regulating vesicular pH, a function important for the correct maturation of the HA glycoprotein. M2 protein has a role in maintaining the appropriate ionic environment for the matu-

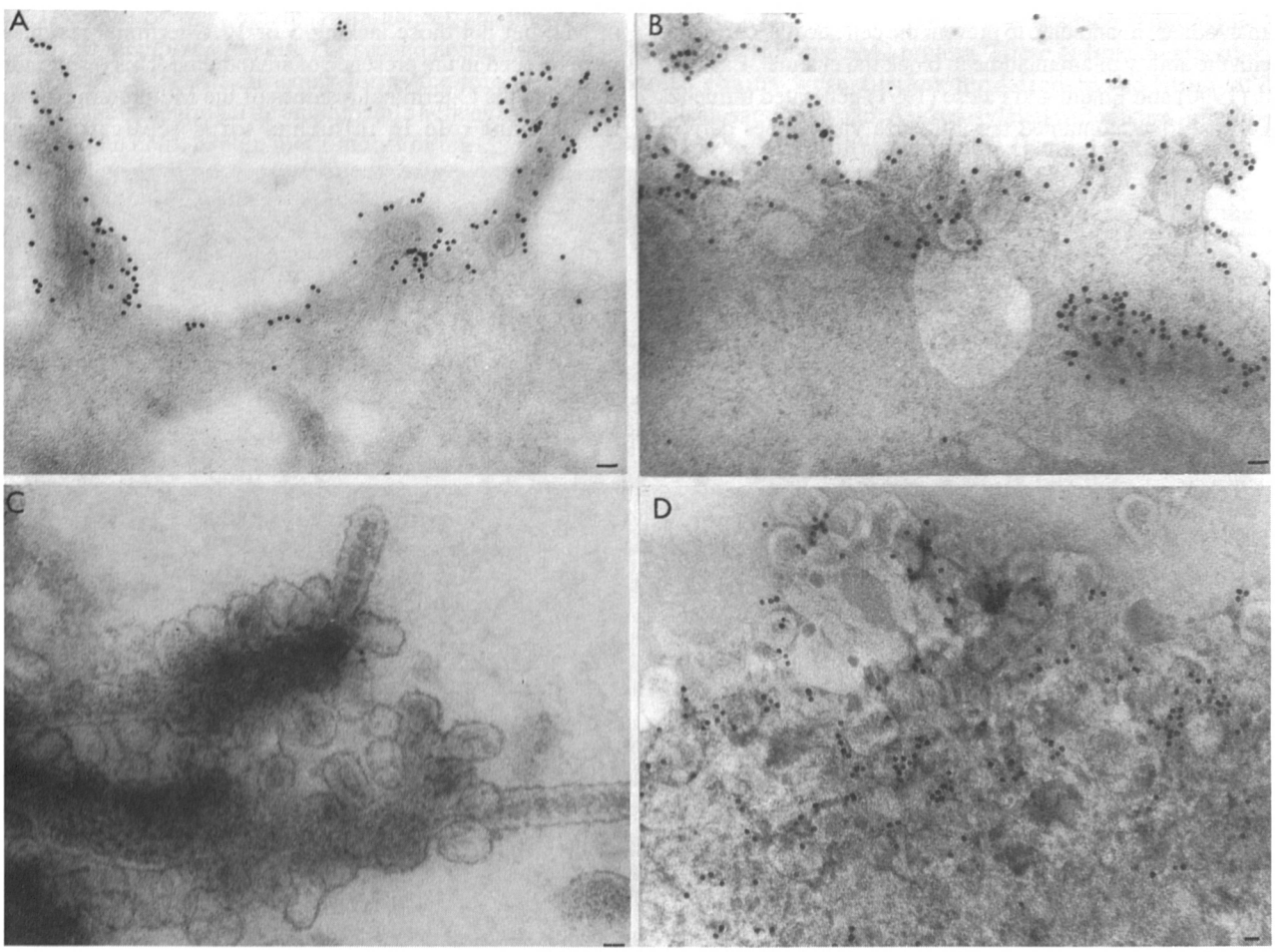

Fig. 2
Ultracryotomy of MDCK FPV-infected cells
A: MoAb HC2-treatment, amantadine not present; B: MoAb HC58-treatment (it recognizes site D close to the interface between HA1 subunits of the trimer, is specific for the native conformation and does not react with low pH HA), amantadine not present; C: MoAb HC58-treatment, amantadine present; D: MoAb H9-treatment (it recognizes low pH HA and also an epitope close to the interface region, and fails to react with native cleaved HA), amantadine present.

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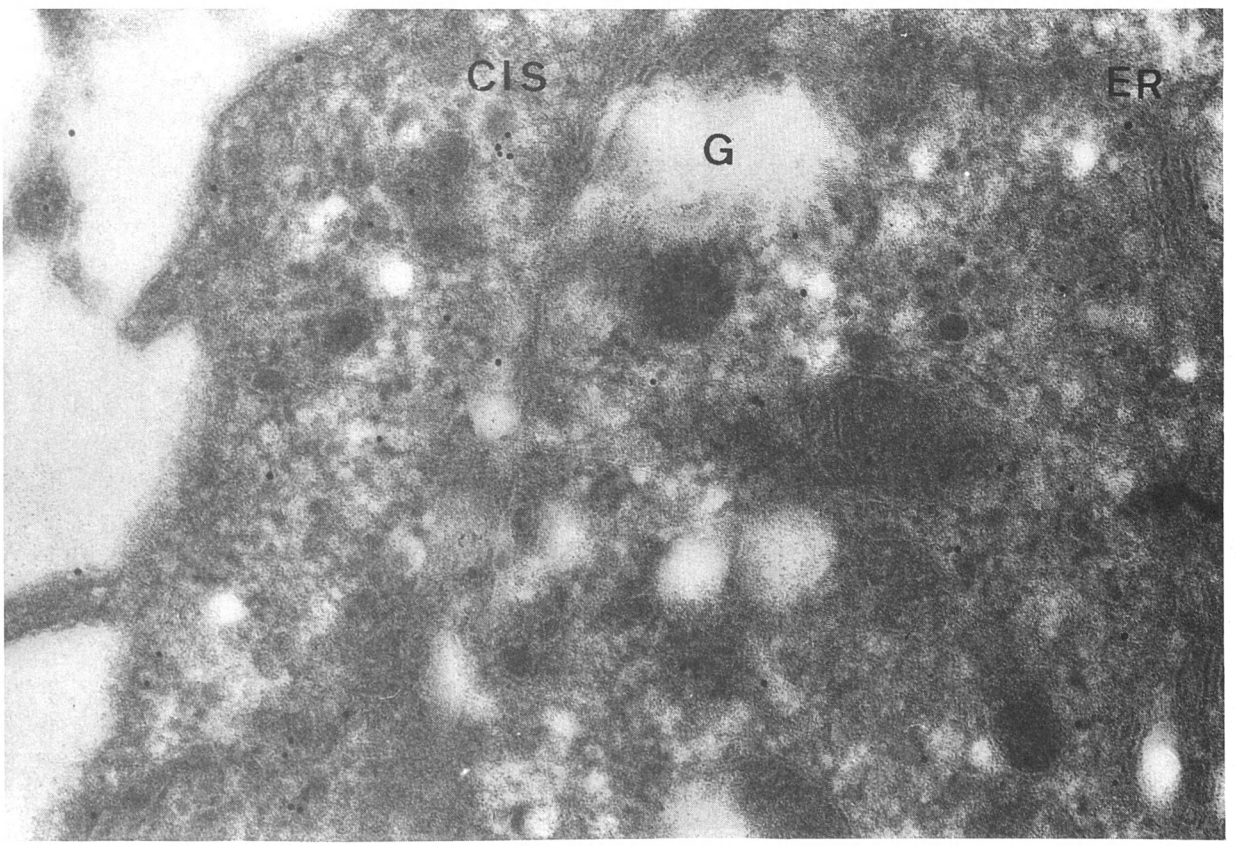

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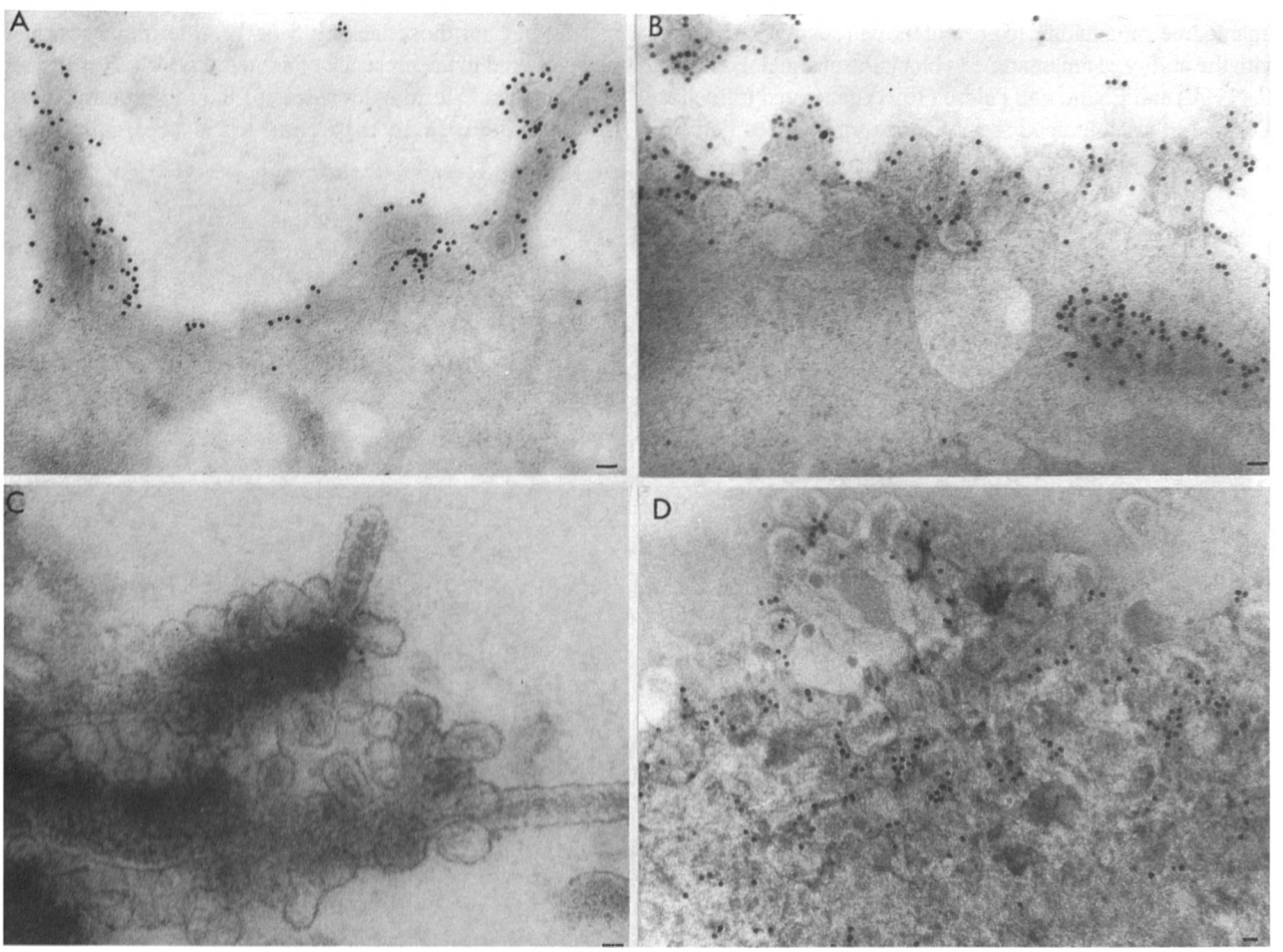

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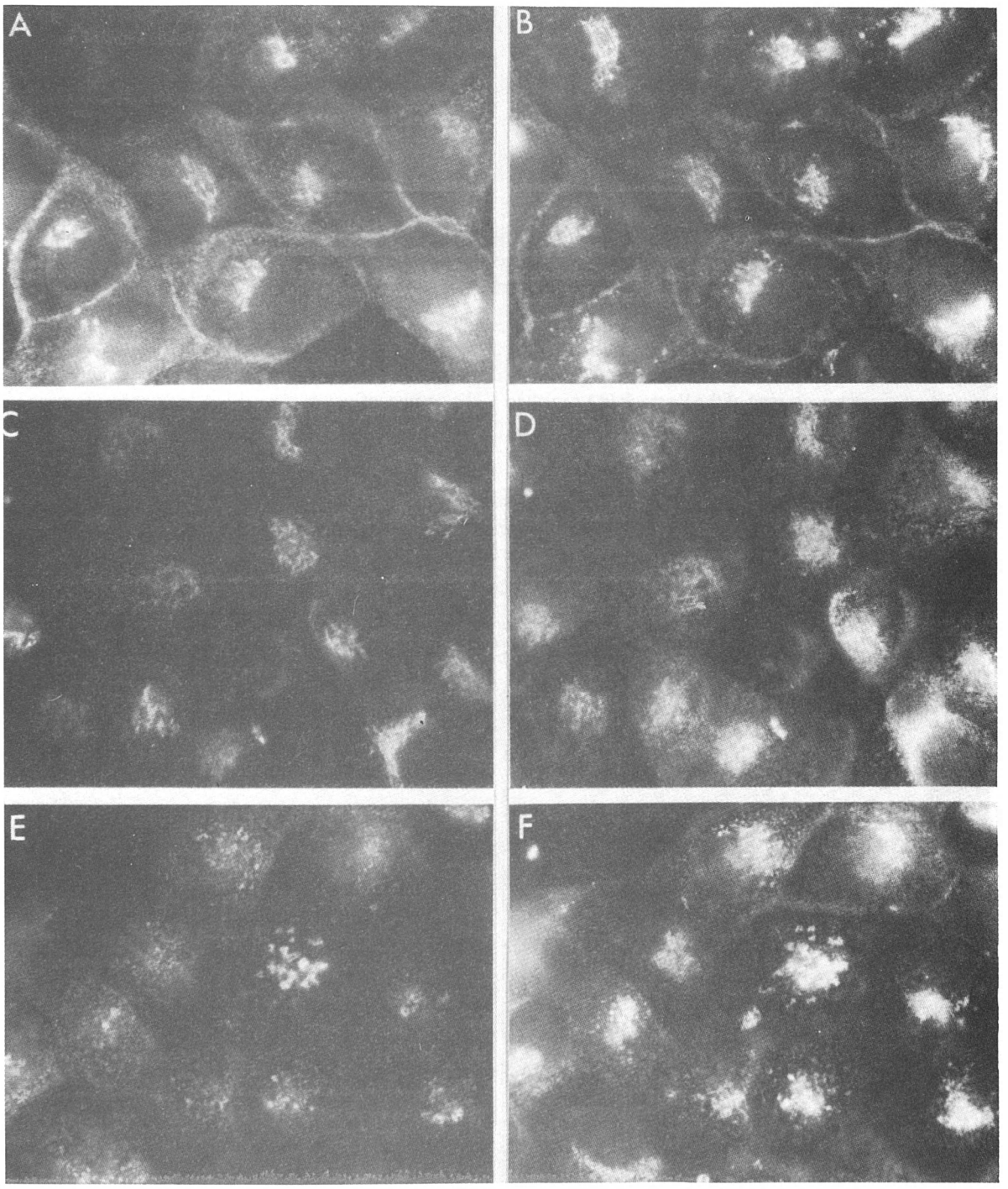

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MDCK cell fixed 5.5 hrs p.i. and reacted with wheat germ agglutinin-TRITC, MoAbs HC58 (A-D) or H9 (E-F), and rabbit anti-mouse-FITC. A,B: no amantadine added. C-F: 5 μmol/l amantadine added 1 hr p.i. A,C,E: FITC fluorescence. B,D,F: TRITC fluorescence.

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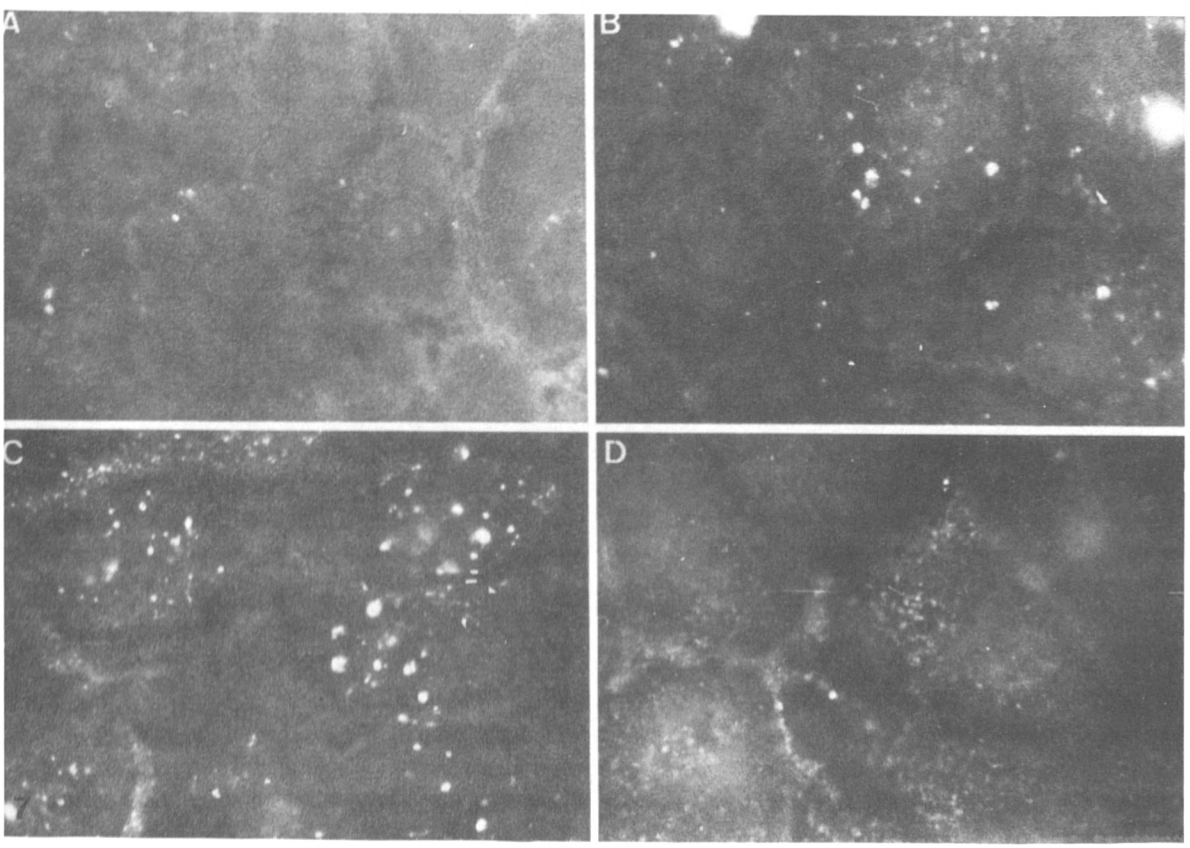

Fig. 4

Immunofluorescent localization of acidic compartments in uninfected and FPV-infected MDCK cells

A: Uninfected cells. B,D: Infected cells. C: Infected cells, 5 μmol/l amantadine added 1 hr p.i. Cells incubated with 30 μmol/l DAMP, and stained with anti-DNP MoAb and rabbit anti-mouse-FITC. D: 25 μmol/l monensin added to DAMP-treated infected cells 5 mins prior to fixation.

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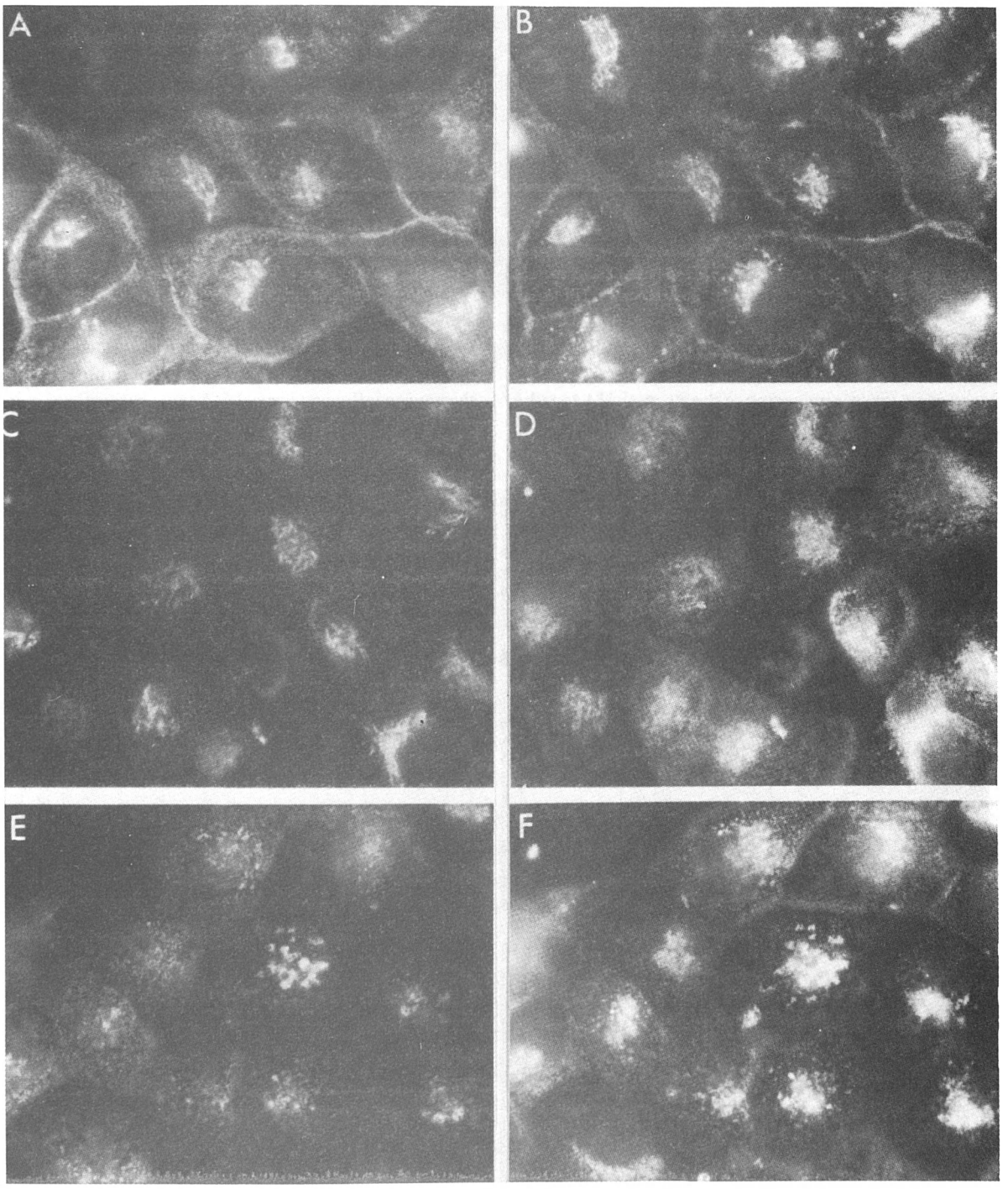

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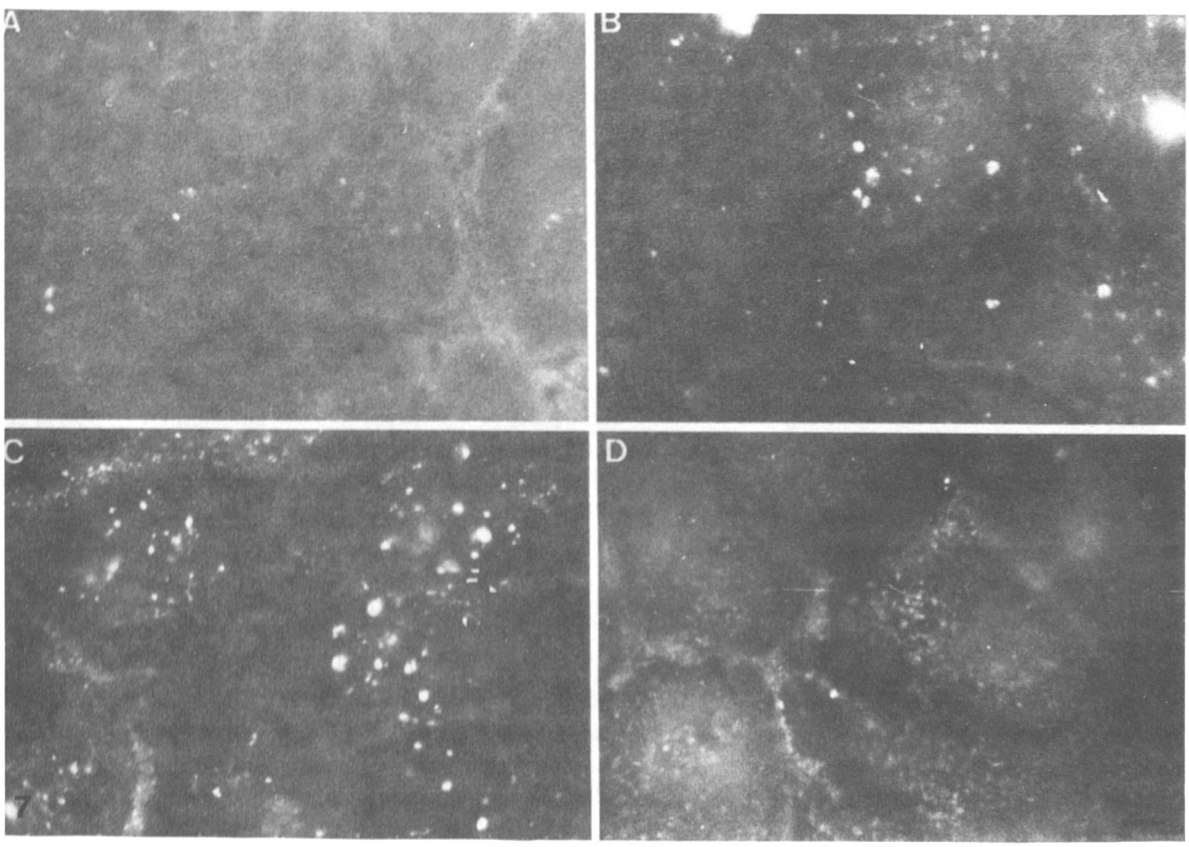

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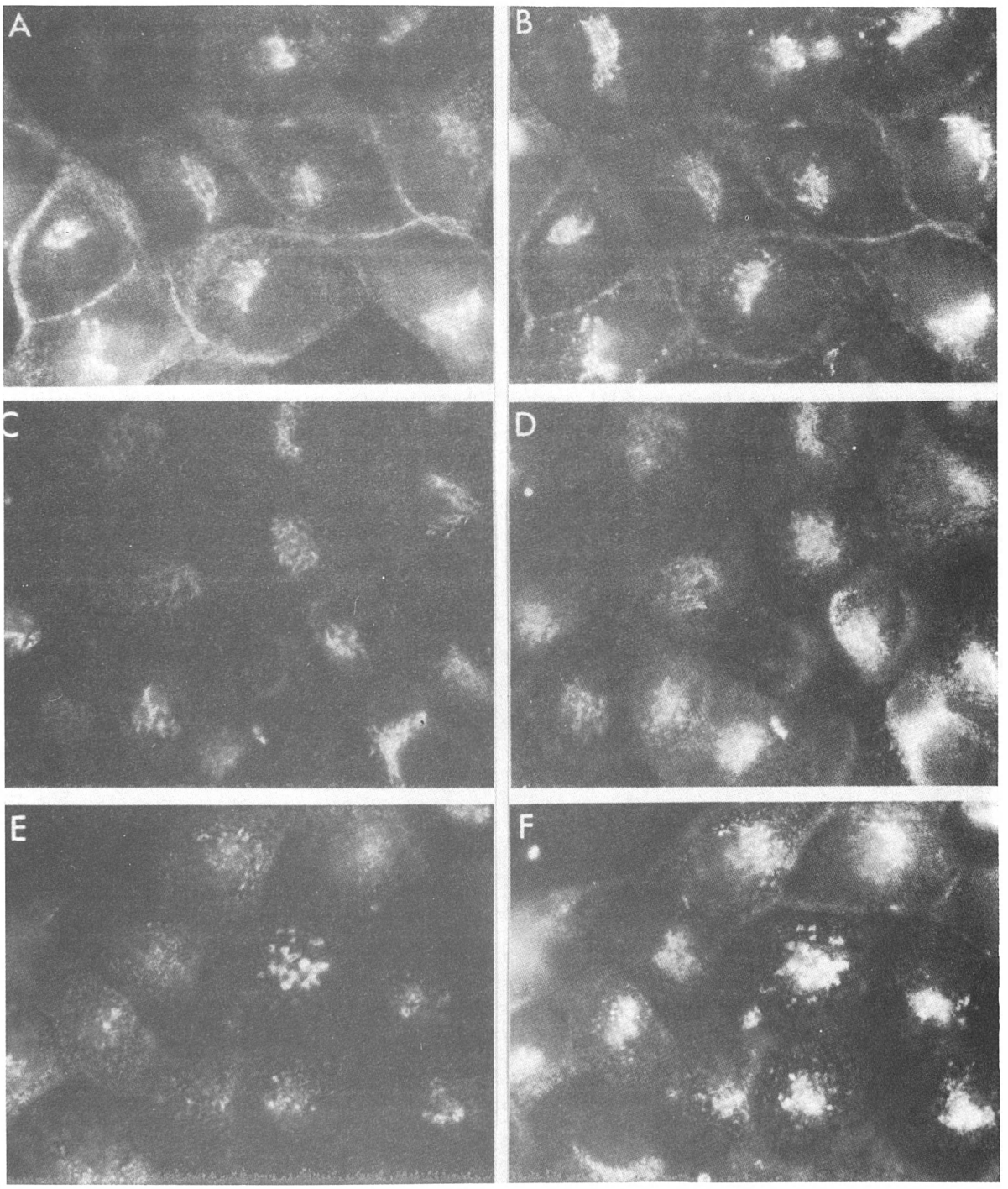

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Reassortant viruses possesing of the Rostock HA together with the Weybridge M gene have not been isolated even when subjected to stringent selection conditions, whereas viruses with the opposite combination have readily been isolated (Hay *et al.*, 1985). M gene of an amantadine-resistant variant of Weybridge strain which encoded a mutant M2 (G34E) protein did, however, readily reassort with the Rostock HA, suggesting that the increased activity of this M2 protein with respect to pH regulation (Grambas *et al.*, 1992) rendered it more compatible with the lower pH stability of the Rostock HA.

Experiments with H7 influenza A viruses emphasized the essential relationship between the characteristics of the two virus proteins, HA and M2, as well as the subtle role of M2 in regulating the pH of the transport pathway to protect the structural integrity of the HA glycoprotein (Grambas and Hay, 1992).

Takeuchi and Lamb (1994) and Takeuchi *et al.* (1994) have shown that the influenza virus M2 protein ion channel activity stabilizes the native form of fowl plague virus (FPV) HA during intracellular transport, but is not required to maintain the equine influenza virus-1 HA in its native form in infected cells. The equine influenza virus-1 A/Cornell/74 (H7N7) HA

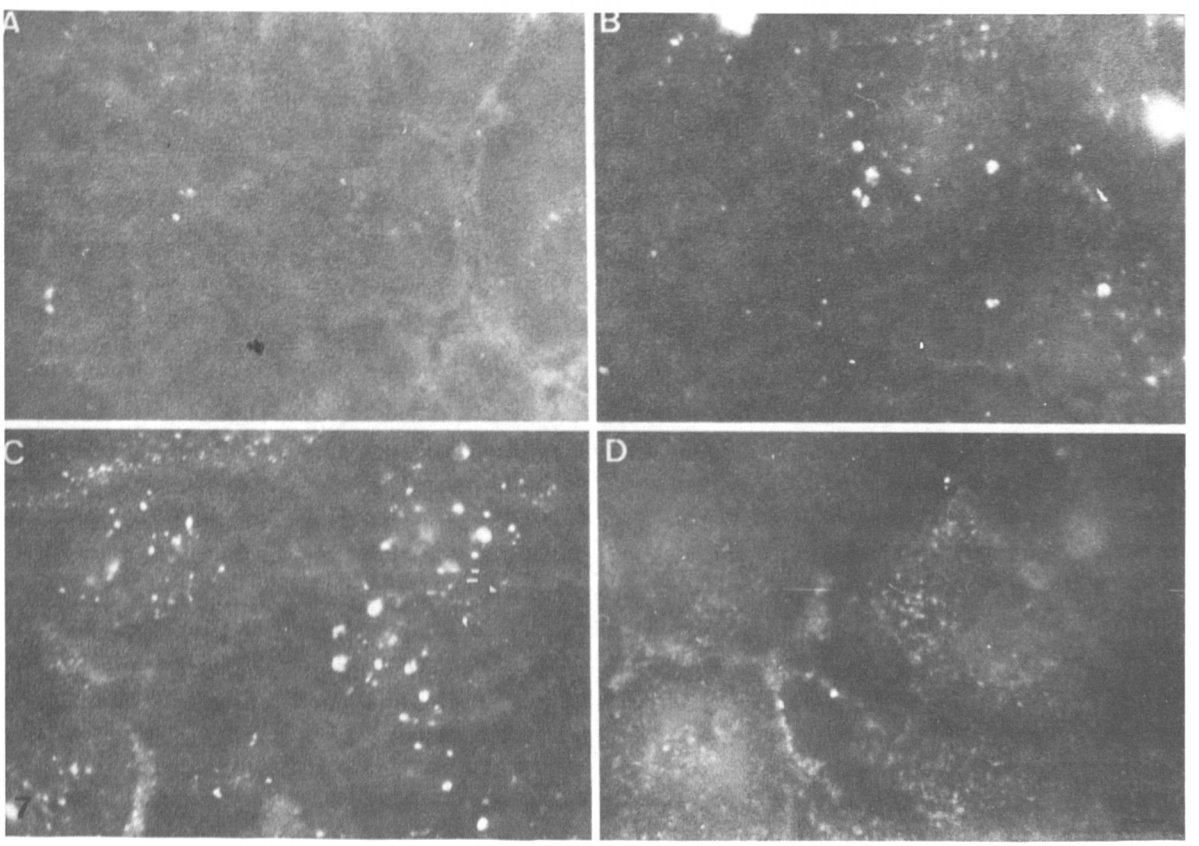

Fig. 4

Immunofluorescent localization of acidic compartments in uninfected and FPV-infected MDCK cells

A: Uninfected cells. B,D: Infected cells. C: Infected cells, 5 μmol/l amantadine added 1 hr p.i. Cells incubated with 30 μmol/l DAMP, and stained with anti-DNP MoAb and rabbit anti-mouse-FITC. D: 25 μmol/l monensin added to DAMP-treated infected cells 5 mins prior to fixation.

is cleaved to HA1 and HA2 in the trans-Golgi network of infected cells in the similar way as avian influenza virus A/chicken/Germany/34 (H7) (FPV), strain Rostock. The pH of transition to the low-pH form of the equine influenza virus-1 HA is ~pH 5.3 and the M2 protein ion channel blocker amantadine does not have a discernable effect on the native conformation of HA during transport through the trans-Golgi network. Moreover, this HA expressed from cDNA does not require coexpression of a functional M2 protein to maintain its native conformation. Also, the findings concerning the protease sensitivity of this HA expressed by vaccinia-HA recombinant virus are in contrast to the protease sensitivity of cDNA-expressed fowl FPV Rostock HA during transport through the trans-Golgi network. Thus, it is possible that the intralumenal pH of the trans-Golgi network does not decrease below pH 5.3 in MDCK cells infected with equine influenza virus-1.

The fusion assay was used to examine HA of the Rostock strain of FPV expressed in CV-1 cells by a SV40 vector and its stability in the exocytotic pathway (Ohuchi *et al.*, 1994). A 50-fold increase in the fusion activity of HA was observed when the expression occured in the presence of ammonium chloride, Tris-HCl, or high doses of amantadine. When chloroquine,another acidotropic agent, was used, HA exposed at the cell surface had to be activated by trypsin, because its intracellular cleavage was inhibited by this compound. HA mutants resistant to intracellular cleavage did not require acidotropic agents for full expression of the fusion activity, when treated with trypsin after contacting the cell surface. These facts indicate that FPV HA is denaturated in the acidic environment of the exocytotic pathway and that its cleavage is a major factor responsible for its pH instability. The coexpression of HA and M2 protein also significantly enhanced the fusion activity of HA, and this effect was inhibited by low doses of amantadine. M2 protein protects HA from denaturation by regulating the pH value in the exocytotic transport process.

The ultrastructural localization of M2 protein in influenza virus-infected MDCK cells was investigated by immunoelectron microscopy using M2-antibody and a gold conjugate (Hughey *et al.*, 1992; Čiampor, 1993). In cells in which extensive virus budding was occuring, the apical cell membrane was labelled with gold particles evenly distributed between microvilli and the surrounding membrane. In addition, a significant fraction of the M2 label was apparently associated with virions. M2 protein undergous directional transport in the absence of other viral proteins and has structural features required for apical transport in polarized epithelial cells. M2 protein is located in close proximity to budding and assembled virions (Fig. 8)

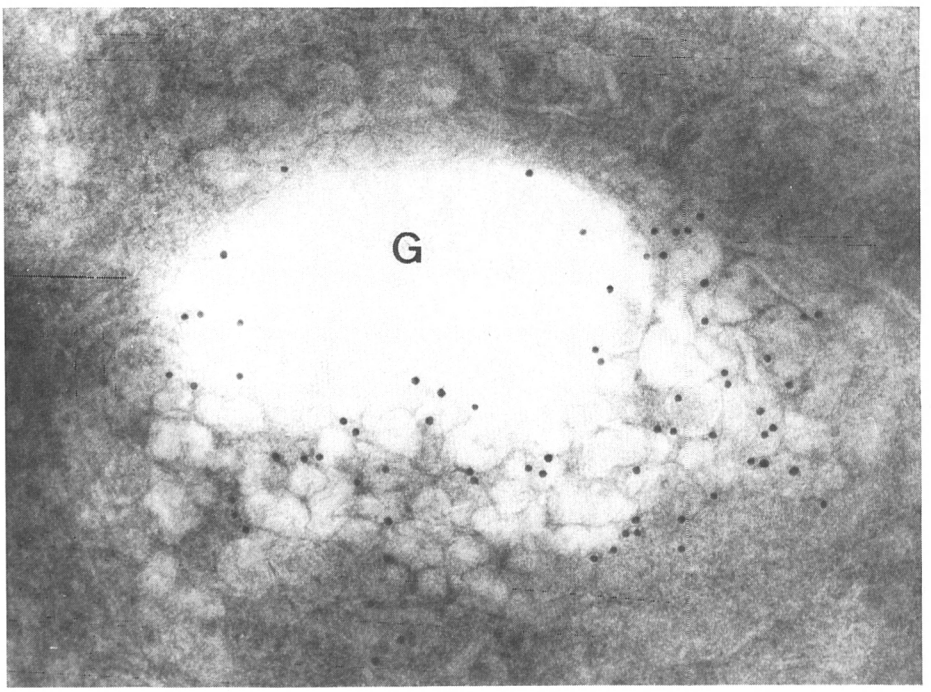

Fig. 5
Localization of acidic compartments in FPV-infected MDCK cells
Trans-Golgi (G) vesicle in an ultrathin cryosection of amantadine-treated cell labelled with anti-DNP MoAb, rabbit anti-mouse MoAb and anti-rabbit gold conjugate (10 nm gold).

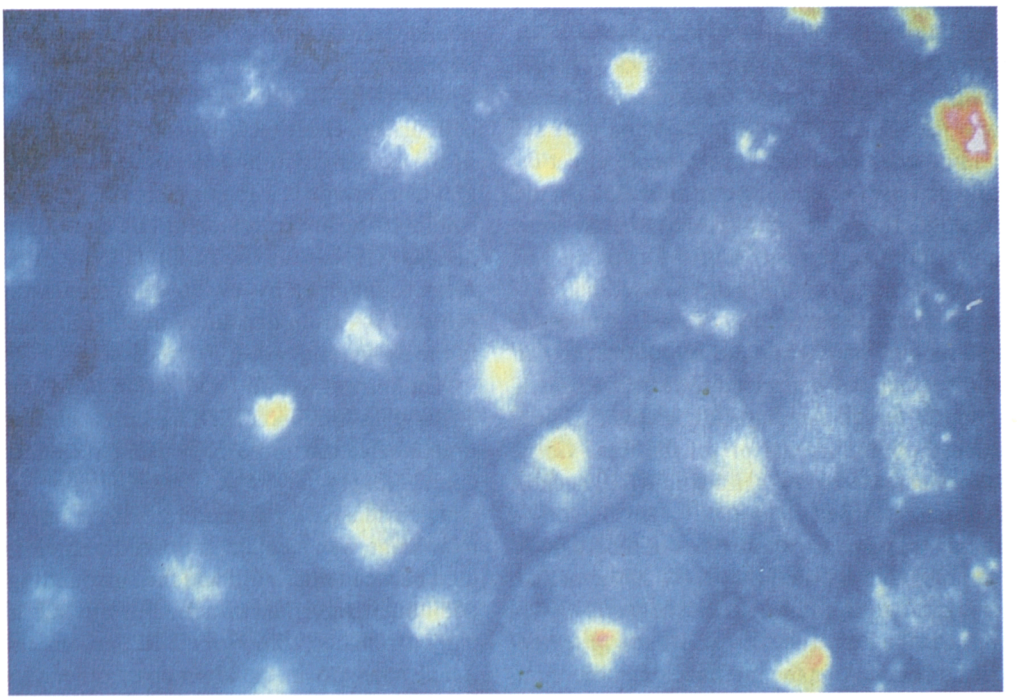

Fig. 6
Confocal optical section of fluorescent amantadine-treated FPV-infected MDCK cells
The section shows exclusive staining of the Golgi stack with the native HA-specific MoAb HC58.

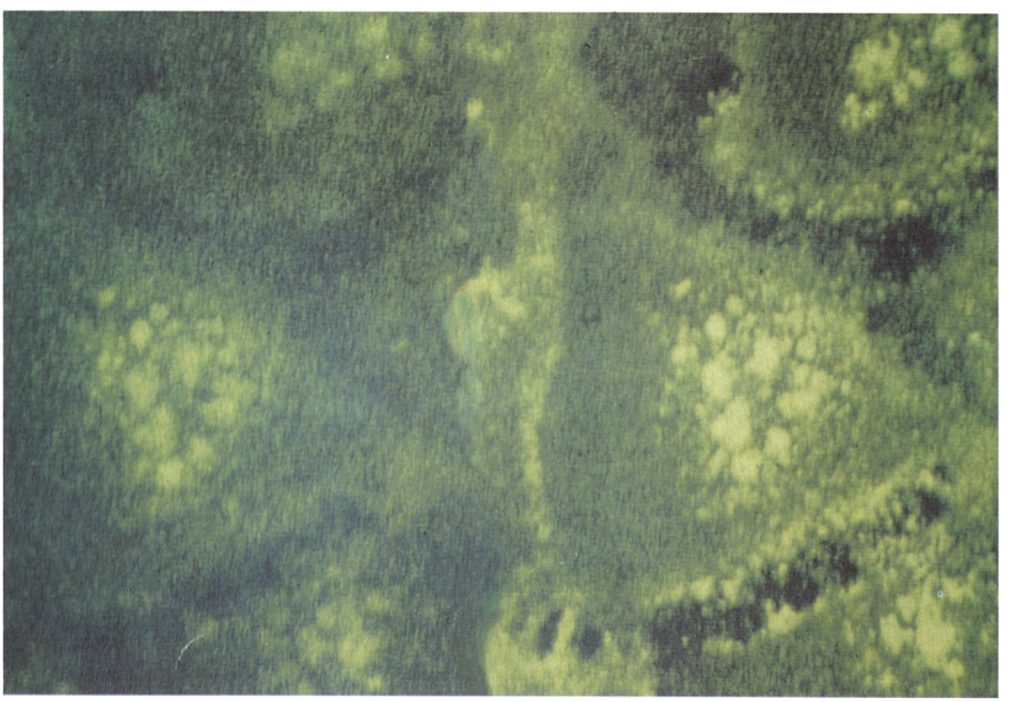

Fig. 7
Confocal optical sections of fluorescent amantadine-treated FPV-infected MDCK cells
The sections show the characteristic staining of vesicles with low pH HA-specific MoAb H9 (section planes separated by 1 μm).

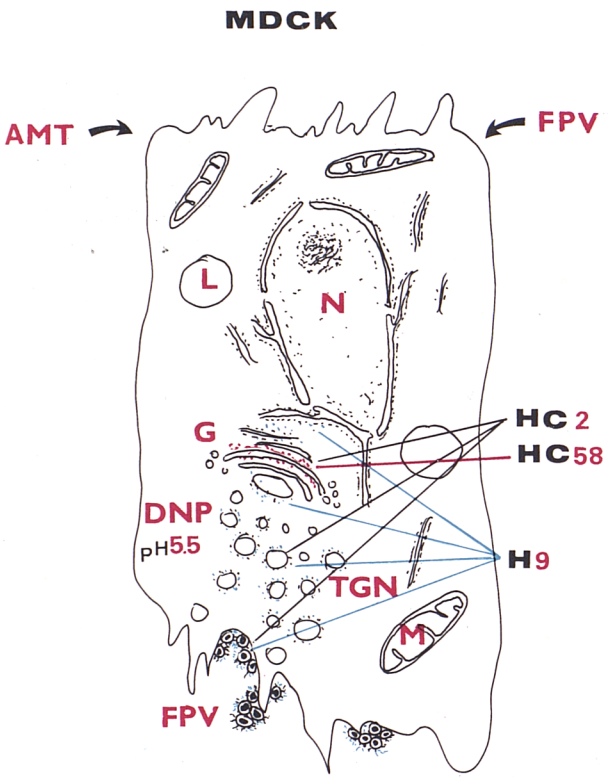

Fig. 8
Summarizing scheme of amantadine-treated FPV-infected MDCK cell
AMT – amantadine; L – lysozome; N – nucleus; TGN – trans-Golgi-network; M – mitochondria; G – Golgi stack; DNP, HC2, HC58, H9 various MoAbs.

Role of M2 protein in influenza virus membrane fusion

Influenza HA membrane glycoproteins have two functions in the initial stages of virus infection. They bind viruses to cells by recognizing sialic acid residues of cell surface glycoconjugates and, following endocytosis, they mediate fusion of virus and endosomal membranes, allowing transfer of the genome into the cell (Wiley and Skehel, 1987).

The activation of membrane fusion potential of influenza virus HA at endosomal pH requires changes in its structure. The structural changes in membrane-anchored and soluble HA preparations at the pH of fusion appear to be same. In the absence of a target membrane, the „fusion peptide" of HA in virosomes associates with the virosome membrane so that HA2 is membrane-bound at both the N- and C-termini, which implies that the structural changes do not result from the proteolytic digestion (Wharton *et al.*, 1995).

Using quantitative evaluation it has been demonstrated that HA in virions (Kostolanský *et al.*, 1988) and on the cell surface (Varečková *et al.*,1993) does not represent a homogenous population of molecules. Only a minor subpopulation of HA, representing approximately 7% of all molecules, contains a single epitope accesible to monoclonal antibody (MoAb) recognizing HA2. There is a high probability that a minor subpopulation of HA molecules containing an epitope for MoAb recognizing HA2 freely available for interaction with antibody represents in fact HA in conformation which is as a rule described as pH 5 form. The pH 5 form of HA does not necessarily include only HA molecules exposed to a low pH. Misfolding of HA molecules also leads to an increased binding of acid-specific antibodies. Amantadine and monensin have an effect on fusion kinetics. Bron *et al.* (1993) investigated the effects of the anti-influenza drug amantadine and the proton-ionophore monensin on the membrane fusion activity of influenza virus in a liposomal model system using a kinetic fluorescence lipid mixing assay. Fusion of influenza virus A/turkey/Oregon/71 (H7N3) with liposomes was slowed down in the presence of 2 μmol/l amantadine. The effect of amantadine was not observed with an amantadine-resistant virus mutant. Fusion inhibition by amantadine was reversed by the proton-ionophore monensin. The effects of amantadine and monensin increased with increasing temperature. These results suggest that intraviral low pH facilitates influenza virus fusion, possibly by weakening the interaction of the C-terminus of the viral HA with M1 protein and/or the viral nucleocapsid. The effect of amantadine on the fusion capacity of influenza virus may contribute to the anti-influenza action of this drug in the early stages of cell infection.

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