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Specific structural alteration of the influenza haemagglutinin by amantadine

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Amantadine hydrochloride specifically blocks the release of virus particles from H7 influenza virus infected cells. This appears to be the direct consequence of an amantadine induced change in the haemagglutinin (HA) to its low pH conformation. The effect is indirect and mediated via interaction of the drug with the M2 protein since mutants altered in this component alone are insensitive to amantadine. The timing of drug action, some 15–20 min after synthesis, and its coincidence with proteolytic cleavage indicates that the modifications to HA occur late during transport but prior to insertion into the plasma membrane. Reversal by mM concentrations of amines and 0.1 μM monensin indicates that amantadine action causes a reduction in intravesicular pH which triggers the conformational change in HA. We conclude, therefore, that the function of M2 inhibited by amantadine is involved in counteracting the acidity of vesicular compartments of the exocytic pathway in infected cells and is important in protecting the structural integrity of the acid-sensitive glycoprotein.

Key words: amantadine/influenza haemagglutinin/M2 protein

Introduction

Amantadine (1-aminoadamantane hydrochloride) and related compounds, at micromolar concentrations, specifically inhibit the replication of influenza A viruses in tissue culture (Hoffman, 1973; Appleyard, 1977; Hay *et al.*, 1986) and amantadine is effective when used prophylactically in preventing infections in humans, animals and birds (reviewed by Tominack and Hayden, 1987; Bryans *et al.*, 1966; Webster *et al.*, 1985). The characterization of drug resistant mutants isolated either following passage of virus in tissue culture in the presence of drug (Hay *et al.*, 1985) or from humans or birds treated with either rimantadine (α-methyl-1-adamantane methylamine) or amantadine, respectively (Belshe *et al.*, 1988; Bean *et al.*, 1989; Hayden *et al.*, 1989) has indicated that the target of drug action is the membrane-spanning domain of the M2 protein. The similarities in the single amino acid substitutions which confer drug resistance demonstrates the correspondence between the mechanisms of action *in vivo* and *in vitro*. In cell culture two stages in the replicative cycle have been shown to be susceptible to drug action: (i) an essential aspect of virus uncoating which appears to be distinct from membrane fusion *per se* (Kato and Eggers, 1969; Bukrinskaya *et al.*, 1982; Wharton *et al.*, 1990) and (ii) a feature important for virus maturation. Although susceptibility to the former action appears to be determined largely by the M gene (Lubeck *et al.*, 1978; Hay *et al.*, 1979), analyses of reassortant viruses showed that susceptibility of certain H5 and H7 viruses to the latter action is dependent also on properties of their haemagglutinins (Scholtissek and Faulkner, 1979; Hay and Zambon, 1984; Bean *et al.*, 1989). Furthermore, inhibition of virus maturation has been shown to be associated with changes in the expression of the haemagglutinin (HA) on drug-treated infected cells (Hay *et al.*, 1986).

From the data presented here, it is apparent that amantadine treatment results in the expression of the low pH form of HA on the surface of H7 virus infected cells. This is due to an M2 protein mediated action since drug resistant viruses containing mutations in M2 alone are insensitive. The evidence indicates that the drug acts late in the transport pathway, close to the site of proteolytic cleavage of HA, by causing a reduction in intravesicular pH.

Results

Whereas most influenza A viruses tested exhibit significant sensitivity to amantadine only when the drug is present prior to or coincident with virus infection, the replication of several viruses of the H7 and H5 subtypes is specifically inhibited by low concentrations of amantadine (0.5–5 μM) added shortly after infection (Hay and Zambon, 1984; Hay *et al.*, 1986). The Rostock strain used in the present experiments exhibited the greatest sensitivity both with respect to inhibition of virus production and modification of HA. The simplest and most sensitive assay for monitoring changes in HA was an ELISA of infected cell monolayers using conformation specific monoclonal antibodies.

Table I. Amino acid substitutions in HA1 of monoclonal antibody selected variants

Monoclonal antibody	Amino acid substitution[a]
HC1	128 Ser → Asn
HC2	144 Gly → Glu
HC3	128 Ser → Ileu
HC10	161 Ala → Asp
HC58[b]	198 Gly → Glu
	250 Ala → Ser
HC61	210 Hist → Asn
H9[c]	205 Gly → Arg

[a]Amino acid residues are numbered according to analogous positions in the X-31 sequence (depicted in Figure 1) (Wilson *et al.*, 1981).
[b]Two separate mutants were characterized.
[c]A mutant which fails to react with H9 was isolated following passage in the presence of biperiden hydrochloride (30 μg/ml) (unpublished data).

Specificities of monoclonal antibodies

The epitopes recognized by six monoclonal antibodies against H7 HA were mapped by RNA sequence analyses of the HA genes of escape mutants isolated following growth of Rostock virus in chick fibroblasts in the presence of each antibody (Table I, Figure 1). Each variant was characterized by a single amino acid substitution in HA1 which, by reference to the structure of the H3 HA (Wilson *et al.*, 1981) occurs in the previously identified antigenic regions A (HC1, HC2, HC3), B (HC10) and D (HC58, HC61). Of particular interest in the present context are HC2 which recognizes the peripheral loop (Figure 1) and reacts efficiently with most conformations of both cleaved and uncleaved forms of the molecule (Figure 2); and HC58 and HC61 which react with regions close to the interface between subunits of the native trimer and discriminate most clearly between the low pH and native forms. A monoclonal antibody (H9) specific for the low pH form of HA (Skehel *et al.*, 1982) (Figure 2), fails to interact with a mutant HA containing a single amino acid substitution, Gly205→Arg in HA1, indicating that it too recognizes a site close to the interface region of the protein.

Antigenic characteristics of HA on amantadine-treated cells

Consistent with earlier observations that amantadine (5 μM) added 1 h after infection does not reduce the synthesis of HA, this treatment caused no detectable alteration in the ELISA of HA on the surface of infected cells using HC2 (Table II). In contrast, reaction of HC58 and HC61 with HA on amantadine-treated cells was virtually abolished, while recognition by HC3 and HC10 were also reduced. On the other hand, reaction of H9 was substantially increased to a level equivalent to that of virus infected cells treated with PBS-citrate pH 5.0. The similarities between low pH HA and HA on amantadine-treated infected cells is further emphasized by the similarities in the spectrum of reactivities to the five monoclonal antibodies. The specificity of the action was confirmed by the absence of any effect of equivalent concentrations of amantadine on the HA of amantadine resistant viruses.

The results of quantitative immunoprecipitation experiments, as those shown in Figure 2A–C, confirmed the absence of native, HC58-reactive, HA on amantadine-treated cells and its replacement with low pH, H9 reactive, HA. As with the ELISA it was consistently observed that in the absence of amantadine a proportion, ~20–30%, of HA on virus infected chick embryo fibroblasts is in the low pH

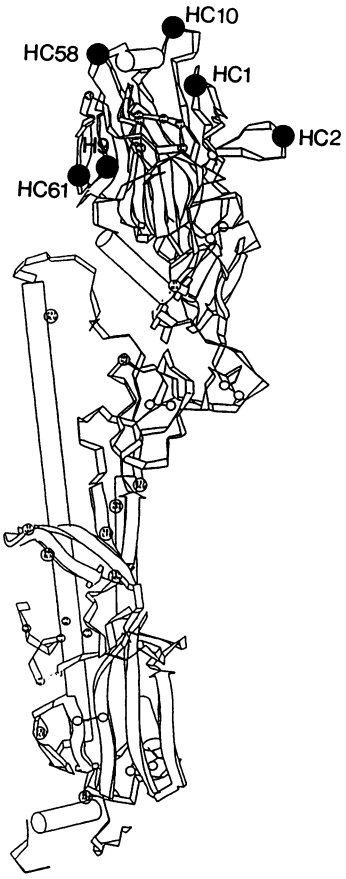

Fig. 1. Locations on the haemagglutinin subunit of amino acid changes (Table I) which abolish recognition by monoclonal antibodies. The inter-subunit interface is to the left of the diagram.

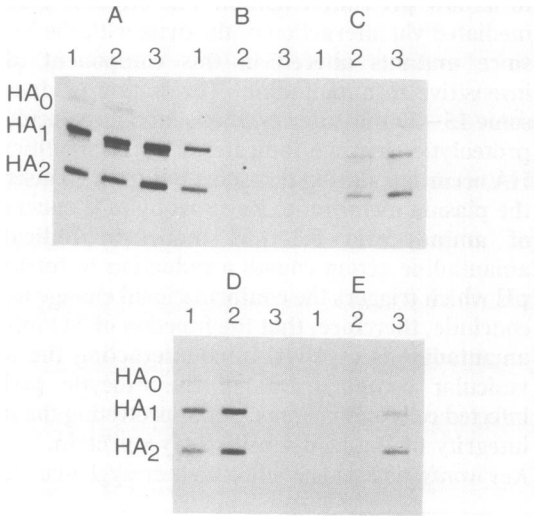

Fig. 2. Immune precipitation of HA from [^{35}S]methionine-labelled infected cells (1), infected cells incubated with amantadine (5 μM) from 1 h after infection (2) and infected cells treated for 5 min with PBS-citrate pH 5.0 at 5.5 h post infection (p.i.), prior to lysis (3). Lysates were immunoprecipitated with HC2 (A), HC58 (B and D) or H9 (C and E) monoclonal antibodies. Chick cells were infected with Rostock (A–C) or Rostock^{R} (D and E).

Table II. Antigenicity of HA expressed on amantadine-treated infected cells and cells exposed to pH 5

Antibody	ELISA (percentage of control)			
	pH 7		pH 5	
	−A	+A[a]	−A	+A
HC2	100	96	96	96
HC3	100	16	16	5
HC10	100	70	56	58
HC58	100	8	3	0
HC61	100	12	2	0
H9	25	116	100	139

[a]Amantadine (5 μM).
The data are expressed as a percentage of the OD_{450} in the non-drug-treated controls, except for H9 in which case they are expressed relative to the pH 5-treated, non-drug-treated cells.

form. The HA of an amantadine resistant mutant, Rostock^R, was completely refractory to amantadine treatment (Figure 2D and E).

Tryptic cleavage and reductive dissociation

Two other criteria which distinguish the low pH conformation of HA from its native structure include the accessibility of specific sites to cleavage by trypsin (Skehel *et al.*, 1982) and the loss of stabilizing interactions between the HA1 and HA2 subunits (Graves *et al.*, 1983).

From the data shown in Figure 3A it is apparent that the HA on Rostock infected cells incubated with amantadine or on control cells exposed briefly to PBS-citrate pH 5.0 is similarly susceptible to specific digestion by trypsin. The loss of >50% of HA1 (estimated by microdensitometry) was accompanied by the release into the supernatant of specific fragments with apparent molecular weights of ~39 kd and 34 kd. In view of the differences in amino acid sequence of the HAs of X-31 and Rostock it is not possible to relate the observed digestion products to the previously identified 40 kd and 25 kd fragments of X-31 HA (Skehel *et al.*, 1982). The low, but detectable, level of cleaved fragments released from control cells, evident in autoradiographs after longer exposure (not shown), presumably reflects the presence of low amounts of the low pH HA on the surface of these cells. HA on cells infected with Rostock^R in contrast exhibited no susceptibility to tryptic digestion as a result of equivalent amantadine treatment (Figure 3B).

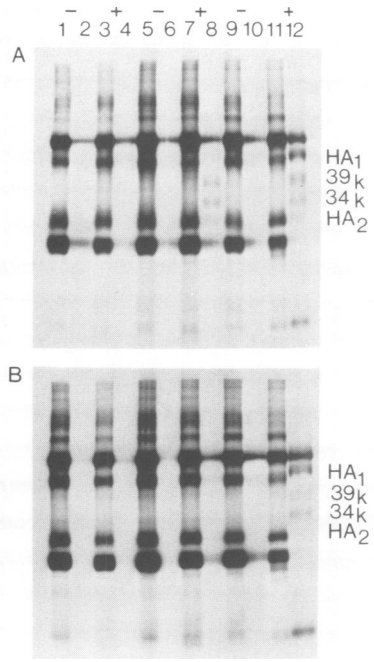

Fig. 3. Trypsin susceptibility of HA on amantadine-treated infected cells. Chick cells infected with Rostock (**A**) or Rostock^R (**B**) were labelled with [^{35}S]methionine between 5 and 5.5 h p.i. and subsequently incubated at 37° for 30 min in PBS with (+) or without (−) trypsin (20 μg/ml). Soyabean trypsin inhibitor (100 μg/ml) was added and cell supernatants clarified. Cells were washed in PBS and both fractions solubilized in SDS, urea, β-mercaptoethanol and analysed on 16% polyacrylamide gels. Odd numbers, cell pellet; even numbers, cell supernatant. Lanes 1−4, untreated cells; lanes 5−8, cells incubated with amantadine from 1 h p.i.; lanes 9−12, cells treated for 5 min with PBS-citrate pH 5.0 at 5.5 h p.i.

Amantadine treatment of infected cells also rendered HA1 susceptible to removal by incubation with dithiothreitol (DTT) (20 mM for 10 min) in the absence of any additional pH 5 treatment in contrast to control cells (Figure 4). Again, this effect was shown to be specific to amantadine by the absence of any influence on the HA of cells infected with Rostock^R.

ELISA of HA on DTT-treated infected cells incubated in the presence of amantadine also showed the removal of H9 reactivity—confirming that this monoclonal antibody recognizes a site on the HA1 component, as already discussed.

Sedimentation analysis

Sucrose gradient analysis of HA labelled in a pulse−chase experiment showed the conversion of HA monomers (~4S) (Figure 5A and B) to trimers of HA1/HA2 (~9S) (Figure 5C−F). Neither incubation with amantadine nor brief pH 5 treatment of infected cells affected this conversion. The only difference noted relative to control cells was the tendency of HA from either set of cells to sediment more diffusely (Figure 5D)—the faster sedimenting HA present in the pellet and lower gradient fractions, in addition to undissociated nucleoprotein and matrix protein, presumably reflecting aggregation of low pH HA (Skehel *et al.*, 1982). This modification, also noted with pH 5-treated HA, was amantadine specific and was absent in analyses of HA from amantadine-treated, Rostock^R infected cells (Figure 5F).

Post-translational modification of HA

Addition of carbohydrate side chains and palmitate moieties to HA was not significantly affected by incubation of Rostock infected cells in the presence of 5 μM amantadine. [^{3}H]palmitate labelling of M2 (Sugrue *et al.*, 1990) as well

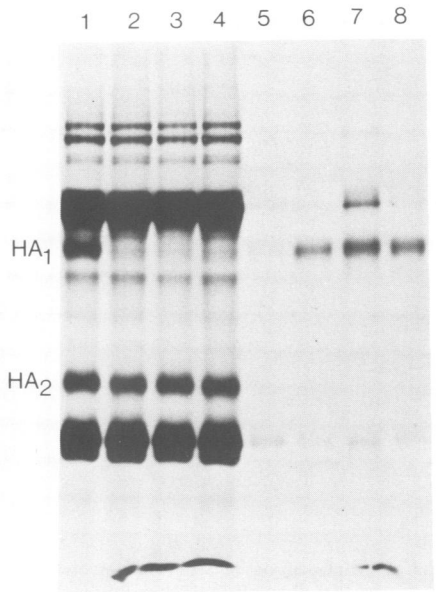

Fig. 4. Reductive dissociation of HA on amantadine-treated cells. Pairs of amantadine-treated and control infected cultures, labelled with [^{35}S]methionine, were treated with PBS or PBS-citrate pH 5.0 for 5 min at room temperature (RT) prior to incubation for 10 min at RT with PBS containing 20 mM DTT. Equivalent samples of solubilized cells (1−4) and supernatants (5−8) were analysed. 1 and 5, control; 2 and 6, amantadine-treated; 3 and 7, pH 5-treated; 4 and 8, pH 5-treated, amantadine-treated.

as HA (Schmidt, 1982) was not reduced by drug treatment. Labelling of HA with [^{3}H]mannose and [^{3}H]glucosamine was unaltered by the presence of the drug and there was only a small, inexplicable increase in the ratio of label associated with HA0 as compared with HA1/HA2 following incubation with the terminal sugars [^{3}H]fucose and [^{3}H]galactose. No difference in endoglycosidase H susceptibility was detected. The presence of amantadine also did not influence proteolytic cleavage of HA and as shown by the results of a pulse–chase experiment (Figure 6) the rate of transport of HA to the site of cleavage was apparently also unaffected. The alteration in HA caused by the drug in this instance, as noted in a number of other experiments, was evident from the slightly faster migration of HA1. The equivalent increase in electrophoretic mobility of pH 5-treated HA1 (see Figure 2) indicates that it is a consequence of the conformational change, rather than of an alteration in e.g. glycosylation or proteolytic cleavage.

Site of action

Based on inhibition of virus production it was previously concluded that to be effective amantadine had to be present prior to synthesis of HA. More direct examination of the effect upon HA by immune precipitation, however, showed this not to be the case. The results of pulse–chase experiments, as shown in Figures 7 and 8 indicated that provided the drug was added within ~20 min of the pulse, i.e. synthesis of HA, >50% of HA was converted to the low pH form. Addition later than 40 min after synthesis has little effect, showing that HA already on the cell surface was unaffected. Comparison of this data with a time course of HA cleavage, done in parallel, indicates coincidence between the 'sites' of amantadine action and HA cleavage (Figure 8).

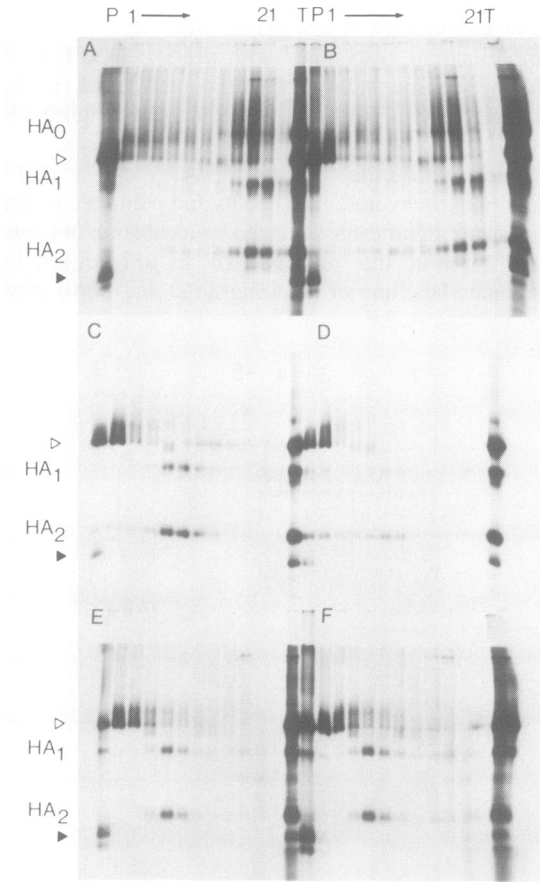

Fig. 5. Effect of amantadine on the sedimentation of HA. Chick cell monolayers infected with Rostock (**A**–**D**) or Rostock^R (**E** and **F**) and incubated in the presence (B,D,F) or absence (A,C,E) of 5 μM amantadine from 1 h p.i. were pulsed at 5 h for 5 min with [^{35}S]methionine (20 μCi/ml) and either harvested immediately (A and B) or chased for 60 min in medium containing 1 mM methionine (C–F). Octyl glucoside extracts were analysed as described in Materials and methods. Equivalent samples of total extract (T), pellet (P) and alternate fractions, 1 (bottom) to 21 of the gradient, were analysed on 16% polyacrylamide gels. ◁ and ◀ indicate the positions of nucleoprotein and matrix protein, respectively.

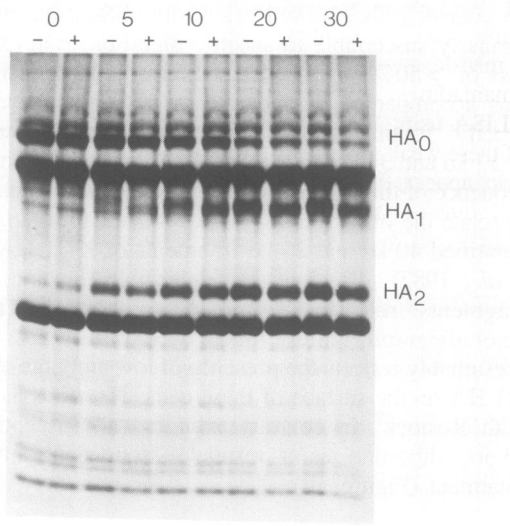

Fig. 6. Proteolytic cleavage of HA in the presence of amantadine. At 5 h after infection, cells incubated in the presence (+) or absence (–) of amantadine (5 μM) from 1 h p.i., were pulse-labelled (5 min) with [^{35}S]methionine (20 μCi/ml) and chased for 0, 5, 10, 20 or 30 min.

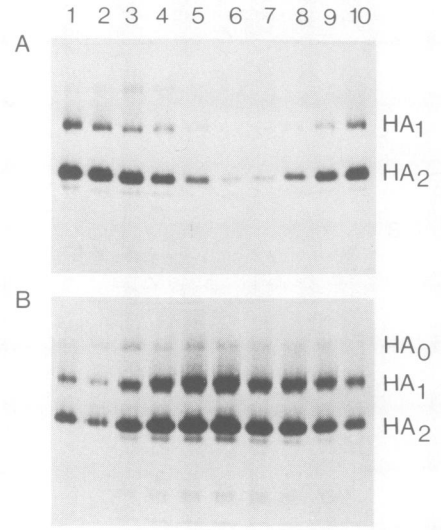

Fig. 7. Temporal localization of the site of amantadine action and its reversal by monensin. Monolayers infected for 5 h were pulse-labelled for 5 min with [^{35}S]methionine (20 μCi/ml) and chased for 60 min in the presence of 1 mM methionine. Lane 1, control cells; lane 2, cells treated with 0.1 μM monensin from 1 h p.i; lanes 3–6, cells treated with amantadine from 1 h p.i. and monensin (0.1 μM) added at 0(3), 10(4), 20(5) and 40(6) min, respectively after the start of the chase; lanes 7–10, amantadine (5 μM) added at 0(7), 10(8), 20(9) and 40(10) min, respectively, after the start of the chase. NP-40 lysates were immunoprecipitated with HC58 (**A**) or H9 (**B**).

Furthermore the data shown in Figure 6 are consistent with only the altered form of HA1/HA2, the HA1 of which exhibits an increased migration, being produced in drug-treated cells.

Reversal by amines and ionophores

In view of the nature of the alteration in HA structure one of the simplest explanations for the action of amantadine would be that it causes a reduction in the pH to which the HA is exposed during its transport to the plasma membrane. This question was investigated by examining the effects of conditions and agents likely to cause an elevation in the pH of intracellular vesicular compartments (Figure 9). Ammonium chloride and methylamine at concentrations of 4 mM largely abolished the production of low pH HA in amantadine-treated Rostock infected cells, as assessed by ELISA using HC58 and H9. By analogy with the effects of these weak bases concentrations of amantadine >0.1 mM also appear to antagonize the specific anti-M2 action of micromolar drug concentrations (Figure 9D) providing an explanation for the 'anomalous' concentration dependence of amantadine action. Incubation of cells in medium with a pH of between 8.4 and 8.9 also partially antagonized the action of amantadine in Rostock infected cells without affecting the level of HA synthesis. Low concentrations (~0.1 μM) of the ionophores monensin (Figure 9C) and nigericin (data not shown) also abolished the action of amantadine without affecting significantly the synthesis or transport of HA. Pulse–chase experiments as described in Figure 7 showed a reciprocal relationship between the point of reversal by monensin and the point of action of amantadine.

Discussion

The structure of HA expressed on the surface of amantadine-treated Rostock virus infected CEF cells was, by all criteria used, indistinguishable from the low pH form which results from exposure of the native molecule to pH 5 (Skehel *et al.*, 1982). Earlier studies (Hay *et al.*, 1986) had shown that although the amount of HA produced was not reduced as a result of amantadine action, HA2 was no longer labelled by lactoperoxidase catalysed iodination. Neither did drug-treated infected cells exhibit cell–cell fusion as indicated by cell lysis (CEF and MDCK cells) or heterokaryon formation (VERO cells) following brief exposure to pH 5 as observed in control infected cultures. No significant alterations were noted in post-translational modifications of the polypeptide either in glycosylation or palmitoylation or their association to form trimers. Transport to the site of proteolytic cleavage and subsequent insertion into the plasma membrane were also not impaired. Thus in the absence of any other amantadine specific changes in the production or localization of virus components (Hay *et al.*, 1986) it would appear that inhibition of virus release by amantadine is the direct consequence of HA being in its low pH conformation. Electron microscopic observations (Ruigrok *et al.*, 1990) have revealed that amantadine treatment does not prevent the formation of budding virus particles but blocks their release. Whether this is due simply to the hydrophobic nature of low pH HA and its tendency to aggregate has yet to be resolved.

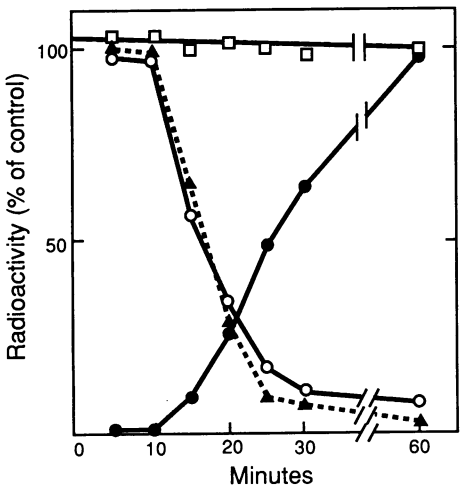

Fig. 8. Temporal comparison of the proteolytic cleavage of HA and its susceptibility to amantadine. Data was obtained from a pulse–chase experiment similar to those described in Figures 6 and 7, which compared cleavage of HA(▲) into HA1/HA2 after different chase periods with the proportion of HA immunoprecipitated by HC2 (□), HC58 (●) or H9 (○) after 60 min chase following addition of amantadine at different times during the chase period. Data from autoradiographs was quantitated by microdensitometry.

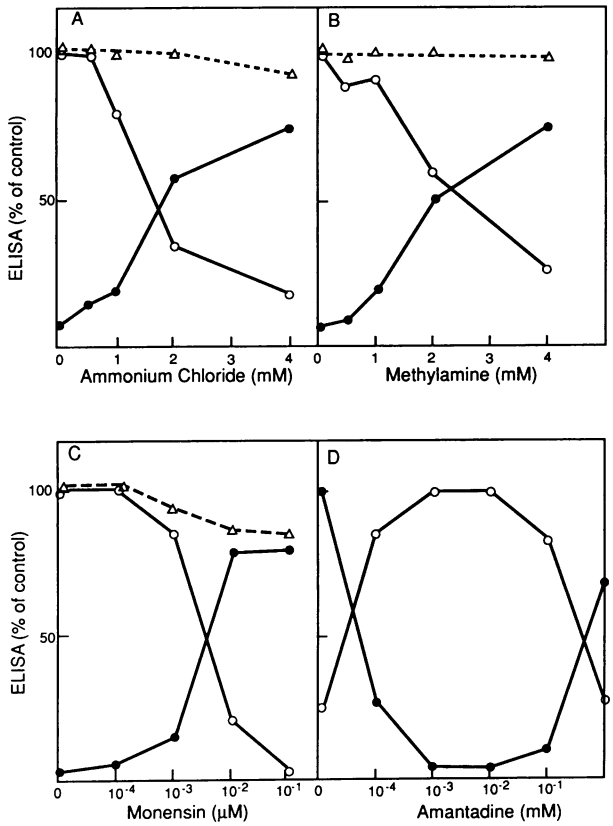

Fig. 9. Reversal of amantadine action by ammonium chloride, methylamine and monensin. **A**, **B** and **C**, amantadine (5 μM) was added at 1 h after infection and different concentrations of ammonium chloride (A), methylamine (B) or monensin (C) added to amantadine-treated or untreated cells at 1.5 h. Cells were fixed at 5.5 h and HA on the cell surface was analysed by ELISA using HC58 and H9 antibodies. (**D**) different concentrations of amantadine were added to cells 1 h after infection. ELISA titres are expressed as a percentage of the values for untreated infected cells (HC58) or infected cells treated with 5 μM amantadine (H9). ○, H9 and ●, HC58, amantadine treated; △, HC58, no amantadine treatment.

Reversal of amantadine action by agents which increase the pH of acidic intracellular compartments, such as μM concentrations of the ionophores monensin and nigericin and mM concentrations of various amines implies that the effect upon HA is due to exposure to a pH capable of triggering the conformational transition rather than due simply to impeding the normal maturation process. In the latter regard pulse−chase experiments did not reveal any 'intermediate', cleaved or uncleaved, forms of HA which react strongly with antibody specific for the low pH conformation. The apparent antagonism of the specific action of amantadine by drug concentrations >0.1 mM explains the observed concentration dependence with an optimum around 1 μM amantadine. Thus, in this situation, the action of mM concentrations of amantadine is contrary to its inhibitory effect upon virus infection, whereby elevation of the pH in endosomes inhibits low pH mediated membrane fusion.

Both the action of amantadine and its reversal by monensin appear to occur rapidly and thus allow pulse−chase experiments to localize the point of action some 15−20 min after synthesis, relatively late in the transport pathway but prior to insertion of HA into the plasma membrane. Once present on the cell surface HA is refractory to drug action. Further evidence from immune fluorescence and immunoelectron microscopic studies (F.Ciampor and A.Hay, in preparation) have indicated that the drug acts after HA leaves the Golgi. The apparent temporal coincidence between the sensitivity of HA to amantadine action and its proteolytic cleavage to HA1/HA2 is not particularly surprising since only the cleaved form undergoes an irreversible conformational change to the low pH form (Daniels *et al.*, 1983b; Ruigrok *et al.*, 1984). Boulay *et al.* (1987) have shown that uncleaved HA0 of H3 subtype viruses also undergoes an irreversible acid induced conformational change, though less extensive than that exhibited by the cleaved molecule. The lack of significant intracellular cleavage of the HAs of most influenza A virus subtypes may in part account for their insensitivity of this particular action of amantadine. Incubation of chick cells infected with certain human virus strains e.g. A/Singapore/1/57(H2N2) in the presence of trypsin (2.5 μg/ml) in addition to amantadine (5 μM) did not affect production of virus, fusion activity of HA or virus infectivity (unpublished data). The pH at which the conformational transition occurs is also important in determining sensitivity to amantadine (A.Hay, in preparation). The exquisite sensitivity of Rostock reflects the relatively high pH (~6.1) at which the conformational transition is triggered and contrasts with the lower sensitivity of the Weybridge strain (A/chicken/German/27, H7N7), the HA of which undergoes the change at ~pH 5.6 similar to the HAs of viruses of the H2 and H3 subtypes (Daniels *et al.*, 1985).

It is evident that amantadine does not act by direct interaction with HA since amino acid changes in the M2 proteins of drug resistant mutants can, in the absence of any change in HA, abolish sensitivity to drug action. Amantadine treatment of infected cells did not alter the synthesis of M2, its assembly into tetramers or the post-translational addition of palmitate or phosphorylation (unpublished data). Initial considerations that the M2 mediated action may result from interference with a structural interaction between M2 and HA have not been borne out. Attempts to demonstrate association between the two molecules using a variety of cross-linking reagents, which promoted substantial cross-linking of the HA trimer and M2 tetramer, in conjunction with specific immunoprecipitation of either HA or M2, were negative (unpublished data). The low ratio of M2:HA in virus particles (Zebedee and Lamb, 1988) contrasts with their similar levels of production in infected cells and is inconsistent with a stable association of M2 with HA, although not incompatible with a transient interaction during transport of HA to the cell surface.

An explanation more consistent with the available information is that M2 is capable of regulating the pH of certain acidic compartments of the exocytic pathway in infected cells. Contrary to the apparent consequence of amantadine action on M2 in reducing pH, the net influence of M2 function would therefore be to counteract increased acidity. pH probes have been used to show that in a variety of endocrine and exocrine cells including human fibroblasts, the vesicles and cisternae of the *trans*-Golgi are acidic (Anderson *et al.*, 1984; Schwartz *et al.*, 1985; Anderson and Orci, 1988). Although estimates of the pH of these compartments of ~6.5 would be insufficient to induce the conformational change in HA, it has been reported that virus infection, e.g. Sindbis virus infection of BHK cells, can cause a reduction in cytoplasmic pH of 0.5 pH units (Moore *et al.*, 1988). Preliminary studies using the pH probe DMAP [3-(2,4-dinitroanilino)-3′-amino-*N*-methyldipropylamine], (Anderson *et al.*, 1984) do indeed indicate that the pH of *trans*-Golgi cisternae in Rostock infected MDCK cells is substantially lower than in uninfected cells (F.Ciampor and A.Hay, in preparation). Thus the protein may well have a role in counteracting certain detrimental consequences of virus infection resulting from for example the down-regulation of host cell protein synthesis.

There is as yet no direct evidence as to the function of M2. However, the homotetrameric nature of the protein (Sugrue and Hay, 1990) and the locations of polar and charged amino acids as well as the drug resistance-determining amino acid changes at residues 27, 30, 31 or 34 on the hydrophilic side of the α-helical transmembrane domain are consistent with the formation of an aqueous channel which is blocked by direct interaction of amantadine (Hay, 1989; Belshe and Hay, 1989). Such an action is analogous to the anticholinergic activities of amantadine and various derivatives which block neuromuscular transmission by interacting with the ionic channel of the nicotinic acetylcholine receptor (Warnick *et al.*, 1982).

In addition to the M2 mediated effects of amantadine, evidence from studies of mutants with alterations in HA or M2 and reassortant viruses containing a heterologous M gene also indicates that compatibility between the properties of HA and M2 is important for the maturation of the functional, native H7 HA molecule (A.Hay, in preparation). In this context studies by Copeland *et al.*, (1986) showed that maturation of the uncleaved H3 HA was completed only after the protein left the Golgi complex. Skibbens *et al.* (1989) have also noted that at this stage of the exocytic pathway changes occur in the HA which affect its detergent solubility. It is not known, however, whether the HA is simply responding to the particular ionic environment or whether interaction with some protein component or cytoskeletal element of the cell is important for maturation of the glycoprotein.

We conclude, therefore, that the M2 of influenza A viruses

provides an example of a protein capable of modulating the pH of compartments of the exocytic pathway which in addition to protecting the integrity of the acid sensitive HA glycoprotein may also be important for promoting the maturation of the active structure.

Materials and methods

Cells and viruses

Primary chick embryo fibroblasts (CEF) were cultured in Tris–Gey's medium supplemented with 10% calf serum. Viruses were grown in 10 day old fertile hen's eggs. The influenza viruses used included A/chicken/Germany/34 (H7N1, 'Rostock' strain) and an amantadine resistant variant Rostock^R isolated following passage in the presence of amantadine (5 μM), as described in Hay *et al.* (1985). Antigenic variants of Rostock were isolated following passage in CEF in the presence of varying dilutions (10^2-10^4) of ascitic fluid containing monoclonal antibodies, by plaque titration on CEF monolayers in the presence of 10^3 dilutions of the appropriate antiserum.

Antibody production

Monoclonal antibodies were produced in BALB/C mice, as described by Daniels *et al.* (1983a) using SP2/0-Ag14 myeloma cells, against purified Rostock virus (HC2, HC3, HC10, HC58, HC61) or low pH HA rosettes (H9) derived from purified Weybridge virus as described by Ruigrok *et al.* (1986). Antibodies against a peptide MSLLTEVETPIR corresponding to the N-terminus of the M2 protein was produced in a rabbit injected with a peptide–BSA conjugate, coupled with glutaraldehyde.

Nucleotide sequence analyses

Sequences of the HA genes of mutant viruses were determined using the dideoxynucleotide chain terminating procedure as described previously (Daniels *et al.*, 1985). The primers used corresponded to the sequences of nucleotides 5–15, 229–239, 427–437, 573–583, 832–843, 1130–1141, 1314–1323 and 1525–1536.

ELISA of HA on virus infected cells

CEF cells in microtitre plates were infected with virus at a multiplicity of infection (m.o.i.) of ~10 plaque-forming units (p.f.u.) per cell. After 5 h incubation in Tris–Gey's medium containing appropriate additions, cells were fixed with 0.05% glutaraldehyde in PBS. ELISA was carried out as described by Belshe *et al.* (1988) using ascitic fluid containing antibodies HC2, HC3, HC10, HC58 or HC61 at 10^3 dilutions or H9 at 10^2 dilutions.

^{35}S-labelling of infected cells

CEF monolayers in 2.5 cm or 5 cm Petri dishes or 24 × 1 cm LIMBRO plates were infected with allantoic fluid at a m.o.i. of ~50 p.f.u. per cell, washed and incubated in Tris–Gey's medium. Cells were labelled for 30 min at 5 h post-infection in medium containing 25 μCi/ml [^{35}S]methionine (>1000 Ci/mmol) or [^{35}S]cysteine (>1000 Ci/mmol) (Amersham International). In pulse–chase experiments cells were labelled for 2–5 min with [^{35}S]methionine (25 μCi/ml) and subsequently washed and incubated with medium containing 1 mM methionine for varying periods. Cells were lysed in either 1% SDS, 8 M urea, 1% β-mercaptoethanol for total protein analyses, or NP-40 buffer containing 1% NP-40, 150 mM NaCl, 0.2 mM PMSF, 1 μM leupeptin, 1 μM aprotinin, 20 mM Tris–HCl pH 7.5 at 4°C for 10 min followed by centrifugation at 13 000 *g* for 10 min for subsequent immune precipitation.

Immune precipitation

Lysates (100 μl) were preadsorbed with 30 μl of a 10% suspension of protein A–Sepharose for 1 h at 4°C and clarified by centrifugation at 13 000 *g* for 10 min. Lysates were mixed with 10 μl ascitic fluid containing anti-HA antibody or 2 μl of rabbit antipeptide antiserum in 600 μl of binding buffer (0.5% NP-40, 150 mM NaCl, 1 mM EDTA, 0.25% BSA, 20 mM Tris–HCl pH 7.5) for 12–16 h at 4°C. Immune complexes were isolated following the addition of 50 μl of a 10% suspension of protein A–Sepharose for 2 h at 4°C and washed three times with high salt buffer (1% Triton X-100, 1 mM EDTA, 10 mM phosphate buffer pH 7 containing 650 mM NaCl) and once with low salt buffer containing 150 mM NaCl and resuspended in 2% SDS, 2% β-mercaptoethanol.

Sucrose gradient centrifugation

^{35}S-labelled, virus infected cells (5 cm monolayers) were extracted with 0.5 ml, 40 mM octyl glucoside, 150 mM NaCl, 20 mM Tris–HCl pH 7.5. Lysates were applied to 5–20% sucrose gradients containing 40 mM octyl glucoside, 150 mM NaCl, 20 mM Tris–HCl pH 7.5 and centrifuged at 40 000 r.p.m. for 16 h at 17°C in a Beckman SW41 rotor.

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