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The molecular basis of the specific anti-influenza action of amantadine

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Amantadine (1-aminoadamantane hydrochloride) is effective in the prophylaxis and treatment of influenza A infections. In tissue culture this selective, strain-specific antiviral activity occurs at relatively low concentrations (5 μM or less), which inhibit either the initiation of infection or virus assembly. The data reported here demonstrate that the basis of these actions is similar and resides in the virus-coded M_2 membrane protein, the product of a spliced transcript of RNA segment 7. Mutations which confer resistance to amantadine are restricted to four amino acids within a hydrophobic sequence, indicating that the drug is targetted against the putative membrane-associated portion of the molecule. The influence of the virus haemagglutinin on the amantadine sensitivity of virus strains implies that the drug may interfere with interactions between these two virus proteins.

Key words: amantadine action/antiviral/influenza virus/M_2 protein/resistant mutants

Introduction

Amantadine (1-aminoadamantane hydrochloride) and rimantadine (α-methyl-1-adamantane methylamine hydrochloride) have been established as effective in the prophylaxis and treatment of influenza A infections (reviewed by Oxford and Galbraith, 1980; Dolin *et al.*, 1982). Although initially licensed in 1966 the clinical use of amantadine has been rather limited, while rimantadine, considered by some to be more effective with fewer side effects, is more widely used in the USSR (Zlydnikov *et al.*, 1981).

In cell culture, amantadine exhibits two concentration-dependent inhibitory actions against virus replication (Hay and Zambon, 1984). A non-specific action of concentrations >0.1 mM indirectly inhibits activation of the membrane fusion activity of the virus haemagglutinin involved in endocytosis (Daniels *et al.*, 1985). This action, which results from an elevation in the pH of endosomes, is not peculiar to amantadine but is effected by a variety of amines (Jensen and Liu, 1963; Helenius *et al.*, 1982; Yoshimura *et al.*, 1982; Hay and Zambon, 1984). Nor does it reflect the clinical spectrum of antiviral activity, since the replication of all influenza viruses, including influenza B strains as well as a number of other enveloped RNA viruses, e.g., paramyxoviruses, togaviruses and retroviruses, are also inhibited (Skehel *et al.*, 1978; Helenius *et al.*, 1980; Wallbank *et al.*, 1966).

In contrast, lower concentrations of 0.1 – 5 μM exert a selective, strain-specific inhibition of stages involved either in initiating infection or in virus assembly (Hoffman, 1973; Appleyard, 1977; Hay and Zambon, 1984) and genetic analyses have indicated the importance of the RNA genes encoding the matrix (M_1) and M_2 proteins (Lubeck *et al.*, 1978; Hay *et al.*, 1979) or haemagglutinin (Scholtissek and Faulkner, 1979; Hay and Zambon, 1984). The characterization of drug-resistant mutants reported here demonstrates that the primary target of these actions is the M_2 protein, the product of a spliced transcript of genome RNA segment 7 (Lamb *et al.*, 1981) recently shown to be an integral membrane protein (Lamb *et al.*, 1985), although the haemagglutinin also influences the particular susceptibility of a virus strain.

Results

Selective action of amantadine

Two features of the selective action of amantadine, the stage of infection inhibited and optimum inhibitory concentration, are illustrated by the data in Table I which shows the concentration-dependence of the inhibition of ^{35}S-labelled virus production during a single cycle of virus replication. Infection by A/Singapore, a human H2N2 isolate, is inhibited at an early, presynthetic stage since the drug must be present prior to infection and early synthetic events, in particular primary transcription, are inhibited (Hay and Zambon, 1984).

In contrast, the replication of two avian strains, Rostock and Weybridge, is similarly affected whether the drug (optimum concentration of 5 μM and 0.5 μM, respectively) is administered before or after virus infection. There is no significant impairment of virus-specified macromolecular synthesis suggesting a block in some late, post-synthetic process, probably involved in virus assembly (Hay and Zambon, 1984; M.C.Zambon and A.J.Hay, in preparation). The replication of resistant mutants (see below) like BEL^R was unaffected by comparable concentrations and only succumbed to the general action of concentrations >50 μM.

These specific inhibitory actions correlate closely with the particular structural attributes of the molecule and require an amine with a hydrocarbon ring of greater than five carbons (Table II). Cyclooctylamine exhibits an activity similar to amantadine (Appleyard and Maber, 1975), whereas cyclopentylamine, like the aliphatic analogues (e.g., octylamine), is inactive. Cyclohexylamine and cycloheptylamine have intermediate activities and the somewhat higher optimum concentrations for these compounds may reflect their reduced tendency to partition into membranes.

Table I. Concentration dependence of the inhibition of virus production by amantadine

Amantadine concentration (μM)	Virus yield (% of control)							
	Rostock		Weybridge		Singapore		BEL^R	
	a	b	a	b	a	b	a	b
0.05	95	92	20	30	100	100	92	100
0.5	21	25	7	7	55	92	95	98
5	4	5	30	45	9	66	81	93
50	25	22	45	100	3	71	38	100
500	17	70	<2	100	<2	87	<2	100

Amantadine was present from 30 min prior to infection (a), or added 60 min after infection (b).

Table II. Effect of cyclooctylamine and related compounds on virus production

Compound	Virus yield (% of control)				
	Singapore[a]	Rostock[b]		Weybridge[b]	
	5 μM	5 μM	50 μM	0.5 μM	5 μM
Amantadine	6	7	8	3	15
Cyclooctylamine	5	5	5	10	5
Cycloheptylamine	22	30	15	35	9
Cyclohexylamine	60	70	30	67	60
Cyclopentylamine	95	100	100	90	95
Cyclooctanol	100	100	100	ND	ND
Octylamine	98	100	ND	92	ND

ND, Not done. Compound was added to cells 30 min prior to infection (a) or 60 min after infection (b).

Table III. Influence of HA and M genes on amantadine sensitivity of reassortants

Parental virus strains		Genotype[b]		Amantadine sensitivity
Amantadine-sensitive	Amantadine-resistant	HA	M	
Singapore	BEL^{R}[a]	B	S	Sensitive
		S	B	Resistant
Rostock	BEL^{R}	B	R	Resistant
		R	B	None isolated
Rostock	Weybridge^{R}	R	W	Resistant
		W	R	Sensitive, equivalent to Weybridge (*)
Weybridge	BEL^{R}	W	B	Resistant
		B	W	Resistant
Weybridge	Rostock^{R}	W	R	Resistant
		R	W	None isolated

[a]Superscript R indicates resistance to amantadine (5 μM).
[b]S, B, R and W indicate the parental origin of Singapore, BEL, Rostock and Weybridge, respectively. Sensitivity to amantadine was assessed by its effect on virus produced following a single cycle of virus infection or by plaque reduction. In the latter assay the phenotypes of Weybridge and Rostock were readily distinguished since in the presence of amantadine (5 μM) no Rostock plaques developed, while fuzzy plaques, half the size of control, developed in the case of Weybridge (*).

The difference between Rostock and Weybridge in the optimum inhibitory concentration of amantadine indicates that this is also dependent on a virus-specific characteristic.

Influence of HA and M genes on susceptibility to amantadine

Table III summarizes data showing the influence of the genes encoding the haemagglutinin (HA) and the matrix (M_1) and M_2 proteins in determining the phenotypes of genetic reassortants obtained from co-infections of amantadine-resistant isolates and each of the three sensitive viruses. Analyses of a large number of reassortants of each combination indicated that the other six genes did not exert any major influence (Hay *et al.*, 1979; Hay and Zambon, 1984; Zambon and Hay, in preparation). Although not all combinations of HA and M genes were isolated, it is apparent that in each case the M gene of the sensitive parent is required for sensitivity of the reassortant and that this gene alone determines the difference in amantadine sensitivity between Singapore and BEL^{R} strains. In the case of Rostock and Weybridge it is evident that properties of their haemagglutinins are also important and that replacement of either by the haemagglutinin of BEL^{R} confers resistance. In this regard the ability of Rostock-Weybridge^{R} reassortants containing the haemagglutinin of Weybridge to form small, fuzzy plaques in the

Table IV. Amino acid substitutions in the M_2 proteins of amantadine-resistant mutants

Virus	Amino acid substitution			
	27	30	31	34
Singapore	V >A(2)	A >T(6)	S >N(8)	
Weybridge	V >A(3) >G(2) >D(1)	A >T(7) >P(2)	S >N(4)	G >E(29)
Rostock	I >S(17) >T(8) >A(1)			

Amino acid substitutions (single letter code) were deduced from changes in nucleotide sequence of the virus RNA M genes; the numbers in parenthesis indicate the frequency of occurrence.

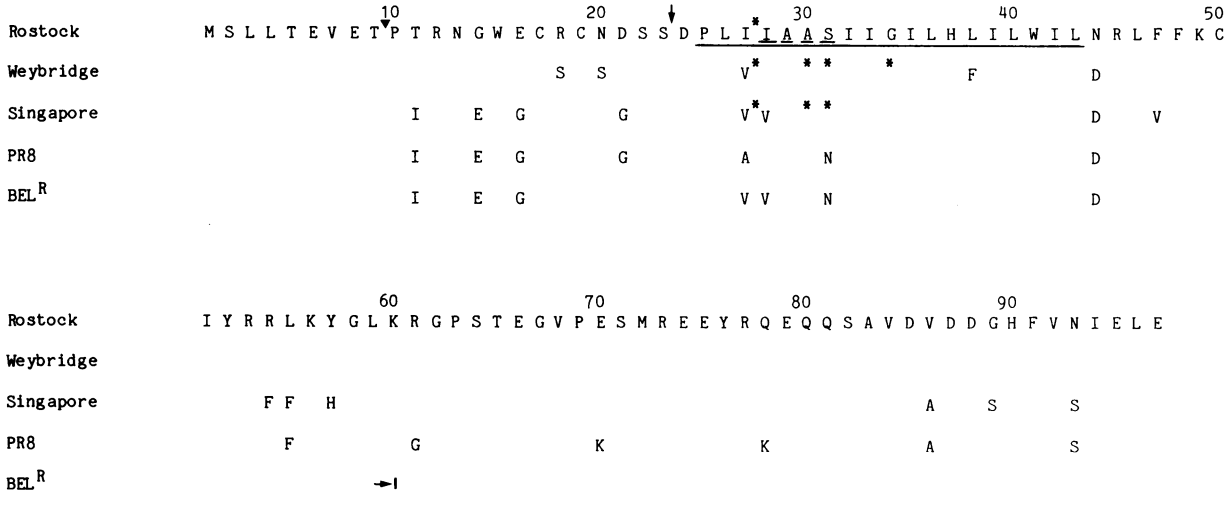

Fig. 1. Location of amino acid substitutions in the M_2 proteins of amantadine-resistant mutants. The amino acid sequence, in single letter code, shown for the M_2 protein of Rostock (McCauley *et al.*, 1982) is as predicted from the data from Lamb *et al.* (1981); ▼ indicates the position of the splice junction and ↓ the corresponding position at which matrix protein terminates. Differences between this sequence and those of the other viruses, including PR8 reported by Winters and Fields (1980), are indicated; the sequence of only the first 60 amino acids is available for BEL^{R}; asterisks show the positions of the amino acid substitutions in the proteins of amantadine-resistant mutants, detailed in Table IV. The sequence of hydrophobic amino acids 25–43 is underlined as are the four amino acids (28–31) missing from a deletion mutant of Weybridge obtained following passage in the presence of 0.5 mM amantadine.

presence of amantadine also correlates with this gene. On the other hand, the concentration for maximum inhibition of these viruses reflects the parental origin of their M genes. It appears, therefore, from the information available, that the sensitivity of reassortants derived from 'similar' parent strains is principally determined by the M gene, while substitution of a more distinct haemagglutinin can also alter the phenotype. A clearer understanding of the relative importance of these two factors in determining susceptibility to amantadine has come from the characterization of mutations conferring drug resistance.

Amino acid substitution in the M_2 proteins of resistant mutants

Amantadine-resistant mutants of the three viruses were isolated following two passages in the presence of 5 μM amantadine and the nucleotide sequences of the M and HA genes were determined by the dideoxynucleotide chain-terminating procedure of Sanger *et al.* (1977). All mutants of the three viruses contained a mutation in their M genes which resulted in a single amino acid substitution in the M_2 protein (Table IV, Figure 1). No changes were noted in the primary sequence of the matrix protein, the product of the unspliced mRNA. Substitutions of only four amino acid residues, 27, 30, 31 and 34, were observed, their occurrence depending on the virus strain (Table IV). Alterations in Rostock^R mutants involved only isoleucine 27, which was predominantly substituted by a serine or threonine, even though isolation of the same mutant was avoided by selecting only one from individual plaque isolates of the sensitive virus.The changes observed in the Singapore^R variants, valine 27 to alanine, alanine 30 to threonine and serine 31 to asparagine were also present in the Weybridge^R viruses, which exhibited other substitutions of residue 27 by glycine or aspartic acid and residue 30 by proline and most frequently substitution of glycine 34 by glutamic acid. One (asparagine 31) or two (alanine 27 and asparagine 31) of these 'resistant' amino acids appear in the M_2 sequences of two other amantadine-resistant viruses, BEL^R and PR8, respectively (Figure 1), whereas two human H3N2 strains A/Udorn/72 (Lamb *et al.*, 1981) and A/Bangkok/1/79 (Ortin *et al.*, 1983) would, by this criterion, be sensitive to the drug.

Of 22 Rostock^R variants only four contained mutations which resulted in single amino acid substitution in their haemagglutinins: in HA_1 serine 221 to tyrosine and valine 223 to methionine, and in HA_2 alanine 35 to valine and glutamic acid 64 to lysine. Only three out of 12 Weybridge^R viruses possessed single substitutions, arginine 121 to lysine and alanine 168 to threonine in HA_1 and arginine 127 to lysine in HA_2. The infrequent occurrence of these changes and the lack of any correlation with the particular amino acid substitution in the M_2 proteins of these viruses indicate that they are unnecessary in determining resistance to amantadine. That resistant viruses containing changes solely in their haemagglutinins were not isolated serves to emphasize the prime importance of the M_2 protein as the determinant of amantadine sensitivity.

Discussion

The genetic basis of the sensitivity of influenza viruses to amantadine, as defined by the reassortant experiments described here and elsewhere (Lubeck *et al.*, 1978; Hay *et al.*, 1979; Scholtissek and Faulkner, 1979), is associated with the two genes encoding the haemagglutinin and the matrix and M_2 proteins, respectively. The characterization of drug-resistant mutants by determining the nature and location of amino acid substitutions has now pinpointed more precisely the primary target of drug action to the M_2 protein.

The M_2 protein is expressed on the plasma membrane of infected cells and the amino-terminal region of at least 18 amino acids is disposed externally (Lamb *et al.*, 1985). All the amino acid substitutions fall within a sequence of 19 predominantly hydrophobic amino acids (25–43), the suggested membrane-spanning domain and this location and the nature of the substitutions may provide clues as to the mechanism of antiviral action. For example, the introduction of the charged amino acids glutamic acid 34 and aspartic acid 27 in different mutants and the deletion of the four amino acids 28–31 in another (Figure 1) may influence the membrane association or more specific intermolecular interactions required for the function of the wild-type protein. The structural consequences of the changes are not known but it is noteworthy that if the membrane-associated sequence forms an α-helix the positions of the substitutions, which generally involve reductions in hydrophobicity, are located on the same face (Schiffer and Edmundson, 1967).

Our results also indicate that the M_2 protein has a role in virus replication – apparently during assembly of virus particles and at a stage involved in initiating virus infection. The latter role implies that the protein is a structural component of the virus, although to date attempts to demonstrate its presence in preparations of purified virus have been negative (Lamb *et al.*, 1985). With regard to virus assembly, there is as yet little precise information regarding either the way in which amantadine might block this process or the role of M_2, but two observations are of interest. Firstly, to inhibit the participation of virus proteins in the assembly process the drug must be present prior to their synthesis (Hay and Zambon, 1984) suggesting that it interferes with interactions between virus components rather than causing dissociation of structures already formed. Secondly, not only does the drug impair the expression of the haemagglutinin at the surface of cells infected with Rostock, but it also affects its reactivity with antibody, indicating a significant structural alteration in this component (M.C.Zambon and A.J.Hay, in preparation). The available biochemical and genetic data suggest, therefore, that amantadine does not affect directly the haemagglutinin molecule but rather may interfere with interactions between the M_2 protein and this virus component. Finally, in the more general context of antiviral chemotherapy, amantadine provides an example of a drug targetted specifically against a viral membrane protein and underlines the feasibility of selectively inhibiting membrane-associated stages in virus replication.

Materials and methods

Viruses and cells

The influenza strains A/Singapore/1/57 (H2N2), A/chicken/Germany/34 (H7N1, 'Rostock' strain), A/chicken/Germany/27 (H7N7 'Weybridge' strain) and BEL^R an amantadine-resistant variant of A/BEL/42 (H1N1) isolated by Appleyard (1977) were grown in 10-day old fertile hen's eggs and purified as described by Hay (1974). Primary chick embryo fibroblasts were prepared as described by Porterfield (1960). For plaque titration of A/Singapore and BEL^R viruses the overlay contained 4 μg/ml trypsin (Appleyard, 1977). Chorioallantoic membranes taken from 11-day old embryonated eggs were incubated in Earle's medium.

Amantadine-resistant mutants were isolated following two low multiplicity passages of virus in the presence of 5 μM amantadine in chick cells (Rostock and Weybridge) or chorioallantoic membranes (A/Singapore). Cells were infected at a multiplicity of infection of ~0.1 and incubated at 37°C for 24–48 h in medium containing 5 μM amantadine; a 10^3 dilution of this medium was used for the second passage. Resistant plaques were isolated and purified by titration with overlay containing 5 μM amantadine; only one resistant virus was isolated from each sensitive plaque isolate. All viruses isolated in this way were unaffected by 5 μM amantadine in either plaque reduction or virus production assays.

Reassortant viruses were plaque purified from medium taken 16 h after co-infection of chick fibroblasts (multiplicity of infection, ~20) with differing relative

proportions of an amantadine-sensitive and amantadine-resistant strain. Genome compositions were determined as described by Hay *et al.* (1979).

[^{35}S]Virus production

Chick fibroblast monolayers (5×10^6 cells) were infected with a multiplicity of ~50 p.f.u./cell. [^{35}]Methionine (10 μCi) was added at 4 h after infection and virus isolated from medium of duplicate cultures taken at 12 h, by centrifugation on 15–40% sucrose gradients as described previously (Hay, 1974). Virus yields from unlabelled cells were estimated by haemagglutinin titration.

Nucleotide sequence analyses

Nucleotide sequences were determined by the dideoxynucleotide chain terminating procedure of Sanger *et al.* (1977) as described previously (Daniels *et al.*, 1983). The 5′ ^{32}P-labelled primers of reverse transcription were synthesized by the procedure of Patel *et al.* (1982) and were complementary to nucleotides 7–20, 178–190, 392–405 or 405–415 (Weybridge), 592–606 and 813–827, according to the sequence of RNA segment 7, encoding the M proteins, reported by McCauley *et al.* (1982). Analyses of haemagglutinin sequences of Rostock and Weybridge viruses utilized primers complementary to nucleotides 5–15, 229–239, 427–437, 573–583, 848–861 (Rostock) or 832–843 (Weybridge), 1113–1124, 1345–1354 (Rostock) or 1315–1324 (Weybridge) and 1580–1589 (Rostock) or 1526–1537 (Weybridge), according to the sequence of the Rostock RNA reported by Porter *et al.* (1979).

Materials

Amantadine hydrochloride was purchased from Sigma and [^{35}S]methionine (46.4 TBq/mmol) and [γ-^{32}P]ATP (222 TBq/mmol) from Amersham International.

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References

Appleyard,G. (1977) *J. Gen. Virol.*, **36**, 249-255.
Appleyard,G. and Maber,H.B. (1975) *J. Antimicrob. Chemother.*, **1** (Suppl.), 49-53.
Daniels,R.S., Douglas,A.R., Skehel,J.J., Waterfield,M.D., Wilson,I.A. and Wiley,D.C. (1983) in Laver,W.G. (ed.), *The Origin of Pandemic Influenza Viruses*, Elsevier, NY, pp. 1-8.
Daniels,R.S., Downie,J.C., Hay,A.J., Knossow,M., Skehel,J.J., Wang,M.L. and Wiley,D.C. (1985) *Cell*, **40**, 431-439.
Dolin,R., Reichman,R.C., Madore,H.P., Maynard,R., Linton,P.N. and Webber-Jones,J. (1982) *New Engl. J. Med.*, **307**, 580-584.
Hay,A.J. (1974) *Virology*, **60**, 398-418.
Hay,A.J. and Zambon,M.C. (1984) in Becker,Y. (ed.), *Antiviral Drugs and Interferon: The Molecular Basis of their Activity*, Martinus Nijhoff Publishing, Boston, MA, pp. 301-315.
Hay,A.J., Kennedy,N.C.T., Skehel,J.J. and Appleyard,G. (1979) *J. Gen. Virol.*, **42**, 189-191.
Helenius,A., Kartenbeck,J., Simons,K. and Fries,E. (1980) *J. Cell Biol.*, **84**, 404-420.
Helenius,A., Marsh,M. and White,J. (1982) *J. Gen. Virol.*, **58**, 47-61.
Hoffmann,C.E. (1973) in Carter,W.A. (ed.), *Selective Inhibitors of Viral Functions*, CRC Press, Cleveland, OH, pp. 199-211.
Jensen,E.M. and Liu,O.C. (1963) *Proc. Soc. Exp. Biol. Med.*, **112**, 456-459.
Lamb,R.A., Lai,C.-J. and Choppin,P.W. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 4170-4174.
Lamb,R.A., Zebedee,S.L. and Richardson,C.D. (1985) *Cell*, **40**, 627-633.
Lubeck,M.D., Schulman,J.L. and Palese,P. (1978) *J. Virol.*, **28**, 710-716.
McCauley,J.W., Mahy,B.W.J. and Inglis,S.C. (1982) *J. Gen. Virol.*, **58**, 211-215.
Ortin,J., Martinez,C., del Rio,L., Davila,M., Lopez-Galindez,C., Villanueva,N. and Domingo,E. (1983) *Gene*, **23**, 233-239.
Oxford,J.S. and Galbraith,A. (1980) *Pharmacol. Ther.*, **11**, 181-262.
Patel,T.P., Millican,T.A., Bose,C.C., Titmas,R.C., Mock,G.A. and Eaton, M.A.W. (1982) *Nucleic Acids Res.*, **10**, 5605-5620.
Porter,A.G., Barber,C., Carey,N.H., Hallewell,R.A., Threlfall,G. and Emtage, J.S. (1979) *Nature*, **282**, 471-477.
Porterfield,J.S. (1960) *Bull. WHO*, **22**, 373-380.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
Schiffer,M. and Edmundson,A.B. (1967) *Biophys. J.*, **7**, 121-135.
Skehel,J.J., Hay,A.J. and Armstrong,J.A. (1978) *J. Gen. Virol.*, **38**, 97-110.
Scholtissek,C. and Faulkner,G.P. (1979) *J. Gen. Virol.*, **44**, 807-815.
Wallbank,A.M., Matter,R.E. and Klinikowski,N.G. (1966) *Science (Wash.)*, **152**, 1760-1761.
Winters,G. and Fields,S. (1980) *Nucleic Acids Res.*, **8**, 1965-1974.
Yoshimura,A., Kurod,K., Kawasaki,K., Yamashina,S., Maeda,T. and Ohnishi,S. (1982) *J. Virol.*, **43**, 284-293.
Zlydnikov,D.M., Kubar,O.I., Kovaleva,T.P. and Kamforin,L.E. (1981) *Rev. Inf. Dis.*, **3**, 408-421.

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