Functional reconstitution in lipid vesicles of influenza virus M2 protein expressed by baculovirus: evidence for proton transfer activity

Cornelia Schroeder,*† Chris M. Ford,‡ Stephen A. Wharton and Alan J. Hay

Division of Virology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

The influenza virus 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 by a factor of 10, 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 the authentic virus protein. Purified wild-type M2 protein and an amantadine-resistant mutant M2 (M2δ) 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 decrease in intravesicular pH, which was susceptible to inhibition by 0.1 to 1 μM-rimantadine or N-ethyl-rimantadine. M2δ behaved similarly but exhibited the expected drug resistance. These experiments indicate that isolated M2 functions as an ion channel and demonstrates in vitro M2-mediated proton translocation.

Introduction

The influenza virus M2 protein is a minor virus envelope protein expressed from a spliced mRNA encoded by the M gene (Lamb & Choppin, 1981). The 96 amino acid polypeptides associate to form disulphide-linked homotetramers (Sugrue & Hay, 1991; Holsinger & Lamb, 1991) which are modified by the addition of palmitate and phosphate (Sugrue et al., 1990b; Veit et al., 1991). As the target of the antiviral compounds amantadine and rimantadine (Hay et al., 1985) M2 was implicated in virus uncoating (Kato & Eggers, 1969; Bukrinskaya et al., 1982; Wharton et al., 1990; Martin & Helenius, 1991). More recently, it has been proposed that during endocytosis the M2 ion channel is responsible for allowing the passage of protons from the acid endosome into the virion, thereby promoting a low pH-induced dissociation of the matrix protein from the nucleocapsid (Hay, 1989; Wharton et al., 1990). Such a role of M2 would be analogous to its ability to reduce the acidity of the trans Golgi network and thereby protect intracellularly cleaved haemagglutinin (HA) against a premature low pH-induced conformational alteration during transport to the plasma membrane (Sugrue et al., 1990a; Ciampor et al., 1992a; Takeuchi & Lamb, 1994; Ohuchi et al., 1994).

Using the conformation of HA as a probe to monitor the pH modulating activity of M2 in infected cells it was estimated that M2 elevates the pH of trans Golgi vesicles by up to 0.8 pH units (Grambas & Hay, 1992). Mutations in M2 that confer resistance to amantadine may either enhance or reduce the pH-modulating activity of the protein and M2 proteins with a higher intrinsic activity can complement less active M2 proteins in supporting the maturation of acid-labile HAs (Grambas et al., 1992; Grambas & Hay, 1992). The correlation between differences in the pH-modulating activity of M2 and differences in the pH stabilities of the respective HAs of certain influenza virus strains indicates the importance of compatibility between the two proteins. Furthermore, since the amantadine sensitivity of HA mutants increases with the acid lability of their HAs (Grambas & Hay, 1992) it has conversely been possible to select in the presence of amantadine a mutant with an acid-stable HA (Steinhauer et al., 1991). The M2 protein in the plasma membrane can also counteract a trans-membrane pH gradient, reducing the intracellular pH to that of the outside medium (Ciampor et al., 1992b).

Direct measurements of ion channel activity in voltage-clamp experiments demonstrated that M2 expressed in Xenopus laevis oocytes possesses a pH-activated, Na⁺-selective conductance (Pinto et al., 1992; Wang et al., 1993). Under the conditions used in these experiments it
was not possible to resolve proton currents. However, a peptide corresponding to the trans-membrane domain of M2 incorporated into planar lipid bilayers has been shown to promote amantadine-sensitive proton translocation at millimolar proton concentrations (Duff & Ashley, 1992).

We report here the use of a fluorescent dye assay to demonstrate, under mild conditions, the proton translocation activity of purified M2 reconstituted into lipid vesicles. The M2 protein was expressed under the control of the baculovirus polyhedron promoter in Spodoptera frugiperda Sf9 cells. The proton translocation activity of the wild-type M2 protein was specifically inhibited by rimantadine whereas the activity of a drug-resistant mutant protein was unaffected.

**Methods**

**Virus and cells.** Wild-type *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV; polyhedrin-positive) was obtained from Dr M. D. Summers (Texas A & M University, Tex., U.S.A.). BacPAK6 virus was a product of Clontech Laboratories. The Sf9 cell subline RE was provided by Dr H. Reiländer (Max-Planck-Institut für Membranbiophysik, Frankfurt, Germany). *Trichoplusia ni* cells (Hi5) were obtained from JRH Biosciences.

Sf9 cells were grown in TC100 medium (purchased from Gibco or Flow Laboratories) supplemented with l-glutamine (292 mg/l), fetal calf serum (FCS; 10%), penicillin (60 mg/l), streptomycin (100 mg/l) and in some cases, amphotericin (Flow Laboratories; 2.5 mg/l). Hi5 cells were grown in SF-900 II serum-free medium (Gibco). Cell cultivation, virus propagation and titration were carried out as described by Luckow & Summers (1989).

**Antiserum.** Rabbit antiserum was raised against peptides corresponding to the N-terminal 24 amino acids (R7 and R53), to the C-terminal 16 amino acids (R54) and to amino acids 60 to 76 (R15) of the M2 protein described by Luckow & Summers (1989). Amantadine was a product of Serva, rimantadine was a gift from Dr I. Dr M. K. Indulen (August Kirchenstein Institute of Microbiology, Latvian Academy of Sciences, Riga, Latvia).

**Construction of baculovirus recombinants.** The plasmid pVL941 (Luckow & Summers, 1989) was obtained from Dr M. D. Summers. An *EcoRI*-Sall fragment encompassing the M2 sequence of influenza virus A/Weybridge (H7N7) was converted to a *BamHI* fragment by attaching appropriate linkers and inserted into the *BamHI* site of pVL941, generating the recombinant pVLM2. This plasmid was cotransfected with AcMNPV wild-type DNA into Sf9 cells using Lipofectin from BRL (Felgner et al., 1987). Polyclenalin-negative plaques were picked and M2-producing clones were plaque-purified. The M2-expressing recombinant baculovirus used was designated CFM2.

A deletion mutant of M2 (lacking amino acids 28 to 31) was constructed by PCR mutagenesis of pVLM2. Primers complementary to the sequences flanking the intended deletion were extended with Vent polymerase (New England Biolabs). The product was phosphorylated with T4 polynucleotide kinase (Boehringer Mannheim), ligated with T4 DNA ligase (New England Biolabs) and used to transform competent *Escherichia coli* DH5 cells. Mutant plasmid DNA and *Bac* linearized BacPAK6 DNA (Kitts et al., 1990) were cotransfected into S9 cells by lipofection. Plaques were screened for typical M2-specific morphological alterations of the S9 RE cells. One plaque was purified and the recombinant designated CSM2δ.

**Metabolic labelling and immune precipitation.** All incubations were done at 27 °C. S9 cells were seeded onto 24-well plates and infected with CFM2 virus at a m.o.i. of 2 p.f.u./ml. At different times post-infection (p.i.) the medium was exchanged for the appropriate depletion medium supplemented with 10% dialysed FCS: TC100 without cysteine and methionine (Gibco-BRL) for [35S]cysteine incorporation; TC100 supplemented with 5 mM-sodium pyruvate for labelling with [3H]palmitic acid, and phosphate-free TC100 for labelling with [32P]orthophosphate. After 1 h this medium was replaced by 50 or 100 µl of depletion medium containing the appropriate radiolabelled compound (2.2 MBq [35S]cysteine (Amersham); 2 MBq [3H]palmitic acid (Amersham); 2 MBq [32P]orthophosphate (Amersham)) and cells were incubated for 30 min or 1 h. Monolayers were then washed and incubated at 27 °C with chase medium (1 ml TC100 plus 10% FCS). Finally, monolayers were washed with ice-cold PBS pH 6.6, and extracted with 100 µl of 1% NP40 in 150 mM-NaCl, 20 mM-Tris-HCl pH 7.5 for 10 min at 4 °C. For immune precipitation the extract was diluted into 12 volumes of binding buffer (0.5% NP40, 150 mM-NaCl, 1 mM-EDTA, 0.25% BSA, 20 mM-Tris-HCl pH 8.0) containing antigens (1:100) and precipitated with protein A-Sepharose (Pharmacia), as described by Sugrue et al. (1990b).

**Purification of M2 protein.** Suspension cultures of exponentially growing S9 cells (107/ml) were centrifuged and infected with baculovirus CFM2 at a m.o.i. of 2 p.f.u./cell. After a 1 h adsorption period the cells were taken up in fresh medium containing 1 µg/ml amantadine and cultured for 48 h at 27 °C. The cells (2 to 5 per l of cell culture) were harvested, washed three times in HEPES-buffered saline (HBS; 100 mM-HEPES, 150 mM-NaCl pH 7.8), and taken up in 10 volumes of ice-cold 100 mM-HEPES pH 7.8 supplemented with 1 mM-PMSF, 0.02% trypsin inhibitor (soybean) and 10 µg/ml aprotinin (all from Sigma). This protease inhibitor combination was used until the KCl extraction step. The cells were disrupted in a Dounce homogenizer and the nuclei and remaining intact cells were pelleted. The supernatant was made 70% (w/v) in sucrose, overlaid with HBS pH 7.8 and centrifuged for 12 h at 20000 r.p.m. in a Beckman SW-28 rotor. The membrane fraction at the interface was pelleted at 26000 r.p.m. for 90 min, washed once in HBS pH 7.8, and extracted with 1 M-KCl in HBS pH 7.8 for 1 h at 4 °C. The membrane pellet was washed once in HBS pH 7.8 containing a mixture of low Mr protease inhibitors (1 mM-PMSF, 10 µM-E-64 (trans-epoxysuccinyl-l-leucylamido(4-guanidino)-butane), 1 µM-bestatin, 1 µM-pepstatin A (Sigma)), which was used throughout the following purification steps except immunoaffinity chromatography. The membranes were extracted for 1 h at 4 °C with 4 mM-n-octyl glucoside (OG; 1-O-n-octyl β-D-glucopyranoside; Sigma), pelleted once more as above, and finally M2 was extracted with 40 mM-OG for 1 h at 4 °C.

M2 protein was purified either directly using an immunoaffinity column, or by Phosphogel (Pierce) followed by immunoaffinity chromatography. After application to activated Phosphogel at pH 4.0 the column was washed with 100 mM-sodium acetate pH 4 containing 40 mM-OG and eluted with 20 mM-NaH2PO4 or Na2HPO4 pH 7.0 and 40 mM-OG. M2-containing fractions were pooled and the buffer was exchanged for HBS pH 7.0 containing 40 mM-OG. The concentrate was applied to an immunoaffinity column [Affi-Gel HZ (Bio-Rad) to which was coupled immunoglobulin purified from R53 serum]. The column was washed with HBS pH 7.0 and 40 mM-OG, and eluted in 1 ml fractions with 100 mM-glycine–HCl pH 2.8 and 40 mM-OG. The fractions were immediately neutralized, M2-containing fractions were pooled and concentrated and the buffer was exchanged for HBS pH 7.0 with 40 mM-OG.
Protein detection. The concentration of M2 in semipure and purified preparations was estimated from the $A_{280}$ measurement using the formula of Beaven & Holiday (1952). Concentration estimates took into account the level of impurities as detected by silver staining. Estimates of M2 concentrations in membrane and cell extracts were made on the basis of Western blots of purified and crude samples run in parallel on polyacrylamide gels.

Gel electrophoresis and Western blotting. Samples in 2 x Laemmli buffer were boiled for 2 min in the presence or absence of 50 mM DTT and run on 12% or 10% polyacrylamide gels with $[^3]^{14}$C-methylated Rainbow markers (Amersham) as $M_r$ standards. Gels were processed for fluorography by immersing in Amplify (Amersham) or Western-blotted on Immobilon P membranes (Millipore). Blots were blocked overnight in 1% dried skimmed milk in PBS, treated with primary antibodies (diluted 1: 500) for 2 h, washed five times, then treated for 1 h with iodinated donkey anti-rabbit antibody (Amersham) diluted 1:1000 into 1% skimmed milk in PBS. Blots and Amplify-treated gels were dried and exposed to pre-flashed X-ray film at $-70^\circ$C.

Reconstitution into lipid vesicles. M2 was reconstituted into liposomes based on procedures of Ruigrok et al. (1986) and Dencher et al. (1986). A solution containing 0.85 mg of 1,2-dimyristoyl phosphatidylcholine (DMPC; Sigma), 0.15 mg 1,2-dioleoyl-phosphatidyl 1-lysine (Sigma) and 0.7 mol% (with respect to lipids) valinomycin in chloroform/ methanol (95: 5 v/v) in a glass tube was evaporated to a thin film under a stream of argon, dried under vacuum overnight and taken up in 250 lal of potassium phosphate buffer (10 mM-K$_2$HPO$_4$, 50 mM-K$_2$SO$_4$) pH 7.4, supplemented with 40 mM-OG, with or without 50 mg of M2 or M26 protein. The molar ratio of lipid to protein was 250. Vesicles were loaded with $[^32]$P phosphate or with $[^3]$H palmitate demonstrated that the M2 monomer appeared as a doublet by the recombinant baculovirus CFM2. The most resilient Sf9 line, RE, was selected for the production of M2 (Fig. 1). Later in the course of this work a 'high producer' insect cell line, T. ni Hi5, which was superior to Sf9 RE (Fig. 1a, b) became available and was used to produce the amantadine-resistant mutant M2 protein, M2$\delta$ (Fig. 1c). Expression of the wild-type M2 protein was not enhanced appreciably by increasing the m.o.i. above 2. Incubation in the presence of 1 mg/ml amantadine, however, increased synthesis of M2 by about 10-fold whether assessed by $[^3]$S) cysteine incorporation or by Western blot analysis (Fig. 1a, b). The expression of the amantadine-resistant deletion (amino acids 28 to 31) mutant M2$\delta$ (Hay et al., 1985) was not susceptible to amantadine stimulation (data not shown). However, in Hi5 cells the level of M2$\delta$ expression was comparable to that of wild-type M2 in the presence of amantadine (Fig. 1c).

Characteristics of M2

Influenza virus M2 protein is a palmitoylated phosphoprotein (Sugrue et al., 1990b; Sugrue & Hay, 1991). Metabolic labelling of CFM2-infected Sf9 cells with $[^3]$P phosphate or with $[^3]$H palmitate demonstrated that the M2 protein expressed in insect cells was phosphorylated (Fig. 2a, lanes 6 to 8) and palmitoylated (Fig. 2a, lanes 2 and 3). The M2 monomer appeared as a doublet

Results

Effects of amantadine on M2 expression in Sf9 cells

During initial experiments it became clear that laboratory variants of the Sf9 cell line differed in their sensitivity to toxicity caused by the M2 protein expressed during initial experiments.
Fig. 2. Palmitoylation, phosphorylation and oligomerization of the M2 protein expressed in Sf9 cells. (a) Palmitate radiolabelling: non-infected Sf9 cells (lane 1) and CFM2-infected cells in the absence (lane 2) or presence (lane 3) of amantadine (1 µg/ml) were labelled with [14C]palmitate. Phosphate radiolabelling: non-infected (lane 5) and infected (lane 6) amantadine-treated cells were labelled with [32P]phosphate at 24 h p.i. Infected cells without (lane 7) or with (lane 8) amantadine (1 µg/ml) were labelled at 48 h p.i. Lane 9, infected amantadine-treated Sf9 cells were labelled with [35S]cysteine. Lane 4, 35S-labelled influenza virus Weybridge strain M2, immune-precipitated with R7 from influenza virus-infected chick cells. Boiled, reduced samples were run on 12 % polyacrylamide gels. M, standards are on the left. (b) M2 oligomers: boiled, unreduced samples were run on a 10 % polyacrylamide gel. Weybridge strain M2 was immune-precipitated with anti-M2 rabbit sera R7 (lane 1) and R15 (lane 2) from pulse-chase 35S-labelled CFM2-infected Sf9 cells or from influenza virus-infected chick embryo cells (lane 3). The positions of M2 dimers (2), trimers (3) and tetramers (4) are indicated.

(Fig. 2a, lane 4), since a proportion of the palmitate is lost during boiling and reduction. The band of lower mobility contains the palmitoylated species (Sugrue et al., 1990b).

Analyses of M2 synthesized in recombinant baculovirus-infected Sf9 cells showed the presence of monomers (Fig. 2a), dimers, trimers and tetramers (Fig. 2b), as for the M2 synthesized in influenza virus-infected cells. In pure and relatively concentrated M2 preparations (200 µg/ml) high M, species, of approximately 200K, predominated (Fig. 3c); similar bands have been observed in analyses of influenza virus virion proteins. By several criteria, therefore, M2 expressed from recombinant baculovirus in Sf9 cells resembled the authentic influenza virus protein.

Fig. 3. Immunoaffinity purification of M2 protein. A detergent extract (60 ml) from CFM2-infected Sf9 cells was applied to an immunoaffinity column. After washing, 1 ml fractions were eluted as described in Methods and 10 µl samples were applied to 12 % polyacrylamide gels. One gel was silver-stained (a) and the other blotted for 30 min (b) and rebotted for another 90 min (c). Lane 1, detergent extract input; lane u, unbound material; lanes 1 to 7, fraction numbers; lane 3*, unreduced fraction 3. By OD measurements, sample 3 was found to contain 0.9 µg protein.

Purification of M2 protein

M2 protein was purified from Sf9 RE cells as described in Methods. The membrane fraction was treated with 1 M-KCl and 4 mM-OG to remove peripheral proteins and the M2 protein was extracted quantitatively with 40 mM-OG. Immunoaffinity chromatography gave 95 % or greater purification, as judged by silver staining of the protein, analysed on a polyacrylamide gel. The assignment of silver-stained bands of the samples from
Proton translocation activity of M2 protein in lipid vesicles

We examined the suitability of various lipid mixtures and reconstitution techniques for producing M2 vesicles in which ion translocation activity could be assayed. The following method was established: detergent solutions of M2 (purified by Phosphogel followed by immunoaffinity chromatography) and M2δ (immunoaffinity-purified) were reconstituted with a mixture of DMPC, phosphatidyl serine and valinomycin (0.7 mol% with respect to DMPC).

column fractions 2, 3 and 4 (Fig. 3a) as monomers, dimers and tetramers is made on the basis of bands at equivalent positions in a parallel gel (Fig. 3b, c). Most of the monomer transferred within 30 min (Fig. 3b) while the tetramer and the high $M_r$ (> 200K) species in the unreduced, unboiled sample (3*) of fraction 3 transferred more slowly (Fig. 3c).

In larger scale preparations the detergent extract was enriched for phosphoproteins using a column of immobilized Fe$^{3+}$ ions (Phosphogel) before immunoaffinity chromatography.
Table 1. Initial acidification rates of vesicles containing M2 and M2δ

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μM)</th>
<th>Initial acidification rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control M2 M2δ</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>0.15  6.9  7.0</td>
</tr>
<tr>
<td>Rimantadine</td>
<td>0.1</td>
<td>0.28  4.5  7.0</td>
</tr>
<tr>
<td>1</td>
<td>0.67</td>
<td>3.9   6.2</td>
</tr>
<tr>
<td>10</td>
<td>1.5</td>
<td>8.2  6.2</td>
</tr>
<tr>
<td>N-ethylrimantadine</td>
<td>0.1</td>
<td>ND†  4.2  ND†</td>
</tr>
<tr>
<td>0.67</td>
<td>0.78</td>
<td>3.0  7.0</td>
</tr>
<tr>
<td>10</td>
<td>ND</td>
<td>8.2  ND†</td>
</tr>
</tbody>
</table>

* Expressed as 10⁹ times the emission intensity per second, calculated from the data presented in Fig. 4 as described in Methods.
† ND, Not done.

...to lipids) in a potassium phosphate buffer containing K⁺ as the only cation. OG was removed by extensive dialysis. Control vesicles without protein were prepared in parallel. The vesicles were loaded with 2 μM-pyranine (a fluorescent pH indicator; Dencher et al., 1986) and fluorescence caused by residual externally bound dye was quenched by DPX.

The driving force for proton flux across a membrane can either be a pH gradient or a membrane potential (Garcia et al., 1984). The incorporation of valinomycin in the membrane of K⁺-containing vesicles allowed the creation of a diffusion potential on exposure of these vesicles to the sodium buffer. The internal pH was monitored by recording, every 10 s, emission intensities at 510 nm for two excitation wavelengths, 403 and 460 nm (Fig. 4; Dencher et al., 1986). Reactions were terminated by adding an excess of the K⁺/H⁺ ionophore nigericin together with the K⁺ ionophore valinomycin to collapse the proton gradient. This elicited an unexplained transient (10 s) drop in pH in the control vesicles before pH equilibration.

After introducing the vesicles into the sodium buffer, proton influx into M2- and M2δ-containing vesicles was initially rapid and the pH reached an equilibrium within 4 min (Fig. 4b, c). The plot resembles a saturation curve. Initial acidification rates, represented by the slopes, were calculated as described in Methods and are shown in Table 1. The internal pH of M2 and M2δ vesicles, determined using a calibration plot, decreased by about 0.5 units (Fig. 4b, c). Under the same conditions the pH in the control vesicles (Fig. 4a) exhibited a slow linear decrease of 0.06 units over 5 min and did not reach equilibrium. In the presence of rimantadine the pH decreased more rapidly, at 1 μM by 0.19 units and at 10 μM by 0.33 units, indicating that the drug itself promoted detectable vesicle acidification (Fig. 4a).

Both the initial acidification rate and the equilibrium pH of M2-containing vesicles were influenced by inhibitors of M2. However, only the initial rates were obtained under identical ionic conditions. Rimantadine at 0.1 and 1 μM inhibited the acidification of M2 vesicles to a similar extent whereas 10 μM-rimantadine enhanced acidification (Fig. 4b; Table 1). Exactly the same effects of rimantadine (inhibitory at 1 μM but stimulatory at 10 μM) were observed when the reaction was not terminated with ionophores but instead was re-initiated by addition of H₃PO₄, lowering the external pH by about 1 unit and thereby causing a further decrease in the internal pH until a new equilibrium was reached (data not shown).

N-ethylrimantadine causes a similar degree of inhibition of influenza virus infection as rimantadine (Indulen et al., 1979). 0.67 μM-N-ethylrimantadine caused a somewhat greater reduction (2.3-fold) in the initial rate of the pH change in M2-containing vesicles than did 1 μM-rimantadine (1.8-fold). Stimulatory concentrations (10 μM) of both compounds increased the rate 1.2-fold (Fig. 4b; Table 1).

M2δ-containing vesicles responded differently to these inhibitors. Acidification proceeded similarly in the absence and in the presence of rimantadine or N-ethylrimantadine (Fig. 4c; Table 1), consistent with the drug resistance of the protein. However, 1 μM-rimantadine, but not 0.67 μM-N-ethylrimantadine, clearly elevated the equilibrium pH (Fig. 4c), although the initial rate was only slightly reduced; 1.1-fold as compared to 1.8-fold in the case of wild-type M2-containing vesicles (Table 1). The reason for this is not clear.

Discussion

The baculovirus-Sf9 cell expression system was chosen to produce the M2 protein since it has been successfully exploited for the other influenza virus envelope proteins (Kuroda et al., 1986; Weyer & Possee, 1991) as well as for ion channel proteins (e.g. Kamb et al., 1992; Carter et al., 1992). M2 expressed by the recombinant baculovirus resembled the authentic virus protein in that it was phosphorylated and palmitoylated and formed homotramers (Sugrue & Hay, 1991; Sugrue et al., 1990b; Veit et al., 1991; Holsinger & Lamb, 1991). In pure and relatively concentrated preparations very large M2 complexes were observed, the functional significance of which is not known. Under optimum conditions the yield per l of Sf9 cell suspension was 2 to 4 mg of M2 protein, of which about 50% was recovered in purified form. Two- to threefold higher yields were obtained using Hi5 cells.

The alleviation of M2 toxicity to the insect cells by amantadine was due to inhibition of M2 function, since the drug enhanced M2 synthesis about 10-fold. Black et al. (1993) reported a similar drug-induced enhancement of the expression of the M2 protein of influenza virus...
strains A/Ann Arbor/6/60, which differs from the Weybridge strain M2 by 15 amino acids (Ito et al., 1991). In contrast, amantadine treatment has no effect on M2 synthesis in virus-infected cells or M2 expression in mammalian cells or E. coli (F. Geraghty, A. Hayhurst & A. Hay, unpublished results). Thus, the effect appears to result from M2 activity on the one hand and properties of the baculovirus–insect cell system on the other. M2 in the plasma membrane of mammalian cells can dissipate a pH gradient between extra- and intracellular environments, causing a decrease in cytoplasmic pH (Ciampor et al., 1992b). The pH of the insect cell medium is particularly low (pH 6.2 to 6.4); thus changes in the internal ionic environment may well underlie the distinctive M2-related phenomena in these cells. The resemblance between M2-induced changes in cell shape to changes produced by 10 μM-monensin (unpublished data), an ionophore known to substitute for M2 in regulating pH within the transport pathway (Sugrue et al., 1990a), is consistent with this interpretation.

The assay of M2 activity in the reconstituted membrane system employed a K+ gradient rather than a proton gradient, emulating the relationship between the cytoplasm (high [K⁺]) and the trans Golgi compartment (high [Na⁺]). M2- and M2δ-mediated proton fluxes (initial rates) were 46 times faster than fluxes into control vesicles. In view of the estimated purity of M2 and M2δ proteins of 95%, the presence of traces of other channel-forming proteins cannot be excluded. However, the specific inhibition by low concentrations of rimantadine (0.1 to 1 μM) and the drug resistance of the mutant protein M2δ support the interpretation that the proton translocation activity was due to the viral protein.

In response to the K+ diffusion potential and against the proton gradient, rimantadine and N-ethyl-N-methylamantadine lowered the pH in protein-free control vesicles, which is consistent with the ability of the hydrochloride, not only the free base, to penetrate lipid bilayers (Duff et al., 1993). The concentration ranges for the specific anti-M2 and non-specific effects of rimantadine were similar to those observed in virus-infected cells, where rimantadine, at 5 μM and above, counteracts its own specific M2-mediated effect (maximum at 0.5 μM) on trans Golgi pH (Grambas & Hay, 1992). Other amphiphilic primary amines and weak bases have also been shown to act this way (Sugrue et al., 1990).

Though proton influx is driven by the K+ gradient, it makes only a minor contribution to the dissipation of this gradient. Whether, like M2 expressed in X. laevis oocytes (Pinto et al., 1992), the M2 protein in the in vitro system is also capable of forming Na+-permeable channels is not monitored. Recent voltage-clamp studies have, however, indicated that the M2 protein expressed in mouse erythroleukaemia cells does form proton-selective channels (I. Chizmachov, F. Geraghty, D. Ogden & A. Hay, unpublished results).

The data presented here are consistent with the ability of the M2 protein to promote proton translocation in vivo and with its two proposed roles in virus infection, acidification of the virion interior during uncoating and protection of HA against premature acid-induced conformational change during protein transport.

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References


