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Influenza Virus M_2 Protein Has Ion Channel Activity

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Summary

The influenza virus M_2 protein was expressed in Xenopus laevis oocytes and shown to have an associated ion channel activity selective for monovalent ions. The anti-influenza virus drug amantadine hydrochloride significantly attenuated the inward current induced by hyperpolarization of oocyte membranes. Mutations in the M_2 membrane-spanning domain that confer viral resistance to amantadine produced currents that were resistant to the drug. Analysis of the currents of these altered M_2 proteins suggests that the channel pore is formed by the transmembrane domain of the M_2 protein. The wild-type M_2 channel was found to be regulated by pH. The wild-type M_2 ion channel activity is proposed to have a pivotal role in the biology of influenza virus infection.

Introduction

The enveloped influenza A virus is an excellent model system for understanding many aspects of membrane protein structure and for elucidating properties of cellular exocytic and endocytic pathways. The virion lipid envelope contains three integral membrane proteins: hemagglutinin (HA), neuraminidase, and a small integral membrane protein designated M_2. Inside the envelope are the helical viral ribonucleoprotein (RNP) structures, which consist of a genomic RNA segment covered with the nucleocapsid protein and the associated RNA transcriptase protein complex. The viral membrane protein (M_1) is a peripheral membrane protein that associates with both the RNPs and the cytoplasmic face of the lipid bilayer (reviewed in Lamb, 1989). The M_2 integral membrane protein (97 aa) is abundantly expressed at the plasma membrane of virus-infected cells but is greatly underrepresented in virions, as only a few (on average 23–60) molecules are incorporated into virus particles (Lamb et al., 1985; Zebedee et al., 1985; Zebedee and Lamb, 1988, 1989). The M_2 protein spans the membrane once and is orientated such that it has 23 N-terminal extracellular residues and a 54 residue C-terminal cytoplasmic domain, and thus M_2 is a model type III integral membrane protein (nomenclature of von Heijne, 1988) (Lamb et al., 1985; Hull et al., 1988; reviewed in Lamb, 1989). The native form of the M_2 protein is minimally a homotetramer consisting of either a pair of disulfide-linked dimers or disulfide-linked tetramers, the disulfide bonds acting to stabilize the oligomer (Holsinger and Lamb, 1991; Sugrue and Hay, 1991). In studies using chemical cross-linking reagents, a small amount of a large complex (150–180 kd) has been identified that appears to contain only M_2 molecules and thus may represent a higher structure of 10–12 M_2 molecules (Holsinger and Lamb, 1991).

Influenza viruses bind to cell surfaces by means of an interaction between HA and cell surface molecules containing sialic acid, and virions enter cells by receptor-mediated endocytosis (reviewed in Marsh and Helenius, 1989). On transfer of the endocytic vesicles containing virion particles to secondary endosomes, the low intracompartmental pH causes a conformational change in HA, which renders it competent to mediate fusion of the viral envelope with the membrane of the endosome. This fusion event delivers the RNPs into the cytoplasm (reviewed in Wharton et al., 1990).

Amantadine (1-aminoadamantane hydrochloride) and its structural analog rimantadine are antiviral drugs that, at micromolar concentrations, specifically inhibit influenza A virus replication (Davies et al., 1964; Appleyard, 1977). The block to virus replication is thought to occur after the virus has bound to the cell but before uncoating occurs (Bukrinskaya et al., 1982a, 1982b; Skehel et al., 1977; Hay et al., 1986; reviewed in Hay, 1989). In the presence of the drug, intact complexes of the RNPs and the M_1 protein can be isolated from cells but these cannot be found in the absence of the drug (Bukrinskaya et al., 1982a). Recently, in an in vitro analysis of the mechanism of virion uncoating it has been found that the M_1 protein is selectively removed from the RNP structure at acidic pH (pH 5.5), and it was suggested that during virus replication, this change in the RNP–M_1 protein interaction occurs when the virion is in the endosomal compartment (Zhirnov, 1990). However, as the M_1 protein resides inside the viral lipid bilayer, a mechanism, sensitive to amantadine, of making the interior of the virion accessible to a pH change is required.

Amantadine-resistant influenza virus mutants have been isolated (Lubeck et al., 1978; Hay et al., 1979; Scholtissek and Faulkner, 1979; Hay and Zambon, 1984), and genetic studies indicated drug resistance is linked to genome RNA segment 7, which encodes the M_1 and M_2 proteins. Nucleotide sequence analysis of RNA segment 7 indicated that the target of antiviral action is the hydrophobic membrane-spanning domain of the M_2 protein, as amino acid substitutions mapped to 4 residues in this domain (Hay et al., 1985). These data indicate that M_2 has an essential role in the influenza A virus replicative cycle.

In addition to the "early" effect of amantadine on the replication of influenza virus at the stage of uncoating, the drug has a second "late" effect on the growth of fowl plague virus, a strain of influenza virus that has an HA with a high pH optimum of fusion (pH 6), as it blocks release of virus particles without interfering with bud formation (Ruigrok et al., 1991). The available evidence strongly suggests that with fowl plague virus, addition of amantadine to cells late

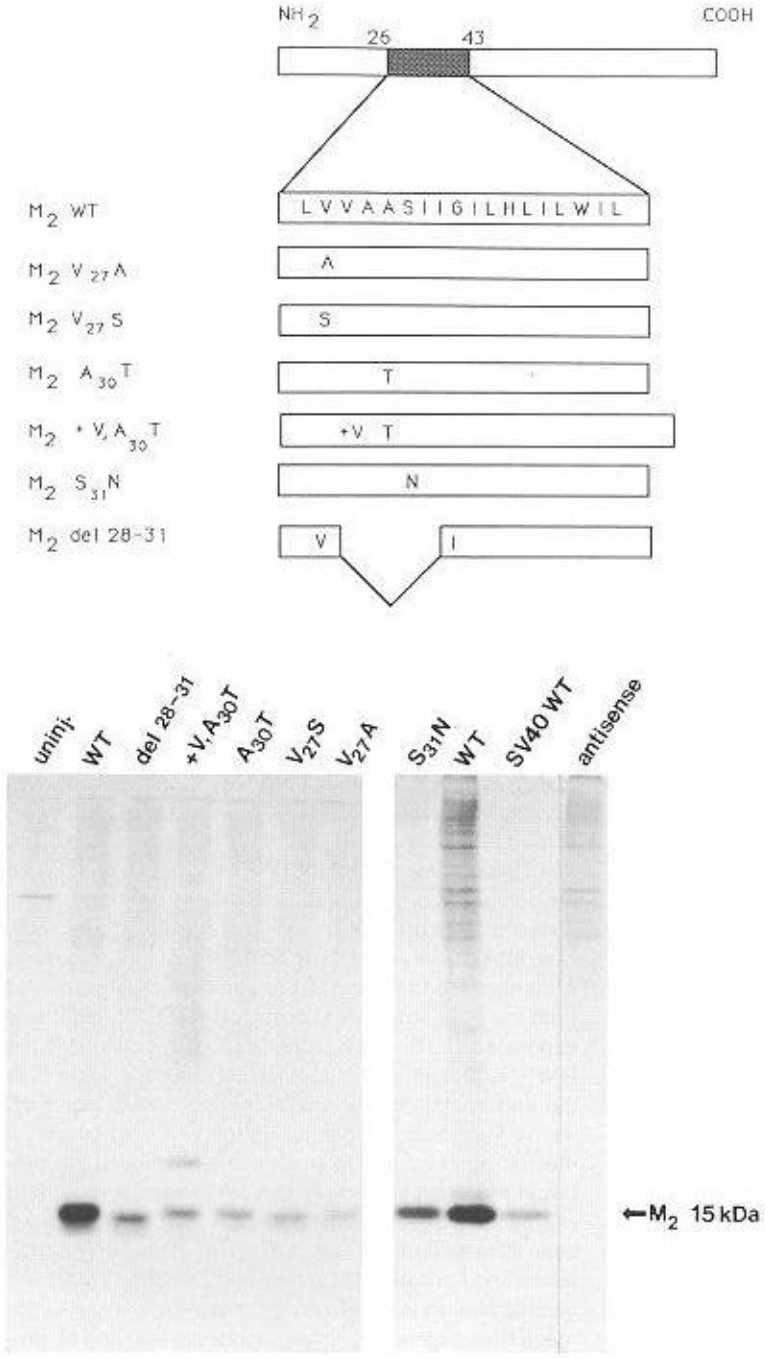

Figure 1. Construction and Expression of Influenza Virus Wild-Type and Mutant M Proteins

(Top) Schematic diagram of the influenza virus M_2 protein and its hydrophobic transmembrane domain residues 26–43 (crosshatched). The amino acid sequence of the transmembrane domain is shown in the expanded section of the diagram using the single-letter code. The mutants M_2–$V_{27}A$, M_2–$V_{27}S$, M_2–$A_{30}T$, M_2–$S_{31}N$, and M_2del_{28-31} were constructed as described in Experimental Procedures. When found in influenza virus, these mutations confer resistance to amantadine (Hay et al., 1985). The mutant M_2+V,A_{30}T contains an insertion of a valine residue between residues 26 and 27 and the rationale for its construction is described in the text.

(Bottom) Expression of wild-type and mutant forms of M_2 in oocytes of Xenopus laevis. Synthetic RNAs were transcribed from pGEM-1 plasmids encoding wild-type or mutant M proteins and microinjected (40 nl of RNA at 0.5 μg/μl) into oocytes of Xenopus laevis. At 24 hr postinjection oocytes were labeled for 3 hr with [^{35}S]methionine and homogenized. M proteins were immunoprecipitated with M-specific antisera and analyzed by SDS-PAGE. Designation of the mutants is as shown in the top panel; uninj, uninjected; antisense, injected with M_2 antisense RNA; SV40WT, marker lane of wild-type M_2 protein synthesized in CV-1 cells, using an SV40 vector to express the M cDNA (Zebedee et al., 1985).

in infection brings about a premature conformational change in HA that occurs in the trans Golgi complex during the transport of HA to the cell surface (Sugrue et al., 1990). By immunological and biochemical criteria, this form of HA is indistinguishable from the "low pH–induced form of HA" (Skehel et al., 1982) that results following exposure of native HA to low pH, which in vivo occurs once the virions are endocytosed and transported to endosomes. This premature conformational change in HA is thought to be detrimental to the release of virus particles (Ruigrok et al., 1991). Alterations in the amino acid sequence of the M_2 transmembrane domain abrogate the effect of amantadine, yet the observable phenotypic changes concern an acid-induced conformational change in HA. It has therefore been suggested that the M_2 protein is an ion channel capable of modulating the pH of intracellular compartments in influenza virus–infected cells (Hay, 1989; Sugrue and Hay, 1991). The same mutations in the M_2 transmembrane domain abolish susceptibility to both "early" and "late" effects of amantadine, suggesting that the M_2 ions must have the same function as in virus-infected cells (Hay, 1989). Thus, in virions, M_2 may be an ion channel

permitting the flow of protons from endosomes into the virion interior to facilitate removal of M_1 protein from RNPs during virion uncoating in endosomes. Here, we describe electrophysiological experiments in which injection of M_2 mRNAs into oocytes of Xenopus laevis induces an ion channel activity that is sensitive to amantadine hydrochloride. We also describe properties of the conductance of altered M_2 molecules that are resistant to the effect of the drug.

Results

Expression of M_2 Proteins in Oocytes

The influenza A virus M_2 protein membrane-spanning domain (residues 26–43 inclusive; Lamb et al., 1985) that is associated with sensitivity of the virus to the antiviral drug amantadine hydrochloride is shown in Figure 1. Several mutants were constructed, using the M_2 cDNA, to encode the amino acid changes in the M_2 membrane-spanning domain that lead to resistance of influenza A virus to amantadine hydrochloride (Hay et al., 1985). These include four single point mutants (M_2–$V_{27}A$, M_2–$V_{27}S$, M_2–$A_{30}T$, and M_2–$S_{31}N$) and a deletion of residues 28–31 (VAAS) from the transmembrane domain (M_2del_{28-31}). If the M_2 protein transmembrane domain is modeled as an α helix, M_2 residues 27, 30, and 31, which are changed in amantadine-resistant mutants, lie on the same face of the α helix, and this region of the protein could involve an interaction with the drug (Hay, 1989). Addition of an amino acid residue to the putative α helix has the potential to disrupt the protein structure, and thus the M_2–$A_{30}T$ mutant was engineered to contain an additional valine residue in the transmembrane domain, a mutant designated M_2+V,$A_{30}T$.

Synthetic mRNA transcripts were synthesized using bacteriophage T_7 RNA polymerase from the wild-type and mutant M_2 cDNA templates cloned in a pGEM3 vector (Zebedee et al., 1985; Hull et al., 1988). The M_2 mRNAs were microinjected into oocytes of Xenopus laevis. To confirm that M_2 mRNAs were translated, at 24 hr postinjection, the oocytes were labeled with [^{35}S]methionine for 24 hr, and lysates were immunoprecipitated with an M_2–specific monoclonal antibody (14C2) (Zebedee and Lamb, 1988). As shown in Figure 1, synthesis of the wild-type and mutant M_2 proteins ($M_r \approx 15{,}000$) could be detected. The additional band of slower mobility detected with M_2+V,$A_{30}T$ is due to addition of asparagine-linked carbohydrate to M_2. This species was found to be sensitive to digestion with peptide N-glycanase to yield the faster migrating band. In addition, the upper band was not observed when oocytes were injected with tunicamycin (data not shown). The addition of N-linked carbohydrate to M_2+V,$A_{30}T$ probably occurs at the site for glycosylation (NDS; M_2 residues 20–22) that is not used in wild-type M_2 protein (Zebedee et al., 1985). A possible explanation for glycosylation is that the structure of the M_2 transmembrane domain is sufficiently rigid that insertion of the valine residue alters the border of the ectodomain/transmembrane domain region to make residues 20–22 accessible to the oligosaccharyltransferase.

Changes of amino acids in the ectodomain and cytoplasmic domain of many integral membrane proteins block their transport out of the endoplasmic reticulum, due to protein misfolding (reviewed in Rose and Doms, 1988). For the work to be described below, it was essential to investigate whether the wild-type and altered M_2 proteins were expressed at the oocyte plasma membrane. Thin frozen sections of oocytes expressing the various M_2 molecules were stained with M_2 ectodomain–specific monoclonal antibody and fluorescein-labeled secondary antibody. As shown in Figure 2, all the M_2 molecules showed a characteristic bright surface staining on the oocyte plasma membrane.

Characterization of an M_2 Protein–Associated Ion Channel Activity

To test for M_2 protein ion channel activity using a two-electrode voltage-clamp procedure, oocytes of Xenopus laevis were injected with the wild-type M_2 mRNA or the mutant M_2 mRNAs, cultured for 1–3 days, and total membrane currents were measured. The currents of oocytes expressing the wild-type M_2 protein were studied by holding the membrane voltage of the oocyte at −40 mV and then changing the membrane voltage to a more negative value (between −60 and −130 mV) with a step voltage-clamp pulse. This hyperpolarization induced an inward current with minimal time dependence (Figure 3A inset) and increased to a steady value immediately after the hyperpolarizing pulse was applied. This current was significantly larger than the endogenous current evoked by identical changes of membrane voltage in control oocytes that either were injected with antisense mRNA or were uninjected (Figure 3B).

We wished to investigate the effect of amantadine hydrochloride on the M_2 protein–associated ion channel activity. The concentrations of the drug used to inhibit influenza virus in tissue culture have ranged from 1–100 μM (Hay, 1989). However, we had no prior knowledge of the concentration of amantadine hydrochloride to use with oocytes or the duration of the treatment. When oocytes expressing wild-type M_2 were bathed for 2 min in a solution that contained 10 or 100 μM amantadine hydrochloride, it was found that the component of the current induced by hyperpolarization of oocytes that was associated with M_2 expression was completely attenuated (Figure 3A; Table 1), whereas the drug had very little effect on the small currents measured in control oocytes (Figure 3B).

Amantadine-Resistant M_2 Proteins Exhibit Drug-Resistant Conductances

We were interested in testing the associated ion channel activity of M_2 proteins containing amino acid changes in their hydrophobic domain that, when found in influenza virus, lead to resistance to amantadine hydrochloride. The membrane currents of oocytes expressing five such naturally occuring proteins, M_2–$V_{27}A$, M_2–$V_{27}S$, M_2–$A_{30}T$, M_2–$S_{31}N$, and M_2del_{28-31} (and protein M_2+V,$A_{30}T$), were measured, and the currents were found to exhibit a range of amplitudes: these amplitudes were all larger than that of the wild-type M_2 protein except for the current associated with expression of mutant M_2–$S_{31}N$, which was smaller. The relative surface expression levels of the mu-

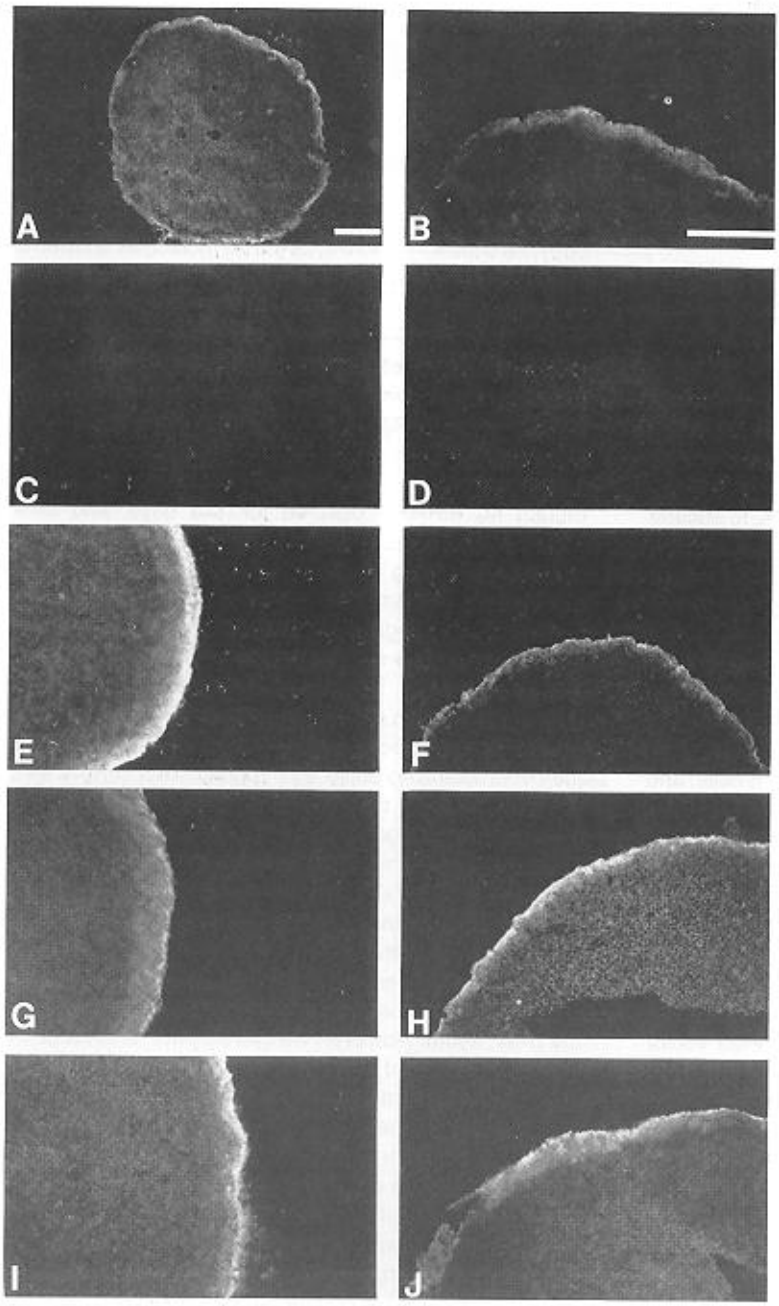

Figure 2. Cell Surface Expression of Wild-Type and Mutant M_2 Proteins Expressed in Xenopus laevis Oocytes

Indirect immunofluorescent microscopy of injections of oocytes was done as described in Experimental Procedures. The M_2 protein was stained with M_2–specific monoclonal antibody (14C2) and fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody. (A and B) Wild-type M_2 protein; (C and D) injection of antisense RNA; (E) M_2–$V_{27}A$; (F) M_2–$V_{27}A$; (G) M_2–$A_{30}T$; (H) M_2+$V_{27}A_{30}T$; (I) M_2–$S_{31}N$; (J) M_2del_{29-31}. (A) and (C) were photographed at 87× magnification. (B), (D), (E), (F), (G), (H), (I) and (J) were photographed at 174× magnification. Bar, 150 μm.

tant M_2 proteins have not been determined and, therefore, could not be compared with current amplitude. However, the difference in amplitude of membrane currents associated with expression of the mutant M_2 proteins did not correlate with the amount of labeled M_2 protein that accumulated in a 20 hr labeling period (see Figure 1; Table 1). Other explanations for the reproducible differences in current amplitude observed from expression of the mutant M_2 proteins include a greater ionic flux through a single channel and a greater probability of a channel being open. Most importantly toward understanding the molecular action of the anti-viral drug amantadine hydrochloride, the membrane currents associated with expression of the mutant M_2 proteins were not significantly attenuated by the drug and in two cases, M_2–$A_{30}T$ and M_2+$V_{27}A_{30}T$, were increased in the presence of the drug, in contrast to oocytes expressing wild-type M_2 protein (Table 1). Thus, these data provide direct evidence that amantadine affects M_2 protein function.

Analysis of Ion Selectivities and Activation Properties of Mutant M_2 Channels

The M_2 protein has a single membrane-spanning domain, and M_2 bears no similarity to the structure of most ion channels cloned to date. It is therefore possible that, rather than being a channel per se, the M_2 protein is a regulatory

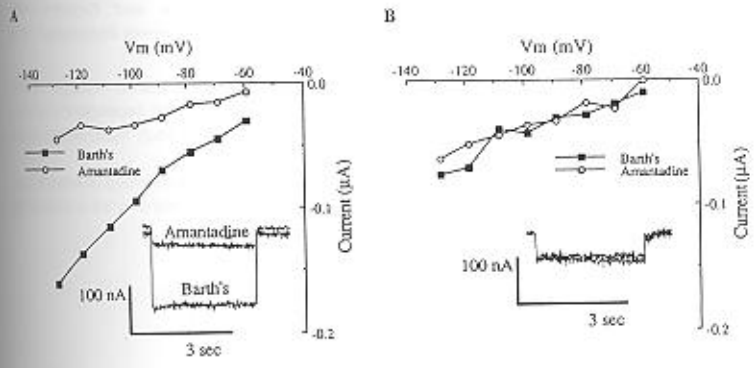

Figure 3. Ion Channel Activity Associated with M_2 Protein Expressed in Oocytes Is Amantadine Sensitive

The current–voltage relationship was determined for oocytes injected with wild-type M_2 mRNA (A) or control oocytes injected with distilled water (B). The data shown in (A) is typical of oocytes expressing wild-type M_2 protein that had a membrane current with only a small time-dependent component (shown in insert). Closed squares, current of oocytes bathed in Barth's solution. Open circles, current of oocytes after bathing in Barth's solution containing 100 μM amantadine for 2 min. Currents were measured at the end of the activating voltage pulse.

protein that activates a normally silent channel endogenous to oocytes. It has been argued similarly that the cystic fibrosis transmembrane conductance regulator and I_{SK} (minK) K^+ channels could be modifiers of existing host cell channels (Kartner et al., 1991; Goldstein and Miller, 1991). The pore is an integral part of every channel, and therefore compelling evidence for a direct role of the amino acids in the M_2 membrane-spanning domain in channel function can be provided if mutations are introduced into this domain that alter ion selectivity without grossly affecting the overall structure and functional integrity of the channel. This analysis has been applied, for example, to K^+ channels, the nicotinic acetylcholine receptor, the cystic fibrosis transmembrane conductance regulator, and I_{SK} (Yool and Schwarz, 1991; Leonard et al., 1988; Anderson et al., 1991; Goldstein and Miller, 1991). We have used a similar approach of comparing the channel behavior associated with mutant M_2 proteins to show that both the ion selectivity and activation properties differ with the amino acid composition of the outermost region of the M_2 transmembrane domain. We used tail current analysis to compare the ion selectivity of the membrane currents of oocytes expressing two amantadine-resistant mutant M_2 proteins, M_2del_{28-31} and M_2+V,A_{30}T. These currents were suitable for the tail current analysis because they were large in amplitude and had two kinetic components (Figure 4; see Figure 7A), one which appeared immediately after the application of a hyperpolarizing step of voltage and a second component that increased slowly with time.

To evaluate the ion selectivity of oocytes expressing these two mutant M_2 proteins with the method of tail current analysis, we applied an activating pulse to −110 mV followed by test pulses between −40 and +40 mV and measured the amplitude of the decaying membrane current after the test pulses (see Figure 5) in order to determine the reversal voltage, V_{rev}. This analysis was performed on a minimum of five oocytes expressing each mutant M_2 protein while the oocyte was bathed in normal Barth's medium and in media in which the ionic composition was altered.

If only one ion were responsible for the membrane current, then replacement of that ion by an impermeant ion ought to alter V_{rev} according to the predicted change in the Nernst equilibrium potential, and alterations in concentrations of other ions ought to be without effect. For oocytes that expressed the M_2del_{28-31} protein and oocytes that expressed the M_2+V,A_{30}T protein, V_{rev} varied with the activities of both Na^+ and Cl^- (Figure 6), suggesting that a nonspecific increase in monovalent ion conductance occurs. However, the dependence of V_{rev} upon Na^+ activity was stronger for the oocytes that expressed the M_2del_{28-31} mutant protein than for oocytes that expressed the M_2+V,A_{30}T protein (Figure 6A). When [Na^+] was decreased from 77 to 2 mEq/l, V_{rev} decreased by 19 mV for oocytes expressing the M_2del_{28-31} mutant protein, while V_{rev} decreased by only 5 mV for oocytes expressing the M_2+V,A_{30}T ($P < 0.01$). On the other hand, when [Cl^-] was decreased from 77 to 9 mEq/l (Figure 6B), V_{rev} increased by 30 mV for oocytes

Table 1. Amplitude of Inward Currents

Protein Genotype	Inward Current (μA) (Mean ± SEM)[a]		
	No Drug n = 5	+ 100 μM Amantadine n = 5	+ 10 μM Amantadine n = 5
Wild-type M_2	0.08 ± 0.02	0.00 ± 0.012	0.00 ± 0.004
M_2-V_{27}A	0.42 ± 0.03	0.28 ± 0.004	
M_2-V_{27}S	0.43 ± 0.18	0.40 ± 0.040	
M_2-A_{30}T	0.45 ± 0.10	0.61 ± 0.050	
M_2-S_{31}N	0.01 ± 0.01	0.01 ± 0.004	
M_2-V,A_{30}T	0.86 ± 0.20	0.92 ± 0.070	
M_2del_{28-31}	2.71 ± 0.23	2.70 ± 0.100	

[a] Current of control oocytes injected with antisense RNA (endogenous current) was subtracted from total current measured to yield current reported. See Figure 3B for endogenous currents. Currents measured at −130 mV.

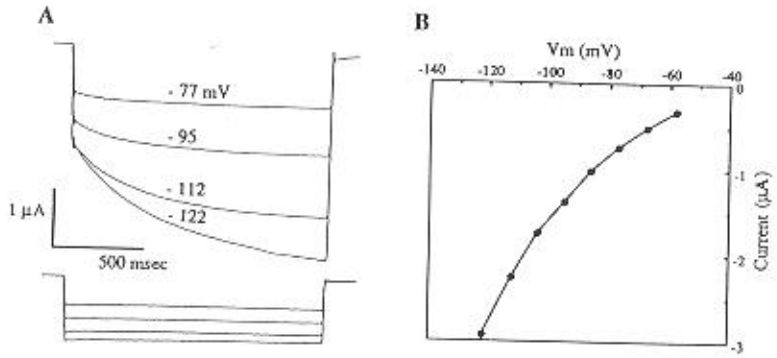

Figure 4. Time Course and Curr
age Relationship of Currents Associa
M_2del_{28-31} Protein

(A) Bottom, hyperpolarization voltag
tained with time; top, time dependenc
rents associated with each hyperpol
voltage step.

(B) Current–voltage relationship of inw
rent measured at the end of a 2.5 s a
pulse.

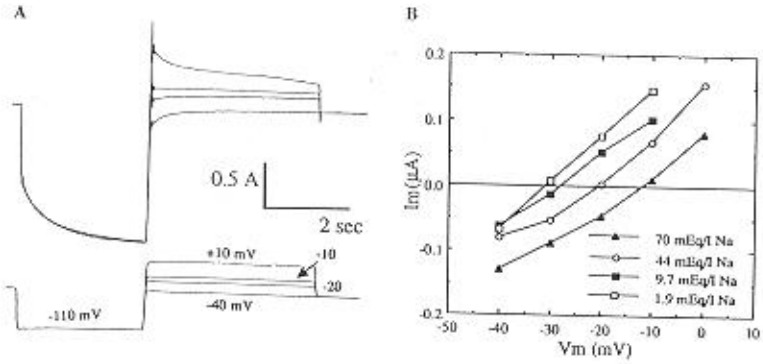

Figure 5. Analysis of Ionic Mechanism
rent Carried by M_2del_{28-31} Protein In
Nonspecific Increase in Na^+ and K^+ C
tance

(A) Time course of the tail currents of o
expressing the M_2del_{28-31} protein evoked
voltages between −40 and +40 mV tha
applied after an activation pulse of −11
measured in Barth's solution (Na^+ activ
mEq/l). Reversal voltage indicated by a

(B) Tail current amplitude as a function
voltage for each of the five values of
noted.

expressing the M_2+V,A_{30}T protein, while V_{rev} increased by only 10 mV for oocytes expressing the M_2del_{28-31} protein ($P < 0.01$). We were not able to fit values for V_{rev} calculated from the Goldman–Hodgkin–Katz relationship to the measured values of V_{rev} (Hille, 1991) to arrive at a calculated value for the Cl^- and Na^+ permeabilities. However, the data shown in Figure 6 indicate that the Na^+ permeability is greater for the M_2del_{28-31} mutant protein-associated channel activity than for M_2+V,A_{30}T, while the Cl^- permeability is reversed for the channels associated with expression of these two mutant M_2 proteins. We found that no detectable alteration in V_{rev} occurred for either mutant when Na^+ was replaced by K^+ in the bathing medium (Table 2), the bi-ionic condition (Hille, 1991). This finding of invariant V_{rev} under these latter conditions suggests that the permeabilities for Na^+ and K^+ are similar for both of the mutants. When Cl^- was replaced by other ions from the halide series (Table 2), V_{rev} varied in a similar manner for both mutants: $F^- > Cl^- > Br^- > I^-$. This finding suggests that the halide permeabilities for the conductances associated with expression of the mutant proteins are determined by hydration energy ($I^- > Br^- > Cl^- > F^-$) (Eisenman and Horn, 1983). The difference in ion selectivities for the channels functioning in oocytes expressing the M_2del_{28-31} or M_2+V,A_{30}T proteins indicates the mutations affect, in some way, the pore-forming region of the channel.

To provide further evidence that the M_2 protein is a channel per se, aspects of the activation properties of oocytes that expressed the M_2del_{28-31} and M_2+V,A_{30}T proteins were compared. After removal of an activating pulse, the cess of deactivation of an ion channel occurs, and channel conductance returns to the value in the re unactivated state. When applying repeated, identical vating pulses, we observed that the currents of ooc expressing the M_2+V,A_{30}T protein increased with suc sive pulses. This suggests that some channel activa evoked during the first activating pulse survived the moval of this pulse, resulting in a greater current ampli at the onset of the next identical pulse (Figure 7). fraction of the increase in current that occurred during activating pulse that remained at the onset of the activating pulse is defined as residual activation. For cytes expressing the M_2+V,A_{30}T protein, the residual vation carried from the first to the second pulse was 0.4 0.03 SEM, and the current increased with each succes pulse. However, the residual activation observed when identical train of pulses was applied to oocytes expres the M_2del_{28-31} protein was only 0.22 ± 0.02 SEM ($P < 0$ for the first pulse and decayed for each successive pu Thus, oocytes expressing the M_2+V,A_{30}T protein disp considerably more residual activation than those expre ing the M_2del_{28-31} protein.

The voltage dependence of activation of the curren oocytes expressing the M_2del_{28-31} and M_2+V,A_{30}T prote was also compared. To do so, we determined the logari mic potential sensitivity, defined as the change in me brane voltage required for an e-fold increase in cond tance. This property has been used as it is an index of t

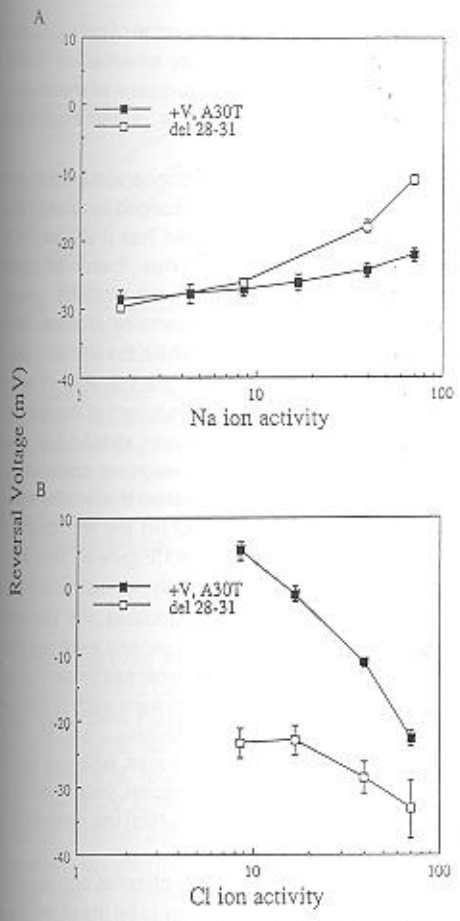

Figure 6. Ion Selectivity of Oocytes That Expressed M_2del_{28-31} and $M_2+V,A_{30}T$ Mutant Proteins

The reversal voltage for tail currents, measured as shown in Figure 5, is plotted against the activity for Na^+ and Cl^-. For the experiments with Na^+, the activities of K^+ and Cl^- were equal to the values in normal Barth's solution. Na^+ was replaced by N-methyl-D-glucamine$^+$, and Cl^- was replaced by methanesulfonate$^-$; when Na^+ was replaced, $[Cl^-]$ was kept at the value of Barth's solution (77 mEq/l), and when Cl^- was replaced, $[Na^+]$ was reduced to 1.8 mEq/l by replacement with N-methyl-D-glucamine$^+$. Note that V_{rev} depended more strongly upon $[Na^+]$ for the oocytes expressing the M_2del_{28-31} mutant protein and more strongly upon $[Cl^-]$ for the oocytes expressing the $M_2+V,A_{30}T$ mutant protein.

Table 2. Ionic Selectivity of Mutants

	Reversal Voltage (mV) (Mean ± SEM)	
Ion Varied	M_2del_{28-31} n = 4	M_2-V,$A_{30}T$ n = 3
NaF	+16.0 ± 2.5	+1.0 ± 4.0
NaCl	−11.0 ± 0.8	−18.0 ± 1.8
NaBr	−17.8 ± 0.7	−30.0 ± 3.8
NaI	−31.0 ± 2.1	−43.0 ± 2.9
88 mM K^+*	−10.6 ± 0.3	−18.8 ± 1.0

* K^+ (88 mM) satisfied the biionic condition (Hille, 1991).

gating charge (or the dipole moment) of the voltage-sensing region of the ion channel (see Hille, 1991). We measured the amplitude of the slowly increasing kinetic component of current at the cessation of closely spaced steps of membrane voltage (3 s duration). When ln(conductance) was plotted against the membrane voltage (V_m), the values fell about a straight line for V_m between −70 and −100 mV (data not shown). The logarithmic potential sensitivity was 50 mV ± 1.6 mV SEM for oocytes expressing the M_2del_{28-31} protein and 80 mV ± 5.7 mV SEM for oocytes expressing the $M_2+V,A_{30}T$ protein ($P < 0.01$). Thus, the voltage dependence of activation differed for oocytes expressing these two mutant proteins.

Activation of M_2 Channel Activity by H^+

In mammalian cells infected with influenza virus approximately 10^6–10^7 molecules of the M_2 protein accumulate at the cell surface (Zebedee et al., 1985), and if these were all active channels (made active by the resting membrane voltage of the cell) it seems likely that the continuous ion flux through the channel (see Table 1) would be extremely deleterious to the cell. Two observations indicated that the conductance of the ion channel in oocytes expressing the wild-type M_2 protein is not activated by changes in membrane voltage: the current–voltage relationship was linear throughout the range of membrane voltages that were applied (see Figure 3), and the current amplitude did not change with time (compare inset of Figure 3A with Figure 4, in which the voltage-activated, time-dependent increase

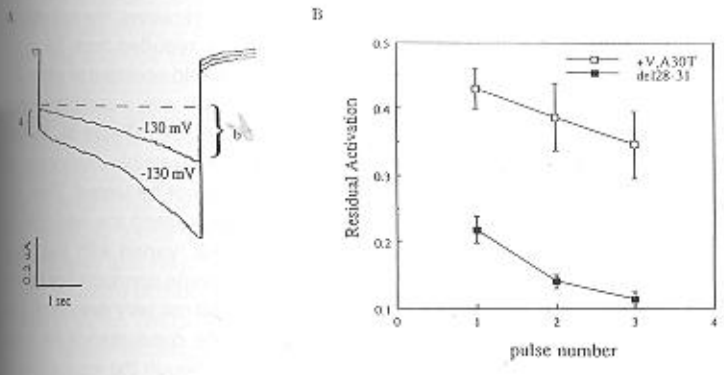

Figure 7. Residual Activation in Oocytes That Expressed M_2del_{28-31} and $M_2+V,A_{30}T$ Mutant Proteins

(A) The fraction of the current developed during a given pulse, (b) that appeared immediately after the onset of a second identical pulse (a) is the residual activation, a/b. (B) Residual activation is plotted as a function of pulse number and was larger for oocytes that expressed the $M_2+V,A_{30}T$ mutant protein. Resting voltage, −20 mV; voltage during pulse, −130 mV. Pulse duration, 3 s. Interpulse interval, 0.5 s.

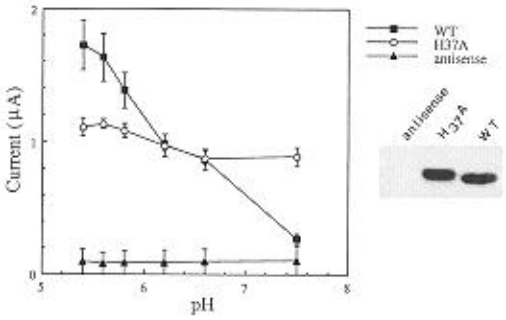

Figure 8. Modulation of the Membrane Currents of Oocytes Expressing M_2 Proteins by H^+

The peak amplitude of the current during a hyperpolarizing pulse (−130 mV) is plotted against extracellular pH. Note that the amplitude for wild type increased by 8-fold when pH was decreased to 5.8. The current of oocytes expressing the M_2–$H_{37}A$ protein remained constant over the entire pH range. (Inset) Expression of wild-type M_2 and M_2–$H_{37}A$ proteins analyzed on SDS–PAGE.

in current of an oocyte expressing the M_2del_{28-31} mutant is shown). This led us to investigate other possible means of activation of the wild-type M_2–associated conductance. The intracellular site of action of M_2 is thought to be in secondary endosomes, to permit ions to flow into the virion particle to facilitate virion uncoating. In addition, as described above in the Introduction, the fowl plague virus M_2 protein is required to be functional in the trans Golgi network. As both of these intracellular compartments are acidic (pH 5–6), it seemed possible that M_2 could be regulated by changes in pH. We tested this notion by measuring the currents of oocytes that expressed the wild-type M_2 protein while the oocytes were bathed in Barth's solution in which the pH had been lowered from the normal value of pH 7.4 to values as low as pH 5.4. The amplitude of the currents increased monotonically with decreasing pH for the oocytes that expressed the wild-type M_2 protein, whereas control oocytes had only a small, pH-independent current (Figure 8). Below pH 5.4 irreversible changes in membrane current occurred and thus were not measured. The currents were linearly related to membrane voltage,

Table 3. Inward Currents of Wild-Type M_2 Channel Activity in Amantadine and Low pH

Treatment	Inward Current (μA) (Mean ± SEM)	n
pH 7.5	0.110 ± 0.05	4
pH 6.2	0.770 ± 0.07	4
pH 6.2 + 100 μM Amantadine	0.0 ± 0.03	4

Table 4. Inward Currents at Low pH

Genotype	Inward Current (μA) (mean ± SEM) pH 7.5	pH 5.8	n
Wild-Type M_2	0.18 ± 0.01	1.68 ± 0.16	4
M_2+V,A_{30}T	1.84 ± 0.41	4.75 ± 0.91	3
M_2del_{28-31}	2.12 ± 0.57	2.22 ± 0.58	6

were not time dependent for any value of pH studied (data not shown), and were blocked by amantadine (Table 3). Thus, the amplitude of the conductance of the channel in oocytes expressing the wild-type M_2 protein can be modulated by H^+.

Histidine residue 37 is highly conserved among strains of influenza A virus, is the only charged residue in the M_2 membrane-spanning domain, and has a pK_a of pH 5.2–6.0, depending on the protein. Thus, it seemed possible that histidine residue 37 might be a titratable group involved in activation of the M_2 channel by protons. Thus, a mutant $H_{37}A$ was constructed in which the histidine residue was changed to alanine. When the currents of oocytes expressing the $H_{37}A$ protein were studied at the normal pH 7.4, the amplitude of the current was about 5-fold greater than for oocytes expressing the wild-type protein (Figure 8). However, more importantly, when these oocytes were bathed in solutions with lowered pH the membrane currents were not markedly increased (Figure 8), thus indicating a role, either directly or indirectly, for histidine 37 in the pH regulation of M_2 activity. In addition, the currents of oocytes expressing the M_2del_{28-31} protein were not altered by bathing in solutions of low pH, whereas those of oocytes expressing the M_2+V,A_{30}T protein were slightly increased with low pH (Table 4). Because M_2del_{28-31} and M_2+V,A_{30}T channel activities are voltage activated, whereas wild-type M_2 is pH activated, the data suggest these amino acid alterations modify the manner in which the channel is regulated.

The ion flux of the wild-type M_2 channel activated at pH 5–6, as opposed to pH 7.4, is likely to be the most relevant measurement for the life cycle of influenza virus. However, the ion selectivity of the currents that flowed at low pH could not be examined by the method of tail current analysis because these currents were not voltage activated. Thus, to obtain a qualitative estimate of the ion selectivity of wild-type M_2 channel activity at low pH, we used an alternative approach and measured the current-voltage relationship with slowly varying command voltages (5 mV/s). The voltage at which no net membrane current flowed, the null voltage, was determined while the oocyte was bathed in media of various ionic compositions. The currents for oocytes bathed in solutions at pH 6.2 were found to vary linearly with membrane voltage (Figure 9). When oocytes were bathed in Barth's solution at pH 6.2, the null voltage was +27 mV (Table 5) and became less positive when $[Na^+]$ was decreased from 88 to 1.76 mEq/l while $[Cl^-]$ and $[K^+]$ were held constant. However, the null voltage did not change when $[Cl^-]$ was reduced from 88 to 4.4 mEq/l while $[Na^+]$ and $[K^+]$ were held constant or when $[K^+]$ was increased from 2 to 20 mEq/l with a complementary decrease in $[Na^+]$ and constant $[Cl^-]$ (Table 5). Further increases in $[K^+]$ brought about irreversible changes in the oocyte at this low pH and could not be tested. Thus, the null voltage for oocytes that expressed the wild-type M_2 protein, when studied at pH 6.2, varied with $[Na^+]$ in a manner consistent with a membrane conductance for this ion. However, the null voltage did not vary detectably with $[Cl^-]$ or $[K^+]$, suggesting that the conductance for these ions is less than that for Na^+. Although the insensitivity of

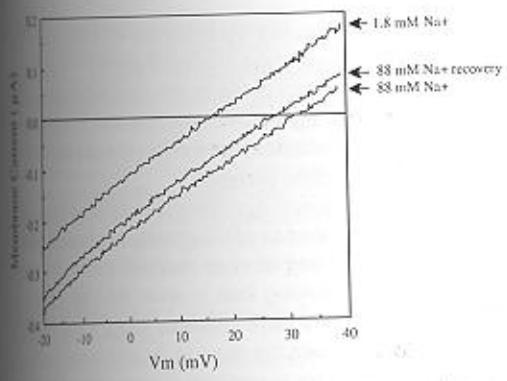

Figure 9. Current–Voltage Relationship of Oocytes Expressing the Wild-Type M_2 Protein Studied at pH 6.2

The current induced by a slowly changing membrane voltage (5 mV/s) is plotted before, during, and after bathing the oocyte in a medium (pH 6.2) in which Na^+ was replaced by N-methyl-D-glucamine$^+$. Note that the null voltage (current axis intercept; see text) decreased when $[Na^+]$ was reduced and that the current–voltage relationships were linear.

Table 5. Ionic Selectivity of Wild-Type M_2

Ion Varied	n	Ion Concentration (mM)	Null Voltage (Mean ± SEM) (mV)
Cl^- [a]	4	88	+27.0 ± 1.3
		4.4	+27.0 ± 3.3
Na^+ [b]	4	88	+33.0 ± 3.8
		1.76	+19.5 ± 2.5
K^+ [c]	3	20	+28.0 ± 2.8
		2	+32.0 ± 3.2

[a] $[Na^+]$ and $[K^+]$ were held constant and Cl^- replaced by methanesulfonate.
[b] $[Cl^-]$ and $[K^+]$ were held constant and Na^+ replaced by N-methyl-D-glucamine$^+$.
[c] $[K^+]$ increased by reducing $[Na^+]$, $[Cl^-]$ held constant.

this method does not allow us to exclude the possibility that some membrane conductance for Cl^- and K^+ exists at low pH, these data indicate that the ion selectivity of the channel activity in oocytes expressing wild-type M_2 is different from that found in oocytes expressing either M_2del_{28-31} or M_2+V,A_{30}T proteins. While the data indicate that the ion channel activity associated with wild-type M_2 expression in oocytes causes permeability to Na^+ ions, we were not able to measure the H^+ conductance, as it is difficult to distinguish the flow of protons from the currents generated by the flow of other ions because of the low concentration (and thus low conductance) of protons in the pH range that oocytes will tolerate. However, it would not be surprising if the monovalent cation conductance we found extends to H^+.

Discussion

The data reported here provide direct evidence that the influenza virus M_2 protein forms an ion channel. Injection of wild-type M_2 mRNA into oocytes produces an inward current that can be blocked by addition of the anti-viral drug amantadine. However, amantadine did not block the conductance of oocytes expressing M_2 proteins that contained mutations that confer viral resistance to amantadine. The amantadine-resistant M_2–S_{31}N protein conductance was seemingly greatly diminished as compared with that of the wild-type M_2 protein: an observation that indicates the conductance associated with the other M_2 proteins is specific and not an artifactual result of microinjection of M_2 mRNA into oocytes. Possible reasons for the lowered conductance of M_2–S_{31}N are discussed below. The M_2 protein has a single membrane-spanning domain and minimally forms a tetramer (Holsinger and Lamb, 1991; Sugrue and Hay, 1991) and does not have the molecular structure of most ion channels cloned to date (reviewed in Miller, 1991). Thus, it was acutely necessary to provide further evidence that the M_2 protein is a channel and to eliminate the possibility that it is a regulatory protein that activates a normally silent channel endogenous to oocytes.

The pore is intrinsic to a channel molecule, and electrostatic forces generated by amino acids that line the pore determine ion selectivity. Thus, a prima facia case that a protein is a channel can be obtained if mutations introduced into the transmembrane domain alter properties of the channel. When mutations were introduced into the transmembrane domain of the M_2 protein, several altered characteristics could be measured. First, the amino acid alterations caused changes in kinetics; some altered M_2 proteins were activated by membrane hyperpolarization (M_2+V,A_{30}T and M_2del_{28-31}) whereas others were not (e.g., wild-type M_2) (see Figure 3; Figure 4). Second, a change in amino acid sequence of the M_2 transmembrane domain produced different ion selectivities for wild-type M_2, M_2+V,A_{30}T, and M_2del_{28-31} (Figure 6; Figure 9). Third, the M_2+V,A_{30}T and M_2del_{28-31} channel conductances had different voltage dependencies of activation and different residual activation properties. Fourth, different mutants had varying pH sensitivities. Thus, although final proof of the M_2 channel activity will require purification and reconstitution of the protein into artificial bilayers, the data reported here, when taken together, provide strong evidence that the influenza virus M_2 protein is a bona fide ion channel.

The whole-cell currents measured in oocytes expressing the various altered M_2 proteins were found to be reproducibly different (Table 1), and the size of the current did not correlate with the amount of radioactively labeled M_2 protein that accumulated in a 20 hr labeling period (see Figure 1). The different current amplitudes could reflect relative surface expression levels, but the levels of surface fluorescence staining for each altered M_2 protein, although only a qualitative estimate, did not suggest that the surface expression levels varied by orders of magnitude. More likely, the differences in current observed reflect a change in ion flux or a greater probability of the channel being open, and these factors can be determined once recordings from single channels can be made. The M_2–S_{31}N protein had a current in the presence and absence of amantadine that was lower than that found with expression of the wild-type M_2 protein in the absence of amantadine. Assuming the explanation for the low current is a direct

reflection of channel activity, we suggest that the serine to asparagine change is deleterious to total ion flux but the remaining activity is not affected by amantadine. This is consistent with the observation that influenza virus containing the M_2–$S_{31}N$ mutation grows poorly (Sugrue and Hay, 1991). In addition, it has been found in cells infected with influenza virus containing the M_2–$S_{31}N$ change that the amount of HA that undergoes a premature acid-induced conformational change is large (50%–75%), even in the absence of amantadine. This premature conformational change suggests that the M_2–$S_{31}N$ protein is functioning poorly (Ciampor et al., 1992).

The data reported here also have general implications toward understanding properties of transmembrane domains. Operationally, transmembrane domains are defined as the hydrophobic domain bounded by 2 charged residues, and using this criterion, the M_2 protein transmembrane domain contains 19 residues. Transmembrane domains are usually thought to be α helical regions, based on theoretical predictions and the observation that membrane-spanning domains of bacterial photosynthetic reaction centers form α helices (Deisenhofer et al., 1985; Allen et al., 1987; Roth et al., 1989). When the M_2 transmembrane domain is modeled as an α helix, residues 27, 30, 31, and 34, which are changed in amantadine-resistant mutants, and histidine residue 37, shown in this report to be implicated in pH regulation of M_2, all map to the same face of an α helix (Sugrue and Hay, 1991) However, there are several observations, which although individually do not rule out an α helical structure for this domain, when taken together need consideration. First, an amantadine-resistant influenza virus has been isolated that contains the M_2 transmembrane domain change of A_{30} to proline (Hay et al., 1985), a residue that can be helix breaking. Second, the insertion of an amino acid into the putative α helix would have the potential to disrupt the conformation of the protein, yet the mutant M_2+V,A_{30}T retains ion channel activity (Table 1). Third, deletion of 4 residues from the 19 residue M_2 transmembrane domain in M_2del_{28-31} still permits this altered protein to function as an ion channel. When residues are deleted from a transmembrane domain it cannot be ruled out that other residues, previously exterior to the bilayer, are pulled into the membrane: if they are not then the remaining 15 residues of the M_2del_{28-31} is too short a region to span the lipid bilayer as an α helix (Adams and Rose, 1985 and references therein). In this regard it has been suggested that the presumed pore region of the Shaker K^+ channel is formed by a conserved stretch of only 18 residues (Yellen et al., 1991). Site-directed mutagenesis shows that the blocker tetraethylammonium binds in this region and that a stretch of only 8 aa may span the membrane. This latter stretch has been suggested to consist of antiparallel β strands (Hartmann et al., 1991; Guy and Conti, 1990). Clearly it is of importance to determine the structure of the transmembrane domain of the influenza virus M_2 ion channel.

Another known channel that has a single membrane-spanning domain is the 130 aa I_{Sx} (minK) K^+ channel (Takumi et al., 1988; Folander et al., 1990; Pragnell et al., 1990; Sugimoto et al., 1990; Goldstein and Miller, 1991), and interestingly I_{Sx}, like the influenza virus M_2 protein, is one of the few known type III integral membrane proteins. However, comparison of the amino acid sequence of the M_2 protein membrane-spanning domain with the data base of protein sequences does not indicate any obvious homology with other known proteins.

The ion channel activity of the influenza virus M_2 protein that can be regulated by pH is expected to have a critical role in the uncoating of virus particles internalized into endosomes by allowing ions to enter the virion particle. In vitro experiments have indicated that a lowered pH is required to dissociate the virion M_1 protein from the RNPs (Zhirnov, 1990). This is probably directly related to the in vivo experiments that indicated that, in the presence of amantadine, M_1 fails to dissociate from the RNPs (Bukrinskaya et al., 1982a; Martin and Helenius, 1991), and transport of the RNPs to the nucleus does not occur (Martin and Helenius, 1991). In addition, for those strains of influenza A virus that have both an HA that is cleaved intracellularly and a high pH optimum of fusion (e.g., fowl plague virus), the M_2 ion channel may regulate the ion balance of the trans Golgi network. This prevents HA from undergoing prematurely, its pH-sensitive conformational change in the trans Golgi network (Sugrue et al., 1990; Steinhauer et al., 1991). These presumed activities of the M_2 ion channel suggest that the M_2 protein presents an important target for a point of intervention by drugs, in addition to amantadine, in the influenza virus replicative cycle.

Experimental Procedures

Site-Specific Mutagenesis, Construction of Recombinant Plasmids, and In Vitro RNA Synthesis

The cDNA to the A/Udorn/72 M_2 mRNA (Zebedee et al., 1985; Hull et al., 1988) was cloned into the BamHI site of the replicative form of M13mp19 and used as template DNA for site-specific mutagenesis as described previously (Holsinger and Lamb, 1991). Oligonucleotides were synthesized by the Northwestern University Biotechnology Facility on a DNA synthesizer (Model 380B, Applied Biosystems, Inc., Foster City, CA). Mutant cDNAs encoding the altered M_2 genes were excised from the replicative form of M13 by BamHI digestion and subcloned into the BamHI site of a pGEM3 vector such that mRNA sense transcripts could be generated using the bacteriophage T_7 RNA polymerase promotor and T_7 RNA polymerase. The nucleotide sequence of the altered cDNAs was confirmed by dideoxynucleotide chain-terminating sequencing (Sanger et al., 1977). For in vitro transcription, plasmid DNAs were linearized downstream of the T_7 promotor and the M_2 cDNA with XbaI. In vitro synthesis of ^{7m}G(5')ppp(5')G-capped mRNA was carried out as previously described (Hull et al., 1988). The RNA yield was determined by measuring the incorporation of [^{3}H]GTP into RNA. Bacteriophage T_7 DNA-dependent RNA polymerase was obtained from Bethesda Research Laboratories (Gaithersburg, MD), RNasin™ and RQDNase™ from Promega Biotec (Madison, WI), and ^{7m}G(5')ppp(5')G from Pharmacia (Piscataway, NJ).

Microinjection and Culture of Oocytes

Ovarian lobules from Xenopus laevis females (Nasco, Fort Atkinson, WI) were surgically removed and treated with collagenase B (2 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN) in Ca^{2+}-free OR-2 solution (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES-NaOH [pH 7.5]) at 24°C for 3 hr to liberate oocytes from follicle cells. Defolliculated oocytes were washed in OR-2 and maintained in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM sodium pyruvate, 5 mM HEPES–NaOH, 0.1 mg/ml gentamycin [pH 7.5]) at 17°C 24 hr prior to microinjection. Selected oocytes (stage IV and V) were injected with 40 nl of RNA (0.5 μg/μl) using a 20 μm diameter glass pipette, and oocytes were maintained in ND96 at 17°C.

Isotopic Labeling of Injected Oocytes, Immunoprecipitation, and SDS-PAGE

Oocytes were incubated in ND96 supplemented with [^{35}S]methionine (250 μCi/ml) (Amersham Corp., Arlington Heights, IL) from 24–48 hr postinjection. Labeled oocytes were homogenized in 75 μl of RIPA buffer per oocyte, and extracts were immunoprecipitated as previously described (Lamb et al., 1978) using M_2-specific 14C2 monoclonal antibody ascites fluid (Zebedee and Lamb, 1988). Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) on 17.5% + 4 M urea polyacrylamide gels and processed for fluorography and autoradiography as previously described (Lamb and Choppin, 1976).

Peptide: N-Glycosidase F Digestions and Tunicamycin Treatment

Following immunoprecipitation, labeled oocyte lysates were treated with peptide: N-glycosidase F (Boehringer Mannheim Biochemicals) essentially as described (Williams and Lamb, 1986). Protein A–Sepharose antibody–antigen complexes were boiled in 20 μl of 10 mM Tris (pH 7.4)/0.2% SDS for 4 min, diluted with an equal volume of 10 mM Tris (pH 7.4), and incubated with 50 mU of endo F at 37°C for 16 hr. For tunicamycin treatment, mRNAs were coinjected with 40 μg/ml tunicamycin (Calbiochem-Behring Corp., La Jolla, CA) (1 mg/ml stock in dimethyl sulfoxide) and incubated in ND96 containing 2 μg/ml tunicamycin both prior to and during metabolic labeling. Oocytes were homogenized and extracts immunoprecipitated as described above.

Indirect Immunofluorescence Microscopy

Oocytes were injected with M_2 mRNAs as described above. Oocytes were frozen by plunging them into isopentane cooled to −170°C over liquid nitrogen and embedded in cryoform, and 10 μm sections were cut on a cryostat (Bright Instrument Company, Huntingdon, England) at −22°C. Sections were collected on gelatin-subbed slides and air dried. Dried sections were fixed in 1% formaldehyde essentially as described (Zebedee and Lamb, 1988), stained with M_2-specific 14C2 ascites fluid (diluted 1:300 in PBS/1% bovine serum albumin), and incubated with fluorescein isothiocyanate–conjugated goat anti-mouse secondary antibody. Photomicroscopy was performed on an Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY). All photographic exposure times were equivalent.

Recordings

Whole-cell currents were recorded from oocytes 48–72 hr after mRNA injection with a two-electrode voltage-clamp apparatus consisting of a differential preamplifier (Nihon Kohden MEZ-7101, Tokyo, Japan) that recorded the voltage difference between a pipette (filled with 3 M KCl) located in the cell and another in the surrounding bath. A voltage-clamp amplifier (Nihon Kohden CEZ-1100) provided feedback current to the oocyte through a second intracellular pipette. Oocytes were recorded in standard Barth's solution (Colman, 1984) or modified Barth's solution as indicated. Amantadine hydrochloride (Sigma Chemical Co., St. Louis, MO) (10 mM stock in Barth's solution) was diluted as indicated. Currents and voltages were digitized every 1 ms for the two-electrode voltage-clamp experiments after appropriate low pass filtering (500 Hz) to reduce aliasing.

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