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Reconstitution of the Influenza Virus M_2 Ion Channel in Lipid Bilayers

M.T. Tosteson[1], L.H. Pinto[2], L.J. Holsinger[3], R.A. Lamb[3,4]

[1]Laboratory for Membrane Transport, 240 Longwood Avenue, Harvard Medical School, Boston, Massachusetts 02115
[2]Department of Neurobiology and Physiology, Northwestern University, Evanston, Illinois 60208-3500
[3]Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, Illinois 60208-3500
[4]Howard Hughes Medical Institute, Northwestern University, Evanston, Illinois 60208-3500

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Abstract. M_2, an integral membrane protein of influenza A virus, was purified from either influenza A virus-infected CV-1 cells or from *Spodoptera frugiperda* (*Sf* 9) cells infected with a recombinant-M_2 baculovirus. The purified protein, when incorporated into phospholipid bilayer membranes, produced ion-permeable channels with the following characteristics: (1) The channels appeared in bursts during which unit conductances of diverse magnitudes (25–500 pS) were observed. (2) The most probable open state was usually the lowest unit conductance (25–90 pS). (3) The channels were selective for cations; $t_{Na} = 0.75$ when 150 mM NaCl bathed both sides of the membrane. (4) Amantadine reduced the probability of opening of the high conductance state and also the conductance of the most probable state. (5) Reducing pH increased the mean current through the open channel as well as the conductance of the most probable state. (6) The sequence of selectivity for group IA monovalent cations was Rb > K > Cs ~ Na > Li. The pH activation, amantadine block and ion selectivity of the M_2 protein ion channel in bilayers are consistent with those observed on expression of the M_2 protein in oocytes of *Xenopus laevis* as well as for those predicted for the proposed role of an ion channel in the uncoating process of influenza virus. The finding that the M_2 protein has intrinsic ion channel activity supports the hypothesis that it has ion channel activity in the influenza virus particle.

Key words: Influenza virus M_2 ion channel — Amantadine block — pH activation — M_2 ion channel reconstitution — Planar bilayers

Correspondence to: M.T. Tosteson

Introduction

Influenza A virus is an enveloped virus with the viral lipid bilayer being derived from the plasma membrane of the host cell. Inserted into the influenza virus envelope are three virally encoded integral membrane proteins: hemagglutinin (HA), neuraminidase (NA) and the M_2 protein. Inside the envelope is the genetic information of the virus, eight segments of single-stranded RNA, which are found in the form of helical ribonucleoprotein (RNP) structures with the RNA covered by nucleocapsid protein subunits and the associated RNA transcriptase complex. The other major viral protein located inside the viral envelope is the matrix (M_1) protein which is thought to interact with both the inner face of the lipid bilayer and the RNPs (*reviewed in* Lamb, 1989).

Influenza viruses enter host cells by receptor-mediated endocytosis, a process which is initiated by the binding of HA to sialic acid residues present on the cell surface. The low pH of endosomal compartments triggers a conformational change in HA which leads to HA-mediated fusion of the viral envelope with the endosomal membrane. The consequence of the fusion event is that the RNP complexes are released into the cytoplasm (*reviewed in* Lamb, 1989). As influenza virus RNA replication occurs in the nucleus of infected cells (*reviewed in* Lamb, 1989), the RNPs have to be transported from the cytoplasm to the nucleus, and for transport to occur, the M_1 protein has to dissociate from the RNPs (Martin & Helenius, 1991).

The M_2 protein is a small, 97 amino acid integral membrane protein "M_2" oriented in membranes such that it has 24 N-terminal extracellular residues, a 19 residue transmembrane (TM) domain and a 54 residue cytoplasmic tail (Lamb, Zebedee & Richardson, 1985). The amino acid sequence of the M_2 protein is shown in

A

Met-Ser-Leu-Leu-Thr-Glu-Val-Glu-Thr-Pro-Ile-Arg-Asn-Glu-Trp-Gly- (16)

Cys-Arg-Cys-Asn-Asp-Ser-Ser-Asp-*Pro-Leu-Val-Val-Ala-Ala-Ser-Ile-* (32)

Ile-Gly-Ile-Leu-His-Leu-Ile-Leu-Trp-Ile-Leu-Asp-Arg-Leu-Phe-Phe- (48)

Lys-Cys-Ile-Tyr-Arg-Phe-Phe-Glu-His-Gly-Leu-Lys-Arg-Gly-Pro-Ser- (64)

Thr-Glu-Gly-Val-Pro-Glu-Ser-Met-Arg-Glu-Glu-Tyr-Arg-Lys-Glu-Gln- (80)

Gln-Ser-Ala-Val-Asp-Ala-Asp-Asp-Ser-His-Phe-Val-Ser-Ile-Glu-Leu- (96)

Glu. (97)

B

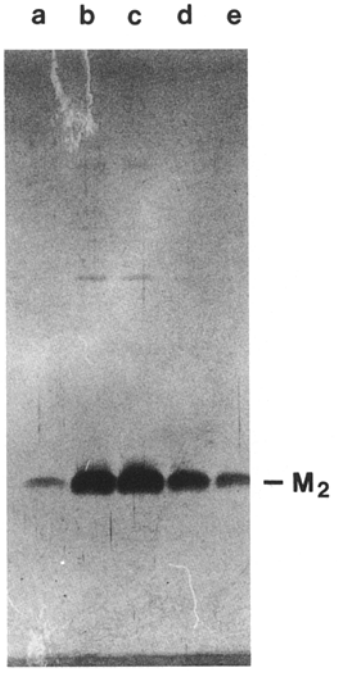


Fig. 1. Amino acid sequence of the M_2 protein and SDS-PAGE of purified M_2 protein. (*A*) The sequence shown of the M_2 protein is that of the human influenza virus A/Udorn/72 subtype. The residues that form the transmembrane domain are boxed and italicized. Data from Lamb, Lai and Choppin, (1981). (*B*) The influenza virus M_2 protein was expressed by infection of *Sf* 9 cells with a baculovirus M_2-recombinant as described in Materials and Methods. The M_2 protein was purified by using M_2-specific monoclonal antibody affinity chromatography as described in Materials and Methods. The M_2 protein was eluted from the affinity column with low pH buffer, aliquots (*a–e*) were subjected to SDS-PAGE under reducing conditions, and the polypeptides visualized by silver stain.

Fig. 1*A*. The native form of the M_2 protein is minimally a homotetramer (Holsinger & Lamb, 1991; Sugrue & Hay, 1991; Panayotov & Schlesinger, 1992). The roles of the M_2 protein in the influenza virus life cycle were inferred from studies with amantadine, an anti-influenza virus drug. Influenza virus "escape" mutants, which are resistant to the effect of the drug, contained amino acid changes that mapped predominantly to the M_2 protein TM domain (Hay et al., 1985). For all subtypes of influenza A virus, the amantadine block to virus replication occurs at an early stage between the steps of virus penetration and uncoating (Skehel, Hay & Armstrong, 1978; Bukrinskaya et al., 1982) and the available evidence indicates that in the presence of amantadine, the M_1 protein fails to dissociate from the RNPs (Martin & Helenius, 1991; Bukrinskaya et al., 1982) and the transport of the RNP complex to the nucleus does not occur (Martin & Helenius, 1991). In addition, it has been found that in the presence of amantadine the rate of fusion of influenza virus with liposomes was slowed down and this effect was not seen with viruses that contained a M_2 protein-specific amantadine-resistant mutation (Bron et al., 1993; Wharton et al., 1994).

In addition to the "early" effect of amantadine, the drug has a second "late" effect on the replication of some subtypes of avian influenza viruses which have an

HA that is cleaved intracellularly and have a high pH optimum of fusion (e.g., influenza A/chicken/Germany/34 (H7N1) (FPV Rostock). A large body of data indicates that addition of amantadine to virus-infected cells causes a premature conformational change in the FPV HA at the time that the HA molecules are transported through the *trans* Golgi network (TGN) (Sugrue et al., 1990; Ciampor et al., 1992*a;* Ciampor et al., 1992*b;* Grambas, Bennett & Hay, 1992; Grambas & Hay, 1992). By immunological and biochemical criteria this form of HA is indistinguishable from the low-pH form of HA (*reviewed in* Wiley & Skehel, 1987). The low-pH conformational transition in HA is thought to occur because the intraluminal pH of the TGN compartment has been lowered below the threshold needed to induce the acid pH transition of HA.

From these and other studies, the hypothesis was developed that the M_2 protein acts as an ion channel. It was proposed that the M_2 ion channel would permit the flow of ions into the virus particle interior to disrupt protein-protein interactions, particularly those between the M_1 protein and the RNPs, and it was proposed that in the TGN the M_2 ion channel acts to keep the pH of the TGN lumen above the threshold for the low pH conformational change (Sugrue et al., 1990; Sugrue & Hay, 1991; Hay, 1992; Helenius, 1992; Marsh, 1992; Skehel, 1992). Support for the hypothesis that the M_2 protein has ion channel activity was obtained by expressing the M_2 protein in oocytes of *Xenopus laevis* and finding that M_2 protein induces an ion channel activity that is blocked by amantadine, regulated by pH and selective for cations (Pinto, Holsinger & Lamb, 1992). The characteristics of the channel activity depend on the amino acid sequence of the M_2 protein TM domain, which presumably forms the pore (Pinto et al., 1992; Wang et al., 1993; Holsinger et al., 1994). The work of Duff and Ashley (1992), who have shown that a 25-residue synthetic peptide corresponding to this domain incorporates into planar bilayers and promotes the formation of channels (at pH 1–2), which close upon addition of amantadine (20 μM), has lent further support to the idea that the TM domain of the M_2 protein forms a pore.

To determine the characteristics of the channel produced by the M_2 protein in a membrane devoid of other proteins, we incorporated the M_2 protein into planar bilayer membranes and measured the resulting channel activity. We report here that the M_2 protein forms multisized channels and that the probability of opening of the large conductance states is drastically reduced by the blocker amantadine. Channel activity was pH regulated and the channel was selective for cations. The amantadine sensitivity, pH regulation and ionic selectivity we have measured are consistent with the characteristics found previously in oocytes (Pinto et al., 1992) and are consistent with the proposed role of the channel in uncoating of influenza A virus.

Materials and Methods

Purification of Influenza Virus M_2 Protein

A baculovirus recombinant that expresses the influenza A/Udorn/72 virus M_2 protein cDNA (Zebedee, Richardson & Lamb, 1985) was constructed by using pBlueBac III (Invitrogen, San Diego, CA) and standard recombinant DNA technology methodologies (Ausubel et al., 1993). As observed previously (Black et al., 1993), addition of 5 μM amantadine to cultures of *Spodoptera frugiperda* (*Sf* 9) cells infected with the baculovirus M_2 recombinant increased the titer of the recombinant virus several fold and also increased the amount of M_2 protein that accumulated in infected *Sf* 9 cells, probably because the M_2 ion channel activity is deleterious to the host cell. However, for purification of M_2 protein to be reconstituted into planar membranes the amantadine was omitted.

To prepare an affinity column for purification of the M_2 protein, purified IgG from the M_2-specific 14C2 hybridoma (Zebedee & Lamb, 1988) was coupled to CNBr-activated Sepharose 4B (Pharmacia, Piscataway, NJ) using methods recommended by the manufacturer. A column (0.75 × 10 cm) was packed with the conjugated Sepharose 4B gel and the affinity column was stored in phosphate-buffered saline containing 0.01% thimerosal at 4°C. Prior to use, the column was equilibrated with column buffer (10 mM HEPES pH 7.7, 0.6% Nonidet P-40, 150 mM KCl, 0.1 mM EDTA, 1 mM phenylmethanelsulfonyl fluoride (PMSF).

For the preparation of M_2 protein from influenza virus-infected cells, CV-1 cells (40 × 10 cm plates) were infected with influenza virus A/Udorn/72 as described previously (Lamb & Choppin, 1979), and at 8 hr post-infection the cells were lysed. For the preparation of recombinant M_2 protein, *Sf* 9 cells (20 × 10 cm plates) were infected with the baculovirus M_2-recombinant and lysed at 48 hr post-infection. Infected CV-1 or *Sf* 9 cells were lysed with 1 ml per plate of ice-cold lysis buffer (2% NP-40, 500 mM KCl, 0.1 mM EDTA, 1 mM PMSF). The plates were scraped with a rubber policeman and the lysates pooled and centrifuged at 100,000 × *g* at 4°C for 30 min in a Ti 60 rotor (Beckman Instruments, Palo Alto, CA) to remove insoluble material. The supernatant was diluted 3.3-fold so that the protein lysate contained 0.6% Nonidet P-40 and 150 mM KCl and loaded onto the affinity column at the rate of 0.5 ml/min. The column was washed with 10 column volumes of column buffer (0.6% Nonidet P-40, 500 mM KCl, 0.1 mM EDTA, 1 mM PMSF) and then the detergent was changed by washing the column with two volumes of 1% octyl glucoside, 500 mM KCl, 0.1 mM EDTA, 1 mM PMSF. The bound M_2 protein was eluted by washing the column with low pH buffer (100 mM glycine, 1% octyl glucoside, 500 mM KCl, 0.1 mM EDTA, 1 mM PMSF, pH 2.5). One milliliter fractions were collected and neutralized immediately with 100 μl 1 M sodium phosphate pH 8.0. Column fractions containing purified protein were pooled and dialyzed against phosphate-buffered saline containing 1% octylglucoside. The purity and concentration of the M_2 protein was determined by SDS-PAGE analysis on a 17.5% polyacrylamide gel (Lamb, Etkind & Choppin, 1978). Known amounts of α-lactalbumin (14.2 kD) and β-lactoglobulin (18.4 kD) were used as concentration standards, and polypeptides were visualized by silver staining by using the Biorad silver stain kit (Biorad Laboratories, Hercules, CA). Where necessary, the M_2 protein was concentrated by immersion of a dialysis bag containing the M_2 protein solution in solid polyethylene glycol 8000. A silver-stained gel showing an example of the purity of the M_2 protein eluted from the antibody affinity column is shown in Fig. 1*B*.

Lipid Bilayers, Data Acquisition and Analysis

The channels induced by M_2 protein were studied using the bilayer technique as previously described (Tosteson, Nibert & Fields, 1993).

The lipid membranes were formed on a hole (50–80 μm diameter) in a Teflon partition which separated the two compartments of a Teflon chamber. Ag-AgCl electrodes were connected to the solution in the chambers via 3 M NaCl-4% agar bridges. The voltage-controlled side was the *cis*-chamber, with the *trans*-compartment the virtual ground. The composition of the solutions surrounding the bilayer could be changed either by perfusion of the chambers or by addition of a small aliquot of a concentrated salt solution (buffered with 10 mM 3-(N-morpholino) ethanesulfonic acid (MOPS) taken to the desired pH with Tris, unless otherwise stated). All experiments were conducted at room temperature (21°C).

The M_2 protein was incorporated by addition of a small aliquot of a concentrated solution to the *cis*-chamber under continuous stirring, to a final concentration between 10 and 20 ng/ml. The salt solutions on both sides of the bilayers at the time of incorporation are described in the text. The channels which are formed from the lipid-protein interaction develop over time and at approximately 30 min after addition of protein, the duration of the channel openings becomes longer than 1 msec and are more frequent, at a given voltage. However, we routinely waited approximately 90 min to start recording the current response to applied voltages, since at this point the characteristics of the M_2-induced channels became constant, presumably reflecting a stable configuration of the protein in the bilayer. Qualitatively similar results were obtained when bilayers were formed using soy bean lecithin (asolectin) purchased from Associated Concentrate (Woodside, NY) and when formed from a 1:1 (molar ratio) of phosphatidyl serine: phosphatidyl ethanolamine (Avanti Polar Lipids, Birmingham, AL). We chose to work with asolectin in most experiments to avoid changes in the surface charge of the membrane as we altered the ionic composition of the solutions surrounding the bilayer.

Single channel currents were filtered at 3 kHz with an eight-pole Bessel filter (Frequency Devices, Haverhill, MA) and the current from the voltage-to-current converter amplifier (Dagan, Minneapolis, MN) was digitized using pCLAMP software with a 12 bit analog to digital converter (Axon Instruments, Burlingame, CA). The data were collected on-line and analyzed using a modified version of the pCLAMP single channel analysis package. Given the multisized nature of the channels, the analysis was done first by taking into account all sizes to obtain a measure of the probability of channel opening, independent of the conductance of its open state and of the dwell times of the state (averaged current). After this was obtained, the analysis of the single channel conductance was then restricted to the lowest-sized state, which was the one with the highest probability of opening at applied voltages <75 mV. For higher voltages, current fluctuations corresponding to channel conductances 50–100 times larger than the minimum would sometimes become the most probable state. The appearance of these fluctuations would also lead, in the majority of cases, to either breakage of the membrane, or to a permanent increase in membrane conductance. Thus, we restricted the analysis to voltages ranging between −70 and +70 mV unless otherwise stated.

The value of the transference number for cations was calculated from measurements of the reversal potential of the single channels in the presence of a salt concentration gradient, using the definition: $V_{rev} = \Sigma(t_i E_i)$ and $\Sigma t_i = 1$, where E_i is the Nernst potential of the *ith* ion and t_i its transference number.

To determine the effects of pH and of addition of amantadine, we tried to complete the following protocol using only one bilayer, to minimize unavoidable differences between membranes. The bilayer was formed in the presence of 150 mM NaCl, 5 mM Tris-MOPS (pH 7.6) and exposed to M_2 as indicated above. Subsequently, the single channel current was determined at three different potentials. The pH of the aqueous solutions was then brought to 5.4 (confirmed at the end of the experiment) by addition of an aliquot of HCl, and the current determined again, at three different potentials. After this was completed, we added an aliquot of a concentrated solution of amantadine, to a final concentration of 50–100 μM, and the single channel current was determined again.

Results

General Characteristics of Currents Induced by the M_2 Protein

The results described in this and following sections were obtained using M_2 protein purified either from influenza-virus-infected CV-1 cells (three preparations) or from the baculovirus recombinant *Sf* 9 cells (two preparations). In all cases the protein incorporation produced the same quantitative results.

Purified influenza virus M_2 protein (*see* Fig. 1*B*) was incorporated into asolectin bilayers in the presence of symmetrical solutions (pH 5.4). Single channel current recordings in response to three different applied voltages from an M_2 channel incorporated into a membrane, in the presence of symmetrical solutions containing 150 mM NH_4Cl (pH 5.4) are shown in Fig. 2. As the single channels induced by the M_2 protein in the presence of symmetrical salt solutions close at negative voltages (*data not shown*), we concentrated our attention on currents obtained at positive membrane voltages. Representative traces of the smallest size fluctuations (selected so that they would not show the larger fluctuations) corresponding to a conductance of 50–90 pS are shown in Fig. 2*A*. The current fluctuations depicted in Fig. 2*B* are shown at a lower amplification than those in Fig. 2*A*, and only for two of the voltages, since the larger conductance states were not present at the lowest voltage applied. Evident in these traces is that, as the applied potential was increased, the current fluctuations at a fixed voltage became larger and the openings of all the states became more frequent. It was also observed that there was an increase in noise when the channel opened, and that the range of conductances which this channel displayed, varied nearly tenfold, from 40 to 600 pS (at +50 mV) (Fig. 2*B*). The current-voltage curve corresponding to the lower channel state is shown in Fig. 2*C* and was found to be sublinear for all cations tested (except Cs^+ for which the current-voltage relation was linear for voltages up to +70 mV). Figure 2*D* depicts the corresponding amplitude distributions at the various applied voltages and shows that, as the voltage is increased, the probability of opening of higher conductance states is increased, leading to a skewing of the amplitude distributions towards the higher voltages. There are at least three alternative explanations for the increased sizes of currents observed as the applied voltage was increased. First, a single M_2 ion channel could have opened to higher conductance states, perhaps through a rearrangement of its structure in

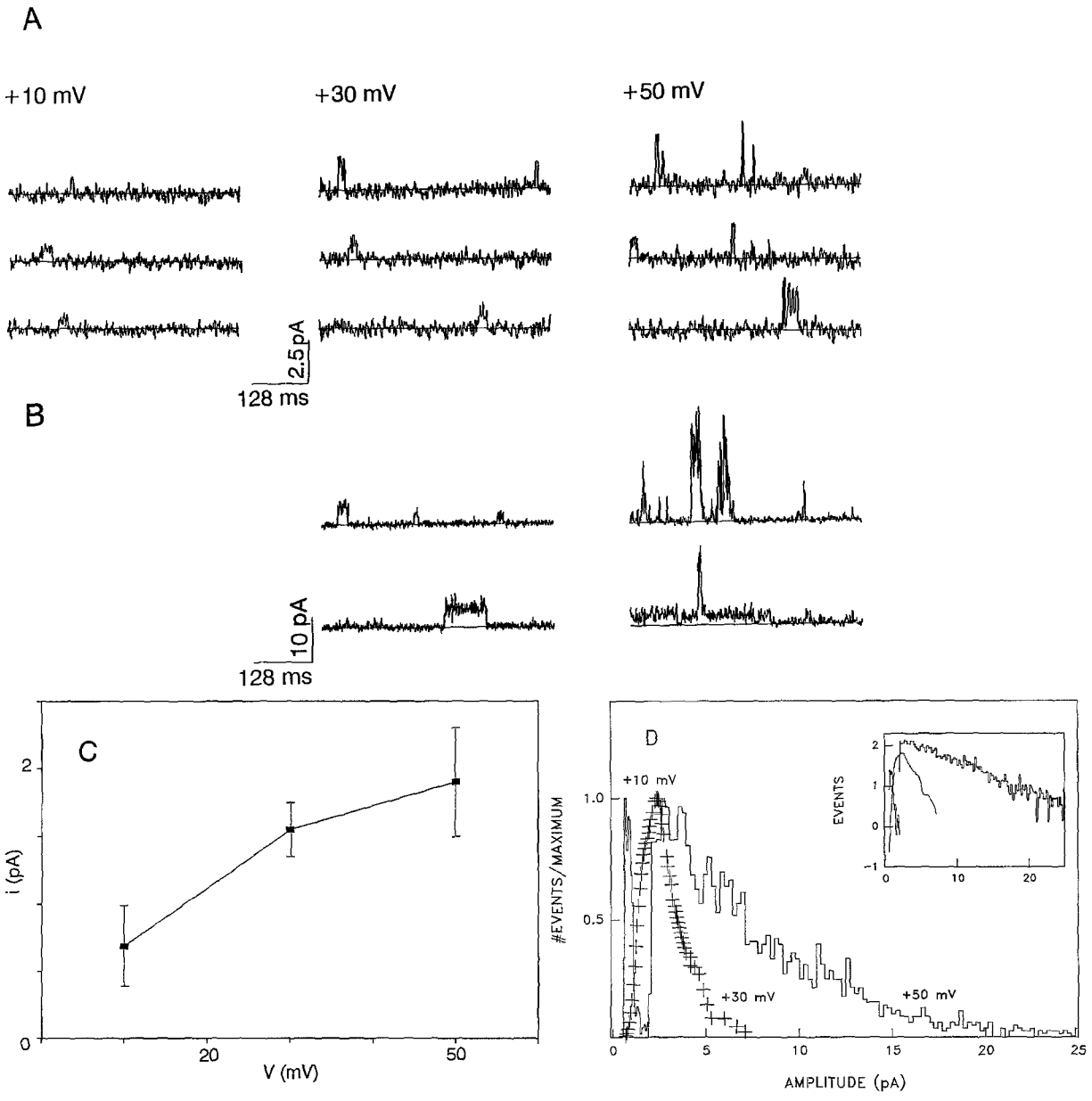


Fig. 2. Current fluctuations induced by incorporated M_2 protein. (*A* and *B*) Time course of the current in response to application of different voltages (numerals above traces). The traces in *A* were chosen to show the current of the lowest state. The traces in *B* are depicted at a lower current sensitivity than those in *A* and were chosen to show the current of higher conductance states. Note that no examples are shown at +10 mV. At +30 and +50 mV it is possible to observe the increased noise of the open channel. (*C*) Current-voltage relationship for the low conductance state. (*D*) Amplitude histogram corresponding to the currents as shown in *A* and normalized to the highest number of events. The inset shows the unscaled amplitude histogram in which the number of events counted at each current bin are shown in a logarithmic scale. Salt: 150 mM NH_4Cl + 10 mM Tris-MOPS (pH 5.4).

a way similar to that which occurs for alamethicin (Eisenberg, Hall & Mead, 1973). Second, the M_2 ion channel could be formed of parallel conductive units, as has been shown for the Cl channel of *Torpedo* electroplax, the anion-selective channel of epithelial cells and for a K^+ channel in renal tubules (Miller, 1982; Krouse, Schneider & Gage, 1986; Hunter & Giebisch, 1987). Third, several M_2 ion channels could have been formed. Our data, as they stand at present, do not allow us to distinguish between the first two possibilities. We believe that the high conductance states are indeed states of one single channel because we have found many instances in which large currents appear or disappear suddenly (within 1 msec) (*cf.* Fig. 2*B*) and the likelihood of

Table 1. Transference number for Na^+

[NaCl] gradient (*cis/trans*) (mM)	*n*	V_{rev} (mV)	t_{Na}	p_+/p_-
150/350	2	8	0.68	2.22
1,000/350	2	−15	0.77	3.78
150/600	2	19	0.76	3.78
1,000/600	1	−7	0.76	3.29
Average ± SD			0.75 ± 0.06	3.5 ± 0.5

The salts used were buffered to pH 5.4 using 10 mM Tris-/MOPS. *n* corresponds to the number of determinations.

the simultaneous opening and closing of many independent channels is extremely low.

As it is necessary for detergent to be present while purifying the M_2 protein, we performed two types of control experiments to determine the effects of the detergents used. In one set, we added an aliquot of eluate from the affinity column used to purify M_2, to the *cis*-side of a bilayer. This sample was both neutralized in phosphate buffer and concentrated (*see* Materials and Methods) in a manner analogous to samples containing protein. We found that there were no current fluctuations induced by the presence of detergent (and absence of protein), even when the volume added was ten times as high as the volume of protein-containing eluate which we used in our studies. In the second set of control experiments, we incubated a portion of the samples containing M_2 protein at 80°C for 30 min, followed by cooling at room temperature. The effect of addition of an aliquot of this control sample was indistinguishable from what we routinely obtain in the presence of the salt solution alone. These results indicate that, under the experimental conditions used in this study, neither the detergents used in the purification and solubilization of M_2, nor potential degradation products of the protein while in the presence of detergent, are capable of producing a change in the electrical properties of the asolectin bilayers.

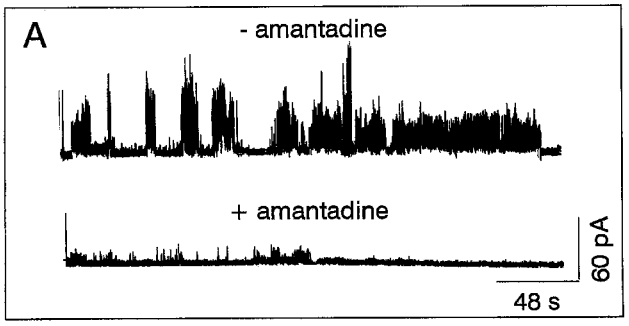
We established that the ion channel formed by the M_2 protein is more permeable to cations than to anions by measuring the reversal potential in the presence of an NaCl concentration gradient. In this case, the bilayers were formed in the presence of 150 mM NaCl + 10 mM Tris-MOPS (pH 5.4) and a salt gradient established as indicated in Materials and Methods, after addition of M_2 protein and establishment of its induced conductance. The value of the transference number (*see* Materials and Methods) for cations was determined in three experiments and found to be: 0.75 ± 0.06 (SD, independent of the size of the gradient imposed (Table 1). This value of transference number indicates that the channel has a permeability for Na^+ that is at least three times as high as that for Cl^-.

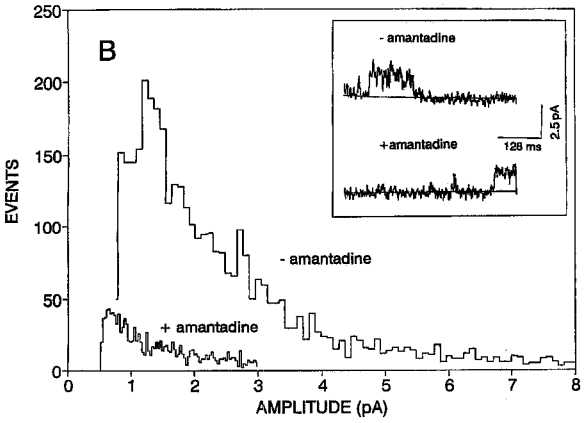
Effect of Amantadine and pH on the Conductance Induced by the M_2 Protein

In experiments with *X. laevis* oocytes that expressed the M_2 protein, the amplitude of the whole-cell currents were drastically lowered by addition of 10–100 μM amantadine and increased (in the absence of amantadine) about eightfold when the pH of the solution bathing the oocytes was lowered from pH 7.6 to pH 5.4 (Pinto et al., 1992; Wang et al., 1993; Holsinger et al., 1994). We therefore studied the effect that addition of amantadine and changes in pH have on the conductance induced by M_2 protein incorporated into lipid bilayers.

Examination of the time course (Fig. 3*A*) and amplitude distribution (Fig. 3*B*) of the currents induced by M_2 showed a marked reduction in both the amplitude and frequency of opening of all states in the presence of amantadine; in particular, the high conductance states which were abolished. The value of the conductance of the most probable peak in the presence of amantadine was also found to be reduced from a value of 65 ± 7 pS in the absence of amantadine to 35 ± 4 pS in the presence of 100 μM amantadine (at +40 mV, 150 mM NaCl). The lower magnitude of the M_2-induced current amplitude recorded in the presence of amantadine does not seem to arise from a fast block of the open channels, since channel openings after amantadine application do not seem to be noisier than those recorded prior to addition of the drug (Fig. 3*B*, inset). Rather, in the presence of amantadine, the frequency of opening to a high conductance state was drastically reduced (Fig. 3*A*). The lower frequency of openings and reduced size of the current of the open state combine to reduce the mean current flowing through the bilayer by approximately fivefold, from 29 to 6 pA (Fig. 3*C*). The effects of amantadine described above were seen in five (out of six) different bilayers made in the presence of NaCl as well as in one bilayer made in the presence of RbCl and another membrane made with LiCl as the main cation.

We studied the effect of lowering the pH of the medium from 7.6 to 5.4 on the change in the conductance of bilayers induced by incorporation of M_2 protein. The complete sequence, as described in Materials and Methods, was successfully repeated on three different membranes. Since we found that the single channel currents at the higher pH were very small, it was necessary to apply voltages of relatively high amplitude to be able to detect and analyze these currents. The result of one of these experiments is shown in Fig. 4, which depicts the distribution of current amplitude at 80 mV before and after changing the pH of the solutions from 7.6 to 5.4. Illustrated in the graph is the fact that at the higher pH, the current distribution is narrow, showing the skewness which indicates the presence of other sizes of single channel currents and with a peak value of 2.0 ± 0.4 pA





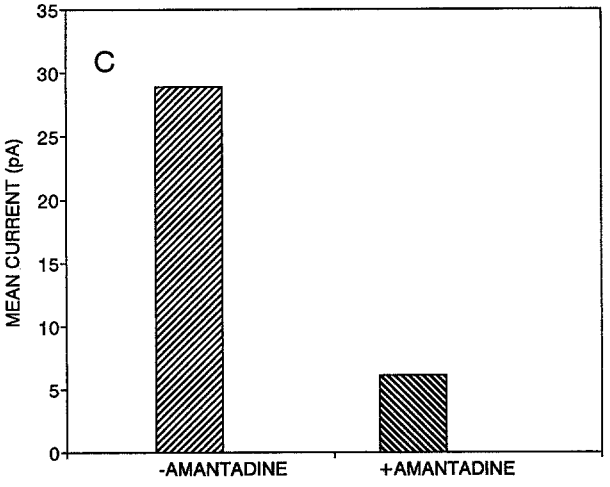


Fig. 3. Effect of amantadine on M_2-induced currents. (*A*) Time course of currents (150 mM NaCl, 10 mM Tris-MOPS, pH 5.4) before and after addition of 100 μM amantadine. Note the sudden onset and closing of large amplitude currents in the absence of amantadine, the lack of large conductance openings in the presence of amantadine (100 μM), and the lower frequency of current openings in the presence of amantadine. (*B*) Distribution of current amplitude in the presence and absence of amantadine (100 μM). The distribution in the absence of amantadine has been truncated to show better the events near the smallest conductance state. Thus, the few large events which dominate the time course in *A* do not appear in the current amplitude distribution (*B*). The inset shows a channel opening in the absence and presence of amantadine, chosen to be of the same current amplitude. Please note that the noise of the open channel is the same in both cases. (*C*) Mean current in the absence and presence of amantadine. The current ratio varied between 4.5 and 8.0 in four determinations. Salt: 150 mM NaCl + 10 mM Tris-MOPS (pH 5.4). $V = +40$ mV.

(mean ± SD in three bilayers). When the pH of the solutions was shifted to 5.4, the current amplitude changed dramatically with the presence of large-size single channel currents which produce an increase in the skewness of the current distribution and a masking of the smallest-size mode of current amplitude. Analysis of this smallest size exclusively, in segments of records devoid of other sizes, revealed that this value is larger than the one obtained at pH 7.6 (4.3 ± 1.1 pA). The presence of larger size conductance present at this voltage and pH is reflected in a tenfold increase in the value of the mean current through the open channel at the lower pH, as shown in the inset of Fig. 4.

Selectivity of the Single Channels Induced by the M_2 Protein

To determine the selectivity among the group IA (alkali) cations, bilayers were formed in the presence of different salts (same concentration) on the two sides of the bilayer, keeping the salt composition of the *cis*-side constant (150 mM NaCl, 10 mM Tris-MOPS, pH 5.4). The reversal potential was determined by interpolation of the current-voltage curve in the region 10–20 mV from the voltage at which no current was detected (Fig. 5). The reversal voltages varied only slightly for the various biionic conditions (Table 2), indicating that the channel is only moderately selective among the group IA cations. Using the values of reversal potentials obtained from these plots, and assuming that the permeability to Cl relative to that of Na is not changed by the presence of a different transcation, we calculated the permeability ratios of the group IA cations relative to Na^+ (150 mM, pH 5.4) from the Goldman-Hodgkin-Katz equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$\frac{P_x}{P_{Na}} = \exp\left[V_{rev}\left(\frac{F}{RT}\right)\right] * \left(1 + \frac{P_{Cl}}{P_{Na}}\right) - \frac{P_{Cl}}{P_{Na}} \quad (1)$$

The concentration of the ions was omitted from the equation because they were all the same in this type of experiment. The results of these measurements indicated that the cations have the following selectivity sequence:

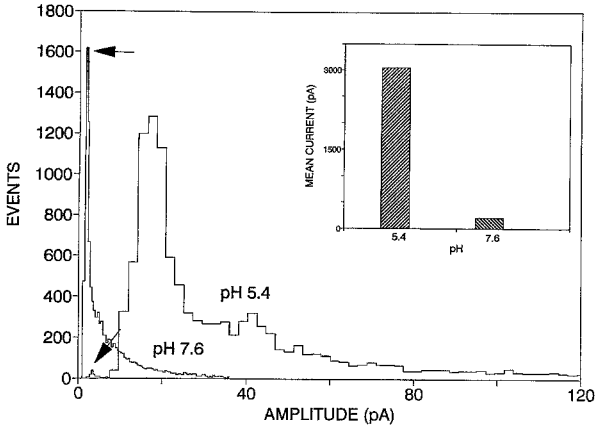


Fig. 4. Effect of changes in pH on the single channel currents induced by M_2. (*A*) Amplitude distribution of currents measured at pH 7.6 and pH 5.4. Note the greater mode and presence of larger currents at the lower pH. The arrows point to the peak of the amplitude distribution which corresponds to the lowest conductance level at each pH. The apparent lower probability of this low conductance level at pH 5.4 is probably an artifact of the analysis, since to obtain this peak we analyzed records which contained only openings ≤12 pS. (*B*) Mean currents obtained at pH 5.4 and 7.6. The same difference in mean current was observed in two more experiments. Salt: 150 mM NaCl + 10 mM Tris-MOPS. $V = +80$ mV.

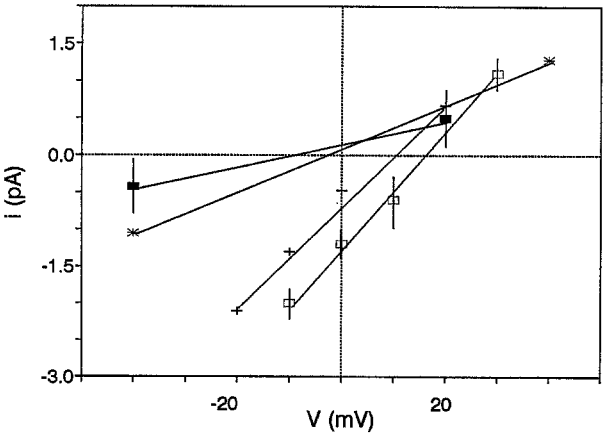


Fig. 5. Ionic selectivity of the M_2 protein channel. Current-voltage relationship plotted near the reversal voltage in the biionic condition with 150 mM NaCl + 10 mM Tris-MOPS (pH 5.4) on the *cis* side of the bilayer and 10 mM Cl salts of: Li^+ [■], K^+ [+], Cs^+ [*], or Rb^+ [□] (pH 5.4). The vertical lines in the figure, above and below experimental points, indicate the SD of the determination.

Rb > K > Cs ~ Na > Li (Table 2), which corresponds to sequence III in the nomenclature of Eisenman and Horn (1983).

Discussion

The properties of the M_2-protein-induced channel activity in bilayers reported in this communication agree well with the properties of the M_2-induced conductances estimated by measuring whole-cell currents in oocytes of *X. laevis* (Pinto et al., 1992; Wang et al., 1993). The conductances are reduced by amantadine, activated by reduced pH, and are cation selective in both systems.

Table 2. Ion selectivity for alkali cations

Cation on trans-compartment	*n*	E_{rev} (mV)	P_X/P_{Na}
Lithium	3	−10 ± 4	0.6
Cesium	5	0 ± 2	1.0
Potassium	2	10 ± 2	1.6
Rubidium	4	16 ± 3	2.2

The composition of the solution in the *cis*-compartment was: 150 mM NaCl + 10 mM Tris-MOPS (pH 5.4). The solution in the *trans*-/ compartment was the Cl salt of the cation indicated in the table (present at 150 mM) and buffered with 10 mM Tris-MOPS (pH 5.4). *n* indicates the number of different membranes where the determination was made.

The use of the bilayer reconstitution system made it possible to obtain certain information about the channel formed by the M_2 protein that could not be obtained from oocytes. The oocyte membrane contains an abundant endogenous channel that is activated by the stretch caused by application of a patch pipette and that has similar ion selectivity to the M_2 protein channel. In the oocyte system, it is therefore difficult to identify single M_2 channels unambiguously. By using the bilayer system, we were able to observe that the size of the M_2 ion channel conductance was variable (Fig. 2). It will be important to test if the multisized nature of M_2 channels in lipid bilayers is also found in mammalian cells expressing the M_2 protein. The data indicating that amantadine reduces (Fig. 3), and that lowered pH increases, the probability of appearance of the high conductance states of the M_2-induced channel (Fig. 4), suggests this is likely. Thus, this study of the M_2 ion channel reconstituted in lipid bilayers suggests that amantadine and pH exert their effects in vivo by acting directly on the M_2 protein, rather than on some other adjacent integral or membrane-associated protein. Whether this suggestion is correct, awaits a comparison of the results presented in the present communication with the results of studies on the properties of the channels of intact virions, as the pH of the medium is changed in the absence and presence of amantadine.

The experiments described here have also provided information relating to the mechanism by which amantadine blocks the M_2 ion channel activity. Amantadine could either be a conventional pore blocker such as tetraethylammonium or Cs^+ or an allosteric blocker interacting with residues of the M_2 protein either inside or outside the pore. Our work on the electrical properties that M_2 protein induces when in membranes supports the notion that amantadine acts as an allosteric blocker rather

than as a blocker of the open channel: (i) measurements of whole-cell currents in oocytes expressing M_2 have shown that the isochronic apparent inhibitory constant ($appK_i$) of amantadine block of these currents (from three subtypes of influenza A virus) was found to be lower when the oocyte was bathed in high pH medium than in low pH medium (Wang et al., 1993). Thus, the $appK_i$ was lower for the closed state of the channel. (ii) In the presence of amantadine, the current-voltage relationship of the whole-cell currents does not show the rectification that would be expected for a charged pore-blocking particle. (iii) The experiments reported in this paper indicate that there is an 80% reduction in the mean current flowing through open channels in the presence of amantadine (*cf.* Fig. 4, inset), due to a reduction in the probability of opening to a large conductance state since the single channel current noise observed when the channel is open is not detectably larger in the presence of amantadine than in its absence (no increased flickering observed). Moreover, we could not detect an overall lowering of the amplitude of the different conductance states as would occur if amantadine produced a very fast flicker undetectable due to bandwidth limitations (Hille, 1992). The failure of rapid closures to occur in the presence of amantadine lends support to the interpretation that amantadine does not function mostly as a blocker of the open pore of the M_2 ion channel, but rather serves as an "allosteric" blocker (Neher & Steinback, 1978).

It is of utmost importance to consider whether the properties of the M_2 protein ion channel measured in heterologous or in vitro systems are appropriate for the channel's purposed role in uncoating influenza virus, as described in the Introduction. The M_2 ion channel hypothesis as originally developed (Sugrue & Hay, 1991) proposed that the M_2 protein itself would be an ion channel that would conduct protons and be blocked by amantadine. Although it may seem obvious that the M_2 protein ion channel activity would be intrinsic to the protein because influenza virus particles are not known to contain any other integral membrane proteins apart from HA, NA and M_2 protein, nonetheless, it had to be shown experimentally.

As described here, the M_2 protein forms an ion channel by itself and is blocked by amantadine. Furthermore, a means of regulation of the channel would be expected and our finding that the M_2 protein channel is activated by the lowered pH found in endosomes (Pinto et al., 1992) provides such a mechanism. pH activation facilitates regulation of the M_2 ion channel in the appropriate intracellular compartments to permit influenza virus replication. We have shown that the M_2 protein ion channel conducts alkali cations, and although we did not test the selectivity for protons in a direct way, we have shown that NH_4 is permeable through the channel (Fig. 2). Taking note that the binding of both H_3O^+ (or H^+) and NH_4^+ to channel ligands yields indistinguishable energy profiles for the transport of these ions (Eisenman & Sandblom, 1984), it is reasonable to surmise that the M_2 ion channel is permeable to H_3O^+.

It is important to note also that the ability of the M_2 protein ion channel to conduct alkali cations is essential for the channel's proposed function. The presence of a *non*selective cation channel in the virion envelope membrane would allow for the equalization of charges across the membrane while protons flow along their electrochemical gradient into the virion. In the absence of this charge equalization, the voltage across the viral membrane would approach the proton equilibrium potential and in this state further proton diffusion would be prevented by the opposing electrical force.

Ion channels are unique among proteins in that their activity can be studied in real time using single channel recording methods. The analysis of the structure-function relationship of ion channels has been greatly advanced by the use of site-directed mutagenesis (*reviewed in* Hille, 1992). However, these methods are not sufficient to understand the function of ion channels because they do not give direct structural information. Two prerequisites to obtaining structural information about a channel are the ability to make wild-type and site-directed mutant proteins in reasonable quantities and the ability to reconstitute channel activity in lipid bilayers. These two prerequisities have now been demonstrated for the M_2 protein. Moreover, despite the small size of the M_2 protein and the simplicity of its membrane topology, the ion channel which it forms is regulated by pH, voltage, drugs like amantadine, and displays substantial ion selectivity. Thus, the present study demonstrates that the M_2 ion channel is an excellent candidate for a direct structure-function analysis of an ion channel of considerable functional complexity.

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