Ion Selectivity and Activation of the M_2 Ion Channel of Influenza Virus

Kiyoshi Shimbo,*‡ Diana L. Brassard,* Robert A. Lamb,*§ and Lawrence H. Pinto‡
*Department of Biochemistry, Molecular Biology and Cell Biology, ‡Department of Neurobiology and Physiology, and §Howard Hughes Medical Institute, Northwestern University, Evanston, Illinois 60208-3500 USA

ABSTRACT The influenza A virus-associated M_2 ion channel is generally believed to function during uncoating of virions in infected cells. On endocytosis of a virion into the lumen of endosomes, the M_2 ion channel is thought to cause acidification of the virion interior. In addition, the influenza virus M_2 ion channel is thought to function in the exocytic pathway by equilibrating the pH gradient between the acidic lumen of the trans-Golgi network and the neutral cytoplasm. A necessary test of the proposed roles of the influenza virus M_2 ion channel in the virus life cycle is to show directly that the M_2 ion channel conducts protons. We have measured the ionic selectivity and activation of three subtypes (Udorn, Weybridge, and Rostock) of the M_2 ion channel in oocytes of *Xenopus laevis* by measurement of 1) the intracellular pH (pH_{in}) of voltage-clamped oocytes, 2) the current-voltage relationship in solutions of various pH and ionic composition, and 3) the flux of ^{86}Rb. We took advantage of the low pH_{in} achieved during incubation in low pH medium to study the effects of low pH_{in} on M_2 activation. Oocytes expressing each of the three subtypes of the M_2 protein a) underwent a slow acidification when incubated in medium of low pH (acidification was blocked by the M_2 ion channel inhibitor, amantadine); b) had current-voltage relationships that shifted to more positive values and had greater conductance when the pH_{out} was lowered (this relationship was modified when Na^+ was replaced by NH_4^+ or Li^+); c) had an amantadine-sensitive influx of Rb^+. The effects on the current-voltage relationship of reduced pH_{in} were opposed to the increased conductance found with reduced pH_{out}. We interpret these results to indicate that the M_2 ion channel is capable of conducting H^+ and that other ions may also be conducted. Moreover, the channel conductance is reduced by decreased pH_{in}. These findings are consistent with the proposed roles of the M_2 protein in the life cycle of influenza A virus.

INTRODUCTION

The influenza A virus M_2 integral membrane protein (Lamb and Choppin, 1981; Lamb et al., 1985) is minimally a homotetramer (Holsinger and Lamb, 1991; Sugrue and Hay, 1991) that is abundantly expressed at the surface of virus-infected cells but is a relatively minor component of virions (Zebedee and Lamb, 1988). The function of the M_2 protein has been proposed to be an ion channel activity that permits ions to enter the virion during uncoating and equilibration of the pH gradient between the acidic lumen of the trans-Golgi network (TGN) and the neutral cytoplasm (Sugrue and Hay, 1991; Hay, 1992).

Influenza virus particles are internalized into cells by receptor-mediated endocytosis. The anti-viral drug amantadine blocks an early step in virus replication between the steps of virus penetration and uncoating (Skehel et al., 1978; Bukrinskaya et al., 1982). In the presence of the drug, the influenza membrane (matrix) (M_1) protein fails to dissociate from the ribonucleoprotein (RNP) core (Martin and Helenius, 1991). Once a virion particle has been endocytosed, the ion channel activity of the virion-associated M_2 protein is thought to permit the flow of ions from the endosome to the virion interior to disrupt protein-protein interactions and free the RNPs from the M_1 protein (reviewed in Hay, 1992; Helenius, 1992; Marsh, 1992; Skehel, 1992; Lamb et al., 1994).

In addition to the early effect of amantadine on influenza virus replication, for some subtypes of avian influenza virus, which have a hemagglutinin (HA) glycoprotein that is cleaved intracellularly and have a high pH optimum of fusion (e.g., fowl plague virus (FPV) Rostock), there is a second effect of the drug late in replication. A large body of data indicates that addition of amantadine to cells late in infection brings about a premature conformational change in HA that occurs in the TGN during the transport of HA to the cell surface (Sugrue et al., 1990; Ciampor et al., 1992a,b; Grambas et al., 1992; Grambas and Hay, 1992; Ohuchi et al., 1994; Takeuchi and Lamb, 1994). Thus, the M_2 ion channel is thought to function in the TGN and associated transport vesicles to regulate intracompartmental pH and keep the pH above the threshold at which the HA conformational change occurs (Sugrue et al., 1990; Ciampor et al., 1992b).

The ion channel activity of the M_2 protein has been demonstrated directly in experiments in which the protein was expressed in oocytes of *Xenopus laevis* (Pinto et al., 1992; Wang et al., 1993; Holsinger et al., 1994, 1995) and mammalian cells (Wang et al., 1994) and in which the ion channel activity was reconstituted in lipid bilayers (Tosteson et al., 1994). The ion channel activity is blocked by the antiviral drug amantadine (Pinto et al., 1992; Wang et al., 1993; Tosteson et al., 1994). The channel has been demonstrated to be activated by low pH, and this activation is dependent on $histidine_{37}$ in the transmembrane domain of the M_2 protein (Wang et al., 1995).

Received for publication 1 November 1995 and in final form 18 December 1995.

Address reprint requests to Dr. Lawrence H. Pinto, Department of Neurobiology and Physiology, Hogan Hall, 2153 North Campus Drive, Northwestern University, Evanston, IL 60208-3500. Tel.: 708-491-7915; Fax: 708-491-5211; E-mail: larry-pinto@nwu.edu.

0006-3495/96/03/1335/12 $2.00

The ion selectivity of the M_2 channel has not been measured in a cellular expression system. Although acidification of vesicles containing purified M_2 protein has been described, the fluorescent indicator method used could not report the conduction of ions other than protons (Schroeder et al., 1994). It is important to determine not only if the M_2 ion channel conducts protons but also if it conducts other ions in addition. If the M_2 ion channel conducted only protons, then it would be necessary for another conducting pathway to provide for electroneutrality of the virion membrane as protons enter the virion.

To test the hypothesis that an ion channel conducts the very same ion that is responsible for its activation is difficult because the properties of conduction and activation can be confused easily and because single-channel activity of the M_2 protein has not yet been reported in a cellular expression system. Therefore, we employed three methods to study the ion selectivity of the M_2 ion channel: measurement of H^+ flux with an intracellular pH electrode, measurement of amantadine-sensitive membrane currents under various ionic conditions and pH, and measurement of the amantadine-sensitive flux of Rb^+ using a radioactive tracer. We found that the M_2 ion channel is capable of acidifying oocytes of *Xenopus laevis* and therefore the M_2 ion channel conducts protons. In addition, our experiments suggest that the M_2 ion channel is also capable of conducting other cations.

MATERIALS AND METHODS

Recombinant plasmids

The cDNA encoding the influenza virus A/Udorn/72 M_2 protein was that described previously (Zebedee et al., 1985; Pinto et al., 1992). The cDNA encoding influenza virus A/chicken/Germany/34 (H7N1) (FPV Rostock) M_2 protein was the cDNA constructed from synthetic oligonucleotides described previously (Takeuchi and Lamb, 1994). The cDNA encoding influenza virus A/chicken/Germany/27 (H7N7) (FPV Weybridge) M_2 protein was derived from the M_2 cDNA to FPV Rostock by serial rounds of site-specific mutagenesis, such that the M_2 protein sequence matched that reported by Hay and colleagues (Hay et al., 1985).

Culture and microinjection of oocytes

Xenopus laevis were identified individually with an implanted microchip marker (Basic Medic Data Systems, Maywood, NJ). Oocytes were removed from female *Xenopus laevis* (Nasco, Fort Atkinson, WI), defolliculated by treatment with collagenase B (2 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN), and incubated in ND96 (96 mM NaCl, 2 mM KCl, 3.6 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM pyruvic acid, 5 mg/ml gentamycin, and 5 mM HEPES), pH 7.6 at 19°C. Oocytes at stage V (clearly demarcated equatorial pigment border, no loss of pigmentation at animal pole; Dumont, 1972) were microinjected with 50 nl of mRNA (1 ng/nl) on the day after defolliculation, incubated for 24 h in ND 96 (pH 7.6), and finally incubated for 24 h in ND 96 (pH 8.5–10.0) at 19°C before use (Colman, 1984; Pinto et al., 1992).

Measurement of pH_{in}

Borosilicate glass micropipettes (~1 μm diameter) were silanized with Sigmacote SL-2 (Sigma, St. Louis, MO), a region of 10–100 μm near their tips was filled with liquid ion exchange ionophore (Fluka 95297, hydrogen ionophore II cocktail A), and their backs were filled with 100 μM MOPS (pH 7.1) (Ammann et al., 1981; Howl et al., 1988). Each electrode was tested in Barth's solution (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $NaNO_3$, 0.71 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 15 mM HEPES, pH 7.5, or 15 mM 2-(*N*-morpholino)ethanesulfonic acid for solutions of pH < 6.8) over the pH range pH 6.2–7.5; only those electrodes that had a response greater than 50 mV/pH unit were used. Several electrodes were also tested for interference by K^+, Li^+, and NH_4^+, but no effect of replacement of Na^+ by any of these ions could be detected. The voltage of the pH electrode was recorded with a high-impedance amplifier (Axopatch 200A; Axon Instruments, Burlingame, CA). A pipette (3 M KCl) was placed in the oocyte to serve as a reference electrode when only pH measurements were made.

Measurement of membrane current

Whole-cell current was measured with a two-electrode voltage clamp (Pinto et al., 1992). The electrodes were filled with 3 M KCl, and the oocytes were bathed in either Barth's solution or a modified solution during the recording. Ramp measurements were made with ramps of membrane voltage that spanned a range of 30 mV in 5 s; voltage-clamp pulses were 100 ms in duration.

Simultaneous measurement of pH_{in} and membrane current were made by impaling the oocyte with a micro pH electrode and two electrodes that were connected to a Dagan 8500 voltage-clamp amplifier. The reading of the voltage-sensing electrode was subtracted from the voltage measured at the pH electrode to calculate pH_{in}. For these simultaneous measurements, the ground of the pH amplifier was connected to the bath with a 3 M KCl pipette located near the oocyte. We found it necessary to use a low-resistance connection between the bath and ground to avoid cross-talk between the current and pH signals. The data were recorded and analyzed using the PCLAMP 6 software package (Axon Instruments).

Measurement of ^{86}Rb flux

The solution containing the radiolabeled Rb was made by adding 10 μCi/μl $^{86}Rb^+$ (New England Nuclear NEZ072, 2.5 mCi/mg) to a modified Barth's solution (pH 6.2) in which the Na^+ was replaced by naturally occurring Rb^+. The labeled Rb^+ in oocyte lysates was counted by adding 5 ml scintillant (POPOP and PPO mixture, Aldrich 32712-3) to 2.5 ml diluted oocyte lysate (Bland and Boyd, 1986; Kemp et al., 1994).

RESULTS

Acidification of the ooplasm

The proposed role of the influenza virus M_2 ion channel in the virus life cycle is to conduct protons into the interior of virions, and in the exocytic pathway to equilibrate the pH gradient between the lumen of the TGN and the cytoplasm. Thus, provided that the M_2 ion channel is sufficiently active, when the M_2 ion channel is expressed in oocytes, the ooplasm ought to become acidified when the oocyte is placed in medium of low pH. As the amino acid sequence of the M_2 protein transmembrane domain, which is believed to encompass the pore of the channel, differs among different subtypes of influenza A virus, we expressed the cDNA of three subtypes, Udorn, Weybridge, and Rostock, to determine whether there were differences in ion selectivity between the M_2 protein subtypes. We measured the pH_{in} of oocytes that expressed the M_2 proteins with a micro pH electrode after incubating the oocytes for 1 h in Barth's

solution at various values of pH from pH 5.5 to 8.0, in the presence and absence of the M_2 ion channel blocker amantadine (Fig. 1). At least three cells of each M_2 protein subtype were measured for each of the six values of pH. We found that pH_{in} did not depend on pH_{out} for the duration of the experiment for either water-injected oocytes (ANOVA $p > 0.68$) or oocytes that were bathed in solutions containing amantadine (Fig. 1). However, oocytes that expressed each of the three subtypes of M_2 protein had pH_{in} that decreased monotonically with pH_{out} over the range that was studied (ANOVA $p < 0.01$), whereas after addition of the M_2 ion channel blocker amantadine, the decrease in pH_{in} was prevented. Oocytes that expressed the Rostock subtype M_2 protein had lower pH_{in} than oocytes that expressed the Weybridge or Udorn subtype M_2 proteins for $pH_{out} \leq 6.5$. It is not known if the lower pH_{in} for the Rostock M_2 protein was due to higher expression levels or higher specific activity of this M_2 protein. The membrane of oocytes that express the M_2 protein depolarizes when the M_2 protein is activated by low pH, and the decrease in pH_{in} that we observed with decreasing pH_{out} might have been due to either the direct action of the M_2 protein or the effect of this depolarization. We therefore measured the time course of pH_{in} for oocytes that expressed the M_2 protein (at least seven cells for each of the three M_2 proteins) while their membrane voltage was clamped to the resting level of about

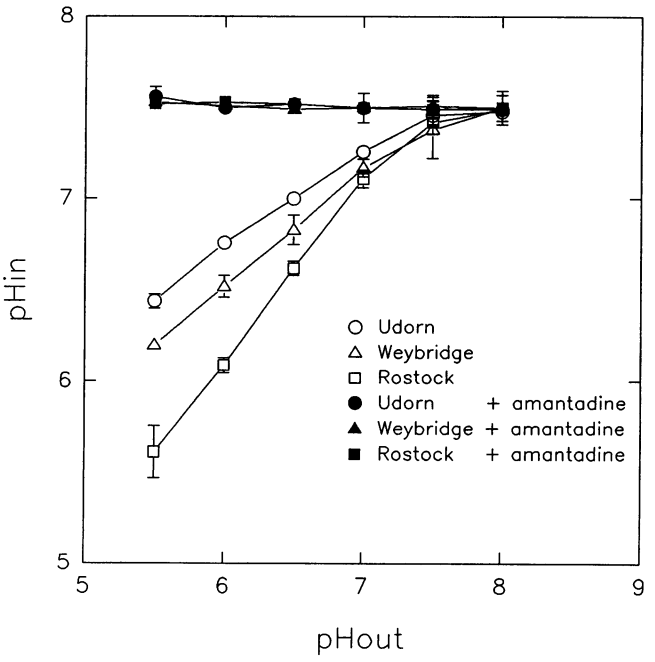

FIGURE 1 Intracellular acidification of oocytes expressing the M_2 protein. (*A*) The intracellular pH, pH_{in}, of oocytes was measured after 1 h of incubation in solutions of the pH indicated (pH_{out}). The intracellular pH decreased for oocytes expressing the M_2 protein from all three influenza virus subtypes: Udorn (●), Weybridge (△), and Rostock (□). This acidification was blocked by amantadine (100 μM, filled symbols) and did not occur for control oocytes (see text).

−40 mV (Fig. 2 *A*). We found that immediately after decreasing pH_{out} a large inward membrane current flowed and persisted for the time that the oocyte was bathed in the low pH solution. While this inward current flowed there was a slow, gradual decrease in pH_{in}. The value of pH_{in} fell to its new steady value in about 45 min; this value was never as low as the pH_{out}. Acidification was never observed in the presence of amantadine (100 μM). We fitted an equation of the form

$$pH_{in}(t) = A \exp(-t/\tau_H) + C, \quad (1)$$

where $pH_{in}(t)$ is the time course of intracellular pH, τ_H is the time constant for acidification, and A and C are constants, to the time course of pH_{in}. The data for each cell were reasonably well fitted by this equation (Fig. 2 *A*), although a plot of intracellular free proton concentration against time was somewhat better fitted with an exponential function of longer time constant than the exponential used to fit $pH_{in}(t)$. The mean values of τ_H were similar for the three M_2 protein subtypes (mean ± SEM): Udorn, 554 ± 176 s; Weybridge, 481 ± 270 s; and Rostock, 540 ± 283 s. The value of the constant C that was fitted to each time course represents the final pH that would have occurred after prolonged acidification. The value of C was consistent with the final pH that was measured for oocytes that were incubated in low pH solutions for 1 h (Fig. 1) (mean ± SEM): Udorn, 6.8 ± 0.2; Weybridge, 6.7 ± 0.3; and Rostock, 6.3 ± 0.1. Thus, the acidification of the ooplasm was achieved for M_2-expressing oocytes for which there was no change in membrane voltage, consistent with the influx of H^+ passing through the M_2 ion channel.

We calculated the average proton flux during the first minute of acidification, neglecting the effect of transport to internal stores. To do this, it was first necessary to determine the buffer capacity of the oocyte, which was done by injecting 50 nl of known dilutions (1–200 mM) of methanesulfonic acid into the oocyte while measuring pH_{in} during the period 0–5 min after injection (Fig. 2 *B*) with an intracellular micro pH electrode. The measured value of pH_{in} was unstable during the period 0–1 min after injection, while the values were nearly constant (<0.1 pH unit) 1–5 min after injection, indicating that the pH_{in} probably did not fall during the interval between injection of acid and measurement. The plot of pH_{in} against injected acid could be described by a single titratable group of pK_a 7.5 and buffer capacity of 26 mEq/pH unit (Fig. 2 *B*, *dotted line*). We calculated the proton flux using the equation

$$F_{H^+} = \frac{1}{60\text{ s}}\int_0^{60\text{ s}} f_{H^+}(t)dt = \frac{V \cdot \beta}{60\text{ s}}(pH_{in}(60\text{ s}) - pH_{in}(0\text{ s})), \quad (2)$$

where F_{H+} is the average H^+ flux in the first 60 s, $f_{H+}(t)$ is the time course of H^+ flux, V is oocyte water volume (half of the geometrical 5.2×10^{-7} liters; see Cicirelli et al., 1983), and β is the buffer capacity of the ooplasm (Fig. 2 *B*). The pH_{in} typically fell by 0.15 pH units (see Fig. 2 *A*) in the

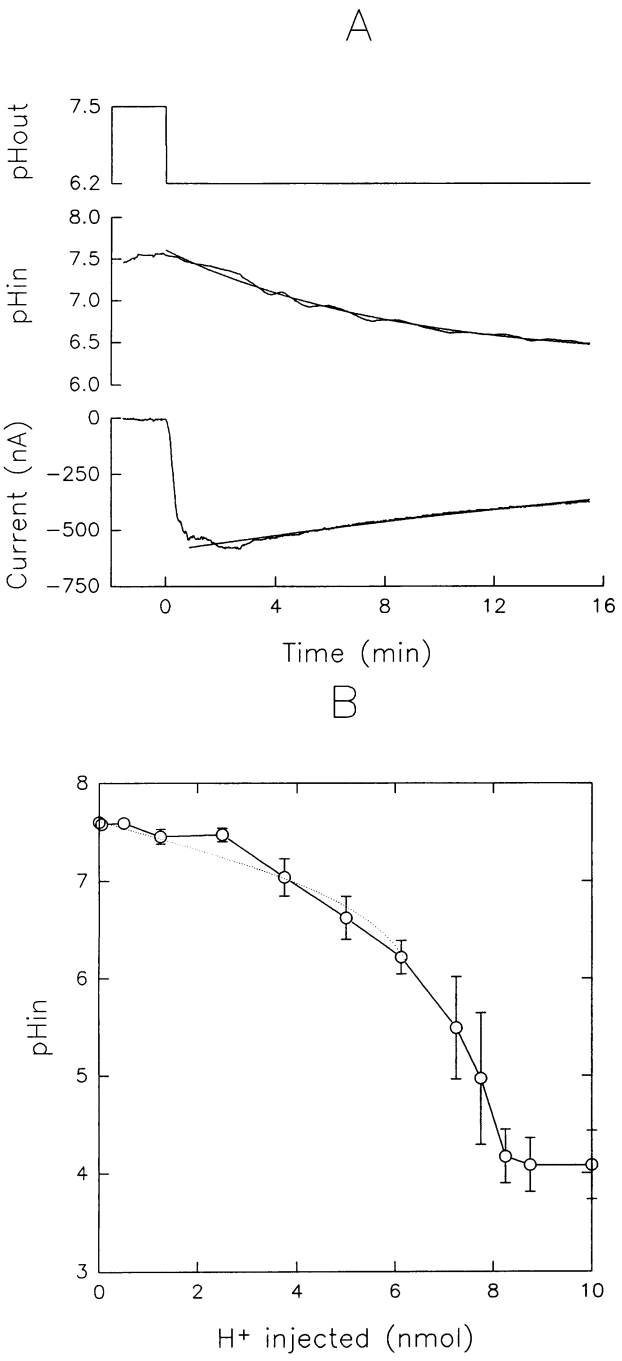

FIGURE 2 (*A*) Time course of intracellular acidification of an oocyte expressing the Rostock subtype M_2 protein upon lowering pH of the bathing medium from pH 7.5 to pH 6.2 while the oocyte's membrane voltage was clamped to −40 mV. The time course of the extracellular pH is shown in the upper record, the time course of the intracellular pH in the middle record, and the time course of the membrane current is shown in the lower record. The smooth line in the middle record shows the result of fitting the equation $pH_{in}(t) = A \exp(-t/\tau_H) + C$ to the time course of the intracellular pH, where $A = 1.36$, $\tau_H = 473$ s, and $C = 6.29$. The smooth line in the bottom record shows the result of fitting the equation $I(t) = I_A \exp(-t/\tau_I) + I_C$ to the time course of the membrane current, where $I_A = -0.85$ μA, $\tau_I = 3120$ s, and $I_C = 0.19$ μA. (*B*) Buffer capacity of the oocyte was determined by measuring intracellular pH from 1 to 5 min after injection of known quantities of methansulfonic acid while the oocyte was bathed in a medium of pH 6.2. The dotted line was fitted by the equation in the range of pH from pH 7.6 to pH 6.2.

first minute, and thus the values of F_{H+} were quite large, typically 1×10^{-11} equivalents/s for oocytes expressing the Rostock subtype of the M_2 protein.

We examined the time course of acidification of oocytes expressing the M_2 protein that were bathed in solutions of altered ionic composition. The altered ionic compositions used were those that in subsequent experiments were found to alter the current-voltage relationship: replacement of all Na^+ in the bathing medium with Li^+ or NH_4^+. Two sets of experiments were performed. In the first set of experiments, the oocytes were not voltage clamped, and the pH_{in} was measured after 1 h of incubation in the Li^+ or NH_4^+ test solution at pH 6.2. This was done for at least three oocytes of each subtype. The pH_{in} measured after 1 h of incubation in the Li^+ or NH_4^+ test solution (pH 6.2) did not differ from the pH_{in} measured after 1 h of incubation in Barth's solution (pH 6.2) for any of the three M_2 protein subtypes. In the second set of experiments, the time course of acidification was measured while the membrane was voltage-clamped to the resting voltage. For these experiments, oocytes expressing the Rostock subtype M_2 protein were initially placed in Barth's solution at pH 6.2 to obtain the initial rate of acidification for later comparison. After 4–10 min, when the rate of acidification was still high (Fig. 2 *A*), the bathing medium was changed and the time course of acidification and membrane current were measured. The time course of acidification of three oocytes bathed in medium in which Na^+ was replaced by Li^+ (pH 6.2) and three oocytes bathed in medium in which Na^+ was replaced by NH_4^+ did not differ significantly from the time course of acidification of oocytes bathed in Barth's solution at pH 6.2 (Fig. 2 *A*) and was well fitted by Eq. 1 (data not shown). The inward membrane current decreased in amplitude when Na^+ was replaced by Li^+ and increased when Na^+ was replaced by NH_4^+. Thus, neither of these ionic substitutions affected the time course or extent of acidification of oocytes expressing the M_2 protein.

We calculated the acidification of the ooplasm that would be expected if all of the voltage-clamp current were carried by H^+. For each oocyte, we fitted an equation of the form

$$I(t) = I_A \exp(-t/\tau_I) + I_C, \quad (3)$$

where $I(t)$ is the time course of membrane current, τ_I is the time constant, and I_A and I_C are constants to the data for each oocyte. The data were well fitted. The values for τ_I did not differ significantly among the three M_2 protein subtypes (mean ± SEM): Udorn, 1076 ± 726 s; Weybridge, 975 ± 648 s; Rostock, 1231 ± 816 s. The magnitude of the current that flowed initially after lowering pH_{out} was quite large, typically −0.7 μA for oocytes expressing the Rostock subtype M_2 protein. For each oocyte, this equation was integrated to yield the total charge that flowed across the membrane during the time of acidification. The time course

of the pH_{in} measured for oocytes bathed in low pH medium was fitted with the following equation:

$$pH_{in}(t) = pH_{in}(0\ s) - \left(\frac{1}{\beta \cdot V \cdot F}\right)\int_0^t I(t)dt, \qquad (4)$$

where $pH_{in}(t)$ is the time course of pH_{in}, F is Faraday's constant, and β is the buffer capacity of the ooplasm (Fig. 2 *B*), assuming that all of the charge was carried by H^+ and neglecting the effects of neutral exchangers of H^+. The second term of Eq. 4 was evaluated using the fitted line in Fig. 2 *B*. In every case, after 15 min of bathing in low pH medium, the theoretical value of pH_{in} was 0.1–0.3 pH units more alkaline than the measured value of pH_{in}. One possible explanation for the discrepancy between the measured value of pH_{in} and the theoretical value of pH_{in} calculated from the membrane current and buffer capacity is that ions in addition to the H^+ flowed across the membrane.

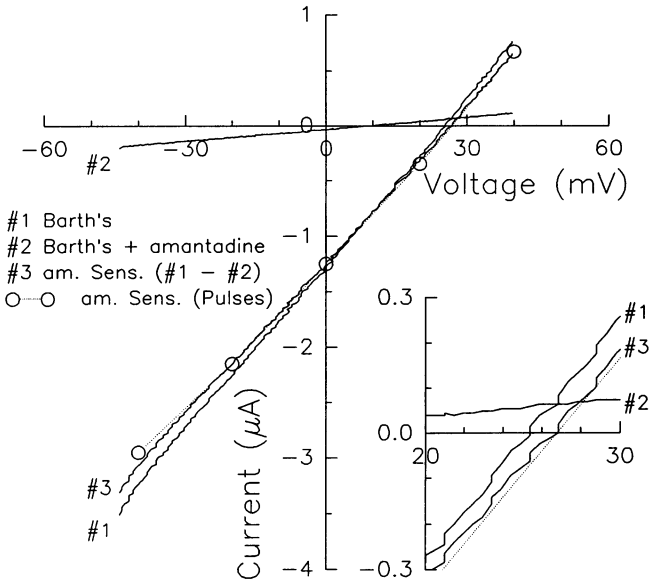

FIGURE 3 Amantadine-sensitive currents of M_2-expressing and control oocytes measured with ramps and pulses. (*A*) Oocyte expressing Rostock subtype M_2 protein. Lines show the total current measured using ramps in Barth's solution (pH 6.2), Barth's solution with 100 μM amantadine (pH 6.2), and the difference between these currents, the amantadine-sensitive current. O, Amantadine-sensitive current measured at pH 6.2 with pulses (100 ms). Inset shows the current-voltage relationship near the reversal voltage without amantadine. Holding voltage was −40 mV in all cases. Note that the current-voltage relationships of the amantadine-sensitive current were indistinguishable for measurements made with ramps and pulses.

Current-voltage relationship of the M_2 ion channel

In an attempt to define more completely the ion selectivity of the M_2 ion channel, we measured its current-voltage relationship under conditions of altered pH and ionic composition. To ensure that currents specific to the M_2 protein, and not endogenous currents, were being measured, we measured the amantadine-sensitive current. The importance of the use of the amantadine-sensitive current is seen by the difference between the reversal voltage for the total and amantadine-sensitive currents that was observed (Fig. 3) for M_2-expressing oocytes. The current-voltage relationships were determined in most cases with voltage ramps that spanned a range of voltage of about 30 mV in about 5 s. The exact range was chosen to encompass the reversal voltage. In several cells, we confirmed that the amantadine-sensitive current obtained with voltage ramps did not differ significantly from the amantadine-sensitive current obtained with steps of voltage (Fig. 3). The current-voltage relationship of control oocytes was not significantly affected by amantadine (100 μM), and the currents of control oocytes were much smaller than the currents of M_2-expressing oocytes at pH 6.2 (less than 0.1 μA in amplitude at −120 mV). However, the currents of M_2-expressing oocytes at pH 7.5 are not very much larger than the currents of control oo-

TABLE 1 Reversal voltage due to replacement of all extracellular Na^+ with various ions

	Principal cation							
M_2 protein subtype	Li^+	Na^+	K^+	Rb^+	Cs^+	$NMDG^+$	TBA^+	NH_4^+
Udorn	11.9* ± 6.7 mV (8)	28.5 ± 6.7 mV (19)	29.1 ± 9.8 mV (6)	29.5 ± 7.6 mV (7)	28.7 ± 6.5 mV (6)	28.3 ± 7.2 mV (8)	36.3 ± 2.8 mV (4)	50.1* ± 4.8 mV (8)
Weybridge	3.3* ± 8.7 mV (9)	25.0 ± 7.4 mV (9)	27.4 ± 3.6 mV (4)	29.6 ± 2.9 mV (4)	29.8 ± 2.3 mV (4)	25.0 ± 4.1 mV (4)	31.3 ± 11.3 mV (4)	50.5* ± 6.9 mV (8)
Rostock	26.9 ± 6.2 mV (8)	27.7 ± 5.5 mV (15)	26.4 + 3.9 mV (6)	27.4 ± 3.6 mV (6)	28.1 ± 4.7 mV (6)	23.8 ± 3.3 mV (9)	29.0 ± 4.0 mV (6)	53.4* ± 5.8 mV (8)

Measurements made in Barth's solution at pH 6.2 (Na^+) or modified Barth's solution (other ions). To determine amantadine-sensitive current, measurements were made within 15 s of solution changes and effects were reversible in all cases. Data are mean ± SEM, with the number of observations shown in parentheses. $NMDG^+$, *N*-methyl-D-glucosamine$^+$; TBA^+, tetrabutyl ammonium$^+$.
* $p < 0.01$.

cytes, so it is important to account for these background currents when making measurements at higher values of pH.

We observed that the current-voltage relationship of the amantadine-sensitive current varied slightly from batch to batch of oocytes. Therefore, all of the measurements shown in each of Figs. 3–6 were recorded from oocytes that came from one batch. For each experiment, however, similar results were obtained from at least two other batches of oocytes. The results from Tables 1 and 2 represent the averages of data obtained from three or more batches of oocytes.

If the M_2 ion channel conducts protons, then the reversal voltage of the amantadine-sensitive current ought to become more positive when the pH_{out} is lowered. Concomitant with this increase in reversal voltage there ought to occur an increase in membrane conductance that results from activation of the channel (Pinto et al., 1992; Wang et al., 1993, 1995). We measured the current-voltage relationship for over 10 cells of each M_2 protein subtype at pH 7.5 and one or more lower values of pH. These experiments were done using the rapidest changes possible in extracellular solution to avoid changes in pH_{in}. The oocytes were bathed initially in a solution of pH 7.5, and the pH was then changed for no more than 15 s; the pH of the bathing solution was then returned to pH 7.5 to check for reversibility. Only reversible changes are reported. In each case, the reversal voltage of the amantadine-sensitive current became more positive and the slope of the current-voltage relationship became greater at the lower pH (see Fig. 4). We measured the reversal voltage and slope conductance of the amantadine-sensitive current as a function of pH for at least three cells of each of the three M_2 protein subtypes for seven values of pH between 6.0 and 8.0 (Fig. 5). The reversal voltage increased monotonically with decreasing pH in this range; however, the theoretical value of the reversal voltage, calculated assuming that protons are the only conducting ion and $pH_{in} = 7.5$ (see above), differed significantly from the measured value throughout the range (Fig. 5 *A*), and this difference became greater for the lower values of pH. The membrane conductance (in a 30-mV range spanning the reversal voltage) increased monotonically with decreasing pH (Fig. 5 *B*). In control experiments, we tested for altered membrane conductance induced by decreased pH_{in}. Sufficient methanesulfonic acid was injected into each of five uninjected oocytes to reduce pH_{in} to ~6.5 (see Fig. 2 *B*). The current-voltage relationship before and after injection was compared, and no significant changes were measured. Thus, the results of measurements of the current-voltage relationship of the amantadine-sensitive current studied at various values of pH_{out} are consistent with the M_2 channel conducting protons, but leave open the possibility that other ions might also be conducted.

The discrepancies between measured and theoretical reversal voltages (Fig. 5 *A*) suggested that ions other than H^+ might be conducted through the M_2 ion channel. Thus, we measured the current-voltage relationship of the amantadine-sensitive current under various ionic conditions. This was done for at least four oocytes of each of the three M_2 protein subtypes for each ion substitution tested. All of the changes we report were reversible. We attempted to omit monovalent ions entirely, but when NaCl was replaced with mannitol or was omitted, the changes were always irreversible. We found that replacement of Cl^- with methanesulfonate$^-$ resulted in no systematic change in either reversal voltage or conductance at pH 6.2 (data not shown). We also found that replacement of Na^+ with any of the following resulted in no systematic change in reversal voltage or conductance: Cs^+, K^+, Rb^+, *N*-methyl-D-glucamine$^+$, or tetrabutylammonium$^+$ (Tables 1 and 2). However, replacement of Na^+ with Li^+ (Fig. 6 and Tables 1 and 2) resulted in a decrease in conductance for all three M_2 protein subtypes and a shift in reversal voltage to more negative values for the Udorn and Weybridge M_2 protein subtypes; the reversal voltage for the Rostock M_2 protein subtype was not altered by this substitution. This difference in the current-voltage relationship is not unexpected in view of the differences in amino acid sequence in the transmembrane domains of the three subtypes of M_2 protein. In addition, the replacement of Na^+ with NH_4^+ (Fig. 6) resulted in an increase in reversal voltage to more positive values for all three M_2 protein subtypes and an increase in conductance for all three M_2 protein subtypes. Oocytes expressing the Rostock and Weybridge M_2 protein subtypes consistently showed and oocytes expressing the Udorn subtype occasionally showed inward rectification when bathed in solutions in which NH_4^+ was substituted for Na^+. Neither of these ionic substitutions altered the acidification of the oocyte when it was incubated in low pH solution (see above). These results suggest that there are other ions in addition to H^+ that are conducted by the M_2 ion channel.

TABLE 2 Fractional change of oocyte conductance due to replacement of all extracellular Na^+ with various ions

	Principal cation						
M_2 protein subtype	Li^+	K^+	Rb^+	Cs^+	$NMDG^+$	TBA^+	NH_4^+
Udorn	0.58* ±0.15	0.98 ± 0.26	1.01 ± 0.26	1.28 ± 0.25	1.07 ± 0.28	1.32 ± 0.38	3.58* ± 0.67
Weybridge	0.43* ± 0.15	0.99 ± 0.21	1.24 ± 0.29	1.28 ± 0.22	1.17 ± 0.25	1.46 ± 0.33	2.32* ± 0.76
Rostock	0.40* ± 0.18	1.11 ± 0.12	1.07 ± 0.13	1.10 ± 0.17	1.24 ± 0.15	1.27 ± 0.16	1.57* ± 0.31

Measurements made as in Table 1. Data are mean ± SEM; number of cells as in Table 1.
* $p < 0.01$.

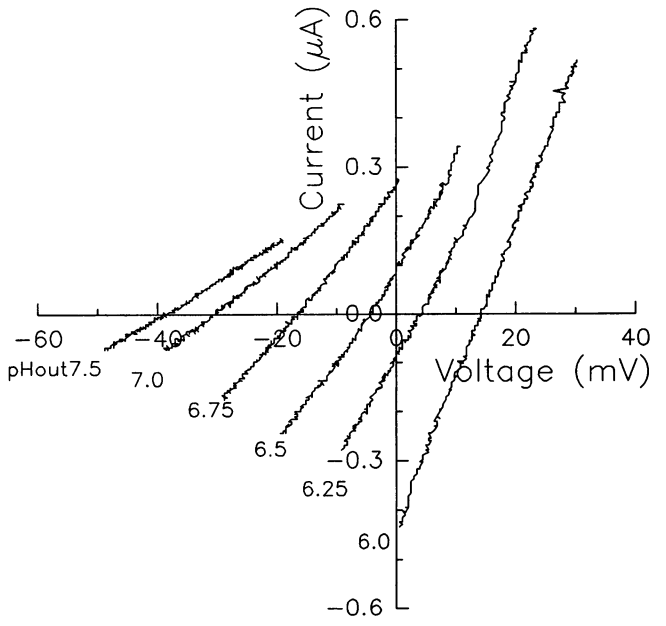

FIGURE 4 pH dependence of the current-voltage relationship of an oocyte expressing the Rostock subtype M_2 protein. The amantadine-sensitive currents were determined at six values of extracellular pH between 6.0 and 7.5. The measurements were begun at pH 7.5 and the changes were reversible. Note the increase in conductance and shift in reversal voltage to more positive values at the lower pH.

Studies with the flux of ^{86}Rb

The finding that replacement of Na^+ with Cs^+, K^+, Rb^+, *N*-methyl-D-glucamine$^+$, or tetrabutylammonium$^+$ did not alter the reversal voltage or conductance of oocytes significantly could be interpreted to mean that these substituent ions are either all impermeant or that they are all nearly as permeant as Na^+. In an effort to distinguish between these possibilities, we measured the influx of Rb^+ into oocytes that expressed the Rostock M_2 protein subtype. This experiment was done in the following way. First, many oocytes from two *Xenopus* were injected with the mRNA for the Rostock M_2 protein subtype and incubated, alongside uninjected control oocytes, for 2 days in ND96. The expression of M_2 protein was checked by testing the currents of oocytes from each *Xenopus*, and the remaining M_2-expressing oocytes were then divided into two groups consisting of equal numbers of oocytes from each *Xenopus*. One group was incubated in Barth's solution at pH 7.5 without amantadine and the other group was incubated in Barth's solution at pH 7.5 with amantadine (100 μM) for 1 h. Both groups were then incubated in a solution of pH 6.2 containing 50 nCi/μl ^{86}Rb and in which all Na^+ was replaced by Rb^+. Amantadine (100 μM) was also added to the one group incubated at pH 6.2 that had been incubated in amantadine at pH 7.5. At 10 time points after the beginning of the incubation at pH 6.2, samples of 20 oocytes were removed from each group, washed 10 times with 5 ml Barth's solution (pH 7.5) within 5 min, homogenized in water, and ^{86}Rb uptake was determined by scintillation counting. The amantadine-sensitive

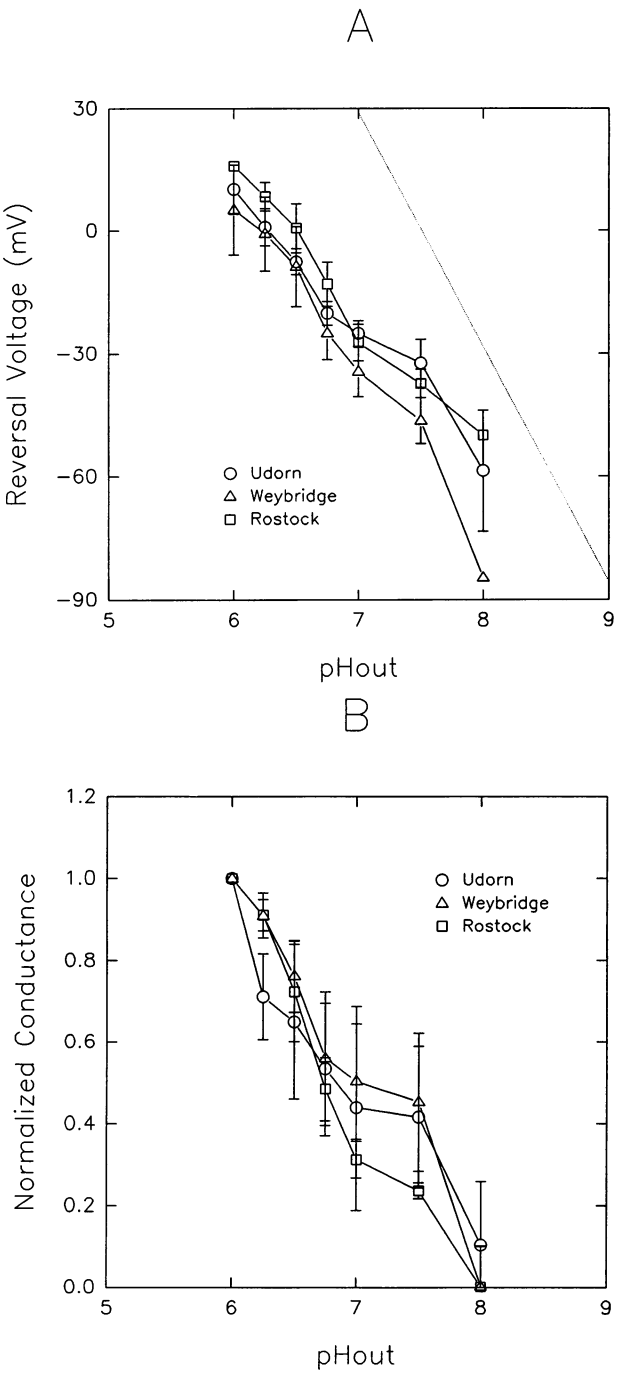

FIGURE 5 pH dependence of the reversal voltage and conductance of oocytes expressing the M_2 protein. The reversal voltage (*A*) and conductance (*B*) of the amantadine-sensitive currents are plotted as a function of extracellular pH for ooyctes expressing the Udorn (○), Weybridge (△), and Rostock (□) subtype M_2 proteins. Measurements were made within 15 s of changing extracellular pH from pH 8.0. All changes were reversible. Details of measurements are given in Materials and Methods. The straight line in *A* shows the theoretical value of reversal voltage, calculated from the applied extracellular pH and intracellular pH 7.5 (see text).

Rb^+ influx (Fig. 7) was calculated as the difference between the radioactivity in the two groups and was seen to occur with a time course that resembled that of the acidification of

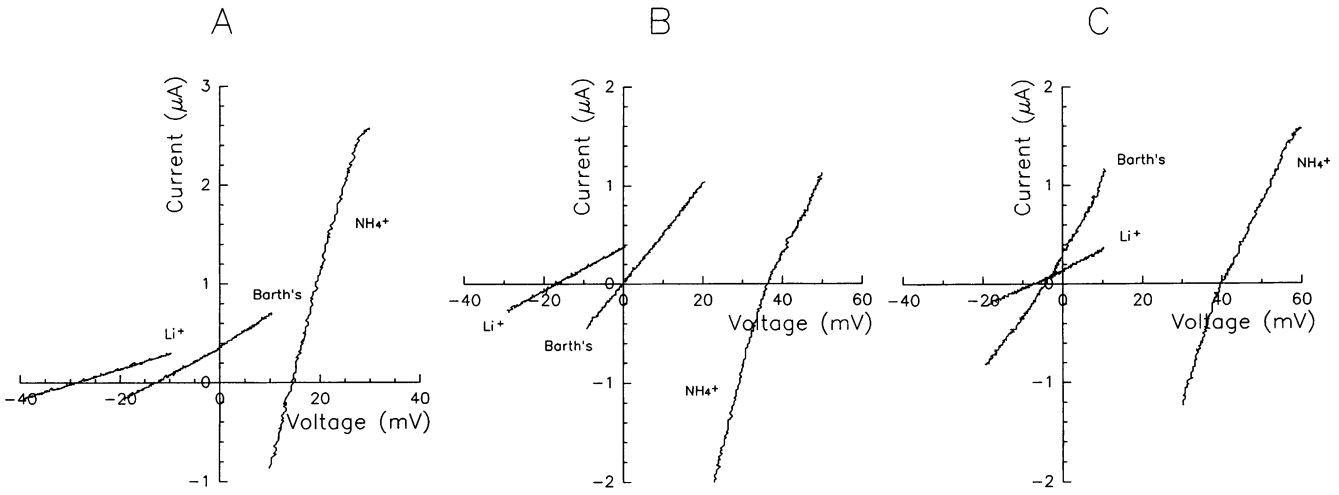

FIGURE 6 Current-voltage relationship of the amantadine-sensitive current of oocytes expressing the M_2 protein bathed in solutions of varied ionic composition at pH 6.5. (*A*) Udorn, (*B*) Weybridge, and (*C*) Rostock subtype M_2 proteins. Note that replacement of all Na^+ with NH_4^+ resulted in a more positive reversal voltage and higher conductance for all three subtypes and that replacement of all Na^+ with Li^+ resulted in a lower conductance for all three subtypes and a less positive reversal voltage for the Udorn and Weybridge subtype M_2 proteins. All measurements were made within 15 s of changing the solution, and all changes were reversible. See Tables 1 and 2 for effects of other ion substitutions.

individual ooyctes (Fig. 2 *A*). Control oocytes also displayed an influx of Rb^+ (about half of the total influx of M_2-expressing oocytes), probably through a ouabain-sensitive Na^+-K^+ ATPase (see O'Connor et al., 1977), but the influx was not amantadine-sensitive (data not shown). In a second experiment, we confirmed the amantadine-sensitive influx of ^{86}Rb after 1 h of incubation and measured the efflux of Rb^+ by pre-loading many cells with ^{86}Rb and then measuring the remaining ^{86}Rb in samples of 20 cells 1 h and 2 h after placing the cells in Barth's solution of pH 7.5 or pH 6.2. The remaining ^{86}Rb fell to about half the original value in 1 h and fell to control values in 2 h for oocytes bathed in solutions of either pH 6.2 or pH 7.5 (data not shown). We were unable to control for the depolarization induced by activation of the M_2 channel in these experiments because the necessity to measure groups of oocytes precluded measurements made in voltage clamp. We attempted to mimic in control oocytes the depolarization of M_2-expressing oocytes by altering the bathing medium. However, only very small changes in resting voltage were evoked by replacing Na^+ with *N*-methyl-D-glucamine (-0.5 ± 2.7 mV SEM, $N = 6$), replacing Cl^- with methanesulfonate$^-$ (3.3 ± 1.6 mV SEM, $N = 6$), or addition of 30 mM K^+ (8.7 ± 5.1 mV SEM, $N = 6$). These results demonstrate the presence of an amantadine-sensitive flux of Rb^+ in oocytes that express the M_2 ion channel.

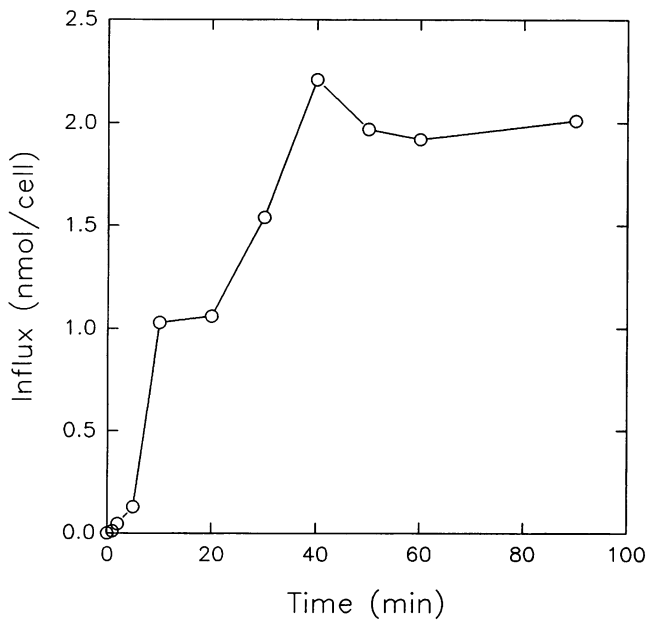

FIGURE 7 Amantadine-sensitive influx of Rb^+ into oocytes that expressed the Rostock subtype M_2 protein. Groups of 20 oocytes (each containing 10 oocytes from two different frogs) were incubated in Barth's solution (pH 7.5) until $t = 0$, at which time they were placed in a solution of pH 6.2, with or without amantadine, containing 50 nCi/μl ^{86}Rb in which Na^+ was completely replaced by Rb^+. The difference between uptake per cell of the group without amantadine and the group with amantadine is plotted against time in the low pH solution.

Effect of altered pH_{in} on the activation of the M_2 ion channel

We took advantage of the decrease in pH_{in} that occurred after prolonged incubation of M_2-expressing oocytes in low pH medium to see if a decrease of pH_{in} was able to activate the channel in a manner similar to that observed with decreased pH_{out} (Pinto et al., 1992; Wang et al., 1995). These experiments were done by measuring the current-voltage relationship and pH_{in} of an M_2-expressing oocyte that was bathed in Barth's solution at pH 7.5, lowering the pH of the incubation solution to pH 6.2, and then remeasuring the current-voltage relationship and pH_{in} at various

times afterward. Finally, the pH of the incubation solution was returned to pH 7.5 and the current-voltage relationship and pH_{in} were remeasured (Fig. 8). This experiment was performed with three cells that expressed the Rostock M_2 protein subtype. For each of the cells, immediately upon lowering pH_{out} from 7.5 to 6.2, the current-voltage relationship shifted to more positive values and the conductance increased, but the pH_{in} did not decrease significantly (Fig. 8). However, while the oocyte was incubated in the low pH solution for increasingly longer times, the pH_{in} decreased and the current-voltage relationship approached that found originally at the higher pH_{out}: the reversal voltage shifted to more negative values and the conductance decreased. In fact, the reversal voltage of the total membrane current of all three cells was negative after acidification ($pH_{in} < 7.0$) of the oocytes and did not have the positive value expected from the H^+ gradient alone. After return of the pH of the incubation solution to pH 7.5, the current-voltage relationship shifted to more negative voltages than the current-voltage relationship that was originally recorded at pH 7.5, although the conductance near the reversal voltage did not undergo a further decrease. Thus, reduced pH_{in} had an effect on the activation of the M_2 protein opposite that of reduced pH_{out}.

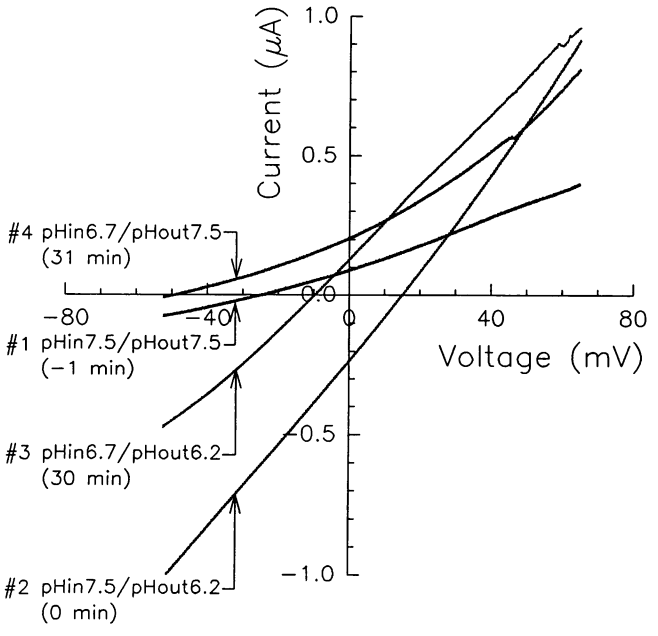

FIGURE 8 Dependence of current-voltage relationship of oocyte expressing M_2 protein on internal pH. The current-voltage relationship was initially measured (using ramps spanning 120 mV in 8 s) at pH 7.5 (#1) and again while the oocyte was incubated for a prolonged period in a solution of pH 6.2 while the internal pH was measured with a pH electrode. Note that the reversal voltage became more positive and the conductance became greater immediately after the extracellular pH was decreased from pH 7.5 to pH 6.2 (#2). However, as the ooycte was incubated in the low pH solution for 30 min, the current-voltage relationship (#3) approached that originally recorded at pH_{out} = 7.5. At the end of 30 min of incubation in pH 6.2 the pH_{in} became 6.7, the pH_{out} was then returned to 7.5, and the current-voltage relationship was again measured (#4).

DISCUSSION

Ion selectivity

The findings that oocytes which express the M_2 protein became acidified when incubated in medium of low pH (Fig. 1) and that this acidification was inhibited by amantadine suggest that the M_2 channel conducts protons. However, this result has to be interpreted cautiously because activation of the M_2 channel of oocytes that are not voltage-clamped results in membrane depolarization, and this depolarization may activate other channels that conduct protons. Therefore, we measured the time course of acidification of oocytes under voltage-clamp conditions and found that there is an amantadine-sensitive acidification (Fig. 2 *A*). Other workers (Cicirelli et al., 1983; Fei et al., 1994) have found that uninjected oocytes undergo acidification when bathed in a medium of low pH. We found no such acidification; perhaps the difference in results stems from our use of oocytes at stage V as opposed to the stage VI oocytes that were probably used in these studies. The ionophore used in the pH electrode has very high selectivity against all of the ions encountered in this experiment. These results confirm measurements made with the fluorescence indicator method (Schroeder et al., 1994). We therefore conclude that the M_2 ion channel is capable of carrying protons, consistent with the roles proposed for the M_2 ion channel in virion uncoating and in equilibrating the pH of the lumen of the trans-Golgi network with the cytoplasm in virus-infected cells.

Several lines of evidence suggest that the M_2 ion channel may be capable of conducting cations in addition to the H^+. First, the plot of the reversal voltage of the amantadine-sensitive current as a function of pH_{out} departed from the Nernst prediction for H^+ (Fig. 4). Although it is possible that there was a difference between the pH of the cortex and the medulla of the ooplasm (where the tip of the pH electrode lies in our experiments; Fig. 2 *A*), this result is inconsistent with the results of currents measured in transformed MEL cells that were induced to express the M_2 protein (Chizhmakov et al., 1995), perhaps because of the limited range of pH that can be tested with the MEL expression system. Second, we found that the reversal voltage of the current-voltage relationship of the amantadine-sensitive current became more negative when Li^+ replaced Na^+ for two M_2 protein subtypes, and the conductance became smaller for all three M_2 protein subtypes with this ionic substitution (Fig. 6). The effect of Li^+ was not to act as a blocker because the time course of acidification was not slowed in the presence of Li^+ (see above). When NH_4^+ replaced Na^+ in the bathing medium the reversal voltage became more positive and the conductance greater for all three M_2 protein subtypes. The change in reversal voltage was not due to the alkalinizing effects of the NH_3/NH_4^+ buffer because the time course of acidification of oocytes in this solution did not differ from that in Barth's solution. An earlier study (Burckhardt and Frömter, 1992) showed acidification of uninjected oocytes bathed in NH_3/NH_4^+ buffer;

however, we observed no consistent alteration of pH_{in} of uninjected oocytes in this condition. We suspect that a difference in the stage of oocytes used may explain the variation in result. The third line of evidence that the M_2 ion channel may conduct cations in addition to H^+ came from experiments in which the reversal voltage was measured while the oocyte became acidified when bathed in medium of low pH (Fig. 8). As the pH_{in} fell from pH 7.5 to pH 6.7 (while the oocyte was bathed in medium of pH 6.2), the reversal voltage changed from about +15 mV to about −10 mV. If H^+ were the only conducting ion, the reversal voltage should have remained positive. As control oocytes that were injected with acid did not undergo any measurable change in membrane current, it is unlikely that the Na^+-H^+ antiporter (DeCoursey and Cherny, 1994) contributed significantly to the membrane currents we recorded. The fourth line of evidence was the finding of an amantadine-sensitive Rb^+ influx of M_2-expressing oocytes. Thus, ions other than the H^+ are probably conducted through the M_2 ion channel.

In an attempt to determine the identity of other ions that may be carried by the M_2 ion channel, we found that replacement of Na^+ in the bathing medium with Cs^+, K^+, Rb^+, *N*-methyl-D-glucamine$^+$, or tetrabutylammonium$^+$ had little effect on reversal voltage or conductance of the amantadine-sensitive current. Thus, these data lead to the paradox of being unable to conclude, on the basis of this experiment alone, whether these ions had conductance equal to or much lower than that of Na^+. However, the results of the tracer experiments with Rb^+ (Fig. 7; discussed below) are consistent with, but do not prove, the proposition that this ion is capable of being conducted by the M_2 protein. The total membrane current of oocytes expressing the M_2 protein was reported earlier to be affected by replacement of Na^+ with *N*-methyl-D-glucamine$^+$ (Pinto et al., 1992); however, in the present study this observation was not repeated consistently and, of more importance, the amantadine-sensitive current was not consistently shifted by this replacement (Tables 1 and 2). In a study of the reversal voltage of currents of lipid bilayers into which purified M_2 protein was introduced (Tosteson et al., 1994), the following ionic selectivity sequence was found: $Rb^+ > K^+ > Cs^+ \approx Na^+ > Li^+$. The present results are consistent with Li^+ having lower conductance than Na^+ and with an amantadine-sensitive Rb^+ flux, but do not allow a determination of the selectivity of the remaining ions. A rough calculation of the relative ionic permeabilities, made from the reversal voltage at $pH_{out} = 6.2$, shows that protons are much more permeable (about 10^5-fold) than other conducting ions.

A tracer experiment confirmed the presence of a membrane flux of an ion other than protons. We showed that oocytes expressing the M_2 protein have an amantadine-sensitive Rb^+ influx (Fig. 7) and efflux when bathed in a medium of low pH. The time course of the influx resembled the time course of acidification of the ooplasm (Fig. 2 *A*). However, the nature of this experiment required the measurements to be made on pools of 20 oocytes and therefore could not be done under voltage-clamp conditions. Thus, we cannot exclude the possibility that the amantadine-sensitive flux resulted from the activation of another channel by the amantadine-sensitive depolarization or decreased pH_{in} evoked by low pH_{out}. We were not able to control for this latter possibility because there is no simple way to depolarize control oocytes to about +20 mV in the absence of an exogenous channel. Thus, these experiments support, but do not prove, the notion that the M_2 channel is capable of conducting ions other than H^+. If it becomes possible to record single-channel currents from the M_2 ion channel molecular complex, it will be possible to resolve the remaining uncertainly of the ionic mechanism.

The interpretation of these experiments depends on the assumption that the amantadine-sensitive current and the current that flows through the M_2 channel are one and the same. Although it is a formal possibility that these two currents are not equal, we think that this possibility is unlikely for several reasons. First, all of the currents measured were low pH-activated but neither voltage-activated nor time-dependent (Shimbo et al., 1995). Second, the concentration of amantadine routinely used (100 μM) was lower than that needed for inhibition of other channels, and the results obtained were confirmed when a lower (10 μM) concentration of amantadine was used (data not shown). Third, the amplitude of the current of M_2-expressing oocytes is proportional to the mass of M_2 protein expressed (Shimbo et al., 1995). Because contol oocytes had no detectable amantadine-sensitive current, the only way in which these requirements could be met is by a previously unreported endogenous channel or a transporter that is normally silent and is up-regulated in the presence of the M_2 protein to have all of the above properties, a possibility we believe to be remote.

Activation

Previous studies have shown that the M_2 ion channel, expressed in mammalian cells (Wang et al., 1994) or in *Xenopus* oocytes (Pinto et al., 1992; Wang et al., 1993, 1995), is activated by lowered pH_{out} and that this activation depends upon $histidine_{37}$ (Pinto et al., 1992; Wang et al., 1995). The present experiments demonstrated that lowering of pH_{in} was accompanied by a decrease in the amantadine-sensitive conductance (Fig. 8), consistent with a decrease of pH_{in} having an effect opposite the activation due to reduced pH_{out}. In fact, upon return of the pH of the bathing medium to pH 7.5, the reversal voltage became more negative than its original value at pH 7.5. The reduction in activation that was observed with decreased pH_{in} must be effected by a different molecular mechanism from that which is responsible for activation resulting from decreased pH_{out}.

Implications for influenza A virus

The demonstration of H^+ conductance of the M_2 ion channel provides direct data to support the suggestions of previous

studies that the channel is capable of acidifying the interior of the virion as a prerequisite to viral uncoating (reviewed in Hay, 1992; Helenius, 1992; Marsh, 1992; Skehel, 1992; Lamb et al., 1994). Furthermore, the demonstration of H^+ conductance of the M_2 ion channel confirms the notion that the channel can equilibrate the H^+ gradient between the lumen of the TGN and the cytoplasm (Sugrue and Hay, 1991; Hay, 1992; Takeuchi and Lamb, 1994). However, the acidification of the virion would be incomplete if the only ion channel in the virion membrane were a H^+ conducting channel; in the absence of a counter-ion only a few H^+ would enter the virion before an unacceptable membrane potential developed. Our results suggesting that cations other the H^+ are also conducted by the M_2 protein are consistent with the interpretation that the M_2 channel itself is capable of providing the flow of the needed counter-ion.

The finding that reduced pH_{in} attenuates the activation of the M_2 protein that results from reduced pH_{out} is consistent with the roles proposed for the M_2 protein. This attenuation would serve to limit the influx of H^+ once the virion interior was sufficiently acidified. Thus, the ion selectivity and activation properties we found for the M_2 protein are well suited for the proposed roles of the protein in the life cycle of influenza A virus.

We are very grateful to Dr. Kaoru Takeuchi, National Institutes of Health, Japan, for synthesis of the cDNA encoding the authentic influenza A/Chicken/Germany/27 (FPV Weybridge) M_2 protein. We thank Joseph Ahn, Eric Larson, and Aurelia Micko for performing some of the initial experiments as part of undergraduate independent study projects.

This research was supported by Public Health Service Research grants AI-20201 (RAL) and AI-31882 (LHP) from the National Institute of Allergy and Infectious Diseases. KS was supported by a fellowship from ARVO/JNSPB. RAL is an Investigator of the Howard Hughes Medical Institute.

REFERENCES

Ammann, D., F. Lanter, R. A. Steiner, P. Schulthess, Y. Shigo, and W. Simon. 1981. Neutral carrier based hydrogen ion selective microelectrode for extra- and intracellular srudies. *Anal. Chem.* 53:2267–2269.

Bland, R. D., and C. A. Boyd. 1986. Cation transport in lung epithelial cells derived from fetal, newborn, and adult rabbits. *J. Appl. Physiol.* 61: 507–515.

Bukrinskaya, A. G., N. K. Vorkunova, G. V. Kornilayeva, R. A. Narmanbetova, and G. K. Vorkunova. 1982. Influenza virus uncoating in infected cells and effects of rimantadine. *J. Gen. Virol.* 60:49–59.

Burckhardt, B., and E. Frömter. 1992. Pathways of NH_3/NH_4^+ permeation across *Xenopus laevis* oocyte cell membrane. *Pflügers Arch.* 420:83–86.

Chizhmakov, I., F. Geraghty, M. Hayhurst, D. Antoniou, D. Ogden, and A. J. Hay. 1995. Selective proton permeability and proton activation of influenza A virus M_2 protein channels expressed in MEL cells. *Biophys. J.* 68:A204. (Abstr.)

Ciampor, F., P. M. Bayley, M. V. Nermut, E. M. A. Hirst, R. J. Sugrue, and A. J. Hay. 1992a. Evidence that the amantadine-induced, M_2-mediated conversion of influenza A virus hemagglutinin to the low pH conformation occurs in an acidic trans Golgi compartment. *Virology.* 188: 14–24.

Ciampor, F., C. A. Thompson, S. Grambas, and A. J. Hay. 1992b. Regulation of pH by the M_2 protein of influenza A viruses. *Virus Res.* 22:247–258.

Cicirelli, M. F., K. R. Robinson, and L. D. Smith. 1983. Internal pH of *Xenopus* oocytes: a study of the mechanism and role of pH changes during meiotic maturation. *Dev. Biol.* 100:133–146.

Colman, A. 1984. Translation of eukaryotic messenger RNA in *Xenopus* oocytes. *In* Transcription and Translation: A Practical Approach. B. D. Hames and S. J. Higgins, editors. IRL Press, Oxford. 271–302.

DeCoursey, T., and V. V. Cherny. 1994. Na^+-H^+ antiport detected through hydrogen ion currents in rat alveolar epithelial cells and human neutrophils. *J. Gen. Physiol.* 103:755–785.

Dumont, J. N. 1972. Oogenesis in *Xenopus laevis* (Daudin). *J. Morphol.* 136:153–180.

Fei, Y., Y. Kanai, S. Nussberger, V. Ganapathy, F. H. Leibach, M. F. Romero, S. K. Singh, W. F. Boron, and M. A. Hediger. 1994. Expression cloning of a mammalian protein-coupled oligopeptide transporter. *Nature.* 368:563–566.

Grambas, S., M. S. Bennett, and A. J. Hay. 1992. Influence of amantadine resistance mutations on the pH regulatory function of the M_2 protein of influenza A viruses. *Virology.* 191:541–549.

Grambas, S., and A. J. Hay. 1992. Maturation of influenza A virus hemagglutinin-estimates of the pH encountered during transport and its regulation by the M_2 protein. *Virology.* 190:11–18.

Hay, A. J. 1992. The action of adamantanamines against influenza A viruses: inhibition of the M_2 ion channel protein. *Semin. Virol.* 3:21–30.

Hay, A. J., A. Wolstenholme, J. J. Skehel, and M. H. Smith. 1985. The molecular basis of the specific anti-influenza action of amantadine. *EMBO J.* 4:3021–3024.

Helenius, A. 1992. Unpacking the incoming influenza virus. *Cell.* 69: 577–578.

Holsinger, L. J., and R. A. Lamb. 1991. Influenza virus M_2 integral membrane protein is a homotetramer stabilized by formation of disulfide bonds. *Virology.* 183:32–43.

Holsinger, L. J., D. Nichani, L. H. Pinto, and R. A. Lamb. 1994. Influenza A virus M_2 protein: a structure-function analysis. *J. Virol.* 68: 1551–1563.

Holsinger, L. J., M. A. Shaughnessy, A. Micko, L. H. Pinto, and R. A. Lamb. 1995. Analysis of the posttranslational modifications of the influenza virus M_2 protein. *J. Virol.* 69:1219–1225.

Howl, J. D., N. A. E. von Sicard, S. J. Publicover, and M. Anderson. 1988. A method for the manufacture of single barrel liquid ion-selective microelectrodes: an in situ study of ant venom pH. *Pflügers Arch.* 411:212–215.

Kemp, P. J., G. C. Roberts, and C. A. Boyd. 1994. Identification and properties of pathways for K^+ transport in guinea-pig and rat alveolar epithelial type II cells. *J. Physiol.* 476:79–88.

Lamb, R. A., and P. W. Choppin. 1981. Identification of a second protin (M_2) encoded by RNA segment 7 of influenza virus. *Virology.* 112: 729–737.

Lamb, R. A., L. J. Holsinger, and L. H. Pinto. 1994. The influenza A virus M_2 ion channel protein and its role in the influenza virus life cycle. *In* Cellular Receptors for Animal Viruses. E. Wimmer, editor. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 303–321.

Lamb, R. A., S. L. Zebedee, and C. D. Richardson. 1985. Influenza virus M_2 protein is an integral membrane protein expressed on the infected-cell surface. *Cell.* 40:627–633.

Marsh, M. 1992. Keeping the viral coat on. *Curr. Biol.* 2:379–381.

Martin, K., and A. Helenius. 1991. Nuclear transport of influenza virus ribonucleoproteins: the viral matrix protein (M_1) promotes export and inhibits import. *Cell.* 67:117–130.

O'Connor, C. M., K. R. Robinson, and L. D. Smith. 1977. Calcium, potassium, and sodium exchange by full-grown and maturing *Xenopus laevis* oocytes. *Dev. Biol.* 61:28–40.

Ohuchi, M., A. Cramer, M. Vey, R. Ohuchi, and H.-D. Klenk. 1994. Rescue of vector expressed fowl plague virus hemagglutinin in biologically active form by acidotropic agents and co-expressed M_2 protein. *J. Virol.* 68:1551–1563.

Pinto, L. H., L. J. Holsinger, and R. A. Lamb. 1992. Influenza virus M_2 protein has ion channel activity. *Cell.* 69:517–528.

Schroeder, C., C. M. Ford, S. A. Wharton, and A. J. Hay. 1994. Functional reconstitution in lipid vesicles of influenza virus M_2 protein expressed

by baculovirus: evidence for proton transfer activity. *J. Gen. Virol.* 75:3477–3484.

Shimbo, K., D. L. Brassard, R. A. Lamb, and L. H. Pinto. 1995. Viral and cellular small integral membrane proteins can modify ion channels endogenous to *Xenopus* oocytes. *Biophys. J.* 69:1819–1829.

Skehel, J. J. 1992. Influenza virus. Amantadine blocks the channel [news]. *Nature.* 358:110–111.

Skehel, J. J., A. J. Hay, and J. A. Armstrong. 1978. On the mechanism of inhibition of influenza virus replication by amantadine hydrochloride. *J. Gen. Virol.* 38:97–110.

Sugrue, R. J., G. Bahadur, M. C. Zambon, M. Hall-Smith, A. R. Douglas, and A. J. Hay. 1990. Specific structural alteration of the influenza hemagglutinin by amantadine. *EMBO J.* 9:3489–3476.

Sugrue, R. J., and A. J. Hay. 1991. Structural characteristics of the M_2 protein of influenza A viruses: evidence that it forms a tetrameric channel. *Virology.* 180:617–624.

Takeuchi, K., and R. A. Lamb. 1994. Influenza virus M_2 protein ion channel activity stabilizes the native form of fowl plague virus hemagglutinin during intracellular transport. *J. Virol.* 68:911–919.

Tosteson, M. T., L. H. Pinto, L. J. Holsinger, and R. A. Lamb. 1994. Reconstitution of the influenza virus M_2 ion channel in lipid bilayers. *J. Membr. Biol.* 142:117–126.

Wang, C., R. A. Lamb, and L. H. Pinto. 1994. Direct measurement of the influenza A virus M_2 protein ion channel activity in mammalian cells. *Virology.* 205:133–140.

Wang, C., R. A. Lamb, and L. H. Pinto. 1995. Activation of the M_2 ion channel of influenza virus: a role for the transmembrane domain histidine residue. *Biophys. J.* 69:1363–1371.

Wang, C., K. Takeuchi, L. H. Pinto, and R. A. Lamb. 1993. Ion channel activity of influenza A virus M_2 protein: characterization of the amantadine block. *J. Virol.* 67:5585–5594.

Zebedee, S. L., and R. A. Lamb. 1988. Influenza A virus M_2 protein: monoclonal antibody restriction of virus growth and detection of M_2 in virions. *J. Virol.* 62:2762–2772.

Zebedee, S. L., C. D. Richardson, and R. A. Lamb. 1985. Characterization of the influenza virus M_2 integral membrane protein and expression at the infected-cell surface from cloned cDNA. *J. Virol.* 56:502–511.