Permeation and Activation of the M₂ Ion Channel of Influenza A Virus*

Jorgen A. Mould‡, Jason E. Drury‡, Stephan M. Frings§, U. Benjamin Kaupp§, Andrew Pekosz¶, Robert A. Lamb¶‖, and Lawrence H. Pinto‡ ‡‡**

From the ‡Department of Neurobiology and Physiology and the ¶Department of Biochemistry, Molecular Biology, and Cell Biology, ‖Howard Hughes Medical Institute, Northwestern University, Evanston, Illinois 60208-3500 and §Institut für Biologische Informationsverarbeitung, Forschungszentrum, 52425 Juelich, Germany

The M₂ ion channel protein of influenza A virus is essential for mediating protein-protein dissociation during the virus uncoating process that occurs when the virus is in the acidic environment of the lumen of the secondary endosome. The difficulty of determining the ion selectivity of this minimalistic ion channel is due in part to the fact that the channel activity is so great that it causes local acidification in the expressing cells and a consequent alteration of reversal voltage, V_{rev}. We have confirmed the high proton selectivity of the channel (1.5–2.0×10^6) in both oocytes and mammalian cells by using four methods as follows: 1) comparison of V_{rev} with proton equilibrium potential; 2) measurement of pH_{in} and V_{rev} while Na^+_{out} was replaced; 3) measurements with limiting external buffer concentration to limit proton currents specifically; and 4) comparison of measurements of M₂-expressing cells with cells exposed to a protonophore. Increased currents at low pH_{out} are due to true activation and not merely increased $[H^+]_{out}$ because increased pH_{out} stops the outward current of acidified cells. Although the proton conductance is the biologically relevant conductance in an influenza virus-infected cell, experiments employing methods 1–3 show that the channel is also capable of conducting NH_4^+, probably by a different mechanism from H^+.

The M₂ protein of influenza A virus is thought to function as an ion channel that permits protons to enter virus particles during virion uncoating in endosomes. In addition, in influenza virus-infected cells, the M₂ protein causes the equilibration of pH between the acidic lumen of the trans-Golgi network and the cytoplasm (reviewed in Refs. 1 and 2). The activity of the M₂ ion channel is inhibited by the antiviral drug amantadine (3–5). The mature M₂ protein consists of a 23-residue N-terminal extracellular domain, a single internal hydrophobic domain of 19 residues that acts as a transmembrane domain and forms the pore of the channel, and a 54-residue cytoplasmic tail (6). Chemical cross-linking studies showed the M₂ protein to exist minimally as a homotetramer (7–9). Statistical analysis of the ion channel activity of mixed oligomers also indicated that a homotetramer is the minimal active oligomeric form of the protein (10).

Despite the small size of the active M₂ oligomer, several lines of evidence indicate that ion channel activity is intrinsic to the M₂ protein. First, ion channel activity has been observed in three different expression systems, *Xenopus* oocytes (3, 11, 12), mammalian cells (5, 13), and yeast (14). Second, M₂ channel activity has also been recorded in artificial lipid bilayers from a reconstituted peptide corresponding to the transmembrane domain of the M₂ protein (15) and from purified M₂ protein (16). Thus, due to its structural simplicity, the M₂ ion channel is a potentially useful model for the study of ion channels in general.

Although a great deal of evidence indicates H^+ is the biologically relevant ion for the role of M₂ protein in the life cycle of the influenza virus (1, 3, 17–22), other ions have been shown to be capable of flowing through the channel (12). In addition, the ion selectivity measured for the M₂ channel has been found to differ depending on whether the activity was measured in *Xenopus* oocytes or mammalian cells. When M₂ protein was expressed in oocytes, V_{rev} was found to differ from the proton equilibrium potential, E_{H+} as $[H^+]_{out}$ was varied (12). On the other hand, when M₂ protein was expressed in MEL cells, V_{rev} was found to agree with E_{H+} (5). In a recent study (23), we found I_{H+} of the M₂ ion channel to be so large that it was capable of decreasing $[H^+]_{out}$ in the locale of the extracellular pore of the channel if the expressing cells were bathed in medium of low buffer concentration. One possible explanation for the different results may be that the channel is also capable of acidifying the interior of some expressing cells, thereby altering reversal voltage, V_{rev}. Shimbo and co-workers (12) found that replacement of Na^+ with Li^+ decreased currents, and replacement of Na^+ with NH_4^+ increased currents. In principle, these effects could have resulted from one of two mechanisms. Either these ions affected the proton current, I_{H+}, or the replacing ions permeated the M₂ ion channel. In this study, we were able to study the effects of these ion replacements on proton currents specifically by taking advantage of the finding that inward H^+ currents are limited when the concentration of buffer in the bathing medium is reduced (23) to distinguish between these possibilities.

The M₂ ion channel current is increased in amplitude when the pH of the extracellular domain is lowered (3, 5, 24). This increase in current occurs within the range of pH values expected for titration of histidine (24). The only amino acid in the transmembrane domain of the M₂ protein with a titratable group in this pH range is His³⁷, and when His³⁷ is replaced by Ala, Gly, or Glu, the proton selectivity of the channel is greatly

** Investigator of the Howard Hughes Medical Institute.
‡‡ To whom correspondence should be addressed: Dept. of Neurobiology and Physiology, Hogan Hall, 2153 North Campus Dr., Northwestern University, Evanston, IL 60208-3500. Tel.: 847-491-7915; Fax: 847-491-5211; E-mail: larry-pinto@northwestern.edu.

reduced, and the channel is conductive over a wider range of pH (3, 24). It has been proposed that His^{37} forms a selectivity filter for protons and that H$^+$ conduction may occur by tautomerization of the imidazole side chain of His^{37} (25). Although the H$^+$ current of the M$_2$ ion channel protein is increased by elevated [H$^+$] in the extracellular medium, this increased current may be due to either the increased abundance of the conducting species or activation of the channel at low pH, or both factors operating together. One way to distinguish pH-dependent changes in activity from the effects of increased abundance of H$^+$ at low pH is to compare the efflux of H$^+$ from acidified cells that express the M$_2$ protein to the efflux from acidified cells treated with the electrogenic protonophore FCCP.[1] Cell acidification can be achieved by lowering the pH of the medium bathing M$_2$-expressing or FCCP-treated cells. If the M$_2$ ion channel is indeed activated by low pH$_{out}$ and conversely deactivated by neutral or alkaline pH$_{out}$, then the efflux of H$^+$ should be greater for FCCP-treated cells than for M$_2$-expressing cells upon return to a bathing solution of neutral or alkaline pH.

In this study we measured ionic currents and pH$_{in}$ in two M$_2$ expression systems to ensure that the results obtained were not specific to the cell type. The results demonstrate that under normal physiological conditions the M$_2$ ion channel specifically conducts H$^+$. We also demonstrate that NH$_4^+$ can permeate the channel, by a mechanism that differs from that for H$^+$ permeation. Furthermore, by comparison of the outward currents of acidified, M$_2$-expressing cells and FCCP-treated cells, we confirm that M$_2$ ion channel activity is modulated by the pH of the solution bathing the extracellular N-terminal domain of the channel.

EXPERIMENTAL PROCEDURES

mRNA Synthesis—The cDNA to the A/Udorn/72 mRNA was cloned into the *Bam*HI site of pGEM3 such that mRNA sense transcripts could be generated by using the bacteriophage T7 RNA polymerase promoter and T7 RNA polymerase. For *in vitro* transcription, plasmid DNAs were linearized downstream of the T7 promoter and the M$_2$ cDNA with *Xba*I. *In vitro* synthesis and quantification of ^{7m}G(5′)ppp(5′)G-capped mRNA was carried out as described previously (3).

Culture and Microinjection of Oocytes—Oocytes were removed from female *Xenopus laevis* (Nasco, Fort Atkinson, WI), defolliculated by treatment with collagenase B (2 mg/ml; Roche Molecular Biochemicals), 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 gentamicin, 5 mM HEPES, pH 7.5, osmolality ~210 mosmol/kg) at 19 °C. Oocytes at stage V were microinjected with 50 nl of mRNA (1 ng/nl) on the day after defolliculation, incubated for 24 h in ND96, pH 7.5, and finally incubated for 24 h in ND96, pH 8.5, at 19 °C before use.

Culture and Infection of CV-1 Cells—CV-1 cells were cultured and infected with recombinant simian virus 40 expressing the M$_2$ protein from influenza A/Udorn/72 (rSV40-M$_2$), as described previously (13). Briefly, CV-1 cells grown to confluency at 37 °C, 5% CO$_2$ in culture media (Dulbecco's modified Eagle's medium + 10% fetal calf serum + penicillin + streptomycin) were trypsinized, pelleted, and resuspended in culture medium. Resuspended cells were incubated in the presence of high titer SV40-M$_2$ (100 μl of resuspended CV-1 cells + 1 ml of virus stock) for 4 h. Infected cells were then diluted 1:1 in culture medium and seeded onto 5-mm square glass coverslips arranged in 3.5-cm Petri dishes (2 ml total volume/dish). Infected cells were then incubated for 48 h before recording to ensure adequate M$_2$ protein expression.

Measurement of Membrane Current of CV-1 Cells—M$_2$ currents were recorded from CV-1 cells using the whole cell patch clamp technique as described previously (13). Briefly, patch pipettes having tip diameters of ~2–3 μm were pulled from borosilicate capillary glass, fire-polished, and then partially filled with pipette solution which contained, in mM,

145 KCl, 5 EGTA, 1 MgCl$_2$, 5 NaCl, 15 HEPES, pH 7.4, adjusted with KOH, osmolality 300–310 mosmol/Kg. Pipettes filled with this solution typically had resistances of ~3–4 MΩ. CV-1 cells attached to glass coverslips were transferred to a recording chamber filled with a solution that contained, in mM, 140 NaCl, 5.3 KCl, 0.55 MgSO$_4$, 1.8 mM CaCl$_2$, 5.5 glucose, 15 HEPES, pH 7.4, or 15 mM MES, pH 6.2, osmolality ~300 mosmol/kg. Seals (in excess of 10 GΩ) were made by gently pressing the patch pipette against a CV-1 cell and then applying ~12 mm Hg suction without delay. The whole cell configuration was achieved by using a brief pulse of high voltage combined with gentle pipette suction. In the whole cell configuration, cells had access resistances of <10 MΩ. Cells were generally bathed in pH 7.4 solution and held at −20 mV. Whole cell currents were recorded after the bathing solution was changed from pH 7.4 to pH 6.2 using a Fast Step Perfusion System (model SF77B, Warner Instruments Corp., Hamden, CT). By using this system, solution changes could be made in less than 100 ms. M$_2$-specific currents were identified by sensitivity to block by 100 μM amantadine.

Measurement of Membrane Current of Oocytes—Whole cell currents were measured using a two-electrode voltage clamp. Electrodes were filled with 3 M KCl, and the oocytes were bathed in either Barth's solution, which contained, in mM, 88 NaCl, 1 KCl, 2.4 NaHCO$_3$, 0.3 NaNO$_3$, 0.71 CaCl$_2$, 0.82 MgSO$_4$, 15 HEPES, pH 7.5, osmolality ~210 mosmol/kg or a modified solution during the recording. Continuous current-voltage (I-V) relationships were measured with ramps of membrane voltage since the M$_2$ channel shows no rapid voltage- or time-dependent gating. These ramps typically spanned a range of 120 mV in 2 s. Oocyte holding potential was −20 mV unless stated otherwise.

Measurement of pH$_{in}$ of Oocytes—Microelectrodes were silanized and filled with protonophore as described previously (12). The electrodes were calibrated before each experiment with four pH values spanning the range encountered during the experiment. The response time of these electrodes, determined with a stepping motor device that changed solution pH within 100 ms, was less than 10 ms.

Measurement of pH$_{in}$ of CV-1 Cells—We used the fluorometric indicator 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) to measure pH$_{in}$. Control cells or cells infected with rSV40-M$_2$ were incubated (37 °C for 1 h) in a solution containing BCECF-AM in 0.25% Me$_2$SO carrier with a final dye concentration of 0.25 μg/ml. These cells were placed on the stage of an epifluorescence microscope equipped with a × 20, 0.75 NA (Nikon) objective that allowed up to seven CV-1 cells to be imaged in its field at one time, an intensified CCD camera and MagiCal image analysis software (Applied Imaging, Sunderland, UK). The dye was excited with 490 nm illumination to observe pH-induced changes in fluorescence. To measure the intracellular concentration of dye and thus allow calibration of pH$_{in}$ of the CV-1 cells, illumination was applied at 435 nm (the isosbestic wavelength) at the beginning and end of the measurements from one field of cells. Emission was recorded at 520 nm. Calibration of the pH$_{in}$ from fluorescence measurements was done using the FCCP equilibration method (26). Briefly, the cells were treated with the protonophore FCCP to allow equilibration of the [H$^+$] across the plasma membrane. The emission at 520 nm as a result of excitation at 490 and 435 nm was measured while the cell was bathed in solutions with pH spanning the range of pH values expected to be encountered during the measurements (pH 4.0, pH 6.7, and pH 9.0), and the resulting ratios (F_{490}/F_{435}) were used to construct a calibration curve (see Equation 1 of Ref. 26).

RESULTS

Reversal Voltage Changes within a Few Seconds after Lowering the pH of the Bathing Solution

The M$_2$ protein from influenza A/Udorn/72 virus was used for this study. If the M$_2$ ion channel is highly proton-selective, then the reversal voltage of the current (V$_{rev}$) should change according to the equilibrium potential for H$^+$ (E_{H^+}) when the difference between pH$_{in}$ and pH$_{out}$ is altered. The reversal voltage of the currents of cells expressing the M$_2$ protein can be measured from continuous current-voltage relationships measured with ramps of membrane voltage because the M$_2$ ion channel is not voltage-activated on the time scale of the ramps of voltage that are practical to use.

Oocytes—We measured the V$_{rev}$ of amantadine-sensitive currents in M$_2$-expressing *Xenopus* oocytes at 20-s intervals using two-microelectrode voltage clamp. Oocytes whose membrane voltage was clamped to −20 mV produced a large amantadine-

---

[1] The abbreviations used are: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; MES, 4-morpholineethanesulfonic acid; BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester; GHK, Goldman-Hodgkin-Katz; NMD6$^+$, *N*-methyl-D-glucamine.

A
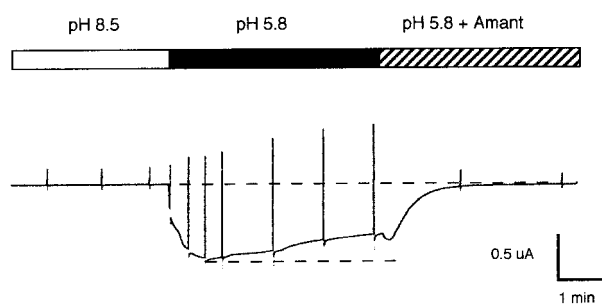
B
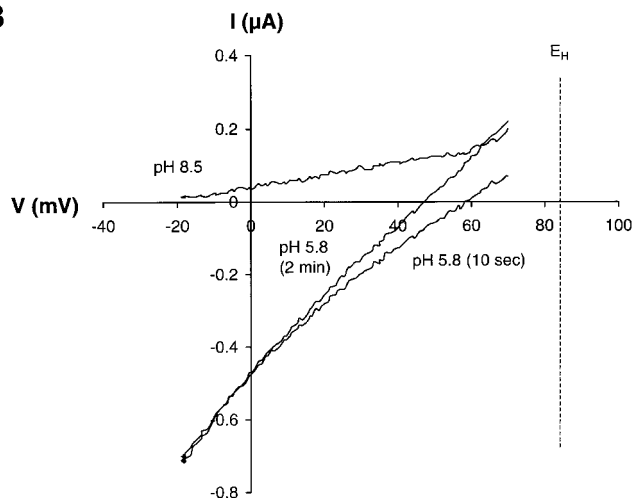
FIG. 1. **Membrane currents and current-voltage relationships recorded from oocytes expressing the M$_2$ protein.** *A,* time course of the membrane current as the pH of the bathing medium was lowered from pH 8.5 to pH 5.8 while membrane voltage was held at -20 mV. Note that the current decreased spontaneously while the bathing solution was held at pH 5.8 and that the current was fully inhibited by 100 μM amantadine (*Amant*). *Upper interrupted line* represents zero current, and *lower interrupted line* represents maximal inward current amplitude. Vertical deflections are the result of the voltage ramps used to measure the current-voltage relationships such as those shown in *B* at the times indicated after pH was reduced. *B,* current-voltage relationship measured in the same cell shown in *A*. Note the return of V$_{rev}$ to negative values and the increase in conductance that occurred after 2 min in low pH bathing medium. E_{H+} is shown with the *vertical interrupted line* and was calculated from the known pH$_{out}$ and pH$_{in}$ measured using an intracellular pH electrode.

sensitive inward current (~ 1 μA) when the pH of the bathing solution was lowered from pH 8.5 to pH 5.8 (Fig. 1*A*). During the time when the oocyte was bathed in low pH medium, V$_{rev}$, measured using voltage ramps, became more positive within a few seconds and reached a maximum prior to the time when the inward current reached its maximum amplitude (Fig. 1*B*). The average peak V$_{rev}$, calculated from the current-voltage relationship of the amantadine-sensitive current, was 51.4 ± 1.22 mV S.E. ($n = 34$), a value more negative than that for E_{H+} (85.2 mV ± 1.86 mV S.E., $n = 34$) which was calculated from the known pH$_{out}$ and the value of pH$_{in}$ measured with a pH microelectrode at the beginning of the experiment. We calculated the permeability of H$^+$ relative to that for Na$^+$ assuming 20 mM intracellular Na$^+$ concentration for these 34 cells, using the Goldman-Hodgkin-Katz (GHK) equation, and we found that the relative permeability was about 1.5×10^6. However, in two cells that had small amantadine-sensitive currents, the peak V$_{rev}$ nearly reached the value calculated for E_{H+} ($+89$ and $+91$ mV). At the time when the peak value of V$_{rev}$ occurred, the current-voltage relationship was steeper than that obtained at

pH 8.5, indicating that the conductance had increased. Shortly after reaching its peak value, the inward current decreased in amplitude over a period of a few minutes (Fig. 1*A*). The current decrease was accompanied by a return of V$_{rev}$ back toward more negative potentials.

CV-1 Cells—The membrane currents and V$_{rev}$ were measured using the whole cell patch clamp technique in CV-1 cells that expressed the M$_2$ protein (Fig. 2), whereas pH$_{out}$ was reduced in less than 100 ms from pH 7.4 to pH 6.2 with a stepping-motor device (see "Experimental Procedures"). The pH$_{in}$ of CV-1 cells, measured fluorimetrically in a separate experiment described below, was used to calculate E_{H+}. The peak V$_{rev}$ measured in a solution of pH 6.2 was 41.8 ± 3.5 mV S.E. ($n = 12$), and E_{H+} was 38.9 ± 4.7 mV S.E. ($n = 13$). In less than 1 min after lowering pH of the bathing solution, V$_{rev}$ reached a peak near E_{H+} and then returned to more negative values (Fig. 2). We calculated the permeability of H$^+$ relative to that for Na$^+$ assuming 2 mM residual intracellular Na$^+$ concentration for these CV-1 cells, using the GHK equation, and found that the relative permeability was about 1.8×10^6.

Since V$_{rev}$ changed after reaching a peak in the low pH solutions in a rapid and systematic manner in both M$_2$-expressing CV-1 cells and oocytes, we studied the reason for the systematic changes in V$_{rev}$, and we employed other means to study the ion selectivity of the channel. There are a number of possible explanations for the decrease of inward current and the return of V$_{rev}$ to more negative voltages after reaching a peak value that was observed in M$_2$-expressing cells bathed in low pH solutions. 1) The influx of ions through the M$_2$ channel could activate an endogenous outward current that opposes the current flowing through the M$_2$ channel. 2) The M$_2$ ion channel might undergo an activity dependent change in ion selectivity. 3) The M$_2$ ion channel might inactivate after long periods in low pH solutions. 4) A constant influx of protons through the M$_2$ channel might cause acidification of the cell cytoplasm and thus decrease the driving force on protons.

For explanations 1 and 2, the possibility was tested that the shift in V$_{rev}$ observed in oocytes and CV-1 cells might be due to activation of an amantadine-insensitive, endogenous current or an activity-dependent change in ion selectivity by measuring currents under ionic conditions chosen to minimize all but H$^+$ currents. The experiments were conducted in CV-1 cells, for which it was possible to control the composition of both the intracellular and extracellular solutions. We found that it was not possible to perform these experiments by changing the internal composition in oocytes using the cut-open technique because small leaks that developed were indistinguishable from M$_2$ currents, except by application of amantadine, the effects of which are not reversible on the time scale of these experiments. The principal endogenous currents of CV-1 cells were found to be similar to those of HEK293 cells, *i.e.* inward Cl$^-$ and outward K$^+$ currents (27). Whole cell currents were measured in CV-1 cells when Cl$^-$ in the bathing solution was replaced with methane sulfonate and KCl in the pipette solution with tetraethylammonium chloride in order to reduce the endogenous currents of the cells. As before, V$_{rev}$ was measured at frequent intervals as the external pH was lowered. Upon lowering the extracellular pH from pH 7.4 to pH 6.2, a large inward current developed in M$_2$-expressing CV-1 cells held at -20 mV, similar to the results obtained with control solutions (Fig. 2). This inward current was accompanied by a shift of V$_{rev}$ to positive values. The peak V$_{rev}$ (38.8 ± 2.01 mV S.E., $n = 5$) was close to the value of E_{H+} (calculated as above, 38.9 ± 4.7 mV S.E., $n = 13$). As observed in the control solution, the inward current began to decrease a few seconds after reaching its maximum amplitude. The decrease of inward current was

FIG. 2. **Current-voltage relationships recorded from a CV-1 cell expressing the M₂ protein at pH 7.4 at various times (*numerals above curves*) after the pH of the bathing medium was lowered from pH 7.4 to pH 6.2.** Note that the return of V_{rev} to more negative values and the increase in conductance that occurred with time in low pH bathing medium were faster than those measured in oocytes (Fig. 1*B*). E_{H^+} (*vertical interrupted line*) was calculated from the known pH_{out} and the pH_{in} was measured fluorometrically in separate experiments.
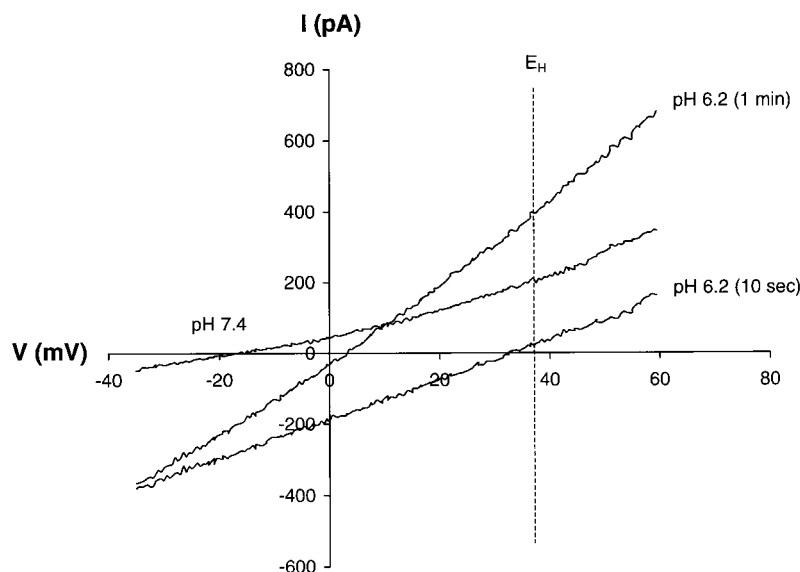
again accompanied by a return of V_{rev} to more negative voltages and an increase in the slope conductance of the I-V relationship. The fact that the return of V_{rev} to negative values still occurred in the absence of other conducting ions suggests that the change of V_{rev} was not the result of either activating an endogenous current or an activity-dependent change in ion selectivity. We calculated the permeability of H^+ relative to that for Na^+ using the mean values of 38.8 and 38.9 mV for the V_{rev} and E_{H^+}, respectively, and assuming 2 mM residual intracellular Na^+ concentration for these CV-1 cells, using the GHK equation, and we found that the relative permeability was about 2×10^7. However, if the actual values differed by as little as 3 mM, the relative permeability would have been about 2×10^6.

For explanation 3, it was also unlikely that the decrease of current and negative shift of V_{rev} observed in low pH was the result of inactivation of the M₂ ion channel. This is because the slope conductance of the I-V relationship actually *increased* in both oocytes and CV-1 cells during the shift (Figs. 1*B* and 2). This observation is the opposite of what would have been expected if the channel had been inactivating. The remaining and most likely explanation for the return of V_{rev} to more negative values for cells bathed in low pH solutions was that intracellular acidification occurred.

For explanation 4, we tested whether the return of V_{rev} to negative potentials and the decrease in amplitude of the inward current observed after their peak values occurred were the result of cell acidification by measuring the shift under conditions designed to minimize acidification. These conditions were achieved as discussed in the following two paragraphs.

If the return of V_{rev} to more negative values is due to acidification, then a larger negative shift should be observed when there is a larger inward driving force on H^+. This can be obtained either by lowering the pH of the bathing solution further or by making the holding voltage still more negative than E_{H^+}. Since most cells become unstable in very low pH solutions (<pH 5.5), we decided to modulate the size of the inward current of oocytes by varying the cell holding voltage while the cells were bathed in Barth's solution of pH 5.8 (Fig. 3). Holding oocytes at voltages more negative than −20 mV produced larger inward currents, accelerated the onset and rate of current decrease, and resulted in a faster rate of negative shift of V_{rev} over time (Fig. 3*A*). It was also found that V_{rev} could be directed to more positive values following the shift in V_{rev} in low external pH by making the holding voltage more
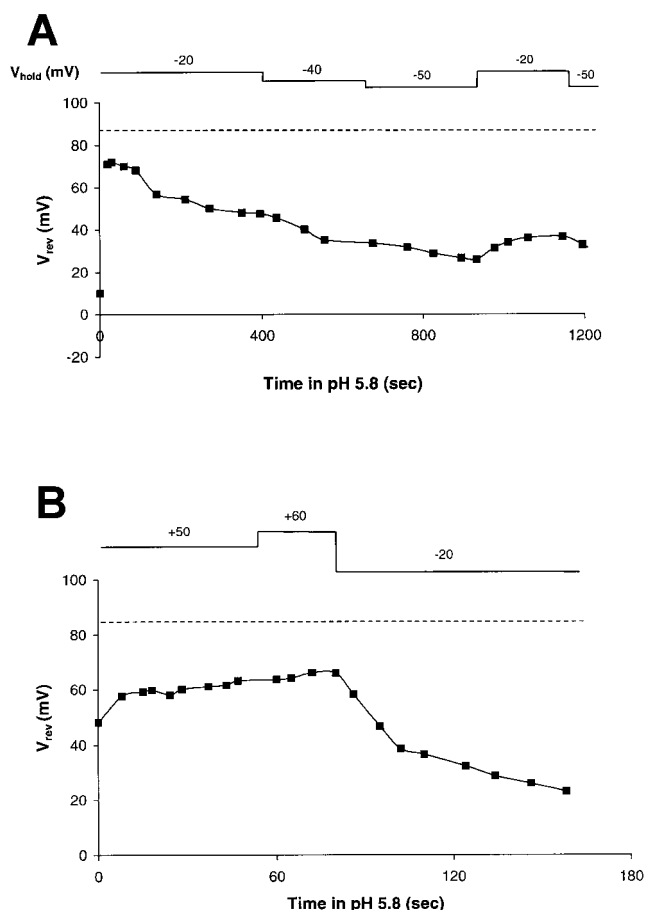
FIG. 3. **Alteration of V_{rev} as a result of manipulation of holding potential.** *A,* holding potential of an oocyte expressing the M₂ protein was adjusted to each of several values (*top record*) and V_{rev} was measured using voltage ramps as shown in Fig. 1*B*. *B*, holding the membrane at +50 mV after reducing pH_{out} from pH 8.5 to pH 5.8 prevented V_{rev} from returning to more negative values. Note that V_{rev} was closer to E_{H^+} for holding potentials closer to E_{H^+} and that V_{rev} was able to be manipulated toward both more negative and more positive voltages. Holding at a membrane voltage of −20 mV caused a rapid decrease in V_{rev}. The interrupted line in *A* and *B* shows E_{H^+} calculated from the known pH_{out} and the pH_{in} measured with an intracellular pH electrode.

positive (Fig. 3*A*). Furthermore, the return of V_{rev} to negative values could be prevented by holding membrane voltage at a large positive value that was close to E_{H+} (Fig. 3*B*). These results demonstrate that V_{rev} can be manipulated by holding voltage, consistent with the current that flows at the holding voltage being capable of altering pH_{in} of the cell.

To minimize the acidification of M_2-expressing cells in low pH solutions, the intracellular buffer concentration was increased. Again, as the ionic composition of the ooplasm could not be controlled, this experiment was performed in M_2-expressing CV-1 cells using the whole cell patch clamp technique. We studied the effect of elevated concentrations of the buffer of the pipette solution. The results obtained from pipettes containing 15 and 120 mM HEPES buffer on V_{rev} and the amplitude of the inward current as pH was lowered from pH 7.4 to pH 6.2 were compared. It was observed that for both 15 and 120 mM buffer in the pipette, lowering the external pH led to an increase of inward current in cells held at -20 mV within a few seconds. It was also observed that for both 15 and 120 mM buffer in the pipette, after lowering the external pH, the amplitude of the inward current reached a peak and then decreased within a few seconds, and during the decrease of inward current V_{rev} returned to more negative voltages. Thus, changes of V_{rev} could not be prevented by increasing the intracellular buffer concentration. Reports in the literature show that in order to control intracellular pH adequately, even with high concentrations of buffer in the pipette solution, the pipette diameter must be at least ⅓ of the diameter of the cell (28). The pipette diameters used in our experiments were on average 3–4 μm diameter, and the CV-1 cells from which we recorded were ~100–150 μm diameter. The small ratio of pipette diameter to cell diameter used in these experiments probably explains why even a high buffer concentration did not stabilize V_{rev}, consistent with poor stabilization of pH_{in}. Taken together, these results indicate that the consistent change of V_{rev} to more negative values is the result of cell acidification while bathed in solutions of low pH.

Measurements of pH_{in} during Exposure to Low pH_{out}

It was found with direct measurements of pH_{in} that the return of V_{rev} to negative values observed in M_2-expressing cells bathed in solutions of low pH was indeed accompanied by cell acidification.

For Oocytes—Intracellular pH was measured using an electrode, and the cells were voltage-clamped to measure the membrane current, V_{rev}, and membrane conductance (Fig. 4*A*). Lowering the pH_{out} from pH 8.5 to pH 5.8 produced a large inward current and a rapid shift of V_{rev} to more positive values, as noted earlier (Fig. 1). Immediately after reaching a peak, the amplitude of the inward current began to decrease. The V_{rev} reached a peak value prior to the amplitude of the inward current and returned to more negative voltages as the amplitude of the inward current decreased. Measurement of pH_{in} during this time (Fig. 4*A*) revealed that pH_{in} did not change immediately after introduction of low pH bathing solution, despite the presence of a detectable inward current. However, after ~120 s bathing in low pH solution, just after the amplitude of the inward current reached a maximum, pH_{in} began to decrease steadily. The onset of this acidification lagged the decrease of inward current and negative shift of V_{rev} by ~100 s. Plots of V_{rev} *versus* pH_{in} (Fig. 4*B*) revealed that the initial shift of V_{rev} to more positive values and subsequent return to more negative values occurred independently of changes in pH_{in} that were recorded with a pH microelectrode. After ~150 s in the low pH solution, the observed changes in V_{rev} and pH_{in} occurred together. Recovery of pH_{in} to control values was ob-

served both when the pH of the bathing solution was returned from pH 5.8 to pH 8.5 and also when 100 μM amantadine was added to the solution (pH 5.8). The recovery of pH under both of these conditions followed an attenuation of the inward current. These results demonstrate that oocytes expressing the M_2 protein acidify when there is a large inward H^+ current. This H^+ influx causes an acidification of the cytoplasmic solution accessible to the pH microelectrode after a delay with respect to the time when V_{rev} reaches its peak value. These results are consistent with a diffusional delay for H^+ in the cytoplasm of the expressing cells, between the membrane and the location of the tip of the pH electrode. This would result in a delay between a decrease of pH at the cytoplasmic opening of the pore of the M_2 channel, which determines V_{rev}, and the decrease of pH at the tip of the pH microelectrode.

For CV-1 Cells—pH_{in} was measured by ratiometric imaging of the fluorescence of the pH-sensitive indicator BCECF. Cells were infected with rSV40-M_2 and loaded with BCECF-AM prior to measuring fluorescence. The pH_{in} of M_2-expressing cells measured in medium of pH 7.4 was pH 6.87 ± 0.81 S.E. ($n = 8$) in one experiment and pH 7.04 ± 0.09 S.E. ($n = 5$) in a second experiment. When the pH of the bathing medium was lowered from pH 7.4 to pH 6.2 for ~200 s, the M_2-expressing cells underwent a rapid decrease in pH_{in} by 0.63 pH units ($\pm$ 0.056 pH units S.E., $n = 8$) in one experiment and by 1.07 pH units ($\pm$ 0.085 pH units S.E., $n = 4$) in a second experiment. These changes were reversible upon return to bathing medium of pH 7.4 (Fig. 5*A*). Cells treated with amantadine did not undergo this change in pH_{in} when bathed in medium of pH 6.2. The membrane voltage of the CV-1 cells expressing the M_2 protein was not clamped in these measurements, and thus it was possible that alterations of driving force might have influenced the membrane proton currents. To control membrane voltage, we took advantage of the potassium ionophore valinomycin to help maintain the membrane voltage at a value determined by the ratio of $[K^+]$ across the membrane. This was done by introducing valinomycin (20 μM final concentration in 0.02% Me_2SO carrier; exposure to carrier alone produced no changes in fluorescence) into the bathing medium. It was found that the rate of acidification increased for lower $[K^+]$ of the bathing medium, consistent with increased driving force for protons caused by a more negative membrane potential (Fig. 5*B*).

The systematic variation of V_{rev} observed with both M_2-expressing oocytes and CV-1 cells has important implications for determining the ion selectivity of this channel. When measured in low pH solutions, the value obtained for V_{rev} will depend upon the time when it is measured. This time dependence of V_{rev} is probably the result of acidification of the bulk solution accessible to the cytoplasmic mouth of the pore of the M_2 ion channel. Thus, even the most appropriate measurement, that of the peak value of V_{rev}, is likely to be distorted by acidification.

Ion Substitution Studies

The possibility that the inward current of the M_2 ion channel might in part be carried by ions other than the proton was tested by replacing other extracellular ions with large, presumably impermeant, ions. We tested for Na^+ permeability by replacing Na^+, the major extracellular cation, with other ions. These experiments were performed in oocytes. We also replaced NaCl with mannitol. Changes in the peak V_{rev}, conductance, and pH were measured after reducing pH of the bathing medium. The principle of this experiment is that if Na^+ normally flows through the channel, replacing Na^+ with an impermeant cation should decrease the amplitude of the inward
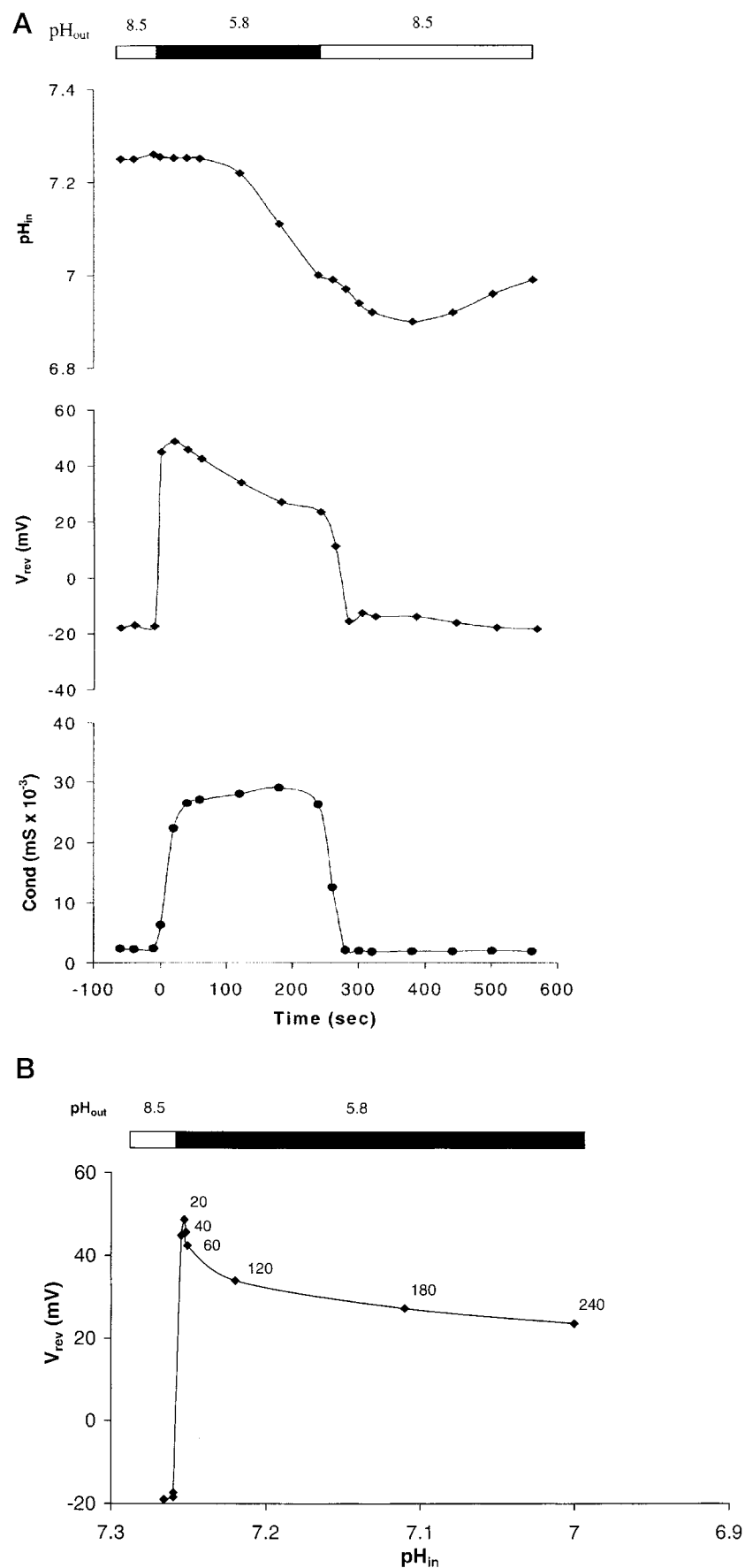
FIG. 4. **Measurement of pH_{in}, V_{rev}, and membrane conductance of an oocyte expressing the M_2 channel as it was exposed to a bathing solution of low pH.** *A,* time course of the changes. Note that pH_{in} (*top graph*) decreased after V_{rev} (*middle graph*) reached its peak value while conductance (*lower graph*) increased steadily during exposure to low pH bathing medium. *B,* plot of V_{rev} against pH_{in} for each of the times shown (in seconds) after lowering pH_{out} from pH 8.5 to pH 5.8. The relationship displayed three phases as follows: (i) from 0 to 20 s, immediately after pH_{out} was reduced, V_{rev} reached a peak value, whereas pH_{in} changed very little; (ii) from 20 to 120 s when V_{rev} returned to more negative values with a small (~0.1 pH unit) change in pH_{in}; and (iii) for $t > 120$ s when pH_{in} changed slowly as V_{rev} reached its plateau value.

FIG. 5. **Measurement of pH$_{in}$ of CV-1 cells that expressed the M$_2$ protein using fluorescence of BCECF.** *A,* the pH$_{in}$ of cells loaded with BCECF decreased when the pH of the control bathing medium (♦, time course in *upper bar*) was decreased from pH 7.4 to pH 6.2 and returned to normal values when pH of the bathing medium was returned to pH 7.4. When bathed in solution containing amantadine (*amant*) (100 μM, ■, time course *upper bar*) at pH 6.2 the pH did not decrease. Acidification did, however, occur as a result of treatment with FCCP (△, time course *lower bar*), following treatment with amantadine. Shown is the average pH from four M$_2$-expressing cells; error bars were omitted for clarity; the largest S.E. was 0.12 pH unit. *B,* changes of pH$_{in}$ for cells that were treated with K$^+$ ionophore valinomycin (*valin*) to stabilize membrane voltage according to the membrane K$^+$ gradient. Note that the acidification was more rapid for membrane voltage of approximately −80 mV (3 mM K$^+$$_{out}$, △, time course in *lower bar*) than for approximately −20 mV (60 mM K$^+$$_{out}$ ♦, time course in *upper bar*), and that lowering pH, in the presence of amantadine (*amant*) (100 μM, ■, time course *upper bar*), did not alter pH$_{in}$. Shown are average pH changes from seven M$_2$-expressing cells; error bars are omitted for clarity.
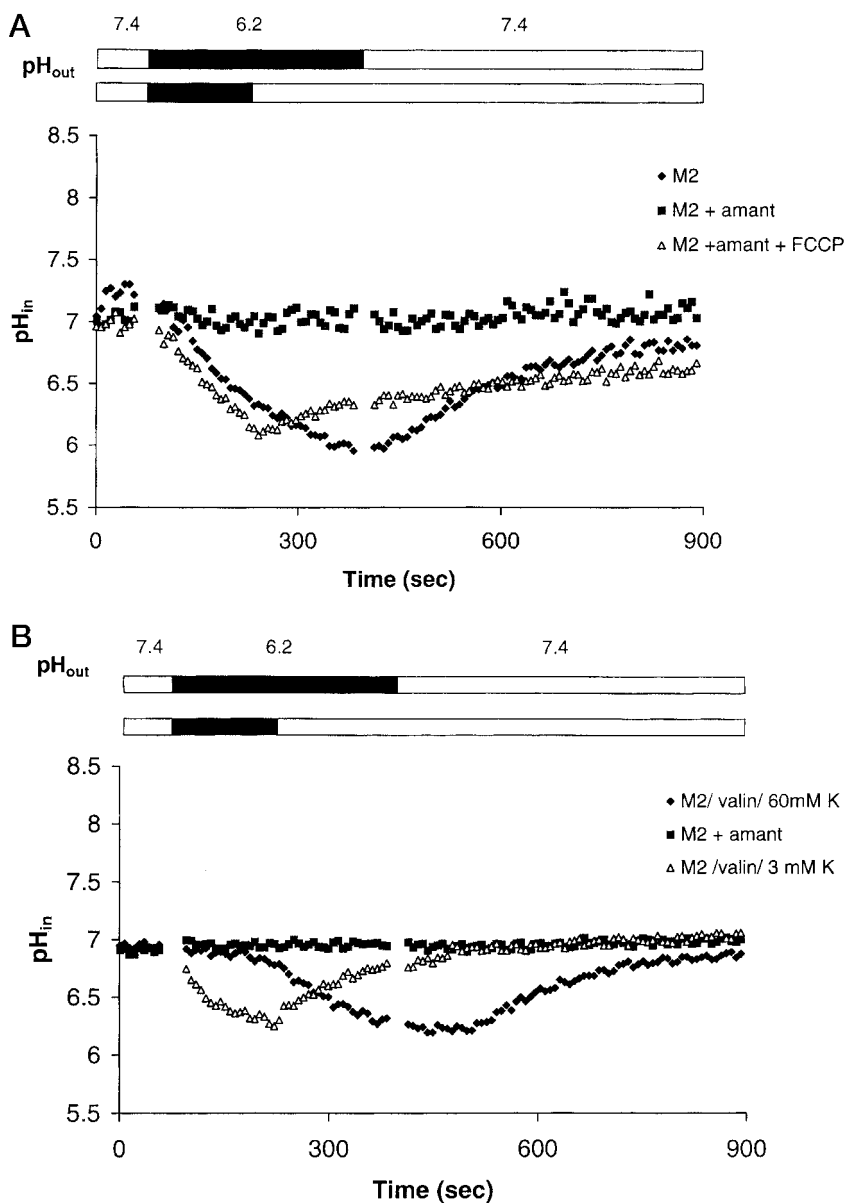
current but not change the acidification rate. NaCl in the extracellular medium was replaced with equimolar concentrations of *N*-methyl-D-glucamine Cl, LiCl, NH$_4$Cl, or iso-osmotically with mannitol. To determine the effect of these Na$^+$ substitutions, we first measured pH$_{in}$, conductance, and V$_{rev}$ in the control solution (containing NaCl), at both pH 8.5 and pH 5.8. This was followed by measurements in a pH 5.8 solution in which NaCl was replaced. Finally, the measurements were repeated in the control solution at pH 5.8 to check for reversibility before applying 100 μM amantadine. Oocytes were bathed in control solution of pH 8.5 between exposures to low pH solutions to allow recovery from intracellular acidification. A full recovery of pH$_{in}$ to control values typically took 15–20 min in the pH 8.5 solution. As the M$_2$ ion channel is closed at this pH and there was no residual current, it is thought that the restoration of pH$_{in}$ to control values was the result of a non-electrogenic endogenous H$^+$ exchanger.

It was found that substitution of Na$^+$ with large, presumably impermeant, cations such as NMDG$^+$ or replacing NaCl iso-osmotically with mannitol had no detectable effect on peak V$_{rev}$, conductance, or oocyte acidification rate in low pH solutions (Table I). This result demonstrates that the M$_2$ ion channel does not conduct detectable amounts of Na$^+$ ions. However,

TABLE I
The effect of Na$^+$ replacement on M$_2$ ion channel activity

Note that changes in all three variables occurred when Na$^+$ was replaced by NH$_4^+$ and changes in acidification rate and conductance occurred when Na$^+$ was replaced by Li$^+$. NMDGCl is *N*-methyl-D-glucamine.

Solution	Change in peak V$_{rev}$ (*x*-Na$^+$) mV	Ratio acidification rate (*x*/Na$^+$)	Ratio conductance (*x*/Na$^+$)
NMDGCl	−2.40 ± 2.8 (4)	1.12 ± 0.56 (4)	1.18 ± .005 (4)
Mannitol	2.72 ± 1.12 (11)	0.99 ± 0.11 (4)	1.11 ± 0.04 (11)
LiCl	5.36 ± 1.72 (18)	0.77 ± 0.05 (9)[a]	0.53 ± .025 (18)[a]
NH$_4$Cl	19.10 ± 4.18 (26)[a]	2.35 ± 0.50 (6)[a]	1.50 ± .085 (26)[a]

[a] Value denotes a significant difference (*p* < 0.05).

when NaCl was replaced with LiCl or NH$_4$Cl the results differed oppositely from those in control solutions. Replacement of NaCl with LiCl decreased conductance and acidification rate (Table I) but had no detectable effect on the peak V$_{rev}$ (Fig. 6*A*), and the peak of V$_{rev}$ occurred at about the same time as it did in Na$^+$-containing solutions, about 20 s after changing solutions. Replacement of NaCl with NH$_4$Cl, on the other hand, increased conductance, increased acidification rate (Table I), and shifted V$_{rev}$ to potentials more positive than those observed
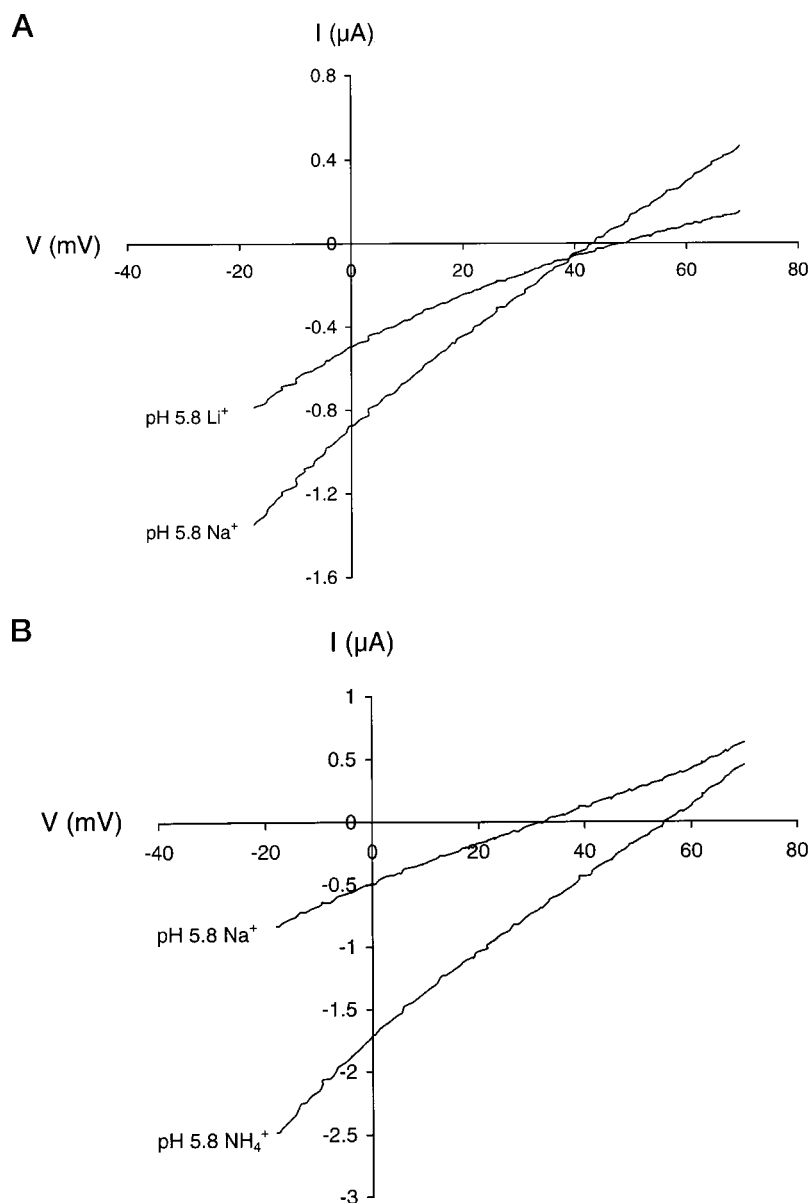
A

B

FIG. 6. **Effect of replacement of Na$^+$ in the bathing medium with Li$^+$ (*A*) or NH$_4^+$ (*B*) on the current-voltage relationship of oocytes expressing M$_2$ protein.** Note the decrease in conductance for the Li$^+$ substitution and the increase in both conductance and V$_{rev}$ for the NH$_4^+$ substitution. Measurements were made 20 s after changing solutions, at the time when V$_{rev}$ reached its peak value (see Fig. 1).

in NaCl (Fig. 6*B*). The peak of V$_{rev}$ also occurred in NH$_4^+$-containing solutions at about the same time as it did in Na$^+$-containing solutions, about 20 s after changing solutions. This increase in conductance was fully sensitive to amantadine (100 μM) and control uninjected oocytes exposed to NH$_4^+$-containing solutions at pH 6.2 or lower did not display inward currents (in contrast, we have found that oocytes bathed in NH$_4^+$ containing solutions at pH 7.5 or above exhibit large endogenous currents (29)). These results can be interpreted by either Li$^+$ and NH$_4^+$ replacing Na$^+$ in permeating the pore, flowing independently through the pore, or Li$^+$ actually interfering with conduction through the pore. To distinguish among these possibilities, low external buffer concentrations were used to limit specifically the component of current carried by protons, I$_{H^+}$.

Currents Measured with Low External Buffer Concentration to Limit I$_{H^+}$

Advantage was taken of the limitation of H$^+$ currents that can be achieved for M$_2$-expressing cells by reducing the buffer concentration of the bathing medium (23). This means was used to limit H$^+$ currents to determine if the alterations in amplitude of the M$_2$ current we observed with Li$^+$ and NH$_4^+$ were due to an effect on H$^+$ currents or due to an effect on other

ionic currents. The limitation of H$^+$ currents from low external buffer concentration results from a decrease in the [H$^+$] near the extracellular mouth of the pore of the M$_2$ ion channel (23). This decrease in [H$^+$] is reflected in a decrease of current seen during a 2-s-long voltage clamp pulse (Fig. 7). If ion substitution inhibits H$^+$ conduction through the channel, then the decrement in amplitude of the inward current during a voltage clamp pulse applied while bathed in a solution of low buffer concentration should be proportional to the decrease of current due to the ion replacement. If, on the other hand, the replacing ion permeates the channel by a mechanism independent of H$^+$ conduction, then the decrement in amplitude of the inward current while bathed in a solution of low buffer concentration should be unaffected by the replacement. The decrease in amplitude of the inward M$_2$ current was recorded during a 2-s hyperpolarizing voltage clamp pulse to -120 mV from a holding voltage of -20 mV at pH 5.8 in the presence of low (0.15 mM) buffer concentration in the bathing medium. We measured the diminution in current amplitude during the pulse and the final current amplitude at the end of the pulse in low buffer, Na$^+$-containing medium. We then measured these variables in low buffer media in which Na$^+$ was replaced by Li$^+$ or NH$_4^+$, and we compared the values. Replacement of Na$^+$ by Li$^+$ in a
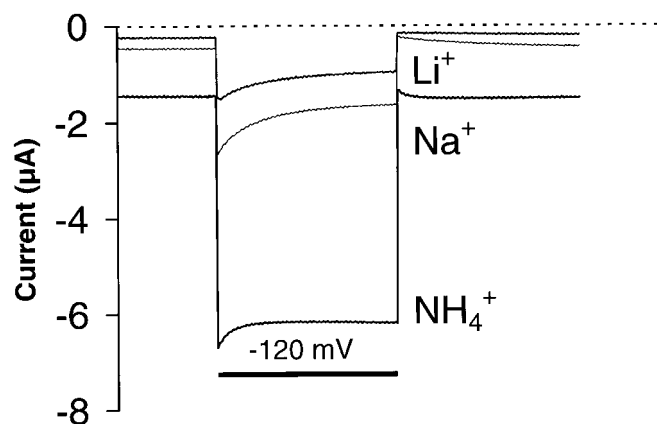
FIG. 7. **Effect of reducing concentration of the buffer in the bathing solution on the currents of oocytes expressing the M₂ protein that were bathed in media containing Na⁺, Li⁺, and NH₄⁺.** The decrease of current amplitude during the 2-s-long pulse serves as a measure of proton current, independent of the current of other ions, as the decay only occurs when the buffer capacity of the outside solution is lowered. Note that the reduction of current in 0.15 mM buffer during a 2-s voltage clamp pulse from a holding potential of −20 to −120 mV was less for oocytes bathed in Li⁺ than for oocytes bathed in Na⁺ and that the reduction in amplitude persisted in NH₄⁺-containing solutions. Note also the smaller final current amplitude at the end of the hyperpolarizing pulse in solutions containing Li⁺ compared with those containing Na⁺ or NH₄⁺.

solution containing 0.15 mM buffer caused a decrease in the steady-state current amplitude, measured at the end of the hyperpolarizing pulse (to $64 \pm 2.7\%$ S.E., $n = 10$). We found that the diminution of current amplitude during the 2-s voltage pulse was reduced proportionally (to $63 \pm 4.5\%$ S.E., $n = 10$) in a medium of low buffer concentration (Fig. 7). This result is consistent with Li⁺ interfering with proton conduction through the M₂ ion channel.

Replacing Na⁺ with NH₄⁺, on the other hand, caused an increase in the steady-state current amplitude in low buffer, measured at the end of the hyperpolarizing pulse (to $315 \pm 20\%$ S.E., $n = 8$). However, the buffering capacity of this solution, which contained 0.15 mM MES at pH 6.2, was unavoidably increased by the presence of 88 mM ammonium buffer (to 0.66 mM total buffering capacity at pH 5.8). The decrement of current amplitude during the voltage pulse was reduced to $61 \pm 5.5\%$ S.E. ($n = 8$) of that observed in Na⁺-containing low buffer (0.15 mM) solutions (Fig. 7), an effect that could be attributed to the modest increase of buffering capacity imparted by the 88 mM NH₄⁺Cl solution (Mould *et al.* (23)). However, the large increase in final current amplitude in the NH₄⁺-containing solution cannot be explained by this modest increase in buffer capacity and demonstrates that an additional current flows which is not affected by external buffer concentration. Similar results were obtained when Na⁺ was replaced with NH₃OH⁺ (data not shown). Finally, replacement of Na⁺ with NMDG⁺ and NaCl with mannitol had no detectable effect on either the final current amplitude at the end of the pulse or the diminution of current during voltage pulses in low external buffer concentration (data not shown).

Comparison of M₂-specific Oocyte Acidification with That Obtained from "Pure" Proton Currents Using an Electrogenic Protonophore

A final way that we tested the ion selectivity of the M₂ ion channel was by comparing the acidification rate, normalized to the amplitude of the inward current, of M₂-expressing cells bathed in solutions of low pH with that obtained in cells exposed to the electrogenic protonophore FCCP (30). If the M₂ channel conducts only protons, then for a given inward current

amplitude the acidification rate of an oocyte expressing the M₂ protein at low pH should be equal to that observed in the presence of FCCP.

Oocytes Treated with FCCP—Membrane current, V_{rev}, and pH_{in} over time were measured in uninjected oocytes bathed in pH 5.8 Barth's solution in the presence or absence of 20 μM FCCP (in 0.02% Me₂SO carrier; exposure to carrier alone produced no membrane currents). Oocytes clamped at a holding voltage of −20 mV developed an inward current at low pH after FCCP was added to the bathing medium (Fig. 8*A, upper record*). The amplitude of the inward current of FCCP-treated cells was generally less than that of M₂-expressing cells studied at the same pH (compare *upper* and *lower records* of Fig. 8*A*), but it was found that applying higher concentrations of FCCP resulted in deterioration of the condition of the cells. The inward current normally appeared within 1 min of exposing cells to FCCP, a delay that was probably due to the time required for incorporation of FCCP into the oocyte plasma membrane. V_{rev} measured using voltage ramps shifted within a few seconds to positive potentials after the pH of the bathing medium was reduced (Fig. 8*B, lower record*). As observed for M₂-expressing oocytes held at −20 mV, the V_{rev} of oocytes exposed to FCCP (70.4 ± 1.6 mV S.E., $n = 10$) was close to E_{H+} predicted from the known pH_{out} and pH_{in} measured with an intracellular pH electrode (81.8 ± 5.2 mV S.E., $n = 10$). After reaching a maximum, V_{rev} began to return to more negative potentials, and the current began to decrease in amplitude a few minutes after the pH of the bathing medium was decreased (Fig. 8). The pH_{in} of oocytes treated with FCCP did not begin to decrease until about 100 s after the changes in current and V_{rev} occurred (Fig. 8*B, upper record*). One important difference was noted between the behavior of oocytes expressing the M₂ protein and those into which FCCP had been incorporated. When the pH of the bathing solution of FCCP-treated oocytes was returned to pH 8.5 following oocyte acidification, a large, transient outward current appeared (Fig. 8*A, upper record*). The appearance of this transient outward current was accompanied by an overshoot of V_{rev} to potentials more negative than those observed before lowering pH of the bathing medium (Fig. 8*B, lower record*). This was in contrast to the measurements of M₂-expressing oocytes for which no outward current flowed upon return to bathing medium of pH 8.5 after prolonged bathing in low pH medium (Fig. 8*A, lower record*). The incorporation of FCCP into the plasma membrane appeared to be reversible, as re-exposure of oocytes to medium of pH 5.8 following washout of FCCP in medium of pH 8.5 failed to produce an inward current (data not shown).

The maximal rate of acidification, normalized to the maximum inward current amplitude, of M₂-expressing oocytes was compared with the same value obtained from uninjected oocytes treated with the FCCP ionophore. It was found that the ratio of maximal rate of acidification to maximal inward current to be similar in both cases (0.085 ± 0.006 pH unit/min/μA for FCCP-treated cells, $n = 8$, *versus* 0.081 ± 0.11 pH unit/min/μA for M₂-expressing cells, $n = 20$).

CV-1 Cells Treated with FCCP—We performed two types of experiments. In the first type of experiment CV-1 cells that did not express the M₂ protein were employed, and membrane currents were measured at low pH_{out} in the presence of FCCP (Fig. 9). In the second type of experiment, we studied the effect of FCCP treatment on the pH_{in} of M₂-expressing cells after inhibiting M₂ currents with amantadine (Fig. 5*A*). The results of both types of experiments were similar to those obtained with oocytes. Lowering the pH of FCCP-treated cells that did not express the M₂ protein from pH 7.4 to pH 6.2 resulted in an inward current flow accompanied by an initial increase in V_{rev}

A

B
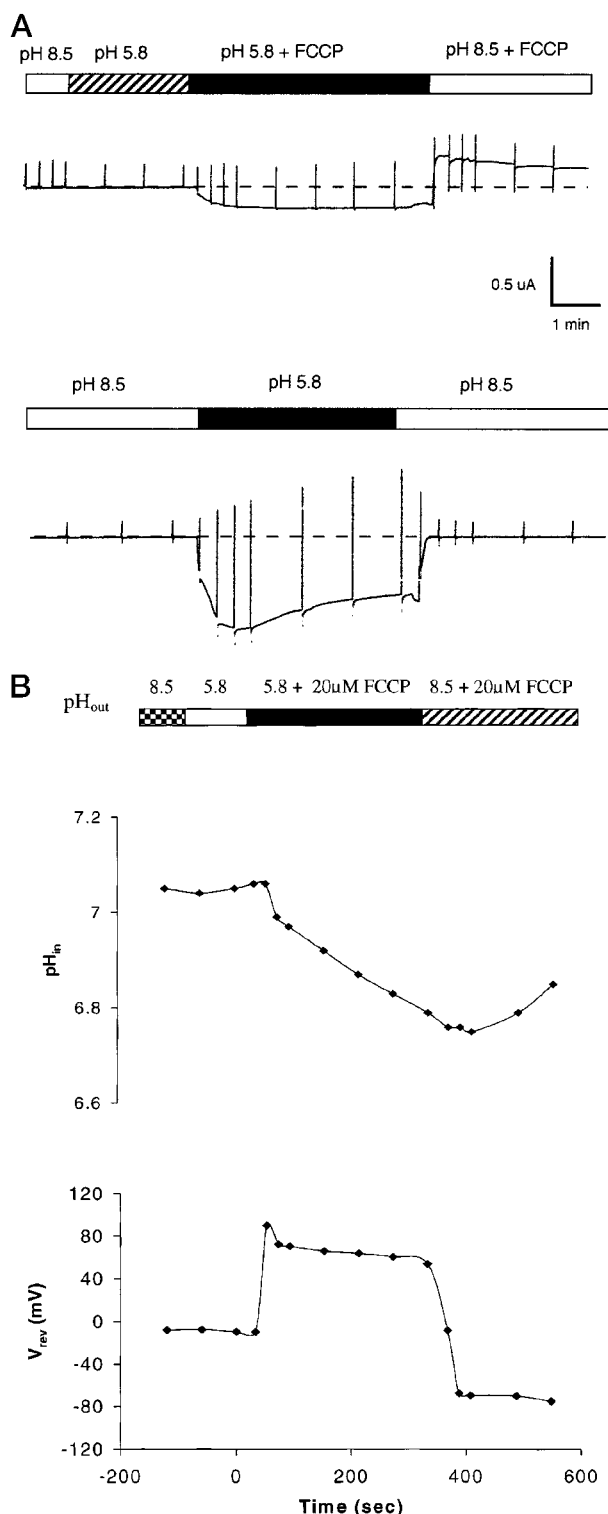
FIG. 8. **Effects of FCCP on oocytes.** *A,* comparison of effects of lowering pH of the bathing medium on the membrane currents of uninjected oocytes exposed to FCCP (*upper record*) and oocytes expressing the M$_2$ protein (*lower record*). Note that when pH$_{out}$ was decreased from pH 8.5 to pH 5.8 the uninjected cell did not display an inward current until FCCP was introduced. Upon return to pH$_{out}$ of pH 8.5, the FCCP-exposed cell developed a large outward current (*upper record*), whereas the M$_2$-expressing cell did not (*lower record*). The *interrupted line* shows the zero current. *B,* pH$_{in}$ and V$_{rev}$ of oocytes treated with FCCP. Time course of pH$_{in}$ and V$_{rev}$ measured in a single uninjected oocyte when pH$_{out}$ was lowered from pH 8.5 to pH 5.8. Note the decrease of pH$_{in}$ after lowering pH$_{out}$ to pH 5.8 only occurred in the presence of FCCP. Note the recovery of pH$_{in}$ when pH$_{out}$ was returned to pH 8.5. Full recovery occurred but is not shown. Note also that V$_{rev}$ increased to a peak positive value after FCCP was applied and returned to more
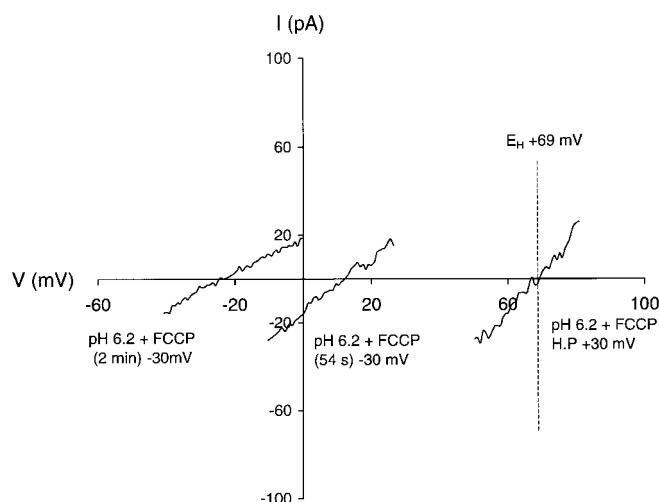
FIG. 9. **Current-voltage relationships of an uninfected CV-1 cell at various times after treatment with 20 μM FCCP at pH 6.2.** Note that V$_{rev}$ was near E_{H+} when cells were held at +30 mV and that V$_{rev}$ became negative when the membrane voltage was held at −30 mV for the times indicated.

to positive voltages and an increase in conductance (Fig. 9). If the holding voltage was adjusted to give very little inward current while the cells were bathed in low pH medium, the measured V$_{rev}$ was 65.8 ± 3.5 mV S.E. ($n = 6$). This value was close to the 69.6 mV value of E_{H+} calculated from the known pH$_{out}$ and an assumed pH$_{in}$ of pH 7.4. However, when the holding voltage was made more negative, the V$_{rev}$ quickly returned to more negative voltages (Fig. 9).

The effect of FCCP on pH$_{in}$ was also measured in CV-1 cells (Fig. 5*A*). In this experiment, M$_2$-expressing cells were exposed first to pH 6.2 for ~200 s and then allowed to recover from acidification in a bathing medium of pH 7.4. The cells were then reexposed to pH 6.2 in the presence of 100 μM amantadine, and in this solution acidification did not occur. Finally, the same cells were exposed to pH 6.2 medium in the presence of FCCP for 200 s. This treatment resulted in acidification once again. Finally, the cells were allowed to recover from acidification in medium of pH 7.4 + FCCP. Bathing FCCP-treated cells in solution of pH 6.2 resulted in an acidification that occurred with a slightly more rapid time course than that observed for M$_2$-expressing cells (Fig. 5*A*). The pH$_{in}$ of the M$_2$-expressing cells was pH 6.96 ± 0.15 pH units S.E. ($n = 5$) at pH 7.4$_{out}$ before application of FCCP, and pH$_{in}$ decreased by 0.88 ± 0.095 pH units ($n = 5$) after reducing pH$_{out}$ from pH 7.4 to pH 6.2 in the presence of FCCP. As also observed with oocytes, these changes of pH$_{in}$ were reversible upon return to bathing medium of pH 7.4. It was not possible to study CV-1 cells in solutions of pH 8.5 because irreversible changes occurred at this alkaline pH value. These two experiments demonstrate that acidification of CV-1 cells can also be achieved by treatment with FCCP and that acidification results from a mechanism that is not affected by the presence of the M$_2$ protein.

DISCUSSION

This study confirms the very high proton selectivity of the M$_2$ ion channel under physiological conditions, demonstrates that Li$^+$ inhibits the channel, and provides additional evidence that the channel allows the permeation of quaternary ammonium ions, probably by a different mechanism than that for H$^+$. These results also provide evidence for the restricted diffusion

negative values during exposure to FCCP at low pH$_{out}$, and the overshoot of V$_{rev}$ to very negative potentials after return to pH$_{out}$ to pH 8.5.

of H$^+$ in the cytoplasm of oocytes and support the notion that the M$_2$ channel is gated by changes in pH$_{out}$.

These results are consistent with the proposed roles for M$_2$ ion channel in the life cycle of the influenza A virus. However, the only known ion channel encoded by influenza A virion is the M$_2$ ion channel, and a pure proton conductance for the M$_2$ channel would depolarize the virion membrane. This depolarization would elevate virion membrane potentials to high enough values to risk dielectric breakdown of the membrane and decrease the driving force on protons. Thus, this depolarization would limit the extent of virion acidification that is possible. A simple calculation can estimate the number of protons that could flow into the virion without causing an excessive virion membrane potential that would lead to dielectric breakdown of its lipid bilayer. If we assume that the virion starts with a membrane potential of about -70 mV (the resting potential of many epithelial cells) and assume that it can withstand a membrane voltage of $+100$ mV, then about $\sim 2.5 \times 10^{-22}$ Eq of protons will be able to enter the virion during acidification, changing its membrane voltage by altering the charge across its membrane capacitance, before placing the virion membrane in danger of dielectric breakdown. Assuming that the volume of the virion is about 1.5×10^{-19} liters and that the interior of the virion is well buffered, the pH inside the virion will decrease by only a few tenths of a pH unit if the pH decrease is distributed uniformly throughout the interior of the virion. The reason that the calculated pH decrease is so small is that every proton that enters the virion carries a charge that increases the membrane voltage of the virion, but only a small fraction of the entering protons contribute to the free proton concentration (lower pH) because the buffering capacity of the virion proteins can sequester the majority of the protons. Thus, the calculated decrease of pH of the virion, if it occurred uniformly throughout the virion, would only lower pH of the virion from an assumed initial value of approximately pH 7.4 to a value slightly higher than pH 7.0. This decrease of pH is inadequate to cause release of ribonucleoprotein complexes from the M1 matrix protein (31) contained within the entire virion. Perhaps the acidification of only a zone of the virion immediately below the virion surface membrane might be adequate (32, 33), as this is the zone where the M1 protein is concentrated. It is intriguing that we found evidence for a localized pH decrease in the zone near the cytoplasmic entrance to the pore of the M$_2$ channel in oocytes.

The Difficulty of Determining M$_2$ Ion Selectivity by Comparing V$_{rev}$ with E$_{H+}$ Calculated from Measured pH$_{in}$—In a previous study Shimbo *et al.* (12) found that when M$_2$-expressing oocytes were bathed in a solution of low pH, V$_{rev}$ was 20–30 mV more negative than the value predicted from E_{H+} calculated from the pH$_{in}$ measured with a micro pH electrode and the pH of the bathing solution. In that study, V$_{rev}$ measurements were made ~ 2 min after the oocytes were exposed to bathing solutions of low pH, at approximately the time when the amplitude of the inward membrane current reached a maximum value, to ensure adequate equilibration of the low pH solution. The relative permeability of H$^+$ to that of Na$^+$ was found to be about 10^5. In the present study it was found that upon lowering extracellular pH, V$_{rev}$ measured in M$_2$-expressing oocytes shifted within a few seconds toward positive potentials near E_{H+} and then fell to more negative values before inward current reached its maximum amplitude (Fig. 1*B,* oocytes). Similar results were obtained in CV-1 cells that expressed the M$_2$ protein (Fig. 2, CV-1 cells). The return of V$_{rev}$ to more negative values was not the result of activating an endogenous current, as the shift still occurred in CV-1 cells expressing the M$_2$ protein in the absence of other conducting ions. The return of

V$_{rev}$ to negative values was also not due to inactivation of the M$_2$ ion channel, as the slope conductance increased as V$_{rev}$ became more negative. This finding is consistent with results obtained for HEK293 cells expressing the Kv2.1 delayed rectifier channel (34). In HEK293 cells the activity of the channel produces changes in $[K^+]_{in}$ that mimic inactivation and results in apparent changes in ion selectivity. By using the peak value of V$_{rev}$ and the pH$_{in}$ measured at the time of peak V$_{rev}$, the permeability of H$^+$ relative to that of Na$^+$ was found to be about $1.5–2.0 \times 10^6$, consistent with the values found by Chizhmakov and co-workers (5) and consistent with results from reconstituted M$_2$ protein in vesicles (35, 36). For CV-1 cells studied in the absence of Cl$^-$ and K$^+$ ions and two oocytes studied in control solution, the measured values of V$_{rev}$ and the calculated values of E_{H+} were within a few mV, consistent with a high proton permeability.

Two lines of evidence indicate that acidification of the cytoplasm of cells expressing the M$_2$ ion channel is the explanation for the return of V$_{rev}$ to more negative values, after reaching a peak positive value, when cells are bathed in medium of low pH. First, the rate of the return of V$_{rev}$ was smaller when cells were held at voltages closer to E_{H+} (Fig. 3). Second, for times longer than 120 s after introduction of the low pH bathing solution, the change in V$_{rev}$ and the change in pH$_{in}$ had a similar time course (Fig. 4).

Several observations indicate that H$^+$ diffusion in oocytes is restricted by the presence of immobile buffers (37–39). First, the decrease in pH$_{in}$ occurred with a delay after the onset of inward current (Fig. 4). Second, the return of V$_{rev}$ to negative values following the occurrence of its peak value near E_{H+} occurred prior to the change in pH$_{in}$ detected using an intracellular pH electrode (Fig. 4). The data presented here are also consistent with previous conclusions that it is very difficult to control the pH$_{in}$ of a cell when the diameter of the patch pipette is much smaller than that of the cell. Even with very high concentrations of buffers, the diameter of the patch pipette needs to be no less than one-third of the cell diameter in order to control pH$_{in}$ adequately (28). As the CV-1 cells used in our patch clamp experiments were 100–150 μm diameter and the largest pipettes we were able to use were 3–4 μm diameter, it would be expected that pH$_{in}$ was not well controlled in our experiments, even when high concentrations of buffer were used. Chizhmakov and co-workers (5) found that the V$_{rev}$ of M$_2$-expressing MEL cells was close to the value predicted for E_{H+} without taking special precautions to minimize I_{H+} at the holding voltage, and a change in V$_{rev}$ with time was not reported. The most likely reasons for these data are that the currents of these cells are much smaller than those of CV-1 cells, that MEL cells are rather small (~ 10 μm diameter) and thus are small in relation to the patch pipettes usually employed, and that the patch pipette contained high concentrations of buffer.

Ion Substitution and Low Buffer Studies—Two other ways that we tested the ion selectivity of the M$_2$ channel were to replace Na$^+$ in the bathing medium with other impermeant cations or NaCl iso-osmotically with mannitol and to reduce the buffer concentration in the bathing medium in order to limit the H$^+$ currents. The effects of these alterations were studied in M$_2$-expressing oocytes while the V$_{rev}$, acidification rate, and conductance were measured. Replacement of NaCl with *N*-methyl-D-glucamine or iso-omotically with mannitol had no detectable effect on V$_{rev}$, acidification rate, or conductance. In addition the decrease of current amplitude due to low external buffer concentration was unchanged by these substitutions. These results are consistent with the interpretation that the M$_2$ ion channel does not conduct detectable amounts of Na$^+$

ions under physiological conditions.

Replacement of NaCl with LiCl, on the other hand, decreased acidification rate, decreased conductance, and had no detectable effect on the peak V$_{rev}$ reached after decreasing the pH of the bathing solution, prior to the time when acidification of the cell was detectable (Table I and Fig. 6*A*). Although the decrease in conductance when Li$^+$ replaced Na$^+$ in the bathing medium could indicate the presence of a detectable initial Na$^+$ conductance, the finding that acidification rate decreased without a change in V$_{rev}$ is not consistent with this explanation. In addition, it was found that the decrease of current that resulted from decreased buffer concentration of the bathing medium was smaller when low buffer was applied in the bathing medium in which Na$^+$ was replaced by Li$^+$ (Fig. 7), suggesting that Li$^+$ substitution attenuated a H$^+$ current. Thus, these results are consistent with Li$^+$ inhibiting the proton conductance of the M$_2$ ion channel.

Although the H$^+$ current of the M$_2$ ion channel is the biologically relevant current, the fact that quaternary ammonium ions have been shown to flow independently of H$^+$ has important implications for the mechanism of permeation of the channel. Replacement of NaCl with NH$_4$Cl caused an increase in acidification rate, an increase in conductance, and a shift of V$_{rev}$ to even more positive potentials than those obtained in NaCl (Table I and Fig. 6*B*). There are several alternative interpretations for these results. First, NH$_4^+$ might traverse the M$_2$ channel, and, once inside the cell, H$^+$ might dissociate from NH$_4^+$, leaving highly membrane-permeable NH$_3$ to diffuse from the cytoplasm. The dissociation of NH$_4^+$ would acidify the cytoplasm of the expressing cell. Second, NH$_4^+$ might act as second proton source for the M$_2$ channel. We distinguished between these possibilities by lowering external buffer concentration to limit I_{H^+} (23). If NH$_4^+$ were acting as an extra proton source, then 88 mM NH$_4$Cl in the extracellular solution would act as a source of protons, much the same as an additional buffer in the bathing medium. If NH$_4^+$ acted in this way, we would not expect to observe reduced proton current upon lowering external buffer concentration. It was found that replacement of NaCl with NH$_4$Cl resulted in a slight decrease in the effect of low buffer concentration on the amplitude of the inward current during a hyperpolarizing pulse (Fig. 7). This decrease was equal to that predicted by the modest increase in buffer capacity of the 88 mM NH$_4$Cl solution used to replace NaCl. However, the large increase in final current amplitude at the end of the pulse in the low buffer, NH$_4^+$-containing solution could not be explained by a small increase in buffer capacity imparted by the 88 mM NH$_4$Cl solution. This suggests that the additional current observed in NH$_4^+$-containing solutions was not a H$^+$ current, and it is therefore likely that NH$_4^+$ itself traverses the M$_2$ ion channel.

Comparison of M$_2$ Currents with Those Obtained Using the FCCP Ionophore—Another way in which the ion selectivity of the M$_2$ channel was tested was by comparing the current and acidification rate of M$_2$-expressing cells with these variables for cells into which the electrogenic protonophore FCCP had been introduced. Addition of 20 μM FCCP to the extracellular medium produced a pH-dependent inward current in oocytes (Fig. 8) and CV-1 cells (Figs. 9). As observed for M$_2$-expressing cells, the peak V$_{rev}$ became very positive at low pH$_{out}$, and came close to E_{H^+} if holding voltage was adjusted to minimize inward current amplitude (Fig. 9). As found for M$_2$-expressing cells bathed in low pH medium, when FCCP-treated cells were held at negative holding voltages, V$_{rev}$ returned in less than 1 min to negative values (Fig. 9). The currents of FCCP-treated cells also caused acidification (Fig. 8*B*, oocytes; Fig. 5*A*, CV-1 cells). These results are consistent with the explanation that the

inward current was carried by H$^+$ and that acidification of the bulk medium accessible to the M$_2$ channel and the FCCP protonophore caused E_{H^+} to become more negative, resulting in a return of V$_{rev}$ to more negative values. When normalized to the maximum amplitude of the inward current, the rate of acidification induced by lowering pH$_{out}$ of FCCP-treated cells was comparable to that obtained with M$_2$-expressing cells. If the M$_2$ channel and the FCCP ionophore carried different ions, the rate of acidification normalized to current would differ between the two. This result suggests that the ion selectivity of the M$_2$ channel, measured under physiological conditions, is similar to that of FCCP and is thus highly proton-selective.

Evidence for Activation of the M$_2$ Channel by Low pH$_{out}$ and a Possible Role for His^{37}—We observed an important difference between cells expressing the M$_2$ ion channel and FCCP-treated cells. In both cases, bathing the cell in low pH solution resulted in acidification of the cytoplasm of the cell. However, for FCCP-treated oocytes, when the pH of the bathing solution was increased to pH 8.5 there was a large outward current, consistent with a reversal of the driving force on H$^+$ (Fig. 8*A, upper record*). This was in contrast to the findings from M$_2$-expressing cells that showed no detectable outward current upon return to pH 8.5 after exposure to low pH bathing medium (Fig. 8*A, lower record*). This finding is consistent with the M$_2$ channel being closed at pH 8.5$_{out}$. As the channel is activated by low pH$_{out}$, for both neutral and low values of pH$_{in}$, this result demonstrates that the channel is gated by pH$_{out}$, independent of pH$_{in}$. Mutants in which His^{37} is replaced have pH-independent activity and reduced selectivity for H$^+$ (24). Cysteine-scanning mutagenesis (25) and inhibition by Cu^{2+} (40) show that His^{37} is a pore-lining residue, suggesting that His^{37} is responsible for H$^+$ selectivity. One possible mechanism is that protonation of His^{37} at low pH might also be responsible for activation of the channel.

Implications of Ammonium Permeability for the Permeation Mechanism of the M$_2$ Ion Channel—Although ammonium and hydroxylamine ions are not the biologically relevant ions of the M$_2$ ion channel, the fact that they are capable of permeating the channel has important implications for the mechanism of M$_2$ ion channel conduction. On the basis of the evidence reviewed above, it has been postulated that the interactions of H$^+$ with His^{37} is essential for H$^+$ transport and H$^+$ selectivity. Permeation of ammonium and hydroxylamine ions would require a different, possibly independent, mechanism of permeation.

REFERENCES

1. Hay, A. J. (1992) *Semin. Virol.* **3,** 21–30
2. Lamb, R. A., Holsinger, L. J., and Pinto, L. H. (1994) in *Receptor-mediated Virus Entry into Cells* (Wimmer, E., ed) pp. 303–321, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
3. Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1992) *Cell* **69,** 517–528
4. Wang, C., Takeuchi, K., Pinto, L. H., and Lamb, R. A. (1993) *J. Virol.* **67,** 5585–5594
5. Chizhmakov, I. V., Geraghty, F. M., Ogden, D. C., Hayhurst, A., Antoniou, M., and Hay, A. J. (1996) *J. Physiol. (Lond.)* **494,** 329–336
6. Lamb, R. A., Zebedee, S. L., and Richardson, C. D. (1985) *Cell* **40,** 627–633
7. Holsinger, L. J., and Lamb, R. A. (1991) *Virology* **183,** 32–43
8. Panayotov, P. P., and Schlesinger, R. W. (1992) *Virology* **186,** 352–355
9. Sugrue, R. J., and Hay, A. J. (1991) *Virology* **180,** 617–624
10. Sakaguchi, T., Tu, Q., Pinto, L. H., and Lamb, R. A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 5000–5005
11. Holsinger, L. J., Nichani, D., Pinto, L. H., and Lamb, R. A. (1994) *J. Virol.* **68,** 1551–1563
12. Shimbo, K., Brassard, D. L., Lamb, R. A., and Pinto, L. H. (1996) *Biophys. J.* **70,** 1336–1346
13. Wang, C., Lamb, R. A., and Pinto, L. H. (1994) *Virology* **205,** 133–140
14. Kurtz, S., Luo, G., Hahnenberger, K. M., Brooks, C., Gecha, O., Ingalls, K., Numata, K.-I., and Krystal, M. (1995) *Anti. Agents Chem.* **39,** 2204–2209

15. Duff, K. C., and Ashley, R. H. (1992) *Virology* **190,** 485–489
16. Tosteson, M. T., Pinto, L. H., Holsinger, L. J., and Lamb, R. A. (1994) *J. Membr. Biol.* **142,** 117–126
17. Sugrue, R. J., Bahadur, G., Zambon, M. C., Hall-Smith, M., Douglas, A. R., and Hay, A. J. (1990) *EMBO J.* **9,** 3469–3476
18. Grambas, S., and Hay, A. J. (1992) *Virology* **190,** 11–18
19. Grambas, S., Bennett, M. S., and Hay, A. J. (1992) *Virology* **191,** 541–549
20. Ciampor, F., Thompson, C. A., Grambas, S., and Hay, A. J. (1992) *Virus Res.* **22,** 247–258
21. Takeuchi, K., and Lamb, R. A. (1994) *J. Virol.* **68,** 911–919
22. Sakaguchi, T., Leser, G. P., and Lamb, R. A. (1996) *J. Cell Biol.* **133,** 733–747
23. Mould, J. A., Li, H.-C., Dudlak, C. S., Lear, J. D., Pekosz, A., Lamb, R. A., and Pinto, L. H. (2000) *J. Biol. Chem.* **275,** 8592–8599
24. Wang, C., Lamb, R. A., and Pinto, L. H. (1995) *Biophys. J.* **69,** 1363–1371
25. Pinto, L. H., Dieckmann, G. R., Gandhi, C. S., Shaughnessy, M. A., Papworth, C. G., Braman, J., Lear, J. D., Lamb, R. A., and DeGrado, W. F. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 11301–11306
26. James-Kracke, M. R. (1992) *J. Cell. Physiol.* **151,** 596–603
27. Zhu, G., Zhang, Y., Xu, H., and Jiang, C. (1998) *J. Neurosci. Methods* **81,** 73–83
28. Byerly, L., and Moody, W. J. (1986) *J. Physiol. (Lond.)* **376,** 477–491
29. Burckhardt, B. C., and Fromter, E. (1992) *Pfluegers Arch.* **420,** 83–86
30. Benz, R., and McLaughlin, S. (1983) *Biophys. J.* **41,** 381–398
31. Zhirnov, O. P. (1992) *Virology* **186,** 324–330
32. Gregoriades, A., and Frangione, B. (1981) *J. Virol.* **40,** 323–328
33. Ruigrok, R. W., Barge, A., Durrer, P., Brunner, J., Ma, K., and Whittaker, G. R. (2000) *Virology* **267,** 289–298
34. Frazier, C. J., George, E. G., and Jones, S. W. (2000) *Biophys. J.* **78,** 1872–1880
35. Lin, T. I., Heider, H., and Schroeder, C. (1997) *J. Gen. Virol.* **78,** 767–774
36. Schroeder, C., Ford, C. M., Wharton, S. A., and Hay, A. J. (1994) *J. Gen. Virol.* **75,** 3477–3484
37. Junge, W., and McLaughlin, S. (1987) *Biochim. Biophys. Acta* **890,** 1–5
38. Irving, M., Maylie, J., Sizto, N. L., and Chandler, W. K. (1990) *Biophys. J.* **57,** 717–721
39. Stewart, A. K., Boyd, C. A., and Vaughan-Jones, R. D. (1999) *J. Physiol. (Lond.)* **516,** 209–217
40. Gandhi, C. S., Shuck, K., Lear, J. D., Dieckmann, G. R., DeGrado, W. F., Lamb, R. A., and Pinto, L. H. (1999) *J. Biol. Chem.* **274,** 5474–5482