Mechanism for Proton Conduction of the M₂ Ion Channel of Influenza A Virus*

The M₂ integral membrane protein of influenza A virus forms a proton-selective ion channel. We investigated the mechanism for proton transport of the M₂ protein in Xenopus oocytes using a two-electrode voltage clamp and in CV-1 cells using the whole cell patch clamp technique. Membrane currents were recorded while manipulating the external solution to alter either the total or free proton concentration or the solvent itself. Membrane conductance decreased by ~50% when D₂O replaced H₂O as the solvent. From this, we conclude that hydrogen ions do not pass through M₂ as hydronium ions, but instead must interact with titratable groups that line the pore of the channel. M₂ currents measured in solutions of low buffer concentration (<15 mM in oocytes and <0.15 mM in CV-1 cells) were smaller than those studied in solutions of high buffer concentration. Furthermore, the reversal voltage measured in low buffer was shifted to a more negative voltage than in high buffer. Also, at a given pH, M₂ current amplitude in 15 mM buffer decreased when pH-pKₐ was increased by changing the buffer pKₐ. Collectively, these results demonstrate that M₂ currents can be limited by external buffer capacity. The data presented in this study were also used to estimate the maximum single channel current of the M₂ ion channel, which was calculated to be on the order of 1–10 fA.

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 (1, 2). The M₂ protein consists of a 24-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 (3). Chemical cross-linking studies showed the M₂ protein to exist minimally as a homotetramer (4–6). More recently, statistical analysis of the ion channel activity of mixed oligomers indicated that a homotetramer is also the minimal active oligomeric form of the protein (7).

Despite the small size of the active M₂ oligomer, several pieces 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 (8–10), mammalian cells (11, 12) and yeast (13). 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 (14) and from purified M₂ protein (15). Thus, due to its structural simplicity, the M₂ ion channel is a potentially useful model for the study of ion channels in general.

Based on calculations using the Goldman-Hodgkin-Katz equation and measurements of current reversal voltage, the M₂ ion channel is thought to be at least 10⁵-fold selective for protons (8, 11), although other monovalent cations may also permeate the channel (8). M₂ ion channel activity is increased when the pH of the extracellular domain is lowered (10, 11, 16). This increase in activity occurs within the range of pH values expected for titration of histidine (17). 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 reduced, and the channel is conductive over a wider range of pH values (10, 11). It has been proposed that His₃⁷ forms a selectivity filter for protons (18).

Two possible mechanisms could account for the high proton selectivity of the M₂ ion channel. First, it is possible that certain residues of the pore form a narrow selectivity filter through which only hydronium ions can pass. Second, a residue in the pore might form part of a conducting pathway, perhaps a proton wire (19), by providing a site highly favorable for interactions with protons. We distinguished between these possibilities by replacing the water solvent with D₂O.

We have observed that inward M₂ currents sometimes decrease during a constant voltage clamp pulse to large negative voltages. Since M₂ currents do not display rapid voltage- or time-dependent activation/inactivation, it is possible that the decrease in current is the result of insufficient buffer capacity at the external mouth of the channel. We tested this possibility by examining the effect of decreased buffer capacity on M₂ current amplitude.

**EXPERIMENTAL PROCEDURES**

**mRNA Synthesis and Site-specific Mutagenesis**

The cDNA to the influenza A/Udorn/72 mRNA was cloned into the BamHI 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 M₂ cDNA with XhoI.

In vitro synthesis and quantification of m⁷G(5′)ppp(5′)G-capped mRNA were conducted as described previously.
Proton Conduction of M₂ Ion Channel

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 CaCl₂, 1 mM MgCl₂, 2.5 mM pyruvic acid, 5 mg/ml gentamicin, and 5 mM HEPES (pH 7.6), osmolality of ~210 mosmol/kg) at 19 °C. Oocytes at stage V were microinjected with 50 nl of mRNA (1 ng/ml) 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 (10, 20). Metabolic labeling of oocytes and analysis of proteins by SDS-polyacrylamide gel electrophoresis were carried out as described previously (9).

Culture and Infection of CV-1 Cells

CV-1 cells were cultured and infected with recombinant simian virus 40 expressing the M₂ protein from influenza A/duck/72 (rSV40-M₂) as described previously (12). Briefly, CV-1 cells grown to confluency at 37 °C and 5% CO₂ in culture medium (Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, penicillin, and streptomycin) were trypsinized, pelleted, and resuspended in culture medium. Resuspended cells were incubated in the presence of high titer rSV40-M₂ (100 μl of resuspended CV-1 cells and 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 incubated for 48 h before recording to ensure adequate M₂ protein expression. The presence of M₂ protein at the surface of infected CV-1 cells was confirmed by indirect immunofluorescence and flow cytometry as described previously (12).

Measurement of Membrane Current

**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 (88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.3 mM NaNO₃, 0.55 mM CaCl₂, 0.82 mM MgSO₄, and 15 mM MES) or a modified solution (140 mM NaCl, 5.3 mM KCl, 0.55 mM MgSO₄, 5.5 mM glucose, and 15 mM HEPES (pH 7.4) or 15 mM MES (pH 6.2), osmolality of ~300 mosmol/kg). Pipettes filled with this solution typically had resistances of ~3–4 megohms. CV-1 cells attached to glass coverslips were transferred to a recording chamber filled with “high buffer” bath solution (140 mM NaCl, 5.3 mM KCl, 0.55 mM MgSO₄, 5.5 mM glucose, and 15 mM HEPES (pH 7.4) or 15 mM MES (pH 6.2), osmolality of ~300 mosmol/kg). Also used in experiments was a “low buffer” pH 6.2 solution that contained 15 mM MES and 150 mM NaCl adjusted to the solution osmolality to 300 mosmol/kg. Seals (in excess of 10 gigahoms) were made by gently pressing the patch pipette against a CV-1 cell and then rapidly applying ~12 mm Hg suction. 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 megohms. 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 model (Model SP77B, Warner Instruments Corp., Hamden, CT). Using this system, solution changes could be made in ~100 ms. M₂-specific currents were identified by sensitivity to block by 100 μM amantadine.

**pD Measurement**

The nominal reading taken from a glass pH electrode (pH nom) deviates from the true pH of DI₂O solution by 0.4 units such that pH = pH nom + 0.4 (21). Our pH meter read 0.41 ± 0.02 units (mean ± S.D., n = 3) higher when 0.01 M HCl was added to DI₂O than when added to D₂O. We therefore corrected the pD in DI₂O solutions by adding 0.41 to the nominal reading of our pH meter.

1 The abbreviation used is: MES, 4-morpholineethanesulfonic acid.

RESULTS

**pH Activation of M₂ Currents**—As we have shown previously (10, 17), whole cell currents recorded in oocytes expressing the M₂ protein increase when the pH of the bathing medium is lowered (Fig. 1). This current is effectively blocked in the presence of 100 μM amantadine, an anti-influenza virus compound and inhibitor of M₂ ion channel activity (10).

**Deuterium Currents of the M₂ Ion Channel**—To test the possibility that protons interact directly with the M₂ ion channel protein when traversing its pore region, we took advantage of some differences in the physical properties of H₂ and D₂.

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age relationships obtained 20 s and 2 min after changing the solvent. To check that irreversible changes did not occur in D$_2$O, the relationship was again determined in water at pH 6.2. Finally, the relationship was determined in the presence of amantadine (pH 6.2). For every value of pL$_{out}$ tested, the current-voltage relationship of the amantadine-sensitive current had the same overall shape in both solvents, with a higher slope conductance for more negative membrane voltages (Fig. 2). Also, for every value of pL$_{out}$ tested, the relationship shifted to more positive voltages (Fig. 3A), and its slope decreased (by 50–60%) when D$_2$O was substituted for H$_2$O (Fig. 3B).

**Effects of Buffer Concentration on M$_2$ Currents**—We have often observed that the inward current of M$_2$-expressing oocytes decreases slightly during a constant voltage clamp pulse to large negative voltages. This effect becomes more prevalent as the flow rate of the solution bathing the oocyte is decreased. One possible explanation for this observation is that the buffer concentration was inadequate to supply the channel with protons. Unlike ion channels that conduct Na$^+$, Cl$^-$, Ca$^{2+}$, etc., proton-selective ion channels are supplied with the conducting ion at a free concentration in the micromolar range. Free protons are supplied by dissociation from buffer molecules, enabling the total concentration of protons to greatly exceed the free concentration. To test this, we measured the dependence of M$_2$ current amplitude on external buffer concentration.

In solutions of low buffer concentration (<15 mM), amantadine-sensitive M$_2$ currents measured in *Xenopus* oocytes decreased with time and had smaller final current amplitudes (Fig. 4A). M$_2$ current decrease in low buffer was measured at a given voltage as the difference between the current amplitude in 15 mM buffer and the final current amplitude measured at lower buffer concentrations. At higher buffer concentrations (>15 mM), amantadine-sensitive M$_2$ currents had approximately the same amplitude as those measured in 15 mM buffer (Fig. 4B). For a given buffer concentration, the magnitude of the current decrease in low buffer was proportional to the inward current amplitude in high buffer (Fig. 5). The relationship between inward current decrease in low buffer and inward current amplitude in high buffer was steeper for lower buffer concentrations (Fig. 5). No change in current amplitude was observed in low buffer when cells were held at a voltage that corresponded to zero current, which for most cells was near the calculated proton equilibrium potential. Low external buffer concentrations also had no effect on the amplitude of outward M$_2$ currents (data not shown).

Are M$_2$ Currents Limited by Buffer Concentration in Mammalian Cells?—To determine whether the limitation of M$_2$
current by external buffer concentration was specific to oocytes, we compared the effects of low buffer concentration on M$_2$ currents recorded in mammalian cells infected with rSV40-M$_2$ (12). M$_2$ currents recorded in rSV40-M$_2$-infected CV-1 cells using the whole cell patch clamp technique were severely reduced in amplitude when the external buffer concentration was decreased from 15 mM to 25 µM using bromcesol purple as a pH buffer (Fig. 6). We chose to use bromcesol purple as a buffer since it undergoes a distinct color change below pH 6.0, allowing the approximate pH of the solution to be easily monitored during the experiment. On average, M$_2$ currents recorded in the low buffer solution at −60 mV were decreased in amplitude to 31.0 ± 8.1% (n = 7) of those recorded in 15 mM buffer. Only a slight decrease in current was observed when the buffer concentration was reduced from 15 to 0.15 mM. On average, M$_2$ currents recorded in 0.15 mM buffer at −60 mV were decreased in amplitude to 88.98 ± 2.17% of those recorded in 15 mM buffer (n = 4). M$_2$ currents measured in CV-1 cells in low buffer were decreased in amplitude at all times during a constant voltage clamp pulse. This result is in contrast to oocytes in which the current in low buffer was seen to decrease over a matter of seconds during a voltage clamp pulse. The results demonstrate that the limitation of M$_2$ current by buffer concentration is not restricted to oocytes and therefore suggest a common mechanism.

**Mechanism of M$_2$ Current Decrease**—The dependence of M$_2$ current amplitude on external buffer concentration suggests that M$_2$ proton currents can exceed the capacity of the buffer to deliver protons at the channel mouth, thus resulting in a transient extracellular alkalinization. The occurrence of a transient alkalinization in low buffer has already been suggested in Fig. 4A (arrow), where there was an “overshoot” of current following a hyperpolarizing pulse. If alkalinization occurs, then the reversal voltage for the M$_2$ current (V$_{rev}$) measured in low buffer should be shifted to a more negative voltage since the pH gradient across the channel would be transiently decreased under this condition. To test this hypothesis, the following experiment was performed. V$_{rev}$ was measured using voltage ramps evoked first while the oocyte was bathed in high buffer, then while it was bathed in low buffer, and finally again while it was bathed in high buffer (Fig. 7). Rapid voltage ramps were necessary to measure the effect of depletion on V$_{rev}$ since there should be no depletion occurring at true V$_{rev}$. We found that at a holding potential of −20 mV, which produces a large inward

*Fig. 4. Decrease in M$_2$ current in Xenopus oocytes expressing the M$_2$ protein at low buffer concentration.* A. M$_2$ currents recorded from a single oocyte in response to a 2-s hyperpolarizing voltage pulse to −120 mV from a holding potential of −20 mV. Currents were recorded in 15 and 0.15 mM MES at pH 5.8. Current decrease was measured as the difference between the final current amplitude at the high buffer concentration and the final current amplitude at the lower buffer concentration. Note the overshoot of current (arrow) that occurred immediately following the hyperpolarizing voltage pulse in 0.15 mM MES. B. Ratio of the final current measured at several MES buffer concentrations to the final current in 50 mM MES (I$_{max}$) plotted as a function of MES concentration. Each data point represents the average value obtained from five cells. Error bars show means ± S.E. The final current was measured at the end of a hyperpolarizing voltage pulse to −120 mV from a holding potential of −20 mV at pH 5.8. The final current amplitude decreased for MES concentrations <15 mM.

*Fig. 5. Dependence of M$_2$ current decrease on buffer concentration and the final current amplitude in M$_2$-expressing oocytes.* Net current decrease for three concentrations of MES buffer is plotted as a function of current amplitude in 15 mM MES. Data are from four oocytes (each represented by a different symbol) in the presence of 15, 1.5, or 0.15 mM MES at pH 5.8. For each oocyte, several final current amplitudes produced by hyperpolarizing voltage pulses of various amplitudes are shown. Lines through the data points are not significant. The inset shows an example of currents recorded in a single oocyte at pH 5.8 at each of the three concentrations of MES buffer. In this case, currents were evoked by a 2-s test pulse to −120 mV from a holding voltage of −20 mV. For a given final current, the decrease in M$_2$ current became larger as the buffer concentration was decreased.
current at pH 5.8, $V_{rev}$ shifted in the low buffer solution toward more negative voltages by $-12.5 \pm 1.0$ mV ($n = 9$). The transient shift of $V_{rev}$ in low buffer solution was larger for more negative holding potentials, which was consistent with greater depletion. The transient shift of $V_{rev}$ in low buffer could be prevented by holding cells at a voltage that corresponded to zero inward current (data not shown). The incomplete recovery of $V_{rev}$ in high buffer following exposure to the low buffer solution is thought to be the result of gradual oocyte acidification while bathed in low pH solutions (8). Consistent with this explanation was a small shift in $V_{rev}$ toward more negative voltages observed in oocytes exposed only to a highly buffered solution (data not shown). The mostly reversible shift of $V_{rev}$ in low buffer to more negative values is consistent with the notion that the $M_2$ current decrease in low buffer is the result of proton depletion in the bulk solution near the extracellular mouth of the $M_2$ channel.

**Does the Vitelline Membrane Limit the Diffusion of Buffer to the $M_2$ Channel?**—Xenopus oocytes are surrounded by the vitelline membrane, which, among other functions, serves to maintain oocyte structural integrity. For this reason, the vitelline membrane is generally left intact for ion channel recording. It was possible that the greater sensitivity of $M_2$ currents in oocytes to low external buffer concentrations and the slower time course of current decrease compared with those recorded in mammalian cells may have been the result of the vitelline membrane acting as a diffusion barrier for protonated buffer molecules. We therefore tested the effect of decreased buffer concentration in oocytes from which the vitelline membranes had been removed and compared the results to those obtained in vitelline-intact oocytes. $M_2$ currents recorded in oocytes with no vitelline membrane decreased in a similar manner to those recorded in vitelline-intact oocytes when bathed in buffer at concentrations below 15 mM ($n = 10$; data not shown). This result demonstrates that the greater sensitivity of $M_2$ currents to low external buffer concentrations seen in oocytes is not the result of the vitelline membrane impeding buffer diffusion to the mouth of the $M_2$ channel.
Is Proton Depletion Localized to the M₂ Channel Macromolecular Complex?—We wished to determine if proton depletion in low external buffer occurred in the macroscopic solution shared by all channels as the result of combined proton flux through multiple M₂ channels or whether the deduced depletion was localized to the bulk solution surrounding the macromolecular complex of each M₂ channel. Assuming the same mean channel conductance for all M₂ channels, oocytes expressing a greater total number of channels (i.e., expression level) should, for a given voltage and pH, have proportionally larger currents. If proton depletion in low buffer is channel-localized, then the ratio of current amplitude in low buffer to current amplitude in high buffer should be constant for a given voltage and pH, regardless of the expression level. On the other hand, if current decrease is due to the cooperative effect of many channels to deplete protons from the macroscopic solution, then the ratio of current amplitude in low buffer to current amplitude in high buffer should decrease with expression level. To distinguish between these two possibilities, M₂ current amplitude was measured at high and low buffer concentrations at a constant voltage and pH in individual oocytes, each with many levels of expression. The ratio of current amplitude in low buffer to current amplitude in high buffer was then plotted for each oocyte against the amplitude of the current measured at pH 6.2 instead of pH 4.4 (the pKₐ of tartaric acid) in order to minimize acidification and subsequent oocyte damage, which occurs at low pH values. The results thus demonstrate that M₂ currents are limited by buffer capacity and not just the concentration of protonated buffer species.

DISCUSSION

Our results are consistent with a model for proton conduction across the M₂ ion channel in which protons donated from buffer molecules interact with a titratable group within the channel pore. At least one such group lies within the electric field of the membrane and is probably His³⁷.

Comparison of the conductance measured in water and D₂O suggests that hydroxonium ions do not pass through the channel in the form of hydronium ions. If this were the case, only a modest decrease in conductance would be expected due to the greater viscosity of D₂O, ~20%. Instead, we found that the slope conductance, measured over a range of −1.5 pH units, decreased by 40–50% (Fig. 3B). The greater decrease in conductance could be explained either by deuterons having a greater affinity than protons for a titratable group lining the M₂ channel pore (22) or by the large difference in the mobilities of protons in H₂O and deuterons in D₂O (19).

Several lines of evidence indicate that one of the ionizable groups of the channel that binds the conducting protons lies within the electric field of the membrane. First, the slope conductance of the current-voltage relationship increases for negative voltages (Fig. 1). The voltage dependence that we measured is similar to that found previously in mouse erythroleukemia cells (11). Second, site-directed mutagenesis experiments have shown that His³⁷ is essential for the activation of the current of the M₂ channel by low pH (17). Experiments with various transition metals have shown that the channel can be inhibited with Cu²⁺ and that the high affinity site for inhibition results from coordination of Cu²⁺ with His³⁷ (23). This inhibition is strongly voltage-dependent, indicating that His³⁷...
lies within the electric field of the membrane. It thus seems likely that His$^{37}$ binds protons as they traverse the pore of the channel (18).

The conclusion that hydrogen ions interact with the pore region of the M$_2$ ion channel has implications for the single channel conductance expected from this proton-selective channel. The M$_2$ single channel current can be estimated in the following three ways.

1) The maximum theoretical flux of hydrogen ions would be limited by the reverse reaction rate constant of the titratable group to which the hydrogen ions bind. If this group is His$^{37}$ ($pK_a = 6$), then the calculated value of the reverse reaction rate constant ($K_{rev} = \frac{[H^+][His^{37}]}{[His^{37+}][H^+]^{10^6}}$) is on the order of $1.7 \times 10^{12}/s$ (22). The maximum single channel current to be expected based upon this value and assuming four His$^{37}$ residues per M$_2$ channel macromolecular complex is $-10$ fA.

2) We found that M$_2$ currents measured at a given voltage in CV-1 cells diminished if the buffer concentration of the oocyte bathing solution was below $0.15$ mM. Thus, the number of protons supplied to a single M$_2$ channel macromolecular complex in $0.15$ mM buffer must be roughly equal to the number of protons traversing the channel during maximum flux. Assuming that the mobility of a proton in H$_2$O is very high, $-10^{-4} \text{ cm}^2/\text{Vs} \cdot \text{cm}(24)$, then the limiting step in the transfer of protons to His$^{37}$ would be the dissociation of protons from the buffer molecules. Assuming that the dissociation rate is $10^{15}/s$ for a buffer of $pK_a = 6$ and that protons diffuse into the channel from a hemispherical sink with a radius of $5 \text{ Å}$, then the maximum current possible from $0.15$ mM buffer would be equal to the number of protonated buffer molecules in the sink multiplied by the proton dissociation rate, a value of $-1$ fA.

3) The final way in which the M$_2$ single channel current can be estimated is by dividing the total current recorded in M$_2$-expressing oocytes by the number of M$_2$ channels per oocyte. The total current measured at pH 6.2 and a membrane voltage of $-130$ mV is $-0.7$ nA. The number of M$_2$ channels per oocyte can be calculated by assuming that there is $-5$ ng of M$_2$ protein expressed per oocyte and by using a molecular weight for the M$_2$ tetrameric channel of 60,000 (9). Given that $-50$% of the M$_2$ protein is present at the cell surface (7), the calculated current per M$_2$ channel is $-0.5$ fA. All three calculations suggest that the M$_2$ single channel current is very small. Two reports of a larger single channel activity of the M$_2$ ion channel have appeared in the literature. The first study used a synthetic transmembrane peptide and reported a conductance of 10 picoemperms (14). However, this value was obtained at pH 2.3 in glycine buffer, far from the pH range accessible to electrophysiological investigations. The second study utilized affinity-puriﬁed M$_2$ protein and did not report consistent single channel conductances (15). Such inconsistency of single channel conductance perhaps resulted from aggregation of the protein in the membrane (25). In a patch clamp study of stably transfected mammalian cells, Chizhmakov et al. (11) reported that M$_2$ single channel currents were not detectable. Our results lend support to the expectation that the M$_2$ single channel current would be below the detectable range of conventional single channel recording techniques.

We found some differences in M$_2$ currents recorded in Xenopus oocytes and CV-1 cells. First, M$_2$ currents in CV-1 cells are not as sensitive to external buffer concentration as those rec-
corded in Xenopus oocytes (Figs. 4–6). Second, M₂ currents recorded in CV-1 cells in low buffer decrease and reach a lower steady-state current almost instantaneously compared with those recorded in Xenopus oocytes, which decrease over a matter of seconds. This was not the result of the oocyte vitelline membrane acting as a diffusion barrier, as removing the vitelline membrane made no difference to the time course of current decrease. One possibility is that there is a greater M₂ current density in oocytes, which leads to more protons being depleted from the surrounding solution. This can be tested by calculating the M₂ current density of M₂-expressing oocytes and comparing this with the equivalent value obtained from CV-1 cells. M₂ current density can be calculated by dividing the whole cell current at a given voltage and pH by the cell-surface area. The latter can be calculated using the standard cell membrane capacitance of 1 microfarad/cm². The calculated values for M₂ current density in oocytes and CV-1 cells are similar, being 0.04 and 0.06 pA/μm², respectively.

A third possibility is that the presence of surface villi in oocytes (but not in CV-1 cells) increases the unstirred layer at the oocyte surface, leading to greater ion depletion through insufficient mixing with the bulk solution (26). If this is the case, then the protonated buffer concentration in the unstirred layer near the mouth of the M₂ channel may be different from the protonated buffer concentration in the macroscopic solution shared by all channels. Thus, the actual concentration of buffer below which oocyte M₂ currents decrease may be closer to the value obtained in CV-1 cells, which have a comparatively smooth surface. The presence of villi causing a diffusion barrier might also explain the slower time course of current decrease observed in oocytes. Although the effect of surface villi on M₂ currents could not be tested directly, the presence of an unstirred layer is suggested by the observation that oocyte currents could be decreased by reducing the bathing solution flow rate even in 15 mM buffer.

M₂ current amplitude at a given pH was found to be strongly dependent on the buffer pKₐ. M₂ currents decreased in amplitude when the buffer pKₐ differed from the solution pH (Fig. 9) even though a relatively high buffer concentration was used. This result can be explained in the same way as the dependence of M₂ current amplitude on external buffer concentration by assuming that a buffer is at maximum capacity to provide protons to the solution only when the pKₐ of the buffer equals the pH of the solution. Our results cannot, however, distinguish between the following mechanisms: 1) protons are transferred from the buffer to water molecules to form hydronium ions, which then donate protons to titratable groups in the channel pore; and 2) protons are transferred directly from the buffer to a group in the channel with a pKₐ similar to that of water.

Our results suggest that protons passing through the M₂ ion channel interact directly with the channel protein instead of passing through the channel as hydronium ions. We demonstrate that M₂ currents can be limited by buffer capacity and are in contrast to those obtained with other proton-specific channels through which proton flux was not limited by modest decreases in buffer capacity (27, 28). In previous work (10, 17), we have demonstrated that His³⁷ is an essential residue for pH sensitivity of the M₂ channel, and thus, this residue would be expected to be a site for proton binding to the channel molecule. Modification of the imidazole side chain of His³⁷ may reveal the atoms that participate in this interaction.

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