Activation of the M_2 Ion Channel of Influenza Virus: A Role for the Transmembrane Domain Histidine Residue

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ABSTRACT To test the hypothesis that transmembrane domain histidine residue 37 of the M_2 ion channel of influenza A virus mediates the low pH-induced activation of the channel, the residue was changed to glycine, glutamate, arginine, or lysine. The wild-type and altered M_2 proteins were expressed in oocytes of *Xenopus laevis* and membrane currents were recorded. The mass of protein expressed in individual oocytes was measured using quantitative immunoblotting and correlated with membrane currents. Oocytes expressing the M_2-H_{37}G protein had a voltage-independent conductance with current-voltage relationship similar to that of the wild-type M_2 channel. The conductance of the M_2-H_{37}G protein was reversibly inhibited by the M_2 ion channel blocker amantadine and was only very slightly modulated by changes in pH_{out} over the range pH 5.4 to pH 8.2. Oocytes expressing the M_2-H_{37}E protein also had a voltage-independent conductance with a current-voltage relationship similar to that of the wild-type M_2 channel. The conductance of the M_2-H_{37}E protein was reversibly inhibited by amantadine and was also only very slightly modulated by changes in pH_{out} over the range pH 5.4 to pH 8.2. These slight alterations in conductance of the mutant ion channels on changes in pH_{out} are in striking contrast to the 50-fold change in conductance seen for the wild-type M_2 channel over the range pH 4.5 to pH 8.2. The specific activity of the M_2-H_{37}G protein was 1.36 ± 0.37 μA/ng and the specific activity of the M_2-H_{37}E protein was 30 ± 3 μA/ng at pH 6.2. These values of specific activity greatly exceed that of the wild-type protein at the same pH (0.16 + 0.01 μA/ng). Oocytes expressing the M_2-H_{37}K and M_2-H_{37}R mutant proteins could not be studied because the oocytes did not survive more than a few hours in culture. Oocytes expressing the M_2-H_{37}E mutant protein also had a voltage-activated Cl^- conductance that was observed only for oocytes that expressed a mass of protein exceeding a large threshold value. These results are consistent with protonation of histidine residue 37 as an essential step in the activation of the wild-type M_2 ion channel.

INTRODUCTION

The low pH-activated M_2 ion channel of influenza A virus (Pinto et al., 1992) is a minor but essential component of virions (Zebedee and Lamb, 1988), and the M_2 ion channel activity is specifically blocked by the antiviral drug amantadine (Pinto et al., 1992; Wang et al., 1993). The M_2 protein is a type III integral membrane protein of 97 amino acids, with a 24-residue N-terminal extracellular domain, a 19-residue transmembrane domain, and a 54-residue C-terminal intracellular cytoplasmic domain (Lamb et al., 1985). Minimally a tetramer, the M_2 oligomeric structure is stabilized by two disulfide bonds such that the homotetramer either consists of a pair of disulfide-linked dimers or is a completely disulfide-linked tetramer (Holsinger and Lamb, 1991; Sugrue and Hay, 1991). The M_2 protein is known to be posttranslationally modified by palmitoylation on cysteine residue 50 (Sugrue et al., 1990b; Veit et al., 1991) and to be phosphorylated on serine residue 64 (Holsinger et al., 1995) but neither modification is essential for the M_2 ion channel activity (Holsinger et al., 1995).

The M_2 ion channel of influenza A virus is thought to play an essential role in the uncoating of the virus. Influenza virus enters cells by binding to cell surfaces, and virus particles are internalized via the endocytic pathway into endosomes. In the acidic environment of the secondary endosome the M_2 ion channel, which is a component of the virion envelope, is thought to be activated by low pH. The activated M_2 channel is thought to permit the passage of protons across the virion membrane into the virion core, weakening protein-protein interactions. When the M_2 ion channel is blocked by amantadine, uncoating of the virion is incomplete (reviewed in Hay, 1992; Lamb et al., 1994). In addition to a role in the uncoating of influenza virus, the M_2 ion channel dissipates the pH gradient across the membrane of the Golgi apparatus, which for some strains of influenza virus is essential to maintain the viral hemagglutinin spike glycoprotein in its pH-neutral and metastable form (Sugrue et al., 1990a; Takeuchi and Lamb, 1994; Ohuchi et al., 1994).

Direct evidence that the M_2 protein has ion channel activity was provided by expressing the M_2 protein in either oocytes of *Xenopus laevis* (Pinto et al., 1992) or in mammalian cells that were infected with recombinant SV40 virus that encoded the M_2 protein (Wang et al., 1993). It was found that cells expressing the M_2 protein exhibited an ion channel activity that was blocked by amantadine and was activated by lowering the extracellular pH from pH 7.5 to pH 5.4. The ion selectivity of the currents depended on the amino acid sequence of the M_2 transmembrane domain (Pinto et al., 1992). In further experiments with purified M_2 protein, introduction of the protein into planar lipid bilayers

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resulted in an amantadine-sensitive ion channel activity that was activated by low pH (Duff and Ashley, 1992; Tosteson et al., 1994). These results demonstrate that the M_2 protein possesses intrinsic ion channel activity.

The only amino acid in the transmembrane domain of the M_2 protein capable of being protonated within the range of pH that results in modulation of the ion channel activity is histidine residue 37 ($pK_a \sim 5.7$). Replacement of this histidine with alanine results in ion channel activity that is not activated by low pH but is partially blocked by amantadine (Pinto et al., 1992), suggesting that protonation of the side chain of this amino acid may be responsible for the activation by low pH. We tested this hypothesis by replacing histidine$_{37}$ with either glutamate or glycine, residues that are negatively charged or uncharged, respectively, throughout the relevant range of pH. According to the hypothesis, mutant ion channels with these substitutions should not be activated by lowered pH within the range pH 5–7. We found that these mutant proteins did indeed form ion channels that were not activated by lowered pH, but possessed amantadine sensitivity and ion selectivity similar to that of the wild-type M_2 ion channel.

MATERIALS AND METHODS

M_2 cDNA site-specific mutagenesis and in vitro synthesis of mRNA were performed as described previously (Pinto et al., 1992; Holsinger et al., 1994; 1995).

Culture and microinjection of oocytes

Oocytes were removed from female *X. laevis* (Nasco, Fort Atkinson, WI), defolliculated by treatment with collagenase B (2 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, IN), and incubated in ND96 at 17°C (Pinto et al., 1992; Wang et al., 1993). Oocytes at stages VI and V were microinjected with 50 nl of mRNA (1 ng/nl) on the day after defoliculation and incubated for 16–24 h before measurements were made (Pinto et al., 1992; Wang et al., 1993).

Electrophysiology

Whole-cell current was measured with a two-electrode voltage-clamp apparatus 16–24 h after mRNA injection (Pinto et al., 1992; Wang et al., 1993). The electrodes were filled with 3 M KCl, and the oocytes were bathed in either Barth's solution (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $NaNO_3$, 0.71 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 15 mM HEPES, pH 7.5), or a modified solution during the recording (Pinto et al., 1992; Wang et al., 1993).

To determine the relationship between pH and current, the current of an oocyte that expressed either the wild-type M_2 or a mutant M_2 ion channel was measured at various values of pH. For the wild-type M_2 ion channel, the current of the oocyte was measured at the pH values in the following sequence: pH 7.5, 4.5, 7.5 (to check for recovery), 5.0, 5.4, 5.8, 6.2, 6.8, 7.5, 8.2, and finally pH 7.5 (to check for recovery). Amantadine was added at the end to measure the leakage current. For the M_2-H_{37}G and the M_2-H_{37}E mutant ion channels, the current of the oocyte was measured at the pH values in the following sequence: 7.5, 5.4, 5.8, 6.2, 7.5, 8.2, and 7.5 (to check for recovery).

Determination of specific activity of the mutant M_2 ion channels

Quantitative immunoblotting was performed to measure the mass of protein in individual oocytes to determine the specific activity of the mutant M_2 ion channel proteins. This method took advantage of the observation that the M_2 immunoreactivity seen in oocytes with the 14C2 monoclonal antibody 1–4 days after injection of mRNA is confined to the cell surface (Pinto et al., 1992), and the calculated value of specific activity would thus apply to the ion channel molecules that are responsible for the measured current flow. After recording the current of an oocyte it was lysed in 50 μl radioimmunoprecipitation assay buffer containing 50 mM iodoacetamide and a cocktail of protease inhibitors (Holsinger et al., 1994). The lysate was extracted with 1,1,2 trichlorotrifluoroethane to remove yolk and pigment proteins. Ten to 40 μl of the extracted lysate was electrophoresed on a 17.5% polyacrylamide-4 M urea gel and then electrotransferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) by using a Trans-Blot semidry transfer cell (Bio-Rad, Richmond, CA). The blot was probed with M_2-specific 14C2 ascites fluid (Zebedee and Lamb, 1988) and horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G secondary antibody (Cappel, Organon Teknika Corp., West Chester, PA). The M_2 protein immune complexes were detected with the enhanced chemiluminescence system, and images on x-ray films were quantified by scanning densitometry (Wang et al., 1994). For the M_2-H_{37}G mutant ion channel, the average specific activity was determined from the ratio of the current that resulted from application of a −120 mV pulse to the mass of the M_2 protein of the oocyte for 7 cells. For the M_2-H_{37}E mutant ion channel the specific activity was determined from the ratio of the current that resulted from the voltage-independent component of conductance at −120 mV (see Fig. 4 *D*) and the mass of the M_2-H_{37}E protein (see Fig. 5 *A*).

RESULTS

Membrane currents of oocytes expressing the M_2-H_{37}G and M_2-H_{37}E proteins. Oocytes were injected with synthetic mRNAs encoding wild-type M_2 and mutant M_2 proteins, and their expression was demonstrated by immunoblotting (Fig. 1). The time course, current-voltage relationship, and amantadine sensitivity of the membrane currents of oocytes expressing the M_2-H_{37}G protein were similar to those of

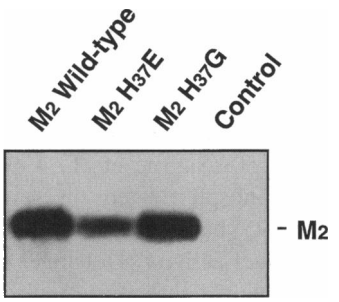

FIGURE 1 Expression of wild-type M_2 and M_2-H_{37}G and M_2-H_{37}E proteins in oocytes of *X. laevis*. Synthetic mRNAs were transcribed from plasmids encoding wild-type or mutant M_2 proteins and microinjected (50 nl of RNA [1 μg/μl]) into oocytes of *X. laevis*. At 24 h postinjection oocytes were homogenized, lysates run on SDS-PAGE, and the polypeptides were transferred to polyvinylidene difluoride membranes. Blots were probed with M_2-specific monoclonal antibody 14C2 and horseradish peroxidase-conjugated secondary antibody, and the immune complex was detected with the enhanced chemiluminescence system. Control: water-injected oocytes.

oocytes expressing the wild-type M_2 protein: the time courses of the currents were rectangular and the currents were inhibited by amantadine (Fig. 2), although to a lesser extent than the wild-type M_2 ion channel. The percentage inhibition of the currents of the mutant ion channel was ~80% (Table 1), whereas the inhibition for wild-type M_2 ion channel under these conditions is complete (Wang et al., 1993). Moreover, the inhibition of the current of oocytes expressing the M_2-H_{37}G protein differed from the inhibition of the current of oocytes expressing the wild-type M_2 ion channel in that the mutant currents could be reversed within 2 min, whereas the inhibition of wild-type M_2 currents cannot be reversed for as long as 25 min (Wang et al., 1993). The specific activity of the M_2-H_{37}G protein measured at pH 6.2 (see Materials and Methods) was much greater than for the wild-type M_2 channel: 1.36 ± 0.37 μA/ng for M_2-H_{37}G versus 0.16 ± 0.01 μA/ng for wild-type M_2 protein (Holsinger et al., 1994).

The nature of the currents of oocytes that expressed the M_2-H_{37}E protein depended on the quantity of mRNA injected. The mass of M_2 protein expressed increased monotonically with the amount of mRNA injected (Fig. 3). For small quantities injected (<3 ng), the conductance was voltage-independent and inhibited by amantadine (Fig. 4 *A* and *B* and Table 1). Thus, these small amplitude currents were similar in time course, current-voltage relationship, and amantadine sensitivity to those of oocytes expressing either the wild-type M_2 protein, the M_2-H_{37}A protein, or the M_2-H_{37}G protein. For larger quantities of mRNA injected (>3 ng; see Fig. 3), the conductance displayed two components, a voltage-independent as well as a voltage-dependent component (Figs. 4, *C* and *D*, and Fig. 3). We quantified the mass of the M_2-H_{37}E protein for individual oocytes from which we first measured the amplitude of both components of current. The amplitude of the voltage-independent component was proportional to the protein mass in the expressing oocyte (Fig. 5 *A*). However, the voltage-dependent component was only evoked from oocytes that were expressing an accumulated mass of M_2-H_{37}E protein of greater than ~60 pg (see Fig. 3) and a voltage-independent

TABLE 1 Amantadine inhibition of current of oocytes expressing the M_2-H_{37}E* and M_2-H_{37}G mutant proteins

pH values	Current (mean ± SEM, μA) Barth's	100 μM amantadine	Barth's recovery	*n*
M_2-H_{37}E				
pH 7.5	−0.16 ± 0.03	−0.01 ± 0.01	−0.07 ± 0.01	6
pH 6.2	−0.2 ± 0.1	−0.04 ± 0.03	−0.09 ± 0.04	5
M_2-H_{37}G				
pH 6.2	−3.0 ± 0.6	−0.50 ± 0.08	−3.0 ± 0.7	4

*The oocytes expressing the M_2-H_{37}E mutant protein had only voltage-independent conductance.

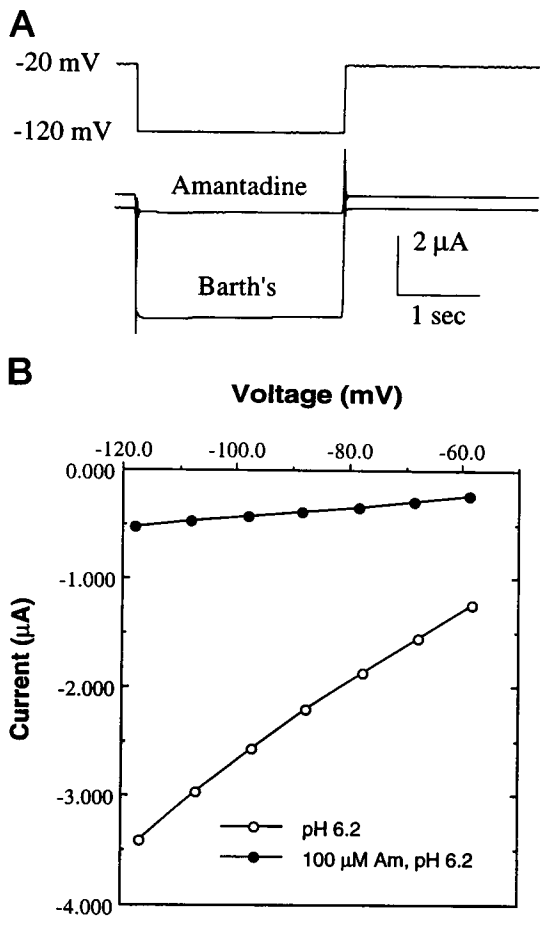

FIGURE 2 Membrane currents of oocytes that expressed the M_2-H_{37}G mutant protein. *A*) Time course of the membrane currents in the absence and presence of amantadine (100 μM). *B*) Current-voltage relationship measured at the end of the voltage pulse. Note that current-voltage relationship was linear and amantadine sensitive (cf. Table 1).

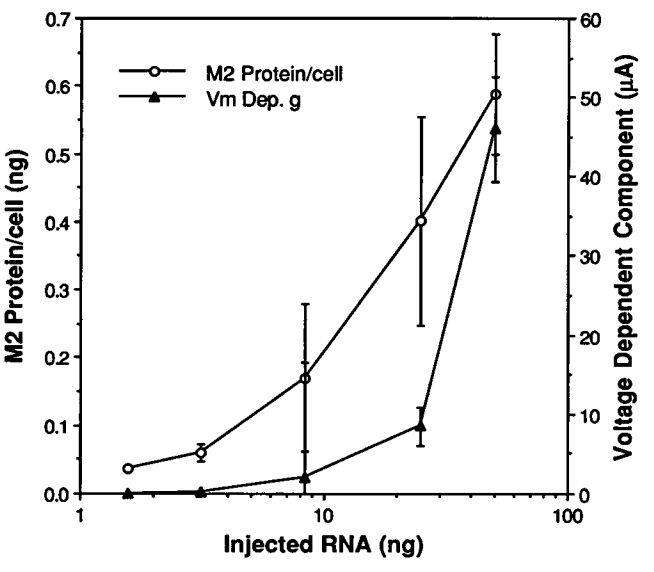

FIGURE 3 Mass of M_2-H_{37}E mutant protein and amplitude of voltage-dependent component of conductance (Fig. 4 *C*) as a function of injected mRNA. Note that the current resulting from the voltage-dependent component of conductance appeared only for mRNA injections >3 ng, corresponding to ~50 pg of mutant protein.

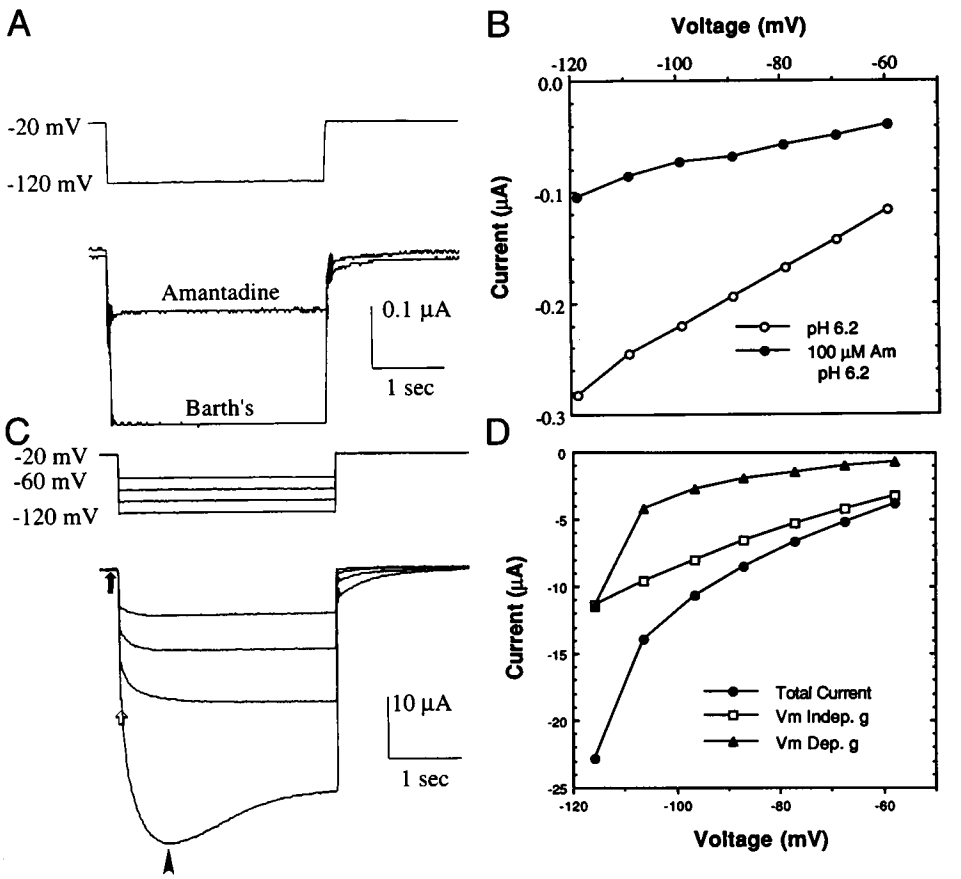

FIGURE 4 Membrane currents of oocytes that expressed low (*A*, *B*) or high (*C*, *D*) levels of the M_2-H_{37}E mutant protein. (*A* and *C*) Time course of the membrane currents. (*B*) Current-voltage relationship for the oocyte expressing the M_2-H_{37}E mutant protein with low currents. Measurements made at the end of the voltage pulse with (●) or without (○) amantadine (100 μM). (*D*) Current-voltage relationship for oocytes expressing the M_2-H_{37}E mutant protein with large currents. (●) Total current, measured between the arrowhead and the filled arrow in *C*. (□) Current resulting from the voltage-independent component of conductance, measured between the open and closed arrows. (▲) Current resulting from the voltage-dependent component of conductance, measured between the arrowhead and open arrow. Note that the voltage-dependent component appears only for oocytes with large membrane currents.

component of more than ~1 μA (Fig. 5 *B*). Thus, there appeared to be a threshold for evoking the voltage-dependent component of current. However, further experiments showed that this voltage-dependent component of current was probably not due to the up-regulation of an endogenous ion channel that has been shown to result from the expression of large amounts of certain proteins (Attali et al., 1993; Shimbo et al., 1995a,b; Tzounopoulos et al., 1995).

Amantadine inhibited both components of the conductance of oocytes expressing the M_2-H_{37}E protein, but the voltage-independent component was inhibited to a greater extent. In contrast to the currents of oocytes expressing the wild-type M_2 protein, the inhibition of both components was partially reversible (Table 2). One remarkable feature of the currents of oocytes expressing the M_2-H_{37}E protein was their large amplitude; we occasionally measured total currents as large as −80 μA (−27 μA for the voltage-independent component of conductance; Fig. 5 *A*) at pH 7.5 for V_m = −120 mV within 20 h after injection of 50 ng of mRNA, in contrast to about −0.2 μA for wild-type M_2 protein under the same conditions. The specific activity of the protein calculated for the voltage-independent component of conductance at pH 6.2 (see Materials and Methods) was also much greater than for wild-type M_2 protein: 30 ± 3 μA/ng for M_2-H_{37}E versus 0.16 μA/ng for wild-type M_2 protein (Holsinger et al., 1994).

Effect of ion channel expression on oocyte morphology

The presence of a large conductance in the membrane of oocytes expressing the M_2-H_{37}E protein was deleterious to the oocytes. This could be seen by comparison of the morphology of the oocytes expressing this mutant protein with that of oocytes expressing the wild-type M_2 protein. For oocytes expressing the wild-type M_2 protein, incubation in medium of low pH activates the channel (Pinto et al., 1992); oocytes under these conditions showed deterioration evidenced by depigmentation of the animal hemisphere and lysis. However, incubation of oocytes expressing the wild-type M_2 protein either at neutral pH (channel inactive) or in the presence of amantadine (channel blocked) did not result in this deterioration. Similar observations have also been made by Giffin and co-workers (1995). In contrast, oocytes expressing the M_2-H_{37}E protein exhibited morphological signs of deterioration even when incubated in solutions of neutral pH; this deterioration was prevented by addition of amantadine (10 μM) to the medium. Thus, it would seem that the continuous activity of the M_2 ion channel is deleterious to the oocytes, but that either deactivation by incubation at neutral pH for the wild-type M_2 protein or inhibition by amantadine prevents cell death. We show below that the pH-dependence of the M_2-H_{37}E mutant protein differs

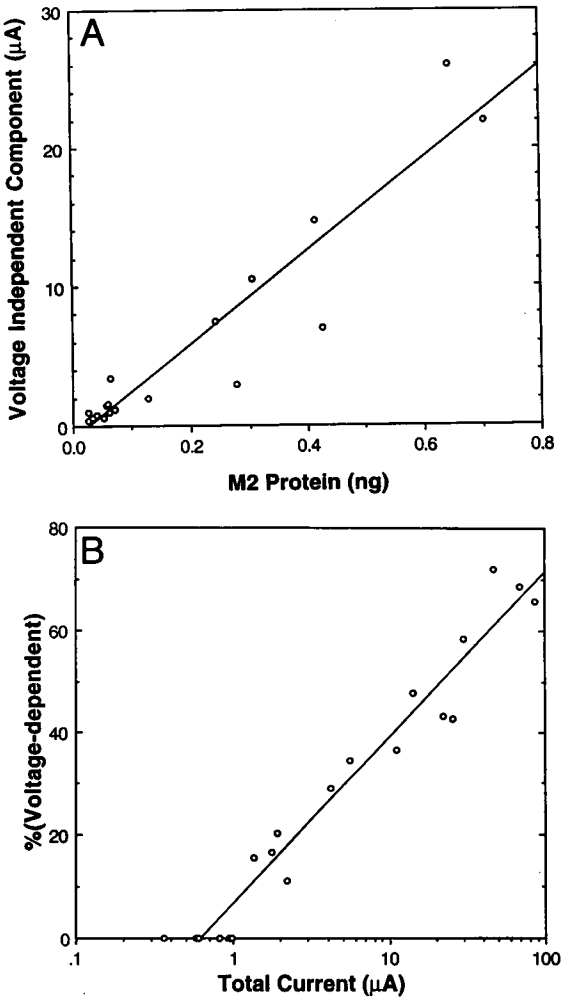

FIGURE 5 (*A*) Dependence of the amplitude of the voltage-independent component of conductance of oocytes expressing the M_2-H_{37}E mutant protein upon the mass of protein expressed in the oocyte. For this analysis the protein was quantified by Western analysis after measurement of current for each individual oocyte. (*B*) The percentage of the total current that resulted from the voltage-dependent component of conductance is plotted as a function of the total current. Note that the amplitude of the current that resulted from the voltage-independent component of conductance was proportional to the mass of the M_2-H_{37}E mutant protein and that the voltage-dependent component was only observed for total currents larger than ~1 μA.

from that of the wild-type M_2 protein, which provides a reasonable explanation for the death of oocytes expressing the M_2-H_{37}E protein even at neutral pH.

We also injected oocytes with synthetic mRNA encoding the mutant proteins M_2-H_{37}R and M_2-H_{37}K. These oocytes displayed morphological signs of deterioration similar to that caused by M_2-H_{37}E mutant protein in the absence of amantadine. A total of 10 batches of oocytes were injected with mRNA from three preparations encoding the M_2-H_{37}R and M_2-H_{37}K mutant proteins, but deterioration was seen in all oocytes in every batch, despite attempts at amelioration by modifying pH, amantadine, and ionic composition in the bathing medium. We were able to record currents from only a few of these oocytes. The currents were qualitatively similar to those of oocytes expressing the M_2-H_{37}E mutant protein (Fig. 4, *C* and *D*), but the poor condition of the oocytes made it impossible to study them further.

TABLE 2 Effect of amantadine upon components of current of the oocytes expressing M_2-H_{37}E mutant protein

	Current (mean ± SEM, μA)			
Conditions	Total	Voltage-independent conductance	Voltage-dependent conductance	*n*
Barth's	−25.4 ± 9.2	−10.8 ± 2.8	−15.7 ± 6.9	5
Barth's, 100 μM amantadine	−5.7 ± 2.0	−1.6 ± 0.4	−4.1 ± 1.6	5
Barth's, 1 min recovery	−16.7 ± 4.8	−7.8 ± 1.7	−9.3 ± 3.1	5

Ion selectivity of the components of the conductance of the M_2-H_{37}E protein

It has been observed that the expression of large amounts of some small integral membrane proteins is associated with a voltage-activated Cl^- conductance (Attali et al., 1993; Shimbo et al., 1995a,b; Tzounopoulos et al., 1995). Thus, we compared the ion selectivity of the voltage-independent and voltage-dependent components of the conductance. For the former component, we performed ramp analysis to determine the reversal potential using slowly changing membrane voltages (1.1 mV/s). For the latter component, we employed tail current analysis to determine reversal voltage. These measurements were performed for both components for Na^+ activity of the bathing medium of 2–70 mEq/l and Cl^- activity of 0–70 mEq/l. The reversal voltage of the voltage-independent component did not vary with Cl^- activity, but did vary from −85 mV to −20 mV over the range of Na^+ activity tested (Fig. 6 *A*). In contrast, the reversal voltage of the voltage-dependent component did not vary with Na^+ activity, but did vary from +30 mV to −10 mV over the range of Cl^- activity tested (Fig. 6 *B*). Thus, the two components of current possess different ion selectivities.

Modulation of the components of conductance of oocytes expressing the M_2-H_{37}E protein by DIDS, Ba^{2+}, and Ca^{2+}

As the voltage-dependent component of conductance was Cl^- selective, we applied the Cl^- channel blocker 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) (1 mM) and found that only the voltage-dependent component of conductance was inhibited (by >50%; see Table 3). Ba^{2+} (2 mM) also inhibited the conductance, but inhibited the voltage-dependent component to a greater extent (Table 4). On the other hand increasing $[Ca^{2+}]_{out}$ increased, and decreasing $[Ca^{2+}]_{out}$ decreased the amplitude of the voltage-dependent component (Table 4). The effects of these modulators on the voltage-dependent conductance of the M_2-H_{37}E protein differed from those seen in oocytes

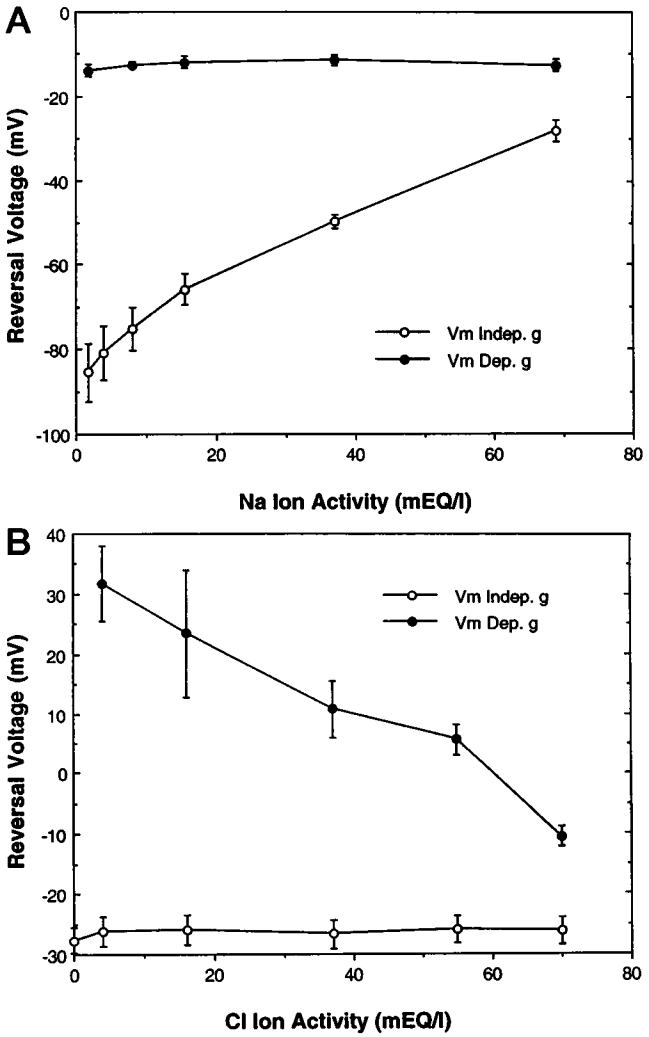

FIGURE 6 Ion selectivity of the components of the current of oocytes expressing the M_2-H_{37}E mutant protein. Reversal voltages were measured using tail currents (voltage-dependent component, ●) or ramp analysis (voltage-independent component, ○) as a function of the activity of Na^+ (*A*) or Cl^- (*B*). Note that the ion selectivities of the two components differed.

with high levels of expression of some small integral membrane proteins (Attali et al., 1993; Shimbo et al., 1995a,b; Tzounopoulos et al., 1995). Thus, the two components of the M_2-H_{37}E current could also be distinguished by their sensitivity to modulators of ion channel activity.

TABLE 3 Effect of 1 mM DIDS upon components of current of oocytes expressing the M_2-H_{37}E mutant protein

Solution to which DIDS was added	Fraction Remaining Current (mean ± SEM)* Voltage-independent conductance	Voltage-dependent conductance	*n*
Barth's, pH 7.5	1.097 ± 0.043	0.493 ± 0.066	5
Barth's, 100 μM amantadine, pH 6.2	1.011 ± 0.080	0.312 ± 0.027	4

*Fraction of the current before application of 1 mM DIDS.

TABLE 4 Effect of divalent cations upon components of current of oocytes expressing the M_2-H_{37}E mutant protein

Conditions	Current (mean ± SEM, μA) Total	Voltage-independent conductance	Voltage-dependent conductance	*n*
Low calcium				
Barth's	−40 ± 13	−11.6 ± 3.7	−26 ± 11	6
0.1 × Ca^{+2}	−20.2 ± 6.3	−11.4 ± 3.4	−7.0 ± 5.0*	6
High calcium				
Barth's	−18.7 ± 7.4	−7.9 ± 2.0	−13.5 ± 6.2	5
10 × Ca^{+2}	−28 ± 11	−10.0 ± 3.3	−19.6 ± 6.3*	5
Barium				
Barth's	−44.3 ± 8.1	−15.6 ± 2.1	−28.5 ± 6.9	5
2 mM Ba^{+2}	−31.4 ± 6.0	−12.3 ± 1.7*	−19.1 ± 4.0*	5

*Statistically significant difference ($p < 0.05$) from the measurement in Barth's.

Modulation of the current of oocytes expressing the M_2-H_{37}G and M_2-H_{37}E proteins by pH_{out}

For oocytes expressing the wild-type M_2 protein, decreasing pH from pH 8.2 to pH 4.5 increases the current by about 50-fold (Fig. 7). The increase in current can be fitted with a single independent site model with $pK_a = 5.77 \pm 0.06$ and Hill coefficient of 0.96 ± 0.14. In contrast, the current of oocytes expressing the M_2-H_{37}G protein (and oocytes expressing M_2-H_{37}A; data from Pinto et al., 1992) were modulated only very slightly by pH_{out}; in the range pH 6.2 to pH 8.2 no significant alteration of amplitude was observed (Fig. 7). When pH was decreased further to pH 5.4 the amplitude increased by only 20%. Similarly, the voltage-independent component current of oocytes expressing the M_2-H_{37}E protein was modulated only slightly by pH (Fig. 7), and the voltage-dependent component of the current was marginally more affected by pH changes (Table 5). The effects of altered pH on the voltage-dependent conductance of the M_2-H_{37}E protein differed from those seen in oocytes with high levels of expression of certain proteins (Attali et al., 1993; Shimbo et al., 1995a,b; Tzounopoulos et al., 1995). Thus, in contrast to the currents of oocytes expressing the wild-type M_2 protein, the currents of oocytes expressing the mutant proteins were not greatly modulated by pH.

DISCUSSION

In this paper, we tested the hypothesis that the M_2 protein transmembrane domain histidine residue 37 plays a crucial rule in the activation of the M_2 ion channel by low pH. We replaced histidine residue 37 with glycine or glutamate; these do not become protonated within the physiological range of pH for oocytes. We found that neither mutant protein was activated by low pH. For one of the mutant proteins, M_2-H_{37}E, strong hyperpolarization of the mem-

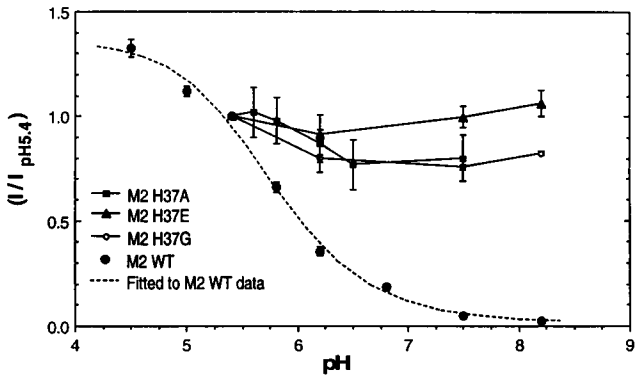

FIGURE 7 pH-dependence of the currents of oocytes expressing the wild-type M_2 (●), M_2-H_{37}G (○), M_2-H_{37}E (▲), and M_2-H_{37}A (■) proteins. (Data for M_2-H_{37}A are from Pinto et al., 1992). For oocytes expressing the M_2-H_{37}E mutant protein, <3 ng of mRNA was injected and thus only the voltage-independent component of conductance was elicited (see Figs. 3–5). The line for wild-type M_2 was fitted by the method of least squares using the equation:

$$(I/I_{pH5.4}) = 1.0/(1 + [H^+]/10^{pK_a})^m \quad (1)$$

and yielding the values of $pK_a = 5.77$ and $m = 0.96$ (the Hill coefficient). The currents of each oocyte had to be measured for many values of pH for this experiment, and the order of measurement probably had a slight influence upon the values obtained (see Materials and Methods), giving a lesser effect of pH upon the voltage-independent conductance for M_2-H_{37}E in this figure than for the experiment of Table 5.

brane activated a conductance that was not related to the normal M_2 ion channel conductance.

Many characteristics of the M_2-H_{37}G ion channel were similar to those of the wild-type M_2 ion channel. 1) The waveform of the current of the M_2-H_{37}G ion channel was rectangular and the current-voltage relationship was linear, as for the wild-type M_2 ion channel. 2) The current of the M_2-H_{37}G ion channel was strongly inhibited by amantadine, as for the wild-type M_2 ion channel, although the inhibition was reversible for the mutant channel. However, in contrast to the wild-type M_2 ion channel, the current of the M_2-H_{37}G ion channel was not increased by low pH. Whereas the current of the wild-type M_2 channel was increased by 25-fold from pH 8.2 to pH 5.4, the current of the M_2-H_{37}G ion channel changed only 20% within this range of pH. The M_2-H_{37}G ion channel has higher specific activity than the wild-type channel throughout the physiological range of pH. This can be seen by comparing the specific activity of the channels at pH 6.2, the value of pH for which the wild-type channel has ~25% of the maximal activity we have observed at pH 4.5 (Fig. 7). At pH 6.2, the specific activity for the M_2-H_{37}G ion channel (1.36 μA/ng) is almost 10-fold higher than the specific activity of the wild-type M_2 ion channel (0.16 μA/ng; Holsinger et al., 1994). These results indicate that replacement of histidine with uncharged glycine results in the formation of an ion channel with properties similar to the wild-type M_2 ion channel, but has higher specific activity than the wild-type channel and is not activated by low pH.

TABLE 5 Effect of pH upon components of current of oocytes expressing the M_2-H_{37}E mutant protein

	Current (mean ± SEM, μA)			
Conditions	Total	Voltage-independent conductance	Voltage-dependent conductance	*n*
Barth's, pH 7.5	−42.7 ± 5.6	−15.4 ± 1.6	−26.4 ± 4.1	9
Barth's, pH 6.2	−12.0 ± 1.5	−8.3 ± 0.9	−3.9 ± 0.7	9
Barth's, pH 7.5 recovery	−24.5 ± 3.0	−12.7 ± 1.4	−12.7 ± 1.8	9

Many characteristics of the voltage-independent conductance of the M_2-H_{37}E ion channel were also similar to those of the wild-type M_2 ion channel. 1) The waveform of the membrane current of oocytes injected with small amounts of the M_2-H_{37}E mRNA was rectangular (Fig. 4 *A*), and the current-voltage relationship was linear (Fig. 4 *B*). 2) Amantadine strongly inhibited the current of the M_2-H_{37}E ion channel in a manner similar to the inhibition of the wild-type M_2 channel (Fig. 4 *B*). 3) The ion selectivity of the mutant ion channel was cationic, as is that of the wild-type M_2 ion channel (Fig. 6). However, unlike the wild-type M_2 channel, the M_2-H_{37}E ion channel was not activated by low pH. The specific activity for the M_2-H_{37}E ion channel, 30 μA/ng, was the highest we have encountered for the M_2 ion channel or its mutants that have been quantified to date (Holsinger et al., 1994). This specific activity is almost 200-fold higher than the specific activity of the wild-type M_2 channel at pH 6.2, at which pH the specific activity of the wild-type channel is ~25% of the maximal value we have observed at pH 4.5. This high level of specific activity can be most easily explained by constitutive activity of the mutant ion channel. Thus, replacement of the M_2 protein transmembrane domain histidine residue with glutamate results in a permanently activated ion channel that is not modulated by low pH.

We observed a voltage-dependent component of current for oocytes that expressed a high level of the M_2-H_{37}E ion channel. There are several possible explanations for this current. First, the voltage-activated current may result from a structurally distorted form of the M_2-H_{37}E mutant protein that only occurs with high levels of expression. Second, the current might result from the activity of an ion channel that is endogenous to the oocyte. However, the properties of the voltage-dependent current we observed for the M_2-H_{37}E mutant ion channel differed significantly from those of previously reported oocyte Cl^- currents (Ackerman et al., 1994; Kowdley et al., 1994) and from those of the Cl^- currents seen with expression of high levels of some small integral membrane proteins (Attali et al., 1993; Shimbo et al., 1995a,b; Tzounopoulos et al., 1995). The Cl^- current associated with the expression of M_2-H_{37}E mutant protein is more strongly dependent on $[Cl^-]$, less affected by DIDS, affected oppositely by altered pH, and partially blocked by amantadine when compared with the current that is seen with expression of large amounts of some small integral membrane proteins (Attali et al., 1993; Shimbo et al.,

1995a,b; Tzounopoulos et al., 1995). Third, the voltage-dependent current we observed with expression of the M_2-$H_{37}E$ mutant ion channel may reflect upregulation of an endogenous channel due to deleterious effects of expression of the M_2-$H_{37}E$ mutant protein. The present results do not provide the basis to determine which of these explanations is correct.

We cannot exclude the possibility that the structures of the mutant M_2 proteins having histidine$_{37}$ replaced with other amino acids were altered significantly. The partial amantadine sensitivity of the voltage-dependent conductance of the M_2-$H_{37}E$ mutant protein suggests that the conductance depends on the M_2 mutant protein. As the conductance of the wild-type M_2 protein is not voltage-dependent, a significant alteration of its structure would have to occur to render the molecule's activity voltage-dependent. Moreover, the charge of the residue 37 of the M_2-$H_{37}E$ mutant protein in the pH range employed is opposite to the charge expected from protonation of histidine. For this negatively charged residue to cause constitutive activation would require a significant alteration of the structure of the wild-type M_2 protein.

The relationship between current amplitude and pH for the wild-type M_2 ion channel can be described as a simple titration curve with activation resulting from protonation of a single (independent) binding site with $pK_a = 5.8$ (Fig. 7). This pK_a is consistent with the protonation of histidine. The data could be fitted with a model having a Hill coefficient of 0.96, consistent with one (independent) binding site. However, the M_2 protein is minimally a homotetramer, possessing four histidine residues. Our data cannot distinguish between the activation resulting from the protonation of one of these histidine residues or the independent protonation of more than one of these residues. An alternative explanation for the increase in inward current amplitude observed when pH was decreased from pH 8.2 to pH 4.5 is that this increase in amplitude resulted only from an increased conductance caused by an increase in the concentration of protons. However, this explanation seems unlikely because the relationship between current and pH followed a single (independent) binding site model (Fig. 8) and did not show an increase in proportion to $[H^+]$. This is readily illustrated by comparing the actual and predicted increase in current amplitude when pH was decreased from pH 8.2 to pH 4.5. If this were the case the predicted increase would have been a factor of 5000, but the observed increase was only a factor of 50. Thus, channel activation is the more likely explanation for the observed increase in current at low pH.

The results, taken together, are consistent with protonation of histidine causing activation of the wild-type M_2 ion channel, but there are several possible explanations for the finding that replacement of histidine by alanine, glycine, or glutamate removes the pH activation of the M_2 ion channel. It is possible that the histidine residue is important for maintaining the conformation of the M_2 ion channel and that the pH sensor resides elsewhere in the molecule. However, we do not believe this explanation to be correct for the following reasons. First, the amantadine sensitivity and current-voltage relationship were preserved in the three mutant ion channels. Second, there are no other amino acids in the transmembrane domain capable of being protonated within the physiological range of pH. Another explanation suggested by the single (independent) binding site curve relating current amplitude to pH (Fig. 7) is that histidine$_{37}$ of the M_2 protein binds ions (perhaps including protons) arriving from one side of the membrane and releases them to the opposite side of the membrane. However, the finding that the specific activity of the M_2-$H_{37}G$ and M_2-$H_{37}E$ mutant proteins is higher than that of the wild-type M_2 protein throughout the physiological range of pH argues against this possibility. Thus, we conclude that protonation of histidine$_{37}$ is directly involved in activation of the M_2 ion channel.

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