Selective proton permeability and pH regulation of the influenza virus M2 channel expressed in mouse erythroleukaemia cells

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1. The M2 protein of influenza A virus is implicated in transmembrane pH regulation during infection. Whole-cell patch clamp of mouse erythroleukaemia cells expressing the M2 protein in the surface membrane showed a conductance due to M2 which was specifically blocked by the anti-influenza drug rimantadine.

2. The ion selectivity of the rimantadine-sensitive current through M2 was determined. Reversal potentials were close to equilibrium potentials for transmembrane pH gradients and not to those for Na^+, K^+ or Cl^- concentration gradients. M2 permeability to Na^+ relative to H^+ was estimated to be less than 6×10^{-7}.

3. The M2 conductance increased as external pH decreased below 8·5 and approached saturation at an external pH of 4, effects attributable to increased permeability due to increased driving potential and to activation by low external pH. Both activation and permeation could be described by interaction of protons with sites on M2, with apparent dissociation constants of approximately 0·1 μM and 1 μM, respectively, under physiological conditions.

4. The M2 protein can transfer protons selectively across membranes with the H^+ electrochemical gradient, properties consistent with its role in modifying virion and *trans*-Golgi pH during virus infection.

The M2 protein of influenza A viruses is a homotetramer of ninety-seven amino acid subunits anchored in the membrane by a hydrophobic domain comprising amino acids 25–43, and is the specific target of the anti-influenza drugs amantadine and rimantadine (reviewed by Hay, 1992). It has been shown to have roles in two stages of virus replication, both of which have been proposed to involve proton transfer across membranes. During virus endocytosis, M2 in the virus membrane may act by mediating H^+ transfer into the virion interior to promote an acid-induced dissociation of the matrix protein from the ribonucleoprotein, which is necessary to facilitate the entry of the latter into the nucleus to initiate replication (Martin & Helenius, 1991). Secondly, in certain avian influenza virus infections M2 is required to reduce the acidity of the *trans*-Golgi to enable passage of acid-sensitive haemagglutinins (HA) to the plasma membrane prior to virus assembly (Sugrue, Bahadur, Zambon, Hall-Smith, Douglas & Hay, 1990; Grambas, Bennett & Hay, 1992; Ciampor, Bayley, Nermut, Hirst, Sugrue & Hay, 1992). After its insertion into the plasma membrane M2 is capable of modifying the pH of the cytoplasm of virus-infected or M2-transfected cells in response to changes in external pH (pH_o; Ciampor, Thompson, Grambas & Hay, 1992; Hay, Thompson, Geraghty, Hayhurst, Grambas & Bennett, 1993).

Direct electrophysiological measurements of ionic permeability due to M2 have been reported to show that: (1) *Xenopus* oocytes and CV-1 cells expressing M2 in the plasma membrane possess a pH-regulated cation conductance with high permeability for Na^+ ions (Pinto, Holsinger & Lamb, 1992; Holsinger, Nichani, Pinto & Lamb, 1994; Wang, Lamb & Pinto, 1994); and (2) purified M2 incorporated into phospholipid bilayers forms cation channels with similar characteristics of pH dependence, Na^+ permeability and amantadine inhibition (Tosteson, Pinto, Holsinger & Lamb, 1994). No measurements of H^+ current were made and it is not apparent how Na^+ permeability accounts for the physiological roles of the protein unless it is presumed that the channels formed by M2 are also permeable to H^+ ions.

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To examine more closely the conductance due to M2 expressed in mammalian cells voltage clamp experiments were made in mouse erythroleukaemia (MEL) cells expressing an M2 protein. These cells have low intrinsic membrane ionic permeability (Shelton *et al.* 1993) and their small size permits control of the ionic composition of the cell interior and makes them suitable for whole-cell patch clamp recording. Furthermore, the M2 protein expressed in MEL cells has been shown to have structural and functional properties similar to those of the protein synthesized during virus infection and has a similar ability to modulate cytoplasmic pH (Hay *et al.* 1993; F. M. Geraghty, unpublished observations). The results presented here show that the M2 protein has selective proton permeability which is modified by pH_o.

Preliminary reports of this work have been presented (Chizhmakov, Geraghty, Odgen, Hayhurst, Antoniou & Hay, 1995).

METHODS

M2-transfected MEL cells

A cDNA copy of the M2 mRNA of the influenza virus A/Chicken/Germany/27 (H7N7, Weybridge strain) was cloned into the Bg1 II site of plasmid pEV3 and the vector was electroporated into MEL C88 cells as previously described (Needham, Gooding, Hudson, Antoniou, Grosveld & Hollis, 1992). MEL C88 cells were derived from the Friend mouse erythroleukaemia cell line (Deisseroth, Barker, Anderson & Nienhuis, 1975). A clone, M2-39, expressing high levels of M2 on induction with 2% DMSO, was selected for study. The amount of M2 was determined by Western blot analysis, as described by Grambas *et al.* (1992).

Electrophysiological recording

M2-transformed MEL cells were used 3 or 4 days after induction with 2% DMSO. Cells were voltage clamped with the whole-cell patch clamp configuration (Hamill, Marty, Neher, Sakmann & Sigworth, 1981). To optimize pH control high concentrations of pH buffer were used as impermeant ions. The patch pipette contained 90 mM *N*-methyl-D-glucamine (NMDG), 10 mM EGTA and either 180 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes) (pH 7·2–7·4), or 180 mM 2-(*N*-morpholino)ethanesulphonic acid (Mes) (pH 6·0 or 6·5). The bath contained a similar solution (280 mosmol kg^{-1}) with 2 mM $CaCl_2$ replacing EGTA. In ion selectivity experiments NaCl and KCl were substituted isosmotically for NMDG–Hepes. The presence of $CaCl_2$ facilitated seal formation; experiments performed using $CaCl_2$-free extracellular solutions showed a similar pH dependence. Membrane current was recorded at room temperature (18–24 °C), digitized at 0·1 kHz and stored and analysed in a PC AT computer. Current was measured at 10 mV increments of membrane potential over the range −100 to +100 mV. Internal pH (pH_i) was constant; pH_o was changed at each potential by fast perfusion close to the cell with the U tube method (Krishtal & Pidoplichko, 1980) and washout by slow perfusion for up to 15 s. Rimantadine hydrochloride was applied by slow perfusion. Chemicals were obtained from BDH or Sigma.

M2 current was determined by subtracting rimantadine-resistant current from total current at each potential and pH_o (see Fig. 2). Reversal potentials (E_{rev}) were estimated by linear interpolation of least-squares fits to two or three data points of the I–V relation on each side of zero current. The zero current intercept and its statistical error were calculated from best-fit parameters. The data recorded between different solutions were corrected for the presence of liquid junction potentials of up to 6 mV.

Values are expressed as means ± S.D.

RESULTS

M2 expression in M2-39 cells

A cDNA encoding the M2 protein of the influenza virus A/chicken/Germany/27 was stably integrated into the genome of mouse erythroleukaemia (MEL) cells under the control of the human β-globin promoter and locus control region (LCR) (Needham *et al.* 1992). Cell differentiation and expression of M2 protein was induced by incubation of cells in 2% DMSO. The amount of M2 protein reached a maximum after 3–4 days and was similar to that produced in virus-infected Madin–Darby canine kidney (MDCK) cells.

Currents induced by changing pH_o and membrane potential

Initially H^+ permeability alone was investigated by measuring whole-cell current in the absence of other monovalent cations, Na^+ and K^+. The pipette and external medium contained only impermeant organic ions *N*-methyl D-glucamine ($NMDG^+$) and $Hepes^-$ or Mes^-. Untransfected MEL cells had a low background permeability. The current–voltage (I–V) relation was linear over the membrane potential range from −100 to +80 mV, intersecting the voltage axis at 0 mV, and was unaffected by pH_o tested between 9·0 and 4·3, with pH_i maintained at either 7·3 or 6·0. These properties were similar to those shown for rimantadine-blocked cells (see Fig. 2*B* and *C*). This background 'leak' conductance through the membrane and membrane–pipette seal (12 GΩ) had a mean of 83 pS (range, 50–200 pS) and was insensitive to the M2 inhibitor rimantadine. The MEL cell clone L16 (kindly provided by Dr R. Daniels, National Institute for Medical Research, London, UK) induced to express a non-channel membrane protein, human T-lymphocyte CD4, gave similar results, showing only leak conductance.

In MEL cells expressing the M2 protein (clone M2-39), with pH 7·4 inside and out, the I–V plot was similar in conductance and shape to that of non-transfected cells. Decreasing pH_o at constant potential, however, caused an increase in the inward current. Figure 1*A* illustrates currents evoked by changing pH_o from an initial value of 7·4 to 5·5, 5·0, 4·5 or 4·2 at membrane potentials of 0 or −40 mV. The current reached a steady state within 200–300 ms, the time estimated for solution change, and remained constant when perfusion was extended for 2–3 s, showing no desensitization on this time scale at any potential. Current returned to the original basal level within 3–7 s, depending on the speed of bath perfusion, upon restoring the original pH. Thus, in cells expressing M2, but not in control cells, a decrease in pH_o below 7·4

evoked an additional membrane conductance, which depended on the H^+ concentration gradient and the membrane potential (Figs 1 and 2).

Selective inhibition by rimantadine

Addition to the external medium of rimantadine, which selectively inhibits viral M2 function (Hay, Wolstenholme, Skehel & Smith, 1985), caused a gradual reduction in current induced by successive pulses of low pH, as shown in Fig. 1*B*. Inhibition was almost complete within 3–5 min incubation in 50 μM rimantadine (exponential time constant, 50–70 s) or after more than 15 min at 5 μM. No reversal of inhibition was observed 10 min after incubation in drug-free medium and the rate of inhibition did not depend on pH_i or pH_o. Addition of 50 μM rimantadine to the solution in the patch pipette did not inhibit, indicating that the drug interacts with M2 only from the extracellular N-terminal region. The specificity of the inhibition was confirmed by similar experiments in cells expressing a rimantadine-resistant mutant M2 protein with glutamic acid in place of glycine 34 (Hay *et al.* 1985), which showed no inhibition with 50 μM rimantadine.

I–V relation for M2

The residual rimantadine-resistant current gives a measure of the background leak current and was used to define the current due to M2, I_H, by subtraction from the total current, as illustrated in Fig. 2 for pH changes from 6·5 to 5·5 with pH_i 6·5. Figure 2*A* and *B* shows current traces at different membrane potentials before (*A*) and after (*B*) inhibition of M2 by rimantadine. The rimantadine-resistant current, like the current in untransfected cells, was insensitive to changes in pH_o (Fig. 2*B*) and was linear with respect to membrane potential (Fig. 2*D*). Figure 2*C* shows a direct comparison of the current, at −80 mV, at pH 6·5 before and 5 min after addition of 50 μM rimantadine; the rimantadine-sensitive current under these conditions accounted for approximately 32% of the initial current of −15 pA. Figure 2*D* shows *I–V* plots for total current (○), rimantadine-resistant leak current (●) and the net rimantadine-sensitive current through M2 (I_H, ▲) obtained by subtraction at pH_i 6·5 and pH_o 5·5.

Ion selectivity

Zero current potentials, E_{rev}, determined from *I–V* plots for M2 current were compared with the H^+ equilibrium potentials, E_H, calculated for the pH gradient with the Nernst equation. These are indicated in the *I–V* plots for M2 current shown in Figs 2*D* and 3*A* for different pH gradients in NMDG–Hepes/Mes solutions. In each case E_{rev} is close to E_H. Data are summarized in Fig. 3*C*, which shows that the reversal potentials (●) determined in NMDG–Hepes/Mes solutions were close to the equilibrium potentials, indicated by the continuous line, calculated for transmembrane H^+ concentration gradients (lower abscissa) in the range 25-fold outward to 100-fold inward. Linear regression of E_{rev} on pH gradient gave a slope of 56·7 ± 1·7 mV compared with the theoretical of 58·2 mV for E_H at 20 °C. The S.D. of the data points around the best-fit line, ±6·8 mV, indicates the overall error in estimating E_{rev}. The equilibrium potentials calculated for other components of the solutions, NMDG, Hepes, Mes, Ca^{2+} and Cl^-, could not account for the E_{rev} measured.

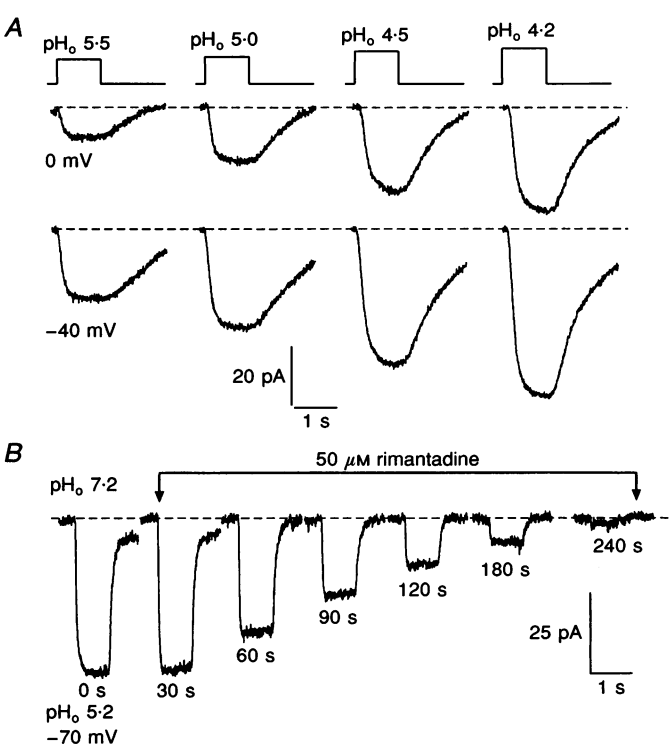

Figure 1. Membrane current induced by low pH in M2-expressing MEL cells and its inhibition by rimantadine

A, membrane current in a M2-39 cell (pH_i, 7·4) in response to 1 s pulses with pH changes from 7·4 to 5·5, 5·0, 4·5 or 4·2 (indicated schematically above traces) at 0 or −40 mV. Currents returned to baseline between pulses. *B*, decrease by 50 μM rimantadine of inward current evoked by pH_o change from 7·2 to 5·2. Times after applying rimantadine are indicated for each trace.

To test the ion selectivity of M2 other inorganic ions were included in internal and external solutions. Figure 3*B* shows that when pH_o was changed from 7·0 to 6·0 with a pH_i of 7·0 in the presence of an outward Na^+ gradient ($[Na^+]_i$, 30 mM; $[Na^+]_o$, 10 mM; Na^+ equilibrium potential, E_{Na}, −28 mV), E_{rev} changed from 0 to +61 mV, close to E_H of +58 mV, in the direction away from an E_{Na} of −28 mV. This result is contrary to the conclusion reported elsewhere (Pinto *et al.* 1992) that Na^+ permeability in M2 increases on lowering pH_o. Figure 3*C* shows the zero current potentials determined at internal and external concentrations of NaCl up to 120 mM but with E_H set at +58 mV (△) or −58 mV (▲), plotted against the Na^+ concentration gradient (upper abscissa). The values did not change with the equilibrium potentials for either the Na^+ gradient (E_{Na}, indicated by the continuous line as for pH) or the Cl^- gradient (E_{Cl}). However, they did correspond to E_H for the pH gradient, either +58 mV (△) or −58 mV (▲) indicated by the dotted lines. Linear regression of E_{rev} on pH gradient in the presence of NaCl gave a slope of 59·2 ± 2·9 mV, which is comparable to the theoretical value of 58·2 mV for E_H at 20 °C, and is not significantly different from the value of 56·7 ± 1·7 mV obtained in the absence of NaCl.

More generally, results obtained with NaCl- and KCl-containing solutions showed that: (i) the amplitude of the

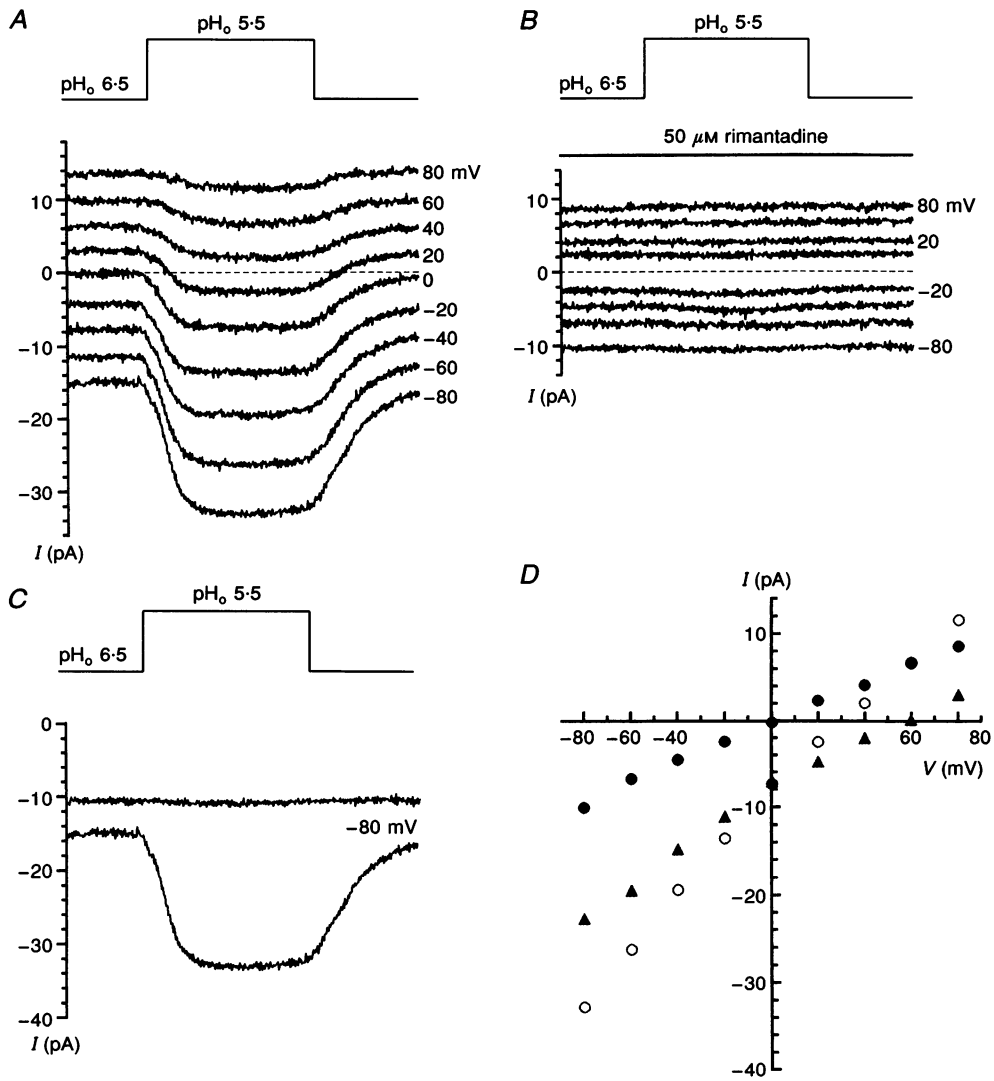

Figure 2. *I–V* analysis of rimantadine-sensitive M2 current

A, superimposed data records of membrane current evoked by pH_o change from 6·5 to 5·5 for 1 s (indicated above traces) at membrane potentials from −80 to +80 mV. *B*, current traces with pH_o change from 6·5 to 5·5 in the same cell in the period 5–10 min after applying 50 μM rimantadine. *C*, current traces with pH_o change from 6·5 to 5·5 at −80 mV just prior to (lower trace) and 5 min after (upper trace) applying 50 μM rimantadine. *D*, *I–V* plots of: ○, total membrane current at pH 5·5 measured 0·5 s after solution change, E_{rev} = +32 mV; ●, rimantadine-resistant current, E_{rev} = 0 mV; ▲, difference current due to M2, E_{rev} = +60 mV. E_H = +58 mV for pH_i 6·5.

pH-induced current was not altered noticeably by the presence of NaCl or KCl; (ii) at $V = 0$, the direction of the current reflected that of the pH gradient and not the Na^+/K^+ gradient; and (iii) E_{rev} was similar to E_H, not to E_{Na}, E_K (the K^+ equilibrium potential) or E_{Cl}. The Goldman–Hodgkin–Katz equation (Hodgkin & Katz, 1949) was used to estimate the maximum permeability to Na^+ relative to H^+ from the maximum difference observed between E_{rev} and E_H, 14·4 mV with 5 mM NaCl pH 6·0 inside and 120 mM NaCl pH 7·0 outside the cell, giving a permeability ratio of Na^+ to H^+ of 6×10^{-7}.

Dependence of M2 conductance on external H^+ concentration

Permeation in ion channels and transporters may be characterized by saturation of the flux at high concentrations of the permeant ion. Plotting the amplitude of inward current, at constant membrane potential, against H^+ concentration showed that current approached saturation as the pH_o was reduced to 5·0. To take account of the change in driving potential for H^+ permeation as pH is altered, results were analysed in terms of the membrane conductance for H^+, defined as $g_H = I_H/(V - E_H)$, and are

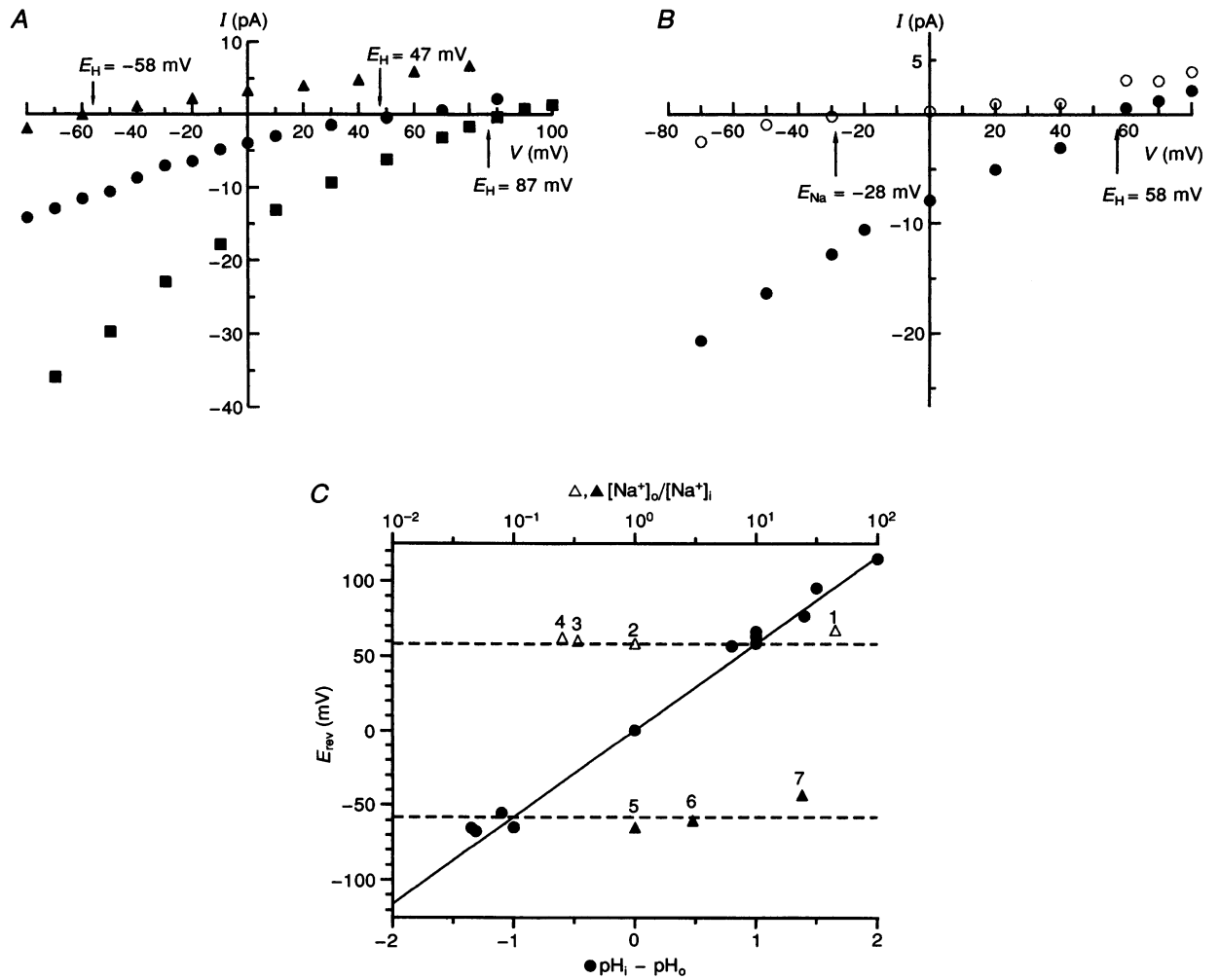

Figure 3. Reversal potentials of M2 current

A, *I–V* plots of current evoked by pH_o change from 7·3 (= pH_i) to 6·5 (●; E_H, 47 mV; E_{rev}, 56 mV) or 5·8 (■; E_H, 87 mV; E_{rev}, 93 mV) in the same cell; and rimantadine-sensitive current on going from pH 6·0 to 7·0 in a different cell (▲; E_H, −58 mV; E_{rev}, −55 mV). *B*, *I–V* plots of rimantadine-sensitive current on going from pH 7·0 internal and external (○; E_H, 0 mV) to pH_o 6·0 (●; E_H, 58 mV). External [NaCl], 10 mM; internal [NaCl], 30 mM; E_{Na}, −28 mV. *C*, reversal potentials plotted against transmembrane pH gradient (●, lower abscissa) and log Na^+ concentration gradient (▲ at E_H of −58 mV; △, at E_H of +58 mV; upper abscissa). For the numbered points (1–7) internal/external $[Na^+]$ (mM) was: 1, 2/90; 2 and 5, 5/5; 3, 30/10; 4, 40/10; 6, 10/30; 7, 5/120. Continuous line has a Nernst slope of 58·2 mV per decade; upper and lower dotted lines indicate +58 mV and −58 mV, respectively. Least-squares regression of E_{rev} on $pH_i - pH_o$ has a slope of 56·7 ± 1·7 mV for Na^+-free solution and 59·2 ± 2·9 mV for Na^+-containing solution. Overall S.D. for the estimation of E_{rev} was ±6·8 mV.

plotted in Fig. 4A. g_H increased from a low value at pH_o 7·2 and reached saturation at pH_o below 4 (100 μM H^+) at each membrane potential tested between −70 and +70 mV. The dependence of g_H on external H^+ concentration can be described by a simple one-site binding model for ion permeation, where both the maximum conductance and the apparent dissociation constant depend on membrane potential. The apparent dissociation constant, K_p, determined by fitting a single site binding curve, was 1·2 μM at −60 mV and 2-fold higher at +60 mV (K_p, 2·3 μM). Evidence was obtained for a separate activation of M2 on lowering pH_o, distinct from changes in H^+ permeation. This is most readily demonstrated by the effect of pH_o on outward current with pH_i of 6 or less. Increasing pH_o would be expected to increase outward current as a result of the larger outward electrochemical gradient for H^+. However, as shown by the I–V data in Fig. 4B, when pH_o was raised from 6·0 (▲) to 8·0 (■) the current was diminished to a level similar to that observed in the same cell after addition of 50 μM rimantadine (●). This effect was analysed at +60 mV for a series of pH_o values between 6 and 9 with a pH_i of 6·0. The conductance, $g = I_H/(V - E_H)$, for outward current in M2 at +60 mV was normalized with respect to the maximum at pH 6·0 (g_{pH6}) in each cell and plotted against pH_o, shown in Fig. 4C. The conductance due to M2 was close to zero above pH_o 8·5 and increased progressively as pH_o decreased, indicating that M2 conductance is activated at a pH_o more acid than pH 8. The fractional conductance was 0·5 at pH 7·0, giving an apparent activation constant, K_a, of approximately 0·1 μM. In contrast, increase of pH_i from 5 to 8 did not inhibit inward current, indicating activation mainly by external H^+.

Because both activation and permeation depend on H^+ it is difficult to distinguish changes in the number of open channels (i.e. open probability) and the permeability of each channel. The decrease in current as driving potential increased allows some distinction between an effect of

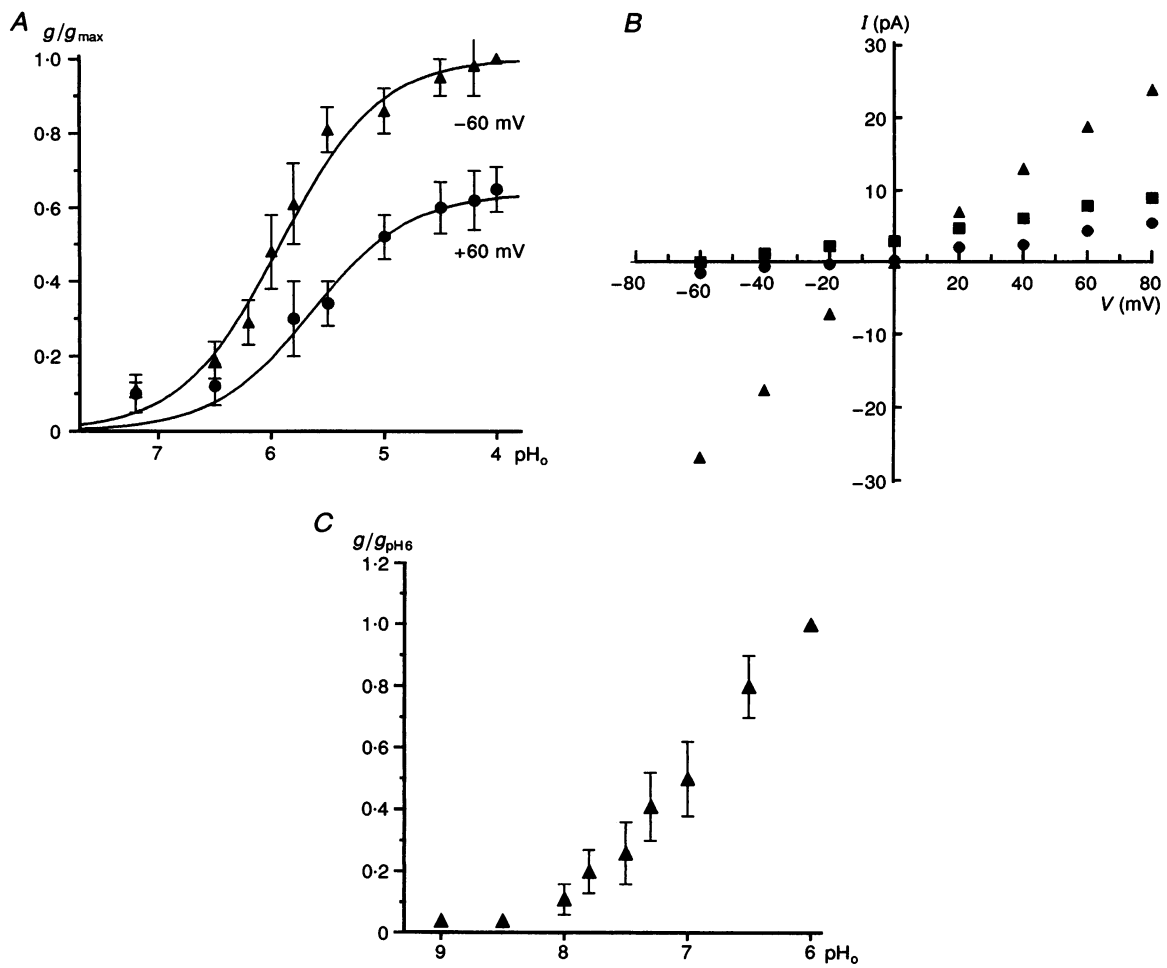

Figure 4. Effect of pH_o on M2 conductance

A, chord conductance $g = I_H/(V - E_H)$ normalized to pH_o 4 at −60 mV plotted against pH_o at −60 and +60 mV; pH_i 7·4. Single site binding function was fitted by least-squares regression with a K_p of 1·2 μM at −60 mV and 2·3 μM at +60 mV. *B*, I–V plots for total current in a cell with pH_i 6·0, pH_o 6·0 (▲), pH_o 8·0 (■) and pH_o 6·0 with 50 μM rimantadine (●). *C*, chord conductance $g = I_H/(V - E_H)$ with pH_i 6·0 at +60 mV normalized to the value in each cell at pH_o 6·0, plotted against pH_o.

external H^+ on single channel permeability, which would if anything increase, and channel open probability, which must therefore have decreased at high pH_o. However, this could not be tested directly by single channel recording because, even under optimal recording conditions, single channel currents were too small to be measured either directly or by analysis of current fluctuations. With large electrochemical gradients the analysis of current variance (assuming a small proportion of channels open) gave estimates of single channel current less than 10 fA and correspondingly very small single channel conductance.

DISCUSSION

The results of these experiments provide direct evidence that in physiological media the M2 protein of influenza A virus forms a proton-selective channel which is regulated by pH_o.

Evidence for proton selectivity is based on three criteria. (1) At zero membrane potential the direction and amplitude of the current depended only on the proton concentration gradient. (2) The zero current potential for permeation through M2 was close to the electrochemical equilibrium potential for H^+ and was not influenced by the electrochemical gradients of other physiological ions. (3) The characteristics of the M2 I–V relationships and saturation at high H^+ concentrations were not affected by physiological concentrations of Na^+, K^+ or Cl^-. This latter observation also indicates that H^+ permeation in M2 is not coupled by cotransport to movement of other ions.

Estimates of the permeability of M2 to Na^+ relative to H^+ gave values less than 6×10^{-7} when expressed in MEL cells. In contrast, it was concluded from studies of the permeation properties of M2 proteins expressed in *Xenopus* oocytes (Pinto *et al.* 1992; Holsinger *et al.* 1994; Shimbo, Brassard, Lamb & Pinto, 1996) and CV-1 cells (Wang *et al.* 1994) that M2 proteins formed cation channels with high permeability to Na^+. However, other characteristics of the M2 proteins in those studies, such as pH dependence, absence of desensitization and inhibition by amantadine, appear similar to those reported here for the M2 protein expressed in MEL cells. Reasons for the apparent difference in ion selectivity between experiments reported here and those reported elsewhere may be as follows. Although whole-cell patch clamp was used to measure currents in M2-expressing CV-1 cells, current was measured only in weakly buffered phosphate-buffered NaCl solution over a limited range of membrane potential, which did not allow direct interpolation of the reversal potential, and rimantadine-resistant leak subtraction was not made, leading to further systematic error in estimating the reversal potential for the wild-type M2 (Wang *et al.* 1994). Furthermore, the conclusion that reducing pH_o below 7 activates M2 permeability mainly to Na^+ (Pinto *et al.* 1992) was tested directly here in the experiments shown in Fig. 3*B* and *C*, which showed that the reversal potential for M2 current followed the change in equilibrium potential for the pH gradient, and not that for Na^+, on decreasing pH_o.

The relationship between conductance and pH_o may be interpreted as evidence for two protonation sites on the M2 channel, one involved in H^+ permeation, the other in activation by pH_o to change the open probability. Interaction of external protons with the activation site, demonstrated clearly for outward current at pH_o greater than 6·0, had an apparent pK_a of 7·0 (at +60 mV) when fitted with a single binding site characteristic, and did not depend on pH_i, consistent with a site on M2 located close to the outside of the membrane. This 'sidedness' of the pH activation also supports a uniform orientation of M2 in the membrane. On the other hand, in experiments at pH_o less than 7·0 with inward current, interaction of external protons with the 'permeation site' showed evidence of saturation of the conductance, with a K_p of 1·2 μM at −60 mV and 2·3 at +60 mV, depending on both membrane potential and pH_i. The greater apparent affinity at more negative membrane potentials indicates that the site is located within the transmembrane domain of the protein. The lower apparent affinity of external protons at lower pH_i (K_p was 3- to 4-fold higher at pH_i 6·0 than pH_i 7·2) may be explained by competition between internal and external protons for the same site. In a similar way, an effect of pH_i on voltage activation of the H^+ conductance in molluscan neurones has been shown (Byerly, Meech & Moody, 1984). With regard to the species of permeant ion, equilibrium potentials for H^+ and OH^- are equal because of the constant ion product for H_2O dissociation. Thus, the reversal potentials do not distinguish between proton and hydroxyl ion permeation.

Proton-selective conductances activated by membrane potential rather than pH have been identified in the plasma membrane of a variety of cells, including snail neurones (Thomas & Meech, 1982), amphibian oocytes (Barish & Baud, 1984) and human neutrophils (DeCoursey & Cherny, 1993), although the proteins responsible have yet to be identified. As in those instances, because of the small unitary current (see Byerly & Suen, 1989) we cannot conclude unequivocally that the M2 protein is a channel rather than some other type of transporter. Although the mechanisms of H^+ conduction may be similar, the differences between the characteristics of cellular conductances, for instance the activation by depolarization and outward rectification (Byerly *et al.* 1984), and the H^+ activated conductance properties of M2 reflect their different roles.

Proton permeability of M2 reflects its physiological activity in modifying transmembrane pH gradients of the *trans*-Golgi during virus export (Ciampor *et al.* 1992), as well as its perceived role in protonating the virion interior during virus uncoating within endosomes during endocytosis early in infection (Martin & Helenius, 1991; Schroeder, Ford, Wharton & Hay, 1994). The responsiveness of conductance to pH_o between 6·5 and 5 and saturation at pH below 5 is consistent with its function in the pH range 5 to 6·5 present

within the *trans*-Golgi and endosomes. In responding to changes in pH_o below 8 but not to pH_i, the activation of M2 in transfected and virus-infected cells (Hay *et al.* 1993) also appears to be related to its function and orientation in viral and endosomal membranes. In viral endocytosis, the acid-sensitive N-terminus of M2 on the viral exterior is oriented to the acidified lumen during uncoating within the endosome. During virus export it again faces into the acidified lumen from the *trans*-Golgi membrane, arriving in the surface membrane by exocytosis oriented to the external face for release in this configuration in virus budding.

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