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Ion Channel Activity of Influenza A Virus M_2 Protein: Characterization of the Amantadine Block

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The influenza A virus M_2 integral membrane protein has ion channel activity which can be blocked by the antiviral drug amantadine. The M_2 protein transmembrane domain is highly conserved in amino acid sequence for all the human, swine, equine, and avian strains of influenza A virus, and thus, known amino acid differences could lead to altered properties of the M_2 ion channel. We have expressed in oocytes of *Xenopus laevis* the M_2 protein of human influenza virus A/Udorn/72 and the avian virus A/chicken/Germany/34 (fowl plague virus, Rostock) and derivatives of the Rostock ion channel altered in the presumed pore region. The pH of activation of the M_2 ion channels and amantadine block of the M_2 ion channels were investigated. The channels were found to be activated by pH in a similar manner but differed in their apparent K_is for amantadine block.

The influenza A virus M_2 protein, which is encoded by a spliced mRNA derived from genome RNA segment 7, is orientated in membranes with 23 N-terminal extracellular residues, a 19-residue transmembrane domain, and a 54-residue C-terminal cytoplasmic domain (24, 26, 27). Minimally, the M_2 protein forms a homotetramer consisting of either a pair of disulfide-linked dimers or a disulfide-linked tetramer (19, 39). The M_2 protein is abundantly expressed at the surface of virus-infected cells, but in comparison with hemagglutinin (HA) and neuraminidase, the M_2 protein is a minor component of virions (27, 43).

The M_2 protein was implicated in having an essential role in the life cycle of influenza virus during studies of the anti-influenza drug amantadine hydrochloride (1-aminoadamantane hydrochloride). Drug-resistant mutants were isolated, and genetic and nucleotide sequencing studies indicated that these mutants contained changes that mapped predominantly to the M_2 transmembrane domain (13). For all influenza virus strains, the amantadine block to virus replication occurs at an early stage between the steps of virus penetration and uncoating (22, 36). In the presence of amantadine, the membrane (matrix) (M_1) protein fails to dissociate from the ribonucleoproteins (4, 29), and transport of the ribonucleoprotein complexes to the nucleus does not occur (29). In addition to the early effect of amantadine, the drug has a second late effect on some subtypes of avian influenza virus which have an HA that is cleaved intracellularly and have a high pH optimum of fusion (e.g., fowl plague virus [FPV] Rostock). A large body of data indicates that addition of amantadine to virus-infected cells causes a premature conformational change in HA that occurs in the *trans* Golgi network. This change occurs because the intralumenal pH of the *trans* Golgi network compartment has been lowered below the threshold needed to induce the acid pH transition of HA (5, 6, 10, 11, 39). This late effect of amantadine can be reversed by addition of the sodium ionophore monensin (39). Moreover, alterations in HA which either increase or decrease the pH at which the HA conformational change occurs also influence susceptibility to drug action (38). Taken together, these data led to the hypothesis that the function of the influenza virus M_2 protein is to act as an ion channel that modulates the pH of intracellular compartments (12, 39). As the same mutations in the M_2 transmembrane domain abolish susceptibility to both the early and the late effects of amantadine, a rational explanation is that the M_2 protein in virions and the M_2 protein in virus-infected cells have the same function. It is generally believed that once the virion particle has been endocytosed, the ion channel activity of the virion-associated M_2 protein permits the flow of ions from the endosome into the virion interior to disrupt protein-protein interactions and free the ribonucleoprotein from the M_1 protein (reviewed in references 12, 15, 28, and 35).

To provide direct evidence for the M_2 protein having ion channel activity, the M_2 protein was expressed in oocytes and voltage-clamp procedures were used to analyze the membrane currents. The data indicated that the M_2 protein has ion channel activity which is regulated by changes in pH, with the channel being activated at the lowered pH found intralumenally in endosomes and the *trans* Golgi network (33). The M_2 protein's ion channel activity was found to be inhibited by amantadine hydrochloride. In addition, mutant M_2 proteins that contain amino acid changes which when found in the influenza virus genome confer drug resistance were not affected by the drug (33), thus providing direct evidence for the mechanism of action of the antiviral drug. Analysis of the ion selectivity of the wild-type M_2 protein's ion channel activity indicated a permeability to Na^+ ions (33). Although it could not be measured in the oocyte expression experiments, it would not be surprising if the monovalent cation conductance of the M_2 ion channel extends to H^+. Under conditions in which the M_2 transmembrane domain was incorporated into planar membranes, a proton translocation that is susceptible to block by amantadine has been identified (8).

Although the M_2 transmembrane domain is highly conserved in sequence for all human, swine, equine, and avian strains of influenza A virus (21, 42), it is possible that some of the known amino acid differences could lead to altered properties of the M_2 ion channel. The two closely related

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strains of avian influenza virus, FPV Rostock [A/chicken/Germany/34 (H7N1)] and FPV Weybridge [A/chicken/Germany/27 (H7N7)], differ in their susceptibilities to amantadine (13). In addition, it is possible that the Rostock and Weybridge M_2 proteins have different response curves to pH activation as it is estimated that Rostock M_2 protein can raise the intracellular pH to a greater extent than can Weybridge M_2 protein (11). Thus, to examine properties of the avian FPV M_2 proteins and a human influenza virus M_2 protein [A/Udorn/72 (H3N2)] and their response to amantadine hydrochloride, we expressed the channels in oocytes of *Xenopus laevis* and measured whole-cell currents under various conditions by a two-electrode voltage-clamp procedure. In addition, we performed experiments bearing upon the nature of the amantadine block of the M_2 ion channels of these subtypes.

MATERIALS AND METHODS

Construction of recombinant plasmids, site-specific mutagenesis, and in vitro synthesis of RNA. The cDNA encoding the influenza virus A/chicken/Germany/34 (H7N1) (FPV Rostock) M_2 protein was constructed from synthetic oligonucleotides with DNA polymerase, DNA ligase, and a polymerase chain reaction; details will be described elsewhere (40). The cDNA was cloned in pGEM3 such that the mRNA sense transcripts could be generated with the bacteriophage T7 polymerase promoter and T7 RNA polymerase. The point mutants, I-27-V and L-38-F, and the double mutant designated Weybridge were constructed by a series of four-primer polymerase chain reactions (16) with the Rostock cDNA as template. The complete nucleotide sequences of all cDNAs were confirmed by dideoxy chain-terminating sequencing using Sequenase (U.S. Biochemical Corp., Cleveland, Ohio). Synthetic RNA transcripts were synthesized as described previously (33).

Culture of oocytes and microinjection of mRNA. Oocytes were removed from female *X. laevis* (Nasco, Fort Atkinson, Wis.). They were then treated with collagenase B (2 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) to remove follicle cells and incubated in ND96 solution (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM sodium pyruvate, 5 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]–NaOH, and 0.1 mg of gentamicin per ml, pH 7.5) at 17°C (33). One day later, selected oocytes (stages IV and V) were injected with 50 nl of RNA (1 μg/μl) with a 20-μm-diameter glass pipette and incubated in ND96 at pH 7.5. A total of 24 h after mRNA injection, oocytes were moved to ND96 at pH 8.5 to limit the deleterious effect of the M_2 ion channel activity on the oocytes.

Electrophysiological recordings. Whole-cell current was measured with a two-electrode voltage-clamp apparatus 48 to 72 h after mRNA injection (33). The electrodes were filled with 3 M KCl, and the oocyte was bathed in Barth's solution (88.0 mM NaCl, 1.0 mM KCl, 2.4 mM $NaHCO_3$, 0.3 mM $NaNO_3$, 0.71 mM $CaCl_2$, 0.82 mM $MgSO_4$, and 15 mM HEPES, pH 7.5) or modified Barth's solution as indicated during recording. Amantadine hydrochloride (Sigma Chemical Co., St. Louis, Mo.) and rimantadine (kindly made available by Hoffmann-La Roche Inc., Nutley, N.J.) stock solutions (10 or 20 mM) were made up freshly.

Data analysis. Whole-cell currents were analyzed with C-Lab software (Indec System, Inc., Sunnyvale, Calif.). The sigmoidal curve of isochronic K_i was fitted as described previously (7). All the exponential curves were fitted by Peakfit software (Jandel Scientific, Corte Madera, Calif.).

Immunoprecipitation. Oocytes were labeled with [^{35}S]methionine (250 μCi/ml) (Amersham Corp., Arlington Heights, Ill.) in ND96 solution from 24 to 48 h postinjection and then homogenized as described previously (33). M_2 protein was immunoprecipitated from oocyte lysates with monoclonal antibody 1F1 (19), and samples were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 17.5% polyacrylamide–4 M urea gels and processed for fluorography and autoradiography as previously described (23).

Indirect immunofluorescence microscopy. Oocytes were frozen and sectioned as previously described (33). Oocytes were sectioned into 10-μm slices, collected on gelatin-subbed slides, and air dried. Sections were fixed in 1% formaldehyde essentially as described previously (41) and incubated with ascites fluid of M_2-specific monoclonal antibody 1F1 (19) (diluted 1:300 in phosphate-buffered saline–bovine serum albumin), a biotinylated goat anti-mouse secondary antibody, and fluorescein isothiocyanate-conjugated streptavidin. Photomicroscopy was performed on a Zeiss Axiophot fluorescent microscope. All photographic exposure times were equivalent.

RESULTS

Expression of Rostock and Weybridge M_2 proteins in oocytes. We have provided evidence previously that the pore of the M_2 ion channel is the transmembrane domain, as mutations that alter both ion selectivity and activation properties can be introduced into the transmembrane domain (33). The FPV Rostock M_2 protein sequence contains 14 amino acids which differ from those of the Udorn M_2 protein sequence, and two of these amino acid changes occur in the transmembrane domain, with Udorn containing valine at residues 27 and 28 and FPV Rostock containing isoleucine at these positions (Fig. 1) (13, 25). To examine directly ion channel activity of the Rostock M_2 protein, a cDNA encoding the M_2 protein was constructed with appropriate oligonucleotides based on the nucleotide sequence of FPV Rostock RNA segment 7 (31), but adjusted so that the M_2 protein sequence matched that reported by Hay and colleagues (13). The FPV Weybridge M_2 protein sequence differs from the Rostock M_2 sequence by five amino acid residues, two in the N-terminal ectodomain, two in the transmembrane domain, and one at the junction of the transmembrane domain and the cytoplasmic tail (13). Thus, to determine whether the amino acid difference in the transmembrane domain between Rostock and Weybridge leads to alterations in channel activity and amantadine sensitivity, the changes I-27-V and L-38-F were introduced into the Rostock cDNA by a mutagenesis procedure. The resulting protein molecule contains the ectodomain and cytoplasmic tail of Rostock M_2 and the transmembrane domain of Weybridge M_2 and for convenience is here designated Weybridge M_2. As described below, there are distinct differences in ion channel activity and amantadine block between Rostock and Weybridge M_2 proteins, which lends credence to the approach taken. In addition to the Rostock and Weybridge M_2 proteins, we also examined Rostock M_2 proteins containing the single residue changes I-27-V and L-38-F. These cDNAs were cloned into pGEM3. Synthetic mRNA transcripts were synthesized with bacteriophage T7 polymerase from the cDNA templates and microinjected into oocytes of *X. laevis*. Translation of proteins was examined by labeling oocytes 24 h postinjection with [^{35}S]methionine for 24 h. Oocyte lysates were immunoprecipitated with a monoclonal antibody (1F1) (19) specific

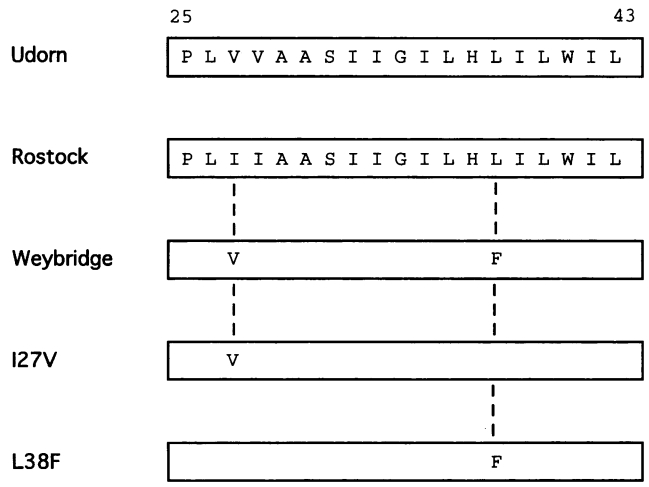

FIG. 1. Schematic diagram indicating the amino acid sequence of the influenza virus M_2 protein transmembrane domain residues 25 to 43 of subtypes Udorn, Rostock and Weybridge and two Rostock point mutants, I-27-V and L-38-F. The Udorn and Rostock M_2 proteins used in these studies have the amino acid sequences reported for the respective subtypes (13, 26). The M_2 proteins designated Weybridge, I-27-V, and L-38-F contain the amino acid sequences indicated and were constructed by site-specific mutagenesis with the Rostock M_2 cDNA as template. Thus, the M_2 proteins designated Weybridge, I-27-V, and L-38-F contain the same residues in the M_2 ectodomain and cytoplasmic tail as those found in the Rostock subtype.

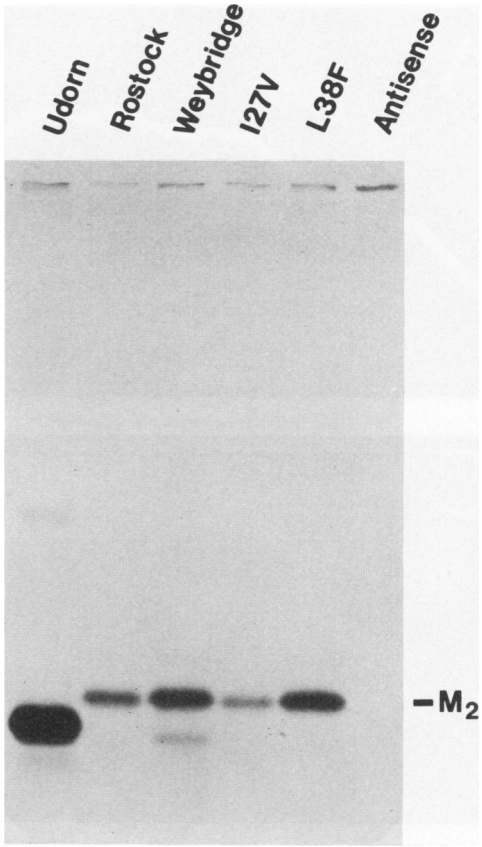

FIG. 2. Expression of wild-type and mutant proteins in oocytes of *X. laevis*. Synthetic mRNAs were transcribed from pGEM3 plasmids encoding wild-type or mutant M_2 proteins and microinjected (50 nl of RNA [1 μg/μl]) into oocytes of *X. laevis*. The Udorn subtype mRNA was transcribed from M_2 cDNA cloned into a derivative of pGEM9Zf(−) (provided by S. Goldstein and Chris Miller) that yields an mRNA containing a 5′ untranslated region derived from rat potassium channel Kv3 and a 3′ poly(A) tract. At 24 h postinjection, oocytes were labeled for 24 h with [^{35}S]methionine and homogenized. M_2 proteins (designated as in the legend to Fig. 1) were immunoprecipitated with an M_2-specific monoclonal antibody (1F1) (19) and analyzed by SDS-PAGE. Antisense, expression of RNA in opposite sense to M_2 mRNA.

for the M_2 ectodomain, and as shown in Fig. 2, M_2 polypeptides with M_rs of ~15,000 could be detected by SDS-PAGE. To examine for expression of the M_2 proteins at the surface of oocytes, thin (10-μm) sections of oocytes were incubated successively with the 1F1 monoclonal antibody, a biotinylated secondary antibody, and fluorescein-conjugated streptavidin. The oocytes showed a characteristic surface staining on the plasma membrane (Fig. 3).

Comparison of responses of different M_2 proteins to activation by lowered pH. The ion channel activity of oocytes expressing the Rostock and Weybridge M_2 proteins was tested by a two-electrode voltage-clamp procedure, and the pH of the medium bathing the oocytes was held at pH 7.5 or 6.2. At pH 7.5, the inward currents of oocytes expressing both Rostock and Weybridge M_2 proteins (-0.15 ± 0.03 and -0.17 ± 0.02 μA, respectively) were slightly larger than those for the M_2 protein of the Udorn subtype (-0.11 ± 0.03 μA). However, to report the pH activation of the M_2 ion channel proteins that is independent of the level of protein expression, the currents were normalized with respect to the current that flowed when the oocyte was bathed in a solution with a pH of 7.5. This was justifiable because cell-to-cell variation in the amplitude of the current was minimal (see standard deviations above). When the pH of the solution in which the oocytes were bathed was decreased to pH 6.2 and measurements were made after 30 s, the currents of oocytes expressing both ion channel proteins increased by approximately sevenfold over the value measured at pH 7.5 and did not increase with longer times of incubation at pH 6.2 (Fig. 4A). A similar current increase upon pH activation was reported previously for the Udorn M_2 protein (33). The amplitude of the current increased monotonically with decreasing pH for the oocytes that expressed either the Rostock or the Weybridge M_2 protein (Fig. 4B), whereas control oocytes had only small, pH-independent currents for all values of pH tested.

Comparison of responses of different M_2 proteins to amantadine block. The ability of amantadine to block the current of oocytes expressing the Rostock and Weybridge M_2 proteins was tested. When applied for 2 min at pH 6.2, amantadine (100 μM) gave a nearly complete block of the Weybridge M_2 channel current but gave only a partial block of the Rostock M_2 channel current (Fig. 5A). To ensure that the oocyte membranes were not made leaky by the low-pH solutions, the oocytes were again bathed in a solution with a pH of 7.5 (pH 7.5 II), and it was found that the currents of the oocytes were very small, indicating that the leakage current was not increased (Fig. 5A). Currents of oocytes did not return to the value observed before amantadine addition because of the very slow reversibility of drug action (see below).

As the transmembrane domains of the Rostock and Weybridge M_2 proteins differ by only two residues, Rostock M_2

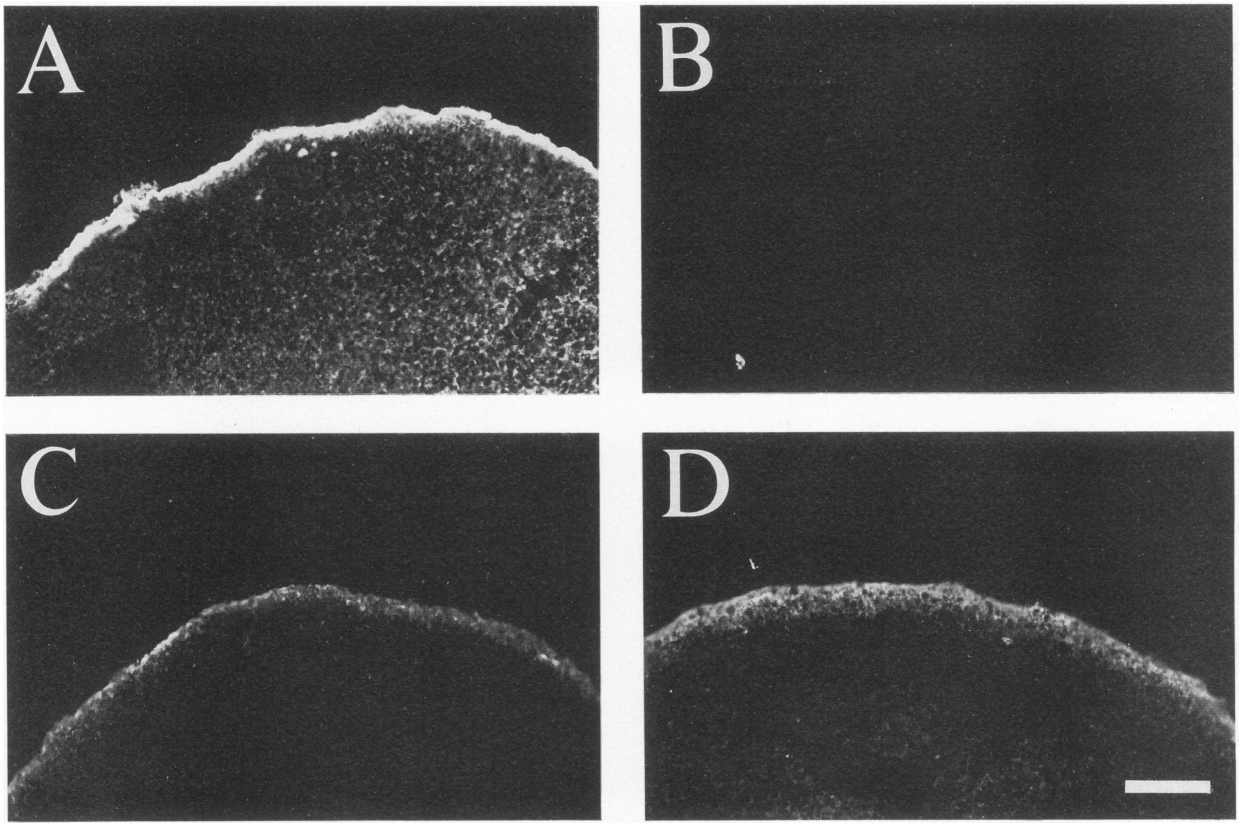

FIG. 3. Cell surface expression of wild-type and mutant proteins in oocytes of *X. laevis*. Indirect immunofluorescent microscopy of sections of oocytes was done with an M_2-specific monoclonal antibody (1F1) (19) and staining with a biotinylated goat anti-mouse secondary antibody and fluorescein isothiocyanate-conjugated streptavidin. A, Udorn M_2; B, antisense RNA-injected oocyte; C, Rostock M_2; D, Weybridge M_2. Bar, 100 μm.

protein mutants containing the single residue changes I-27-V and L-38-F were examined to investigate whether a single residue contributed to the observed difference in response to amantadine between Rostock and Weybridge (Fig. 1). The synthesis (Fig. 2) and expression at the plasma membrane (data not shown) of these point mutant proteins were confirmed, and the extent of the block of their currents by amantadine at pH 6.2 was tested. Substitution of I-27 found in the Rostock M_2 channel for V rendered the channel fully susceptible to block by amantadine in 2 min at pH 6.2 (Fig. 5A). However, substitution of L-38 with F made a channel that was not completely blocked by amantadine in 2 min at pH 6.2 (Fig. 5A).

The ability of 100 μM amantadine to block in 2 min the currents of the Rostock and Weybridge M_2 ion channels at pH 6.2 differentially led us to search for conditions in which the drug could block the Rostock M_2 channel activity completely. Thus, we tested for block at pH 6.2 after prior application of amantadine (100 μM for 2 min) at pH 7.5 (Fig. 5B). It was observed that the Rostock M_2 channel was nearly completely blocked by prior application of amantadine at pH 7.5, a pH at which the channel is nearly closed. Thus, for the Rostock M_2 ion channel amantadine is effective as a closed state channel blocker and is less effective once the channel has been activated (opened) by lowered pH. As the block by amantadine is at best slowly reversible, the short duration of incubation at pH 6.2 without the drug did not permit currents to return to the values observed at pH 6.2 prior to drug addition. The L-38-F mutant was again not completely blocked by amantadine, as was found at pH 6.2. Thus, one interpretation of these data is that the single change L-38-F leads to partial amantadine resistance but in channels that also contain the I-27-V change (as in Weybridge), the effect of the latter substitution is predominant and renders the channel amantadine sensitive.

Stoichiometry of amantadine block. Studies on the amantadine sensitivity of the replication of influenza virus subtypes in tissue culture indicated that the growth of some subtypes (e.g., Weybridge or Udorn) is more sensitive to the drug than that of others (e.g., Rostock) (13). We wished to determine the concentration dependence of the amantadine block on the M_2 ion channel activity of the Rostock, Weybridge, and Udorn subtypes. However, the effect of amantadine is at best only slowly reversible in tissue culture cells (20, 22) and in oocytes (see below), and this would make it difficult to study the drug block under equilibrium conditions because it is possible to record continuously from the same oocyte for only about 1 h. Thus, to study the stoichiometry of the reaction, we measured the time course of the block of M_2 channel currents for each of several concentrations of amantadine. For a first-order reaction between an ion channel protein and a blocker (where the blocker concentration is constant), the time course of current after application of the blocker ought to be characterized by a single exponential decay, the time constant of which is inversely proportional to the concentration of the blocker (see below and reference

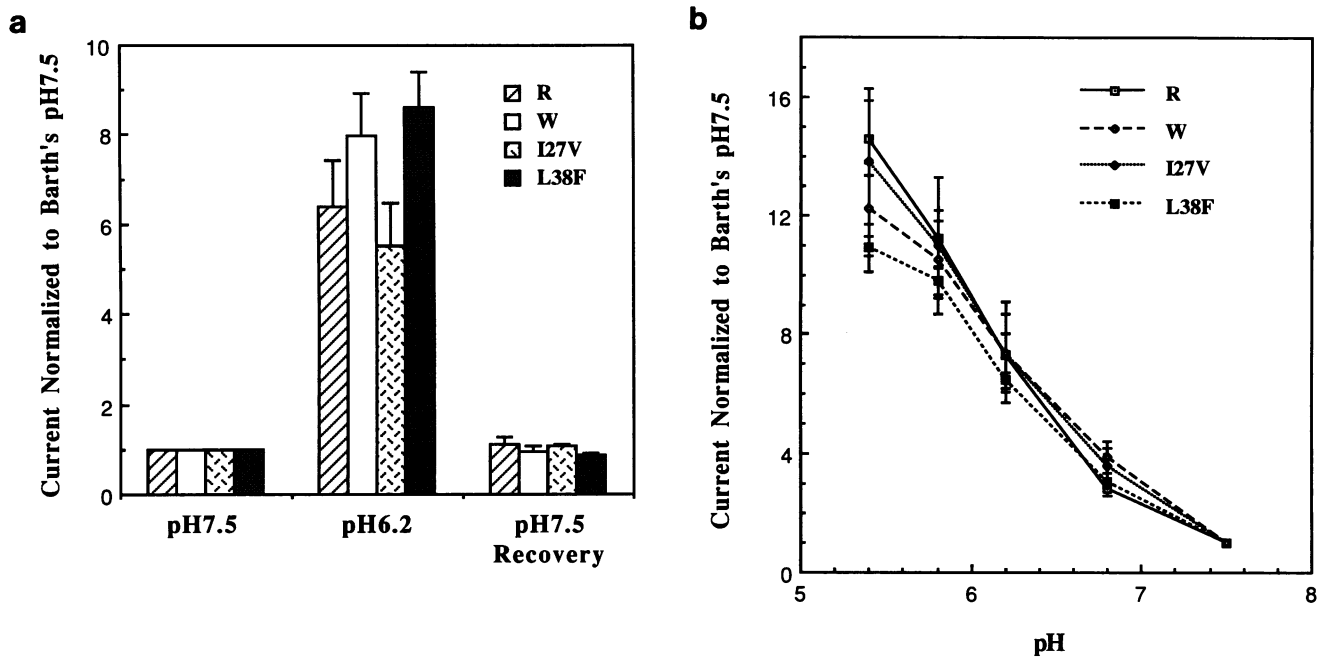

FIG. 4. Activation of M_2 ion channels by lowered pH. (a) Membrane current amplitude measured at pH 7.5, after 30 s of incubation at pH 6.2, and again after 30 s of incubation at pH 7.5 (to check for recovery). Note that the current (plotted as a multiple of the current at pH 7.5) of each channel increased reversibly at pH 6.2. (b) Membrane current (plotted as a multiple of the current at pH 7.5) as a function of pH. Measurements were made 30 s after solution changes. In this and all subsequent figures, the low endogenous current (background), recorded from oocytes injected with antisense mRNA, was subtracted from each datum point. This corrected current was then normalized to the corrected current that flowed at pH 7.5. R, Rostock M_2; W, Weybridge M_2; I27V, I-27-V M_2; L38F, L-38-F M_2.

2). From the experimentally determined time constant of the block, it is possible to calculate the forward reaction rate constant (k_r). For a first-order reaction, k_r ought to have the same value for all concentrations.

We measured the time course of the block of the current of the Rostock M_2 channel for periods as long as 1 h after applying 0.4 to 100 μM amantadine (example data appear in Fig. 6). The time course of the current decay could be fitted by a first-order exponential function in all cases. The time constant of current decay (τ) decreased from about 1,320 s for 4 μM amantadine to about 70 s for 100 μM amantadine (Fig. 7). The Hill coefficient (see Appendix) for the binding of a ligand (amantadine) to a protein is given by the slope of the relationship between $\log(1/\tau)$ against log[ligand]. The data could be fitted by a straight line, and the slope of this line was 0.91 ± 0.02. Thus, the Hill coefficient for amantadine binding to the M_2 ion channel was about 1, consistent with a single drug molecule blocking the channel.

With the knowledge that amantadine blocks M_2 ion channels with a monomolecular reaction, it was possible to calculate the forward reaction rate constant, k_r. It is derived in the Appendix that $k_r = 1/([\text{amantadine}]\,\tau)$ where τ is the time constant of current decay. The calculated values of k_r are reported in Table 1. It should be noted that these calculated values of k_r are fairly constant with amantadine concentration, consistent with a first-order reaction. The values for k_r varied among the three subtypes, with k_r being highest for Udorn, then for Weybridge, and then for Rostock.

We also measured the concentration dependence of the block of the M_2 ion channel by amantadine for the M_2 ion channels of subtypes Rostock, Weybridge, and Udorn. Because the amantadine block is nearly irreversible (Fig. 6), it is not practical to measure the equilibrium inhibitory constant. Thus, we performed measurements after a fixed time (2 min) of exposure to amantadine to allow the calculation of the isochronic apparent inhibitory constant, appK_i. For oocytes expressing M_2 ion channels from each of the subtypes, we applied amantadine in four to six concentrations from 1 to 100 μM. Amantadine was applied while the oocyte was bathed in either a solution with a pH of 7.5 or a solution with a pH of 6.2, and the fraction of current that remained was measured. The fraction of remaining current was plotted as a function of amantadine concentration, and the data could be fitted by the following relationship: fraction of remaining current = $1/\{1 + ([\text{amantadine}]/\text{app}K_i)\}$. The values for app$K_i$ varied among the subtypes of M_2 ion channels (Table 2). The M_2 ion channel of the Udorn subtype required the lowest concentration, and that of the Rostock subtype required the highest concentration of amantadine for half-maximal block. Thus, the value of appK_i was lowest for Udorn, then for Weybridge, and then for Rostock. The value of appK_i also depended upon the pH for the M_2 ion channel of each subtype and was higher for pH 6.2 than for pH 7.5 (Table 2). The finding that appK_i was higher at pH 6.2 (the activated [open] state) than at pH 7.5 for all three M_2 channels suggests that the drug may have a higher affinity for the closed channel than for an activated channel.

Rimantadine, α-methyl-1-adamantane methylamine, is also active as an anti-influenza virus drug and because of a lower incidence of neurological side effects in humans is the preferred drug for use in the prophylaxis of influenza A virus infections (reviewed in reference 12). Influenza virus mutants resistant to the drug show the same spectrum of changes in the M_2 protein transmembrane domain as found with amantadine (reference 3; reviewed in reference 14). The

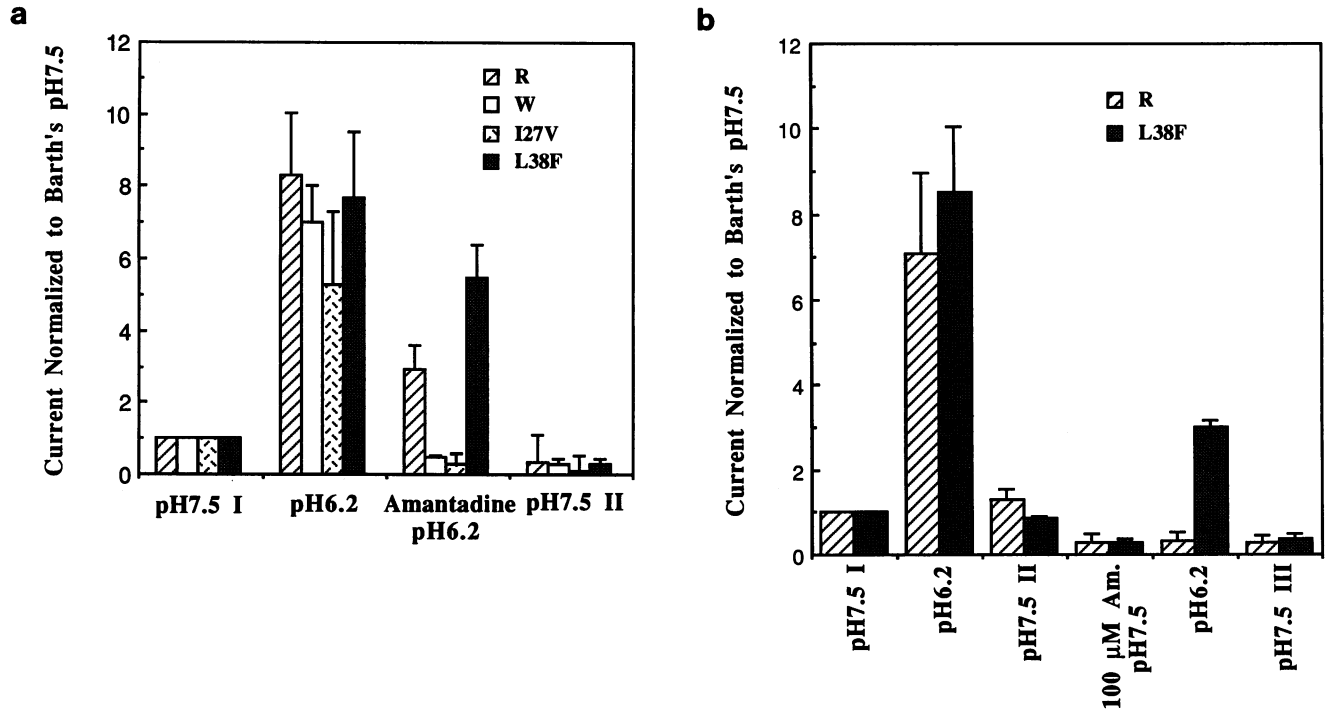

FIG. 5. Differences among M_2 ion channels in their block by amantadine at pH 6.2 and pH 7.5. (a) Membrane currents were measured while oocytes were bathed in Barth's solution, pH 7.5 (first cluster); pH was then reduced to 6.2 to activate the M_2 channels (second cluster) with measurements made 30 s after solution change; amantadine (100 μM) was then added to the low-pH solution for 2 min, and the amantadine sensitivity of the various channels was measured (third cluster); finally, pH was returned to 7.5 to check for leakage of oocyte membranes (fourth cluster). (b) Membrane currents were measured while oocytes were bathed in Barth's solution, pH 7.5 (first cluster); pH was then reduced to 6.2 to activate the M_2 channels (second cluster) with measurements made 30 s after solution change, and reversibility was checked by returning the oocytes to Barth's solution (pH 7.5 II) (third cluster). Amantadine (100 μM) was subsequently added at pH 7.5 for 2 min (cluster 4), and then the pH was reduced to 6.2 in the absence of amantadine to test for sensitivity to previously applied amantadine, and measurements were made after 30 s (cluster 5). Finally, pH was returned to 7.5 (pH 7.5 III) in the absence of amantadine to test for oocyte membrane leakage. Designation of the M_2 ion channels is as in the legend to Fig. 1.

oocyte expression system permits a direct test of the ability of rimantadine (10 and 100 μM) to block the ion channel activity of all three subtypes, and therefore, we tested the extent of rimantadine block at pH 7.5 for all three subtypes, and for the Udorn subtype also at pH 6.2. As shown in Fig. 8 and Table 3, the concentration at which rimantadine blocked the channel activity was essentially indistinguishable from that of amantadine for the respective subtypes.

The block of ion channels by a drug is sometimes directional, particularly for charged blockers such as amantadine. For example, the outward, but not inward, flow of K^+ through the inward rectifier K^+ channel is blocked by internal Mg^{2+} (30) and the outward, but not inward, flow of K^+ through the delayed rectifier K^+ channel of the squid giant axon is blocked by internally added positively charged tetraethylammonium ions (1, 17, 37). Since a recent model of the proposed structure of the M_2 ion channel (34) suggests that amantadine blocks the channel by acting as a plug that is applied from the extracellular entrance to the channel, we investigated the notion that inward but not outward currents might be blocked by amantadine. This was done by applying a slowly increasing (about 1.1 mV/s) membrane voltage to oocytes expressing the M_2 ion channel of all three subtypes, bathed in a solution with a pH of 6.2 to activate the channel, while measuring the membrane current. The current recorded was plotted against the voltage applied (example data appear in Fig. 9), and the voltage at which no net membrane

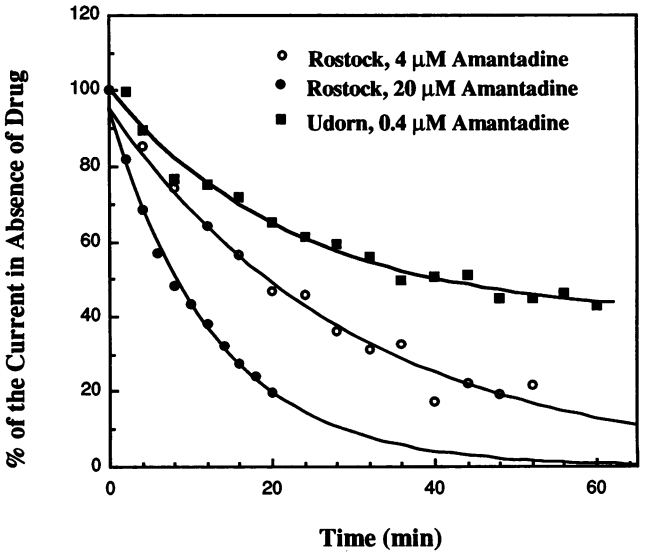

FIG. 6. Time course of inhibition of M_2 ion channels by amantadine. The surface currents of oocytes expressing the Rostock or Udorn M_2 ion channel were measured, and then amantadine was added at the appropriate concentration to the solution in which the oocytes were being bathed, and the currents were measured again after the time shown.

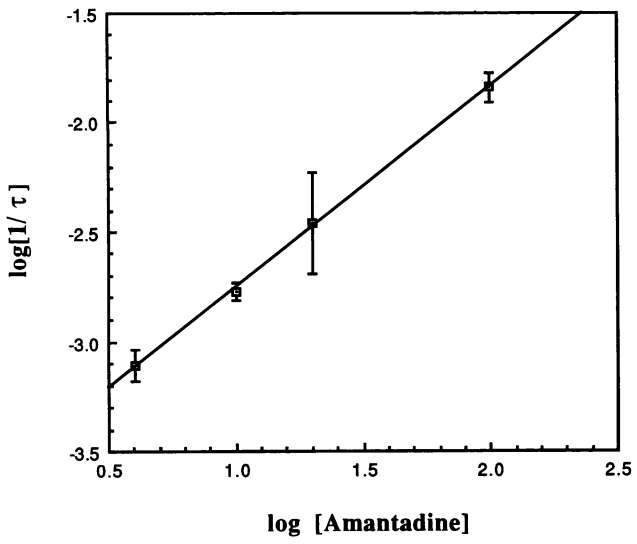

FIG. 7. Hill coefficient for amantadine inhibition of Rostock M_2 ion channel. The Hill coefficient, n, is equal to the slope of the line fitted to the measured values of $\log(1/\tau)$ against log[amantadine], where τ is the time constant of current decay, as measured as in Fig. 6 (see Appendix). The number of cells measured is shown in Table 1.

current flowed (the null voltage) was determined in the presence and absence of amantadine (100 μM). The value for the null voltage varied from oocyte to oocyte but fell within the range of −40 to 0 mV. We noted the slope of the current-voltage relationship in the vicinity of the null voltage as this slope gives the membrane conductance, which is proportional to the extent to which the channel is open. The slope was reduced by amantadine approximately equally for both inward (negative) and outward (positive) membrane currents. Thus, amantadine reduced inward and outward currents nearly equally and did not produce a directional block of the M_2 ion channel.

Reversibility of amantadine block. In principle, there are two methods to demonstrate reversible block of an ion channel. The first is to show that the channel current increases after removal of the blocker from the bathing medium. The second is to demonstrate that an incomplete equilibrium block of the channel can be achieved by continuous bathing in a solution having a low concentration of the blocker. The logic of the second principle is that for an irreversible block, even very low concentrations of the blocker will eventually lead to a complete block of the channel. We applied both of these principles to search for reversible block by amantadine.

We studied currents of the M_2 channels of all three subtypes (Rostock, Weybridge, and Udorn) for as long as 20 min after removal of amantadine from the bathing medium. For none of four to five oocytes studied for each subtype was the recovery of an amantadine-sensitive current observed. We were not able to study oocytes reliably for longer than 30 min because amantadine-insensitive leak currents developed. Because even a small leak current might have been mistaken for the small M_2 ion channel current that would be expected, we were unable to use this approach to search for reversibility. Experiments based upon the second principle are less likely to be influenced by the development of small leak currents. Thus, we studied the time course of block of the Udorn M_2 channel while the expressing oocyte was bathed in a solution having a very low concentration of amantadine (0.4 μM; Fig. 6 and Table 1). For five oocytes expressing the Udorn M_2 channel recorded for as long as 1 h, the block was observed to be incomplete. A single exponential function ($e^{-t/\tau}$ + constant) was able to be fitted to these data very well ($r > 0.94$). The asymptotic value to which this function decayed was 40 to 75% of the original current. In the case of these oocytes, the remaining unblocked current was able to be blocked by subsequent addition of amantadine (100 μM) to the bathing medium, showing that failure to develop complete block was not due to the presence of amantadine-insensitive leakage currents. Oocytes that had measurable amantadine-insensitive currents were discarded. If it is accepted that block of the M_2 channel of the Udorn subtype is a reversible process, then the reverse reaction rate constant of amantadine block can be calculated to be $3 \times 10^{-4}\ s^{-1}$. With this reverse reaction rate constant and the value $\bar{k}_r = 900\ M^{-1}\ s^{-1}$, the apparent K_i for the reversible inhibition of the M_2 currents would be 0.3 μM for the Udorn subtype. Thus, by using a method that allowed us to select for measurement only those oocytes that were not affected by leak currents, we were able to obtain evidence that amantadine block of the M_2 channel is slowly reversible.

TABLE 1. Forward reaction rate constant of amantadine inhibition (k_r) of M_2 ion channels

Amantadine concn (μM)	Value for subtype[a]: Udorn		Rostock		Weybridge	
	n[b]	k_r ($M^{-1}\ s^{-1}$)	n	k_r ($M^{-1}\ s^{-1}$)	n	k_r ($M^{-1}\ s^{-1}$)
0.4	5	900 ± 100				
4	1	865	2	190 ± 23	1	400
10			4	170 ± 8		
20			3	170 ± 46	3	220 ± 68
40	5	600 ± 30				
100			4	140 ± 12	4	300 ± 25

[a] k_r expressed as mean ± standard error of the mean.
[b] n, number of cells measured.

TABLE 2. Isochronic apparent inhibitory binding constant (K_i) of amantadine inhibition[a]

pH	K_i (μM) for subtype (mean ± SE): Udorn	Rostock	Weybridge
7.5	9 ± 2	52 ± 6	15 ± 3
6.2	13 ± 2	78 ± 9	28 ± 1

[a] Measurements were done after 2 min of incubation in amantadine.

DISCUSSION

Mutations found in the M_2 protein transmembrane domain which lead to amantadine resistance occur at residues 26, 27, 30, 31, and 34 (10, 13, 33). Two observations described here indicate that the interrelationship between the ability of amantadine to block the M_2 ion channel and the molecular architecture of the M_2 ion channel is complex. Firstly, the Rostock M_2 ion channel was effectively blocked in 2 min by amantadine only when the channel was closed (at pH 7.5), whereas the change of I-27-V in the M_2 transmembrane domain permitted drug block in 2 min at both the closed (pH 7.5) and the open (pH 6.2) channel states. Secondly, the

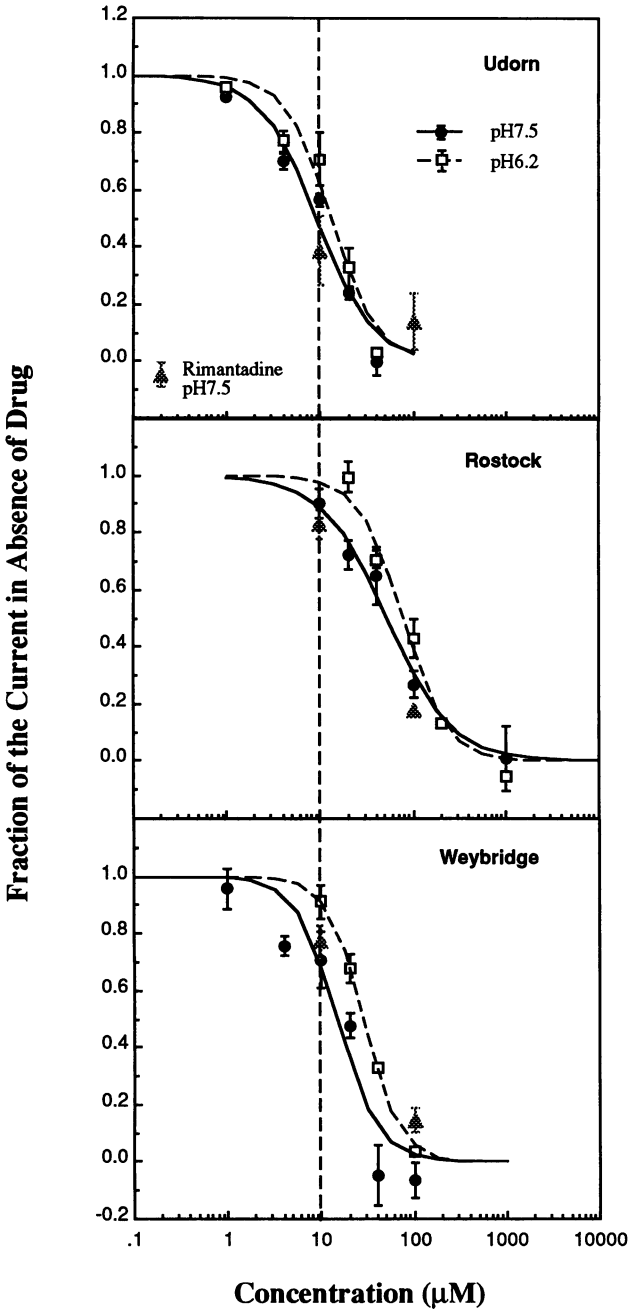

FIG. 8. Isochronic (2-min) inhibition curves for block of M_2 ion channels of Udorn (upper), Rostock (middle), and Weybridge (lower) subtypes of influenza A virus by amantadine. The calculated values of appK_i are tabulated in Table 2. Calculated values for inhibition by rimantadine appear in Table 3.

change L-38-F introduced into the Rostock M_2 protein led to greatly diminished drug susceptibility at both pH 7.5 and pH 6.2. However, when these two point mutations were combined in the Weybridge M_2 protein, the effect of the I-27-V change was predominant over that of L-38-F. In natural isolates of influenza virus, the combination of residues V-27 and F-38 in the M_2 protein transmembrane domain has been reported only for the influenza A/Korea/426/68 (H2N2) virus

TABLE 3. Rimantadine inhibition of M_2 ion channel[a]

Rimantadine concn (μM)	% of current in absence of drug (mean ± SEM) for subtype at pH			
	Udorn		Rostock	Weybridge
	7.5	6.2	7.5	7.5
10	40 ± 10	51 ± 3	83 ± 5	70 ± 6
100	−10 ± 10	1 ± 1	18 ± 1	14 ± 4

[a] Measurements were done after 2 min of incubation in rimantadine.

(21), and it will be interesting to test the sensitivity of this subtype to amantadine.

The calculated values of both the forward rate constant (k_r) for amantadine block (Table 1) and the isochronic apparent inhibitory constant (appK_i) of amantadine block (Table 2) differed among subtypes, with k_r being highest for Udorn, then for Weybridge, and then for Rostock and appK_i being lowest for Udorn, then for Weybridge, and then for Rostock. These data are consistent with the observed difference in susceptibility of the FPV subtypes to inhibition of virus growth by amantadine, with Weybridge being more sensitive than Rostock (13). The direct effect of the clinically useful amantadine derivative rimantadine on M_2 ion channel activity had not been tested previously in the oocyte expression assay, and at the concentrations tested (10 and 100 μM) at pH 7.5, the inhibitory effect (within 2 min) on ion channel activity was the same as that of amantadine for the Rostock, Weybridge, and Udorn subtypes.

Although commonly assumed, and from the observation that amantadine-resistant mutations occur on the outside of the presumed transmembrane domain, there is no report of experimental data to indicate that amantadine binds to the pore region of the M_2 ion channel. However, amantadine is a hydrophobic molecule and can penetrate lipid bilayers (9), and even if radioactively labeled drug of sufficiently high

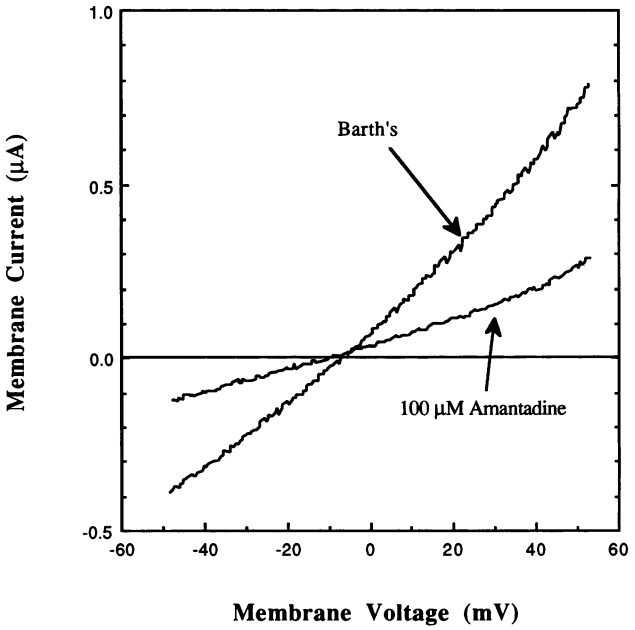

FIG. 9. Current-voltage relationship of the Weybridge M_2 ion channel measured at pH 6.2 with and without amantadine (100 μM).

specific activity could be obtained, drug binding studies with M_2 protein in a lipid bilayer would be difficult to interpret. Thus, we sought indirect evidence for the manner by which amantadine blocks the M_2 ion channel. Our calculation of the Hill coefficient for amantadine block (0.91 ± 0.02) indicates that one amantadine molecule blocks each functional M_2 ion channel complex. Although this is compatible with the drug blocking the pore region of a tetrameric M_2 protein channel, it is still possible that amantadine binds elsewhere on the molecule but modifies the pore region upon binding. Generally, for blockers that bind to the pore region, one of the following two characteristics is often observed: (1) the appK_i for the blocker is lower for the open pore and (2) for charged blockers such as amantadine, the current-voltage relationship of the blocked channel is nonlinear and often shows easier passage of current in one direction (for a review, see reference 18). We tested for these characteristics in our experiments, but the data obtained were not those expected for amantadine binding to the pore. The appK_i was higher for the open channel of each of the three subtypes (Fig. 8), and the current-voltage relationship in the presence of amantadine was linear (Fig. 9). Thus, we did not find two of the characteristics generally associated with a blocker that binds to the pore region. It does not seem likely that amantadine partitions into the membrane more effectively at pH 7.5 than at pH 6.2, thus affecting the results because its pK_a in Barth's solution is approximately 10.8 (data not shown). A third characteristic often observed of blockers that bind rapidly to the pore region is that the currents of single open channels show rapid closures in the presence of the blocker (32), and these measurements await to be determined. From the data obtained to date, a possibility that cannot be eliminated is that amantadine acts as an allosteric blocker which binds to a part of the channel protein that is not in the pore-forming region but that upon binding the drug causes a conformational change in the pore-forming region.

In influenza virus-infected tissue culture cells, the block of virus replication by amantadine is for all practical purposes irreversible (20, 22). Therefore, we searched for reversible amantadine block by two methods. Firstly, amantadine was applied for a short time and the recovery of currents after removal of amantadine was monitored. Secondly, low concentrations of amantadine were applied and we looked for an incomplete block in the equilibrium state. The second method was more reliable than the first because it was possible to select for measurement only those oocytes that were not affected by leakage currents; the method was performed by applying a high concentration of amantadine (100 μM) after the equilibrium state had been reached. Using this latter method with the Udorn M_2 ion channel, we found incomplete block, suggesting that reversible block might occur. However, the reverse reaction rate constant (3×10^{-4} s^{-1}) was very low, and it gives the impression of irreversible block in the time course of ordinary physiological experiments.

Evidence has been obtained that suggests that the Rostock M_2 ion channel can raise the intralumenal pH of the *trans* Golgi network to a pH higher than that achieved by the Weybridge M_2 ion channel (10, 11), which implies that the Rostock ion channel has a higher activity than the Weybridge ion channel. The data reported here indicate that the Rostock and Weybridge M_2 ion channels are activated very similarly by pH and that thus this is not the explanation for their possible difference in activity. The measurements of ion channel activity reported here do not permit the calculation of the flux of an individual ion channel, nor do they provide information as to the mechanism by which whole-cell currents increase when pH is lowered. For example, the increase in whole-cell current when pH is lowered from 7.5 to 6.2 might be due to a conformational change in the M_2 protein pore region or a change in the charged state of the histidine 37 residue, both of which may cause an increase in the unitary conductance of an individual ion channel at the lower pH. Alternatively, the increase in whole-cell current may be due to an increase in the fraction of the time that each individual functional M_2 ion channel complex spends in the open state at the lower pH, giving a greater fraction of open channels at the lower pH. To resolve these questions, it will be necessary to make single channel measurements.

APPENDIX

This derivation shows that the slope of the plot of $\log(1/\tau)$ against log[amantadine] gives the Hill coefficient of amantadine block. Let the reaction of a blocker with a channel be described by

$$[C] + n[B] \rightarrow CB_n$$

where C is the channel, B is the blocker (in this case, amantadine), and n is the Hill coefficient. The concentration of active channels and therefore the channel current can be described by the differential equation

$$-\frac{d[C]}{dt} = k_r[C][B]^r$$

where k_r is the forward reaction rate constant. Therefore,

$$-\frac{d[C]}{[C]} = k_r[B]^r dt$$

If $[B]$ does not change with time (as was the case for the present experiments), integrating the latter equation,

$$C = C_o e^{-k_r[B]^r t}$$

This expression can now be reevaluated in terms of the experimentally measured time constant of decay of the current, τ.

$$1/\tau = k_r[B]^r$$

Taking logarithms of this expression,

$$\log(1/\tau) = n \log(k^r[B])$$
$$= n \log[B] + n \log k^r$$

Thus, the slope of the relationship between $\log(1/\tau)$ and log [amantadine] will be the Hill coefficient for the reaction.

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