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Characterization of Inhibition of M_2 Ion Channel Activity by BL-1743, an Inhibitor of Influenza A Virus

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The influenza A virus M_2 integral membrane protein has ion channel activity that can be inhibited by the antiviral drug amantadine. Recently, a spirene-containing compound, BL-1743 {2-[3-azaspiro (5,5)undecanol]-2-imidazoline}, that inhibits influenza virus growth was identified (S. Kurtz, G. Luo, K. M. Hahnenberger, C. Brooks, O. Gecha, K. Ingalls, K.-I. Numata, and M. Krystal, Antimicrob. Agents Chemother. 39:2204–2209, 1995). We have examined the ability of BL-1743 to inhibit the M_2 ion channel when expressed in oocytes of *Xenopus laevis*. BL-1743 inhibition is complete as far as can be measured by electrophysiological methods and is reversible, with a reverse reaction rate constant of 4.0×10^{-3} s^{-1}. In contrast, amantadine inhibition is irreversible within the time frame of the experiment. However, BL-1743 inhibition and amantadine inhibition have similar properties. The majority of isolated influenza viruses resistant to BL-1743 are also amantadine resistant. In addition, all known amino acid changes which result in amantadine resistance also confer BL-1743 resistance. However, one BL-1743-resistant virus isolated, designated M_2-I_{35}T, contained the change Ile-35→Thr. This virus is >70-fold more resistant to BL-1743 and only 10-fold more resistant to amantadine than the wild-type virus. When the ion channel activity of M_2-I_{35}T was examined in oocytes, it was found that M_2-I_{35}T is BL-1743 resistant but is reversibly inhibited by amantadine. These findings suggest that these two drugs interact differently with the M_2 protein transmembrane pore region.

The low-pH-activated M_2 ion channel of influenza A virus (17) is a minor but essential component of virions (30), and the M_2 ion channel activity is specifically blocked by the antiviral drug amantadine (17, 29). 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 (14). The M_2 protein is a tetramer that 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 (9, 24).

The M_2 ion channel of influenza A virus is thought to play an essential role in the uncoating of the virus by permitting the passage of protons across the virion membrane into the virion core and weakening protein-protein interactions. When the M_2 ion channel is blocked by amantadine, uncoating of the virion is incomplete (reviewed in references 7 and 13). 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 in order to maintain the viral hemagglutinin spike glycoprotein in its pH-neutral metastable form (15, 19, 23, 25).

Direct evidence that the M_2 protein has ion channel activity was provided by expressing the M_2 protein either in oocytes of *Xenopus laevis* (17, 28, 29) or in mammalian cells that were infected with recombinant simian virus 40 that encoded the M_2 protein (27). 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 7.5 to 5.4 (17, 22, 27–29). The ion selectivity of the currents depended on the amino acid sequence of the M_2 transmembrane domain (19). In further experiments with purified M_2 protein, introduction of the protein into planar lipid bilayers or liposomes resulted in an amantadine-sensitive ion channel activity that was activated by low pH (21, 26). Other biophysical studies have suggested that amantadine binds to the transmembrane domain of the M_2 protein (3, 4).

Expression of the M_2 protein is deleterious to several host cell systems. Infection of *Spodoptera frugiperda* Sf9 cells with a recombinant baculovirus expressing the M_2 protein leads to inhibition of the replication of the recombinant baculovirus (1, 26), and expression of M_2 protein in oocytes of *X. laevis* leads to premature cell death (5, 13). In addition, expression of M_2 protein in *Escherichia coli* causes membrane permeability changes (6). Recently, it was observed that high-level expression of the M_2 protein in *Saccharomyces cerevisiae* results in growth impairment (12). It was found that yeast strains expressing wild-type (wt) M_2 protein can be rescued by addition of amantadine to the medium. In contrast, the growth impairment observed in a yeast strain expressing an amantadine-resistant M_2 allele was not remediated by the addition of amantadine. These findings suggested a direct correlation of growth impairment in yeast cells and expression of the M_2 protein ion channel activity. This observation permitted development of a high-capacity assay to permit identification of presumptive inhibitors of the M_2 ion channel activity. From such a screen, a spirene-containing compound, BL-1743 {2-[3-

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azaspiro (5,5)undecanol]-2-imidazoline}, was identified (12). Here we show directly that BL-1743 inhibits the influenza virus M_2 ion channel activity, and we describe the nature of the inhibition and characterize mutants resistant to BL-1743.

MATERIALS AND METHODS

Drugs. BL-1743 was selected from the Bristol-Myers Squibb chemical collection. The drug was pure, as judged by mass spectroscopy. BL-1743 was dissolved at 10 mg/ml in ethanol and diluted in aqueous medium prior to use. Amantadine was purchased from Sigma Chemical Company, St. Louis, Mo. Amantadine was dissolved at 10 mg/ml in ethanol and diluted in aqueous medium prior to use.

Isolation and characterization of BL-1743-resistant influenza virus mutants. Five separate plaques of influenza virus A/Udorn/72 were individually amplified in MDCK cells in the presence of 10 μg of BL-1743 per ml. Resistant virus for each of the five virus stocks was isolated through double plaque purification and subsequent amplification in MDCK cells, all in the presence of 10 μg of BL-1743 per ml. Resistant virus 2 (R-2) was further amplified in MDCK cells and purified by sucrose gradient centrifugation, and viral RNA was isolated (18). The M_2 gene of R-2 was cloned into the *Bam*HI-*Xba*I fragment of pUC19 by reverse transcription-PCR (GeneAmp; Perkin-Elmer, Norwalk, Conn.), using M_2-specific primers UdM2-1 (5′GGATCCGGATCCAGCAAAAGCAGGTAGATAT TGAAAGATG3′) and UdM2-2 (5′TCTAGATCTAGATTACTCCAGCTCTA TGCTGACAAAATG3′), and the cDNA nucleotide sequence was confirmed. For plaque reduction assays, approximately 50 PFU was used to infect MDCK cells. After 1 h at 37°C, agar overlays containing various concentrations of either BL-1743 or amantadine were added. Cells were incubated for 3 days at 37°C prior to staining with crystal violet.

mRNA synthesis and site-specific mutagenesis. The cDNA to the A/Udorn/72 mRNA (11, 31) was cloned into the *Bam*HI site of pGEM3Zf(+) such that mRNA sense transcripts could be generated by using the bacteriophage T7 RNA polymerase promoter and T7 polymerase and was used as a template for phagemid-based mutagenesis (16). The nucleotide sequence of the altered cDNA in pGEM3Zf(+) was confirmed by dideoxynucleotide chain-terminating sequencing (20). For in vitro transcription, plasmid DNAs were linearized downstream of the T7 promoter and the M_2 cDNA with *Xba*I. In vitro synthesis and quantification of ^{7m}G(5′)ppp(5′)G-capped mRNA was carried out as described previously (17).

Culture and microinjection of oocytes. *X. laevis* organisms were identified individually with an implanted microchip marker (Basic Medic Data Systems, Maywood, N.J.). Oocytes were removed from female *X. laevis* (Nasco, Fort Atkinson, Wis.), defolliculated by treatment with collagenase B (2 mg/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.), and incubated in ND96 (96 mM NaCl, 2 mM KCl, 3.6 mM $CaCl_2$, 1 mM $MgCl_2$, 2.5 mM pyruvic acid, 5 mg of gentamicin per ml, 5 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.6]) at 19°C. Oocytes at stage V were microinjected with 50 nl of mRNA (1 ng/nl) on the day after defolliculation, incubated for 24 h in ND96 (pH 7.6), and finally incubated for 24 h in ND96 (pH 8.5) at 19°C before use (2, 17).

Measurement of membrane current. Whole-cell current was measured with a two-electrode voltage clamp (17). 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 $MgSO_4$, 15 mM HEPES [pH 7.5) or a modified solution during the recording. Ramp measurements were made with ramps of membrane voltage that spanned a range of 30 mV in 5 s; voltage clamp pulses were 100 ms in duration.

RESULTS

Direct test of the effect of BL-1743 on the M_2 ion channel activity. Expression of the influenza virus M_2 protein was found to be toxic to yeast cells, but addition of the M_2 ion channel blocker amantadine resulted in growth rescue of the yeast cells. This observation provided a simple and high-throughput assay for identifying potential inhibitors of the M_2 protein ion channel activity (12). Using this assay, a spirene-containing compound, BL-1743, was selected from the Bristol-Myers Squibb chemical collection. BL-1743 was found to rescue growth of a yeast strain expressing an M_2 ion channel that is also sensitive to amantadine (12).

The design of the screen that enabled identification of BL-1743 as a potential anti-influenza virus drug led to the expectation that BL-1743 would be an inhibitor of the M_2 ion channel. To provide proof of principle of the assay, the effect of BL-1743 on the M_2 ion channel activity was tested directly. The cDNA encoding the M_2 protein of influenza virus A/Udorn/72 (a subtype that is amantadine and BL-1743 sensitive) was expressed in *X. laevis* oocytes, and membrane currents were measured while voltage clamp pulses from −60 to −120 mV were applied. The M_2 ion channel is activated by lowered pH (17, 27–29), and thus membrane currents were measured at pH 7.5 and 6.2. BL-1743 (100 μM) was added into the bathing solution of oocytes activated at pH 6.2 (Fig. 1). After 2 min of exposure to BL-1743, the currents were reduced to less than 20% of their prior values for all voltages tested. This residual current was approximately equal to the value found in water-injected control oocytes. The percentage inhibition of current by BL-1743, during brief exposure to a high concentration of the drug, was approximately equal to that obtained with amantadine (Fig. 2).

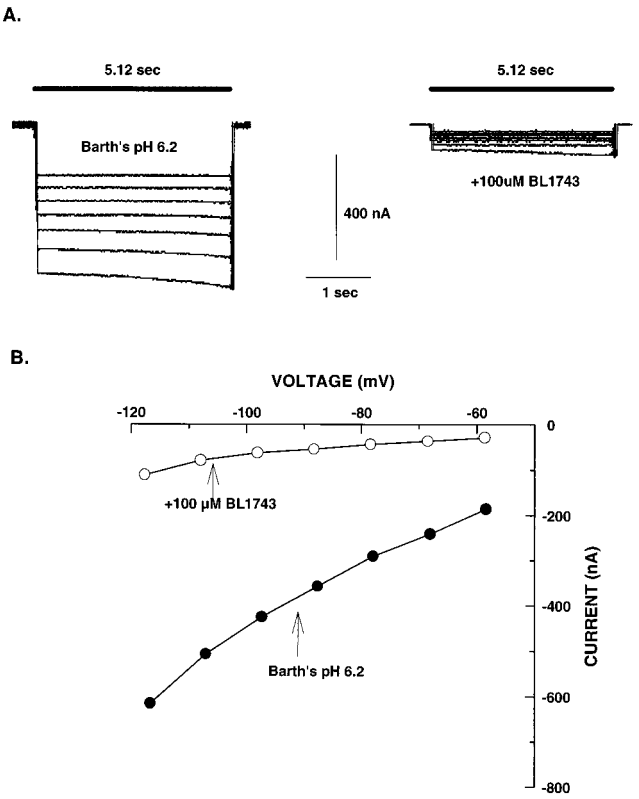

FIG. 1. Ion channel activity of M_2 protein expressed in oocytes can be inhibited by BL-1743. (A) Whole-cell ionic currents from M_2-expressing oocytes bathed in Barth's solution (pH 6.2) without (left) or with (right) 100 μM BL-1743. Holding potential was −20 mV; test pulses (time course indicated by the bar) were from −120 to −60 mV in 10-mV increments. (B) The current-voltage relationship determined from the current at end of the pulses in panel A. ●, currents of oocytes bathed in Barth's solution (pH 6.2); ○, currents of oocytes in the presence of 100 μM BL-1743 for 2 min.

Reversibility of inhibition by BL-1743. One important feature of the inhibition of the M_2 ion channel activity by amantadine is that the inhibition is only slowly reversible. Indeed, reliable recordings cannot be sustained from oocytes for a sufficient duration to demonstrate reversibility after amantadine washout, and reversibility can only be inferred from the finding of incomplete equilibrium inhibition in the presence of low concentrations of drug (29). We observed that after washout of BL-1743, the inhibition of the M_2 ion channel activity was reversed with a time constant of 12 min (Fig. 3), and that 30 min after washout (the longest practical time of recording), the recovery was 90% complete. In these experiments (involving prolonged impalement of the oocytes with the electrodes),

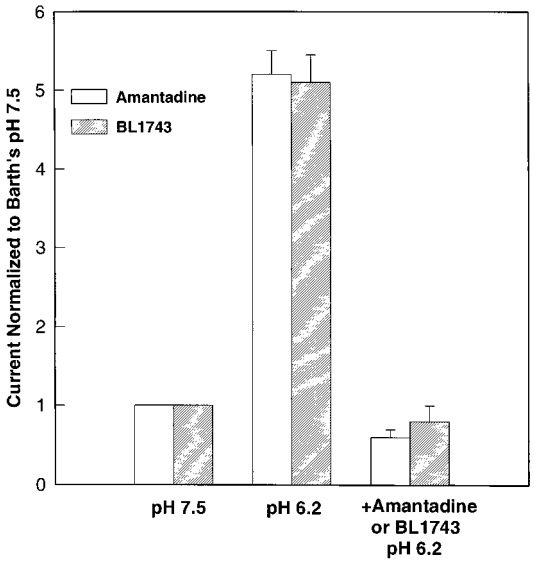

FIG. 2. Comparison of inhibition of M_2 ion channel activity by BL-1743 and amantadine. Membrane currents of one group of cells were measured in Barth's solution at pH 7.5, after 30 s of incubation at pH 6.2, and after 2 min of incubation at pH 6.2 with 100 μM amantadine (open bars). A separate group of cells was measured with Barth's solution at pH 7.5, after 30 s of incubation at pH 6.2, and after 2 min of incubation of pH 6.2 with 100 μM BL-1743 (filled bars). Currents are plotted as multiples of current at pH 7.5. Values are given as means ± standard errors of the means ($n \geq 5$).

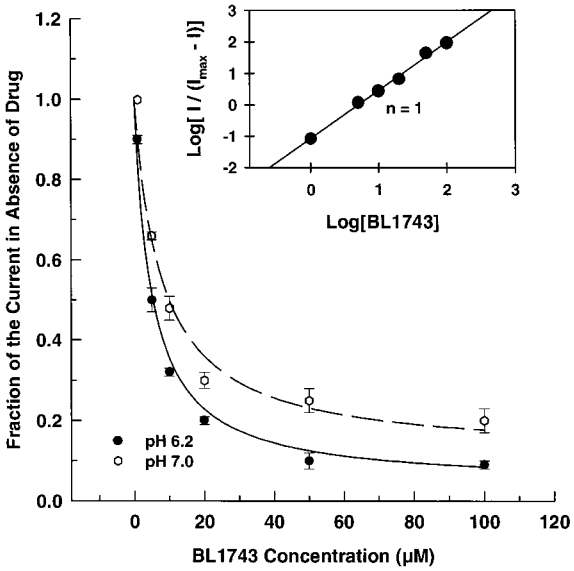

FIG. 4. Equilibrium inhibition curves for inhibition of M_2 ion channel by BL-1743. The oocytes were bathed in Barth's solution at either pH 7.0 or pH 6.2, to which the concentrations of BL-1743 shown on the abscissa were added. The steady-state current (measured for four oocytes at each concentration shown and usually reached in 25 to 30 min after addition of inhibitor) is plotted against inhibitor concentration. The calculated values for apparent K_i were 4.7 μM at pH 6.2 and 7.3 μM at pH 7.0. In the insert, a straight line was fitted to the plot of $\log[I/(I_{max} - I)]$ against log[BL-1743] for pH 6.2. The slope of this line, the Hill coefficient (n), was 1.0.

the reversibility of ion channel activity was distinguished from the development of leakage currents by applying amantadine (100 μM) at the end of the measurement; only amantadine-sensitive currents are reported. Thus, inhibition of ion channel activity by BL-1743 differs from that seen with amantadine in that the inhibition with BL-1743 was reversible on a short time scale.

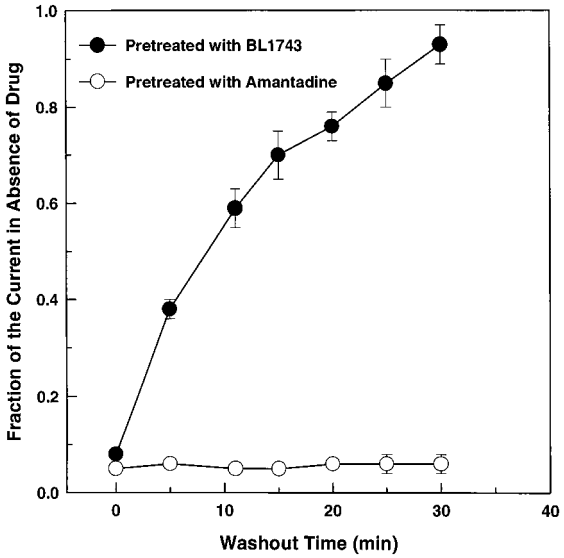

FIG. 3. Inhibition of M_2 ion channel activity by BL-1743 is reversible. The membrane currents of five oocytes expressing the M_2 ion channel were first measured in Barth's solution (pH 6.2) and again after addition of 50 μM BL-1743 or 50 μM amantadine. Two to three minutes after addition of BL-1743 or amantadine, oocytes were washed with inhibitor-free Barth's solution (pH 6.2), and the membrane currents were measured repeatedly at the times shown after the beginning of washout. Current values are given as means ± standard errors of the means of the current in the absence of drug.

Equilibrium binding constants for inhibition and reaction rate constants for BL-1743. As the inhibition of ion channel activity by BL-1743 was reversible, it was possible to measure equilibrium inhibition and determine the apparent K_i. This was done for two values of pH, 6.2 and 7.0, for which the M_2 channel is activated seven- and twofold, respectively, above the value found at pH 7.5 (29). The inhibition for a given condition was obtained by first measuring the current before application of BL-1743, then applying the drug to the bathing medium, and monitoring the current until it reached a new, constant value. Finally, amantadine (100 μM) was applied to check for the development of leakage currents. These measurements showed apparent K_is of 4.7 μM at pH 6.2 and 7.3 μM at pH 7.0 (Fig. 4). These data were analyzed to determine the Hill coefficient for inhibition (I) by plotting $\log[I/(I_{max} - I)]$ against log[BL-1743] for pH 6.2. The slope of a line fitted to the plot, the Hill coefficient, was 1.0 (Fig. 4, insert), indicating that inhibition was governed by a monomolecular reaction. We also determined the forward and reverse reaction rate constants, K_{on} and K_{off}, for the reaction of BL-1743 with the ion channel complex from detailed measurements of the time course of onset of inhibition (Fig. 5) and the measured value of K_i. The onset of inhibition was hastened by increasing the concentration of the drug, and the calculated values for K_{on} and K_{off} (720 and 4.0×10^{-3} s^{-1}, respectively) were identical for drug concentrations from 5 to 20 μM. The forward reaction rate constants for BL-1743 (720 M^{-1} s^{-1}) and amantadine (600 to 900 M^{-1} s^{-1}) are very similar for the Udorn subtype. However, the reverse reaction rate constant for BL-1743 (4.0×10^{-3} s^{-1}) greatly exceeds that calculated for amantadine (3×10^{-4} s^{-1}).

Inhibition by BL-1743 is not directional. The inhibition of an ion channel by a charged molecule is sometimes directional.

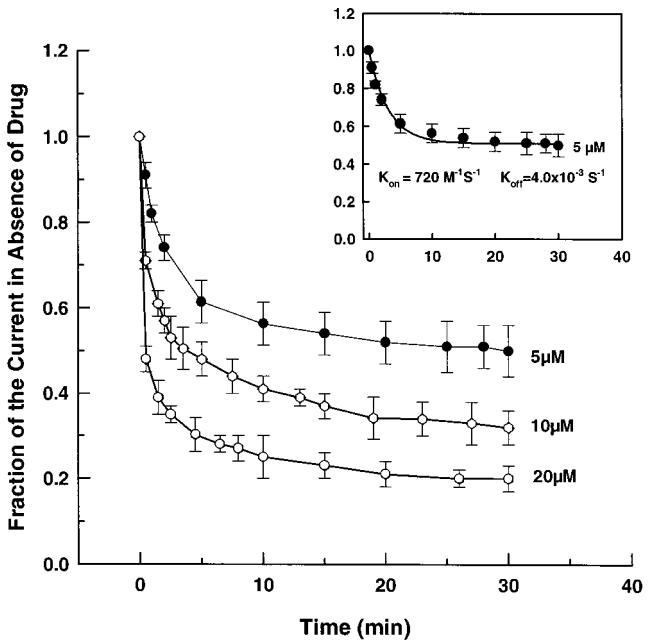

FIG. 5. Time course of inhibition of M_2 ion channel by BL-1743. The membrane currents of at least four oocytes expressing the M_2 ion channel were measured in Barth's solution at pH 6.2 with or without BL-1743 at the concentrations shown. To determine the K_{on} and K_{off} of BL-1743 action, the time courses were fitted by Peakfit software (Jandel Scientific, Corte Madera, Calif.). The results of this fitting are shown in the insert, in which data for 5 μM BL-1743 are plotted to show inhibition shortly after addition of drug; also shown is the fitted line.

Since the guanidinium group of BL-1743 is protonated in the range of pH used in this study, the BL-1743 molecule has a net positive charge. If the molecule, applied extracellularly, acted by blocking the open pore of the M_2 molecular complex, then inward current (positive charge flowing from outside to inside the membrane) would be expected to carry the drug into the pore region and achieve blockage, while outward current would be expected to sweep the drug molecule away, relieving the blockade. We tested the directionality of inhibition by applying a slowly increasing (6 mV/s) membrane voltage to oocytes expressing the M_2 ion channel before and after introducing BL-1743 (100 μM) to the bathing medium (Fig. 6). We observed that inward and outward currents were inhibited equally by the drug, indicating that the inhibition of the ion channel activity by the charged BL-1743 inhibitor is not directional.

Naturally occurring amantadine-resistant mutants are also BL-1743 resistant. The results described above suggest that BL-1743 acts to inhibit M_2 ion channel activity in a manner similar to that of amantadine but with a K_{off} larger than that for amantadine. Thus, we investigated the possibility that BL-1743 interacts with the same residues of the M_2 ion channel complex as amantadine by measuring the inhibition of ion channel activity of several amantadine-resistant mutants by BL-1743. Four amantadine-resistant mutants, M_2-$V_{27}S$, M_2-$A_{30}T$, M_2-$S_{31}N$, and M_2-$G_{34}E$ (8), were tested, and each was found to be resistant to BL-1743 (Fig. 7).

Isolation of BL-1743 resistant A/Udorn/72 virus. Although specific changes at the four M_2 transmembrane domain residues known to cause resistance to amantadine (8) also cause resistance to BL-1743, it is possible that the spectrum of amino acid changes resulting in resistance to BL-1743 is broader than that seen with amantadine. Differences in resistance spectrum may provide insights into the interaction of the compounds with the M_2 channel and the mechanism by which they inhibit ion channel activity. Therefore, a number of viruses resistant to BL-1743 were selected, double plaque purified, and examined for resistance against amantadine. Five independently plaque-isolated BL-1743-resistant A/Udorn/72 viral isolates were analyzed. These viruses were plaqued in the presence of various concentrations of either amantadine or BL-1743 to assess their sensitivities to each compound. Four of these viruses exhibited resistance to both compounds up to 100 μg/ml (287 μM) (data not shown). However, R-2 was found to be unique in that this virus is resistant to BL-1743 but remained sensitive to amantadine. The 50% effective concentrations (EC_{50}) for amantadine, extrapolated from numerous plaque reduction experiments, were approximately 260 to 660 nM for wt A/Udorn/72 and 2.6 to 6.6 μM for A/Udorn R-2. However, wt A/Udorn/72 exhibited an EC_{50} for BL-1743 of approximately 2 μM, while R-2 was completely resistant to the drug at concentrations of up to 144 μM. Thus, although R-2 is approximately 10-fold less sensitive to amantadine than wt virus, it is at least 70-fold more resistant to BL-1743. In addition, R-2 was resistant to BL-1743 in plaque reduction assays to even higher concentrations of drug (up to 570 μM; data not shown), although cellular toxicity was apparent at these high drug levels.

The unique phenotype of R-2 suggested that its M_2 protein transmembrane domain would contain additional amino acid changes specific for BL-1743 resistance. To investigate this possibility, R-2 was amplified and its virion RNA was purified. The M_2 coding region was then cloned by reverse transcription-PCR using specific primers, and the nucleotide sequence of the cDNA was obtained. Two nucleotide differences were found in R-2 M_2 cDNA compared with M_2 of wt A/Udorn/72: at nucleotides 104 and 105, the TC dinucleotide in the wt sequence was transposed to CT in the R-2 sequence. These mutations in the R-2 M_2 protein result in an isoleucine-to-threonine change at amino acid 35 within the M_2 protein transmembrane domain, generating the M_2-$I_{35}T$ mutant.

Mutations that cause differential effects on inhibition by BL-1743 and amantadine. A direct test of the BL-1743 resis-

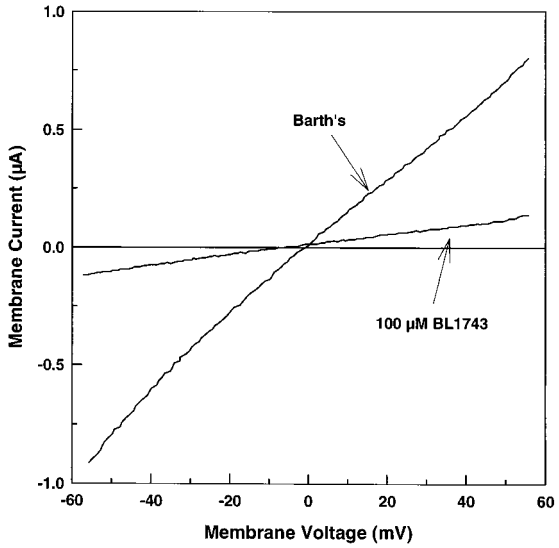

FIG. 6. Current-voltage relationship of the M_2 ion channel measured in Barth's solution at pH 6.2 with and without BL-1743 (100 μM). Note the lack of rectification over a wide voltage range.

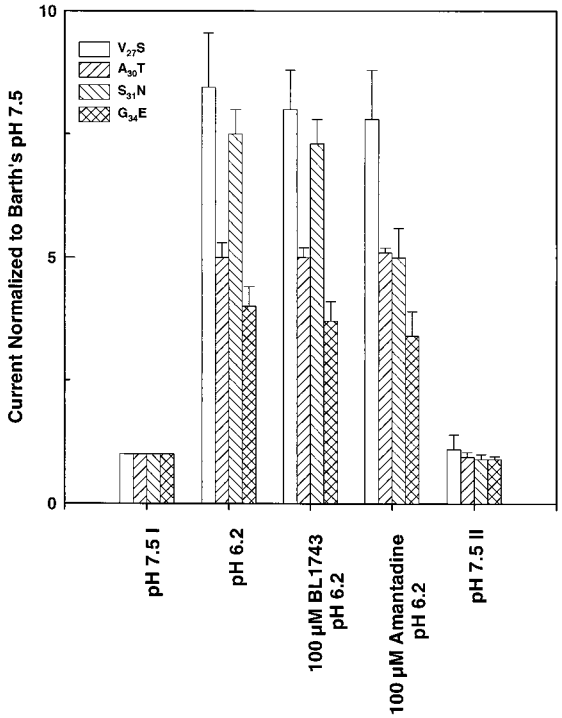

FIG. 7. Amantadine-resistant M_2 mutants are also BL-1743 resistant. Membrane currents were measured initially in Barth's solution at pH 7.5 (pH 7.5 I), after 30 s of incubation of pH 6.2, 2 min after addition of either 100 μM amantadine or 100 μM BL-1743, and finally again at pH 7.5 (pH 7.5 II) (to check for leakage). Currents plotted are the mean ratios of measured current to the current initially measured at pH 7.5 ± standard errors of the means. The M_2 cDNAs expressed in oocytes contained the mutations M_2-$V_{27}S$, M_2-$A_{30}T$, M_2-$S_{31}N$, and M_2-$G_{34}E$ (10, 17).

tance and amantadine sensitivity of the ion channel activity of A/Udorn/72 containing the M_2-$I_{35}T$ substitution was performed by expressing the M_2-$I_{35}T$ protein in oocytes and measuring membrane currents. The currents of oocytes that expressed the mutant protein were largely resistant to BL-1743 (blocked by only 26%) and were fully inhibited by addition of amantadine (Fig. 8). However, unlike the irreversibility of amantadine inhibition of wt M_2 ion channel activity on the short time scale of this measurement (17, 29), the inhibition of M_2-$I_{35}T$ by amantadine could be reversed. The M_2 ion channels of influenza viruses A/chicken/Germany/34 (H7N1) (FPV Rostock) and A/chicken/Germany/27 (H7N7) (FPV Weybridge) differ in sensitivity to amantadine inhibition at low pH (29). It was shown previously that M_2 transmembrane domain residue 38, a leucine in Weybridge and phenylalanine in Rostock, contributes to the lesser inhibition of Rostock by amantadine. Thus, we tested the inhibition by BL-1743 of the ion channel activity of the M_2-$L_{38}F$ mutant protein at low pH (6.2) and found inhibition to be nearly complete (Fig. 9). Thus, a residue in the M_2 protein transmembrane domain which imparted resistance to amantadine but not to BL-1743 was identified.

DISCUSSION

Amantadine and BL-1743 have some structural relationships, and the two compounds presumably inhibit the ion channel activity of the M_2 protein by making specific interactions with residues in the M_2 protein transmembrane domain. The inhibition in both cases is as complete as can be detected by electrophysiological methods. The main difference in properties observed between the two inhibitors is that inhibition by BL-1743 is reversible on the time scale of normal electrophysiological experiments whereas inhibition by amantadine is not reversible. The reason for this difference is that the reverse reaction rate constant for BL-1743 is about 10-fold greater than that for amantadine.

Although mutations in the M_2 transmembrane domain can impart resistance to both amantadine and BL-1743, the two compounds do not interact with the protein in exactly the same way. Mutations known to confer amantadine resistance at M_2 residues 27, 30, 31, and 34, all within the M_2 transmembrane domain, also induce complete resistance to BL-1743. However, a mutant of influenza virus A/Udorn 72 which exhibits complete resistance to BL-1743 (several hundred fold, as determined in plaque reduction experiments) but only 10-fold resistance to amantadine was isolated. The M_2 gene of this virus has a mutation which encodes threonine at transmembrane domain residue 35 of the M_2 protein. Direct measurement of ion channel activity of the M_2-$I_{35}T$ protein indicated it is resistant to BL-1743 but is reversibly inhibited by amantadine, in contrast to the irreversible inhibition seen with the wt M_2 protein. The fact that reversible inhibition by amantadine was observed with the M_2-$I_{35}T$ mutant protein indicates that the reverse reaction constant for the mutant must exceed that of the wt M_2 protein, but reaction kinetics for amantadine were not quantified in the present study. The finding that the M_2-$I_{35}T$ mutant protein is reversibly inhibited by amantadine demonstrates that the binding of amantadine to low-affinity sites in

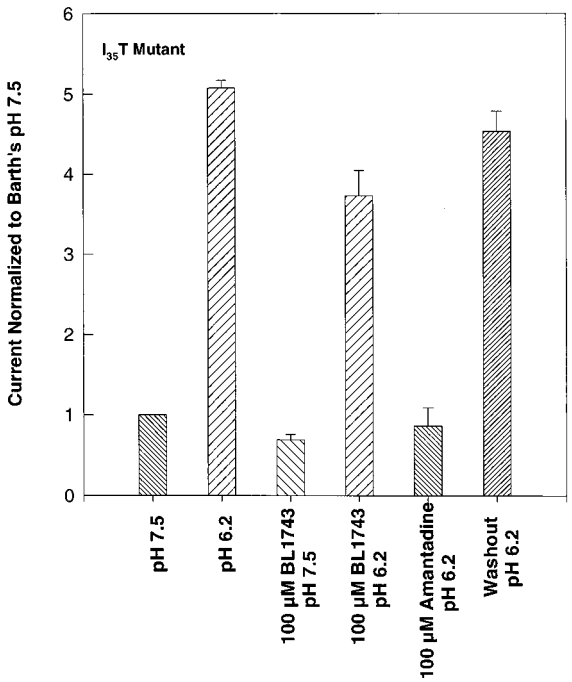

FIG. 8. The M_2-$I_{35}T$ mutant is resistant to BL-1743 but reversibly inhibited by amantadine. Sequentially, membrane currents of oocytes expressing M_2-$I_{35}T$ were measured in Barth's solution at pH 7.5, pH 6.2, and again pH 7.5 with 100 μM BL-1743 (to check for inhibition by BL-1743 at high pH), again at pH 6.2 with BL-1743, and finally with 100 μM amantadine at pH 6.2. Two minutes after addition of amantadine, oocytes were washed with drug-free Barth's solution at pH 6.2 for 2 to 3 min, and membrane currents were then measured. Similar reversibility of inhibition by amantadine was also seen without prior exposure to BL-1743 (see text).

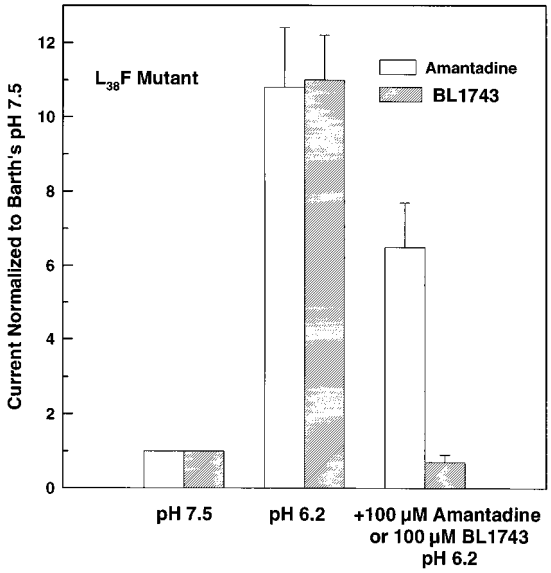

FIG. 9. The M_2-L_{38}F mutant is inhibited by BL-1743 but not amantadine. Membrane currents of oocytes expressing the M_2-L_{38}F mutant were measured in Barth's solution at pH 7.5, after 30 s of incubation at pH 6.2, and after addition of either 100 μM amantadine or 100 μM BL-1743. Currents shown are the mean ratios (± standard errors of the means) of measured current to the current initially measured at pH 7.5.

the oocyte (e.g., the yolk) is not responsible for the inability to observe reversible inhibition with this compound.

Analysis of the properties of another mutant, M_2-L_{38}F, indicated that this mutant ion channel protein had drug sensitivity/resistance properties that were opposite those of M_2-I_{35}T. The M_2-L_{38}F mutant protein exhibits incomplete resistance to amantadine but complete sensitivity to BL-1743. Thus, although mutants with changes in the M_2 protein transmembrane domain located closer to the presumed outer leaflet of the bilayer (extracellular side) have similar properties with respect to amantadine and BL-1743, changes closer to the presumed inner leaflet of the bilayer (cytoplasmic side) can exhibit major differences, suggesting that the two drugs interact differently with this portion of the transmembrane domain of the M_2 protein.

The reversibility of the inhibition of the M_2 ion channel by BL-1743 is a consequence of its greater reverse reaction rate constant. The forward reaction rate constants for BL-1743 (720 M^{-1} s^{-1}) and amantadine (600 to 900 M^{-1} s^{-1}) are very similar for the Udorn subtype. However, the reverse reaction rate constant for BL-1743 (4.0×10^{-3} s^{-1}) greatly exceeds that calculated for amantadine (3×10^{-4} s^{-1}). This nearly 10-fold difference makes it possible to observe reversibility in electrophysiological experiments using BL-1743.

The Hill coefficient for BL-1743 inhibition of the M_2 ion channel was 1.0, as found previously for amantadine inhibition (29), indicating that inhibition by both drugs is due to a single inhibitor molecule interacting with one ion channel complex. We are not able to determine from the present experiments whether BL-1743 acts by binding to the open pore of the M_2 ion channel or by altering the conformation of the protein allosterically. The apparent K_i is higher when the channel is in the open state (low pH) than when it is in the closed state. However, this does not necessarily point to a open pore binding site, as the act of opening may alter the pK_a of the residues to which the compound binds. One observation suggests that the compound may not bind to the open pore. The current-voltage relationship is modified equally for both inward and outward currents (Fig. 6) in the presence of BL-1743. Since BL-1743 is positively charged at the pH values used in these experiments, the compound, when applied extracellularly, would be swept away from the pore by outward currents. This should cause a greater attenuation of inward currents than of outward currents, inconsistent with our observations. Only by recording the activity of single-ion channel complexes in the presence of the compound will we be able to ascertain the mechanism of action.

The discovery that BL-1743 blocks the influenza A virus M_2 ion channel in a reversible manner is exceedingly useful in electrophysiological studies of the M_2 ion channel. However, the overlapping spectra of amantadine and BL-1743 resistance mutations and the higher apparent K_i (4.7 μM for BL-1743 and 0.3 μM for amantadine) do not indicate that BL-1743 should replace the use of amantadine (or rimantadine) for the prophylaxis or treatment of influenza virus infections in humans.

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