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Growth Impairment Resulting from Expression of Influenza Virus M2 Protein in *Saccharomyces cerevisiae*: Identification of a Novel Inhibitor of Influenza Virus

STEPHEN KURTZ,[1] GUANGXIANG LUO,[2] KAREN M. HAHNENBERGER,[3] CINDY BROOKS,[1] OKSANA GECHA,[2] KIM INGALLS,[1] KEI-ICHI NUMATA,[1] AND MARK KRYSTAL[2]*

Department of Microbial Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, New Jersey 08543[1]; Department of Virology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492[2]; and Molecular Devices Corp., Sunnyvale, California 94089[3]

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The gene encoding M_2, the ion channel-forming protein of influenza virus A, was expressed under the control of an inducible promoter in *Saccharomyces cerevisiae*. By using single and multicopy plasmids containing *GAL* promoter-M_2 fusions, a correlation was observed between plasmid copy number and growth in medium inducing M_2 expression. Cells expressing M_2 from multicopy plasmids have reduced growth rates, suggesting that high levels of M_2 are toxic to growth. The addition of amantadine, a compound known to block the ion channel activity of certain M_2 alleles, restores the growth rates to wild-type levels in cells expressing an amantadine-susceptible allele of M_2 but not an amantadine-resistant allele of M_2, suggesting that M_2 expression in *S. cerevisiae* results in the formation of functional M_2 ion channels. Measurements of extracellular acidification by microphysiometry suggest that proton efflux in M_2-expressing cells is altered and that the addition of amantadine permits the reestablishment of the proton gradient. The growth impairment phenotype resulting from M_2 expression was used to develop a high-capacity screening assay which identified a novel inhibitor possessing an antiviral profile similar to that of amantadine.

Functional expression of ion channels in heterologous cell types provides a general method for studying the properties of channel function. Expression in *Xenopus laevis* oocytes has provided numerous insights into ion channel function (17, 18, 35). More recently, expression of ion channels in *Saccharomyces cerevisiae* strains defective in K^+ uptake has served as a means for isolating new genes encoding functional channels (1, 29). However, expression of ion channels in yeast cells is not restricted to the restoration of uptake functions and may result in a variety of phenotypes, depending on the properties of a particular channel.

The M_2 protein of influenza virus A is one of three integral membrane proteins contained in the viral lipid envelope. The M_2 protein is a 97-amino-acid polypeptide containing a single membrane-spanning region. M_2 polypeptides associate as disulfide-linked homotetramers to form ion channels (15, 34). Direct evidence defining the ion channel function of M_2 has recently been obtained by expression studies in *Xenopus* oocytes (26, 38) and in in vitro studies (6, 28, 36). A therapeutic agent for influenza virus A infections, amantadine, has been shown to function by blocking M_2 ion channel activity (7, 12, 13, 26, 32, 33, 37, 38). Other biophysical studies have confirmed that the transmembrane portion of the molecule is the binding site and suggest that the mechanism of action is binding of the compound within the channel pore (6, 7, 27). Inhibition studies with amantadine suggest that M_2 is required at both early and late stages in the infection cycle of the virus. Early in infection, M_2 permits the flow of protons from the endosome into the virion. The resultant decrease in pH facilitates the dissociation of the matrix protein (M_1) from viral genomic ribonucleoproteins (12, 14, 20, 26) and thereby stimulates viral uncoating. In the presence of amantadine, M_1 is unable to dissociate from the viral ribonucleoproteins and transport of the ribonucleoprotein complex to the nucleus does not occur. At later stages of infection, M_2 polypeptides are abundantly expressed on the cell surface and within the cell in the endoplasmic reticulum and Golgi compartments. It is believed that M_2 functions at late stages of infection to maintain a high pH in these vesicular compartments, thereby preventing the premature activation of intracellularly cleavable hemagglutinin through a low-pH conformational change (5, 8, 9, 33).

We observed that high levels of M_2 expression in yeast cells results in growth impairment, similar to that reported for other expression systems (2, 10). Several lines of evidence suggest that the growth impairment in yeast cells results from the ion channel activity of M_2. *S. cerevisiae* strains with this phenotype allowed for the development of a high-capacity assay which identified a novel inhibitor of M_2 function with anti-influenza virus activity similar to that of amantadine.

MATERIALS AND METHODS

Strains. *S. cerevisiae* W303 (*MAT***a** *ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) was used as the recipient for transformations. The following *S. cerevisiae* strains were used in the study: SGY1442 contains the parent vector pYES2; SGY1443 contains the A/WSN/33 M_2 gene downstream of the *GAL* (galactose) promoter in pYES2; SGY1444 contains the WSN gene with an asparagine-to-serine mutation at position 31 downstream of the *GAL* promoter in pYES2; SGY1492 contains the WSN M_2 gene downstream of the *GAL* promoter in the pYEUra3 vector; SGY1493 contains the Udorn M_2 gene downstream of the *GAL* promoter in pYES2.

Yeast manipulations. Standard yeast medium (yeast extract-peptone-dextrose [YEPD] and supplemented minimal medium) were used for cell growth (31). Cells were made competent for transformation by using the lithium acetate protocol of Ito et al. (16). Unless otherwise noted, transformants were maintained on selective medium containing 2% glucose (noninducing conditions) and were induced to express protein through replacement with minimal medium containing 2% galactose. Protein extracts were prepared as described previously

* Corresponding author. Mailing address: Department of Virology, Bristol-Myers Squibb Pharmaceutical Research Institute, 5 Research Parkway, Wallingford, CT 06492. Phone: (203) 284-7974. Fax: (203) 284-6088. Electronic mail address: MARK R. KRYSTAL@CCMAIL.BMS.COM.

(3). Proteins were detected by reaction with antisera to M_2 (gift of P. Rota) and a secondary antibody conjugated to alkaline phosphatase.

Construction of expression plasmids. The A/WSN/33 and A/Udorn/72 M_2 genes were modified through PCR-directed mutagenesis of cDNA by standard procedures. The A/Udorn/72 cDNA clone of the M_2 protein was a kind gift of R. Lamb (Northwestern University). The genes were cloned into the *Bam*HI-*Xba*I window of pYEUra3 (*CEN*; Clontech, Palo Alto, Calif.) or pYES2 (2μm; Invitrogen, San Diego, Calif.). Conversion of asparagine residue 31 to serine in the WSN (N31S) mutant was also accomplished by PCR-directed mutagenesis. All gene sequences were verified by DNA sequencing.

Assay for detecting M_2 inhibitors. Cultures of strains containing M_2 expression plasmids were grown overnight in minimal medium containing 2% glucose and were then shifted to inducing medium (2% galactose) at a concentration of 10^5 cells per ml and were maintained at 30°C with vigorous aeration. To identify compounds inhibiting M_2 function, cells were distributed into 96-well microtiter plates in the presence of compounds drawn from the Bristol-Myers Squibb compound collection. Compounds were judged to test positive in the assay if they allowed for the restoration of cell growth. Compounds testing positive were verified by examining M_2 protein levels by Western blot (immunoblot) analysis in order to eliminate compounds which affect the expression of M_2. When required, amantadine-HCl (Sigma Chemical Co., St. Louis, Mo.) was prepared fresh and was added to the cultures at the time of induction at the indicated concentrations (see Fig. 3 to 5). Optical density was determined at intervals by measuring the A_{600}.

Microphysiometer analysis. The extracellular acidification rates of the yeast strains containing various M_2 expression plasmids were measured with a Cytosensor microphysiometer (Molecular Devices Corp., Sunnyvale, Calif.) in which cells are immobilized in a microvolume flow chamber in contact with a pH-sensitive silicon sensor (11, 22). Approximately 4×10^5 yeast cells were immobilized in Agarose Cell Entrapment Medium (Molecular Devices Corp.) between two microporous membranes. Flow chambers at 30°C containing the immobilized cells were superfused at a rate of 100 μl/min with minimal medium containing 2% galactose or 2% glucose plus appropriate nutritional supplements. Acidification rates were measured every 80 s by interrupting the flow of medium for 10 s. Amantadine at 100 μM was introduced 20 s prior to a flow-off period. Cells were exposed to amantadine for a total of 8 min. Acidification rates are expressed as a percentage of the basal rates before exposure to amantadine.

In vivo assays. The antiviral activities of the compounds in vivo were assessed in a plaque reduction assay. Approximately 100 PFU of A/WSN/33 or A/Udorn/72 virus was added to MDCK cells in 35-mm^2 dishes, and the dishes were incubated at 37°C for 1 h. The medium was removed and the cells were covered with 2 ml of agar overlay medium containing various amounts of either amantadine-HCl or BL-1743 in the presence of 5 μg of trypsin per ml. After 2 to 3 days of incubation, the monolayer cells were stained with 0.1% crystal violet solution, and the numbers and sizes of the plaques were assessed.

RESULTS

Expression of M_2 in *S. cerevisiae*. Expression plasmids encoding sequences of the M_2 protein of influenza virus under the transcriptional control of an inducible yeast promoter (*GAL1*) were introduced into *S. cerevisiae* W303. M_2 expression was assessed in strains containing either a single copy (centromere) or a high copy (2μm ORI) number and *GAL*-M_2 fusion plasmid after incubation in noninducing (glucose) or inducing (galactose) medium. As shown in Fig. 1, expression of M_2 in *S. cerevisiae* was detectable only in extracts of cells induced with galactose (Fig. 1, lanes 5 and 7). Synthesis of M_2 protein to levels comparable to those in virus-infected MDCK cells was detected in the strain containing the high-copy-number (pYES2-derived) plasmids (Fig. 1, compare lanes 7 and 8). Low levels of M_2 protein were detected in the strain transformed with the single-copy (pYEUra3-derived) vector.

M_2 expression impairs the growth of yeast cells. In the course of culturing the various transformants, it was observed that the growth rate of the transformant expressing the M_2 gene from a high-copy-number plasmid was significantly impaired relative to those of the other strains. Consequently, growth rates were analyzed by shifting equal numbers of log-phase cells previously grown in noninducing glucose-containing medium into medium containing galactose. Growth determinations indicated a correlation between the levels of M_2 expression and growth impairment (Fig. 2). Determination of the doubling times indicated that the strain expressing M_2 from the high-copy-number plasmid divides significantly more slowly than control strains (7.6 versus 4.0 h). This effect is observed only when the strain is grown on galactose (M_2-inducing conditions), because no growth differential is observed in medium containing glucose (noninducing conditions). However, in strains containing single-copy expression plasmids, no significant growth impairment was observed even after M_2 induction.

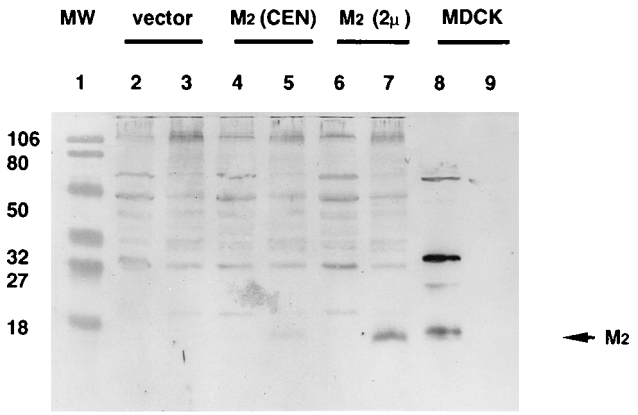

FIG. 1. M_2 is expressed at high levels in yeast cells, as shown by a Western blot indicating the levels of M_2 protein produced in yeast cells. Equivalent amounts of protein extract (100 μg) from each strain were separated on sodium dodecyl sulfate–12.5% polyacrylamide gels. The strains contained either the parental vector (SGY1442; designated vector), a single-copy centromere-containing plasmid expressing M_2 [SGY1492; designated M_2 (*CEN*)], or a high-copy-number 2μm origin-containing plasmid expressing M_2 [SGY1443; designated M_2 (2μ)]. Galactose-induced samples were loaded in lanes 3, 5, and 7, while uninduced samples were loaded in lanes 2, 4, and 6. Extracts prepared from influenza virus-infected and mock-infected MDCK cells are included as controls in lanes 8 and 9, respectively. The migrations of molecular weight standards (lane 1) are indicated.

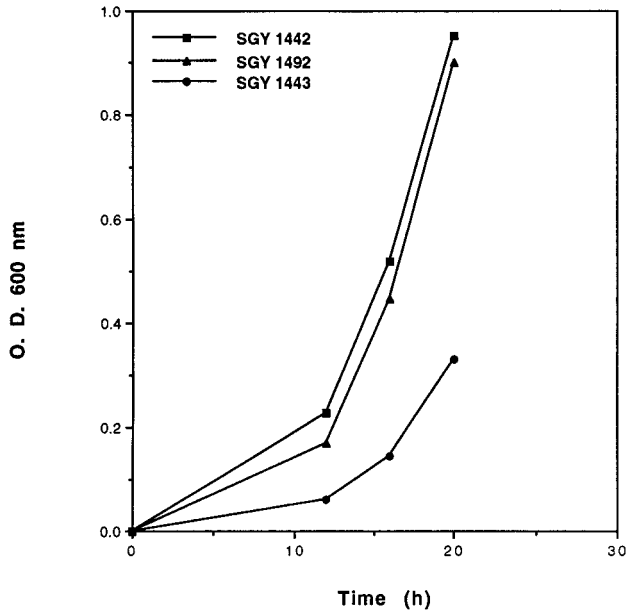

FIG. 2. Growth impairment of cells expressing M_2. Growth of cells expressing M_2 varies with plasmid copy number. Cultures of strains containing vector (SGY 1442), single-copy M_2 plasmid (SGY 1492) or high-copy-number M_2 plasmid (SGY 1443) were grown in inducing medium at 30°C with vigorous aeration. Samples were removed at various intervals, and the optical density (O.D.) at 600 nm was determined. The A_{600} is plotted versus time (in hours).

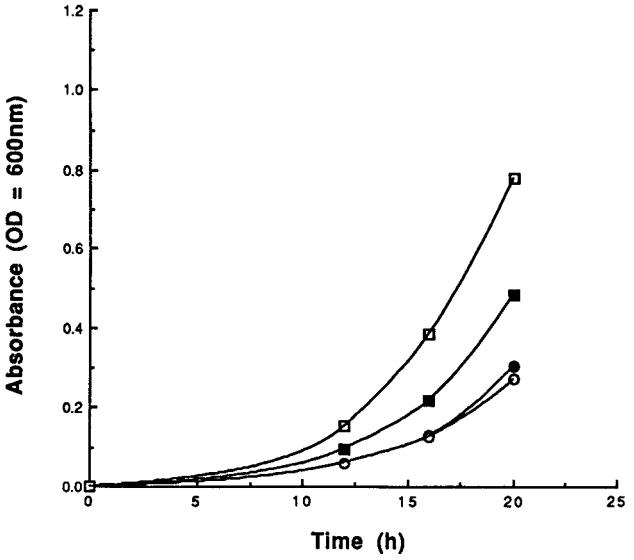

FIG. 3. Amantadine rescues the growth of cells expressing the Udorn M_2 protein. Duplicate cultures of strain SGY1443 containing the amantadine-resistant M_2 plasmid (WSN; ○, ●) or strain SGY1493 containing the amantadine-susceptible M_2 plasmid (Udorn; □, ■) were grown in inducing medium at 30°C with (○, □) or without (●, ■) amantadine. Samples were removed at various intervals, and the optical density (OD) at 600 nm was determined. The A_{600} is plotted versus time (in hours). The amantadine concentration was 100 μM.

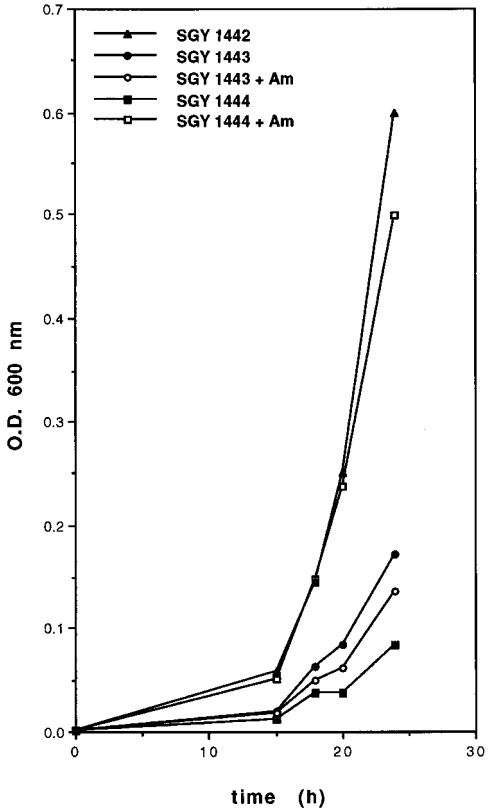

FIG. 4. Conversion of the amantadine-resistant M_2 (WSN) to amantadine (Am) susceptibility as determined by measuring the growth of strains expressing an allele of M_2 converted from amantadine resistance (WSN) to amantadine susceptibility. Cultures of a control vector-containing strain (SGY 1442, ▲); SGY 1443, the strain containing an amantadine-resistant M_2 plasmid (WSN); or strain SGY 1444, the strain containing the amantadine-susceptible M_2 [WSN (N31S)] plasmid, were grown in inducing medium at 30°C with vigorous aeration with 10 μM amantadine or without added drug. Samples were removed at various intervals, and the optical density (O.D.) at 600 nm was determined. The A_{600} is plotted versus time (in hours).

Toxicity is due to ion channel activity of M_2. One explanation to account for the impaired growth rate after M_2 induction is that the ion channel activity of the M_2 protein produced the observed toxicity. The approximate pH of the growth medium for the yeast cells is 6.0, which would be sufficient to activate the M_2 ion channel (26). To examine whether the ion channel activity is responsible for the toxicity, strains were induced for M_2 expression in the presence of amantadine, a known blocker of M_2 channel function (12, 13, 26, 33, 37). Because the M_2 gene used in this initial study was obtained from A/WSN/33 virus, which has the characteristic of amantadine resistance, a strain containing an amantadine-susceptible allele was constructed. The gene encoding the M_2 protein from an amantadine-susceptible strain, A/Udorn/72 virus (26, 37), was cloned into the pYES2 expression vector, and yeast cells were transformed with this construct. The induction of the M_2 protein of A/Udorn/72 virus results in a reduction of the growth rate (Fig. 3). The extent of growth impairment is significant, although not as great as that seen with expression of the WSN M_2 protein (Fig. 3). However, the growth rates of these transformants are substantially rescued when 100 μM amantadine is added to the culture medium at the time of induction (Fig. 3). In contrast, the growth of transformants expressing the amantadine-resistant WSN allele is not remediated with the addition of amantadine at 100 μM (Fig. 3). This result suggests that the toxicity related to M_2 expression correlates with an amantadine-responsive activity, i.e., ion channel function.

Conversion of the WSN M_2 protein to amantadine susceptibility. The predominant mutations which result in amantadine resistance map to one of four amino acids within the membrane-spanning region of the molecule (13). Examination of the WSN M_2 protein sequence suggests that resistance in this particular molecule is conferred by an asparagine residue present at amino acid 31. Therefore, residue 31 was converted from asparagine to serine. Both WSN alleles are toxic when they are overexpressed in yeast cells; however, only the toxicity of the WSN (N31S) allele is remediated by the addition of amantadine (either 10 or 100 μM) to the growth medium (Fig. 4). In the presence of amantadine, transformants expressing the WSN (N31S) allele grow at near wild-type rates, whereas growth of the transformants expressing the amantadine-resistant WSN allele is not remediated with amantadine (Fig. 4). This experiment provides additional genetic evidence that overproduction of M_2 results in growth impairment and is a consequence of M_2 channel function. It also proves that this residue in the wild-type protein, as predicted, is responsible for resistance to amantadine.

Proton flux is altered by expression of M_2. In yeast cells, the plasma membrane H^+-ATPase establishes an electrochemical gradient by pumping protons out of the cell, which maintains ion balances and facilitates the uptake of other ions (30). Perturbations to this gradient permit leakage of essential ions and can result in cell death. We reasoned that M_2 may exert its toxic effect on yeast cells by disrupting the electrochemical proton gradient. To examine whether M_2 expression correlates with changes in proton flux, we used microphysiometry (22) to measure the rates of extracellular acidification of cells expressing this ion channel before and after exposure to the M_2 channel blocker amantadine (Fig. 5A and B). The microphysiometer measures the rates of extracellular acidification of cells in

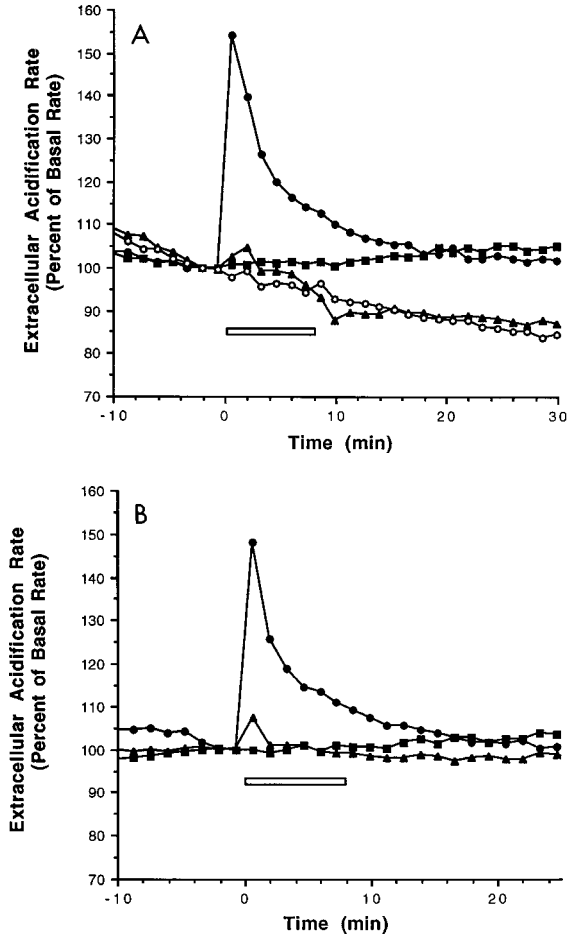

FIG. 5. Proton flux in strains expressing M_2 protein is reduced. (A) Amantadine causes a transient increase in extracellular acidification in yeast cells expressing an amantadine-susceptible channel. Control yeast cells (SGY1442; ■), cells expressing the amantadine-resistant WSN M_2 protein (SGY1443; ▲), or yeast cells expressing the amantadine-susceptible WSN (N31S) protein (SGY1444; ●) were grown overnight in medium containing galactose and were superfused with medium containing 2% galactose. After stable basal acidification rates were obtained, the cells were exposed to the same medium containing 100 μM amantadine for 8 min (indicated by the bar). Normalized extracellular acidification rates are plotted versus time (in minutes). Open circles represent SGY1444 cells grown and superfused in noninducing glucose medium. (B) Effect of amantadine on proton flux varies with external pH. Equal numbers of galactose-induced SGY1444 cells were loaded in three separate flow chambers and were superfused with minimal medium containing galactose at pH 5.5 (●), pH 6.5 (▲), and pH 7.5 (■). After stabilization of the acidification rates, cell were exposed to 100 μM amantadine for an 8-min period (indicated by the bar). Experiments were repeated at least twice, and representative data are shown.

a flow chamber by using a semiconductor-based sensor (11). These rates are sensitive to changes in metabolic activity (excretion of acidic metabolites) and to transmembrane proton flux associated with intracellular pH homeostasis (25). This technique has previously been used with mammalian cells to monitor cholinergic-stimulated changes in Na^+/K^+ ATPase activity (23) as well as activation of the nicotinic acetylcholine receptor (24). If the ion channel function of M_2 is acting to destabilize the gradient, then this should be reflected by changes in extracellular acidification.

The extracellular acidification rates of control strains SGY1442 (containing the vector only) and SGY1443 (expressing the amantadine-resistant WSN protein) are unaffected by the addition of 100 μM amantadine (Fig. 5A). However, exposure of strain SGY1444 (expressing the amantadine-susceptible M_2 protein) to 100 μM amantadine results in an immediate 50 to 60% increase in the rate of extracellular acidification, which is detected within 20 s of exposure to the drug (Fig. 5A). This increase in proton flux is transient and begins to return to basal levels even while the drug is still present. In contrast, strain SGY1444 maintained under conditions in which the M_2 gene is not expressed exhibits no effect on proton flux upon exposure to amantadine, indicating that the transient increase in acidification rates observed for SGY1444 grown under inducing conditions is a result of amantadine blockage of M_2 ion channel activity.

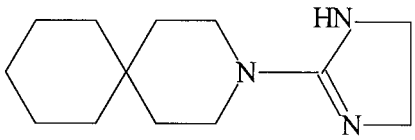

FIG. 6. Structure of BL-1743, 2-[3-azaspiro(5,5)undecano]-2-imidazoline, an inhibitor of M_2 protein function.

Previous studies of the M_2 ion channel expressed in *X. laevis* oocytes (26) or CV-1 cells (38) or reconstituted in a lipid bilayer (36) demonstrated that the activity is modulated by the external pH, with the channel being activated at low pH. We determined the effect of external pH on the activity of the M_2 ion channel expressed in yeast cells by superfusing flow chambers containing SGY1444 with minimal medium containing 2% galactose at pHs of 5.5, 6.5, and 7.5 and then measuring the extracellular acidification rates before and after exposing the cells to 100 μM amantadine (Fig. 5B). At a pH of 5.5, the typical increase in the acidification rate of 50% was observed upon exposure to amantadine. However, at an external pH of 6.5, the observed increase in the acidification rate was less than 10%, and at pH 7.5, no change in the acidification rate was detected (Fig. 5B). This result is consistent with earlier observations that the M_2 ion channel is activated at a pH of 5 to 6 (26).

Identification of BL-1743, an inhibitor of M_2 function. The observation of M_2 expression in yeast cells was adapted into a screen format to identify compounds that inhibit the function of M_2. One compound identified in this assay is BL-1743 (Fig. 6) a spirene-containing lipophilic amine. This compound was able to rescue the growth of WSN (N31S) M_2-expressing yeast cells with a 50% effective dose (ED_{50}) of approximately 250 nM. Amantadine has an ED_{50} of 125 nM in this assay (data not shown). Interestingly, BL-1743 does not rescue the growth impairment of the wild-type WSN M_2 alleles expressed in yeast cells (data not shown), indicating that it may function in a manner similar to that of amantadine. BL-1743 also does not rescue growth by simply reducing M_2 expression in yeast cells; the levels of M_2 protein in cultures exposed to BL-1743 are comparable to those in amantadine-treated cultures (data not shown).

In vivo activity of BL-1743. BL-1743 was next examined as an inhibitor of influenza virus replication in an in vivo plaque reduction assay (Fig. 7). Amantadine was able to inhibit plaque formation in A/Udorn/72-infected cells with an ED_{50} of approximately 260 nM, although it had no effect on plaque formation in WSN-infected cells. More importantly, BL-1743 was also able to inhibit plaque formation in A/Udorn/72 virus-infected cells, with an ED_{50} of approximately 2 μM. BL-1743, as expected from the yeast rescue experiments, had no effect on A/WSN/33 plaque formation.

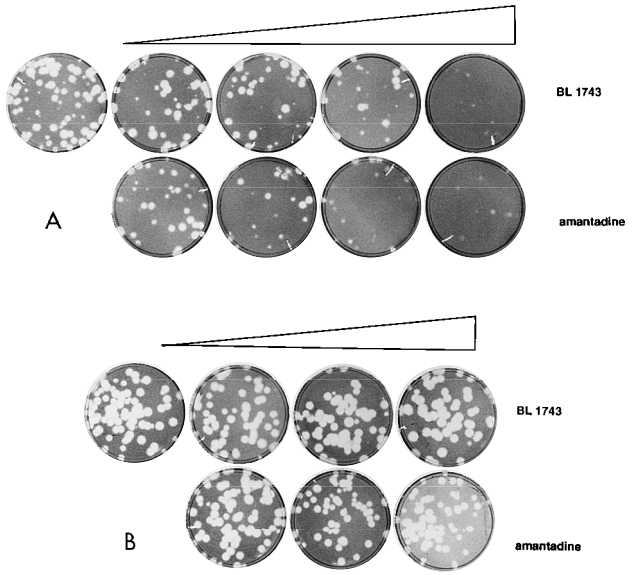

FIG. 7. Influenza virus A infection is blocked by BL-1743 in a plaque assay. A total of 50 to 100 PFU of A/Udorn/72 (A) or A/WSN/33 (WSN) (B) viruses were used in a plaque assay in the presence of increasing concentrations of either amantadine or BL-1743. For the A/Udorn/72 virus, the BL-1743 drug concentrations used were 0, 0.4, 0.8, 1.6, and 10 μg/ml, while the amantadine concentrations were 0.04, 0.08, 0.16, and 1 μg/ml. For the A/WSN/33 virus, both the BL-1743 and amantadine concentrations used were 1, 10, and 50 μg/ml.

DISCUSSION

Phenotypically, yeast cells expressing high levels of M_2 protein exhibited a consistently slower growth rate compared with that of control cells. The observation of growth impairment with M_2 expression is consistent with previous studies concerning M_2 expression in baculovirus-infected insect cells (2) and, more recently, in bacteria (10). In Sf9 cells, high levels of M_2 were produced only upon the addition of amantadine to the growth medium. One interpretation of this phenomenon is that the ion channel activity of M_2 is toxic to insect cells and that high-level expression can only be reached if this activity is blocked, perhaps because the cells remain viable and produce more protein. In our yeast system, several lines of evidence also suggest that toxicity is due to ion channel activity. Our most compelling data supporting this interpretation are from the comparison between amantadine-susceptible and amantadine-resistant alleles of M_2. Amantadine, a known inhibitor of ion channel activity, was able to rescue the growth of an amantadine-susceptible protein (the M_2 protein from A/Udorn/72 virus), but not that of an amantadine-resistant protein (A/WSN/33). In addition, when amino acid 31 of the WSN gene was altered from asparagine to serine and placed back into the yeast expression system, this transformant had a toxicity profile identical to that of the wild-type WSN protein, but the growth impairment was now responsive to amantadine. This proves that the asparagine at amino acid 31 is indeed responsible for the resistance of the WSN M_2 protein to amantadine and also strongly suggests that the toxicity associated with protein expression is directly related to the ion channel activity of the M_2 protein. The susceptibility of the WSN (N31S) M_2 protein to amantadine was also examined. This allele is extremely susceptible to amantadine, with significant rescue of growth observed at concentrations as low as 30 nM (data not shown). Growth rescue is also concentration dependent, suggesting that higher drug concentrations effectively block more channels. Growth rescue levels off at approximately 10 μM (unpublished data), suggesting that at this concentration all channels are blocked.

The M_2 protein may exert its toxic effect on yeast cells by destabilizing membrane function in a manner similar to that of killer toxin, a low-molecular-weight polypeptide encoded by the yeast M1 virus which forms ion channels in the lipid bilayer (4, 19, 21). In yeast cells, ion uptake is accomplished through the concerted actions of transporter proteins with ion selectivity and the electrochemical gradient established by the plasma membrane H^+-ATPase (30). Disruption of the integrity of this gradient leads to cell death by permitting the leakage of essential ions. Exposure to toxin results in a rapid inhibition of net proton pumping which reduces the proton gradient across the plasma membrane. Measurement of extracellular acidification rates of yeast strains expressing the M_2 ion channel before and after exposure to amantadine provides evidence that the open channel impairs the ability of the cell to maintain the proton gradient. Presumably, the plasma membrane H^+-ATPase is more active in the presence of the M_2 channel in order to maintain a gradient. The transient increase in proton flux observed upon exposure of cells expressing an amantadine-susceptible M_2 protein to the drug suggests that blocking the channel stops the leakage of protons into the cells. Previous studies of the M_2 channel expressed in *X. laevis* oocytes determined its cation selectivity but were unable to directly demonstrate proton flow through the channel (26). Recently, however, reconstitution of recombinant M_2 into liposomes has allowed for the demonstration of M_2-mediated proton translocation in vitro (28). The microphysiometer analyses presented here suggest that the M_2-mediated proton translocation occurs in vivo in this heterologous system.

The microphysiometer analysis also indicates that, as observed in other expression systems, the activity of the M_2 channel in yeast cells is modulated by external pH (26, 36, 37). The magnitude of the increase in acidification rates due to amantadine exposure is greatest at pH 5.5, weaker at pH 6.5, and undetectable at pH 7.5, suggesting that amantadine has no effect on acidification rates at the higher pH because the channel is inactive. An alternative explanation for these results is that at the higher pH there is less proton leakage into the cell and that the M_2 channel in the open state would have less of an effect on the maintenance of the proton gradient. In this case, the addition of amantadine would be expected to have little or no effect.

The toxicity profile of M_2-expressing yeast cells allowed for the development of a screening protocol based on the rescue of cell growth in the presence of test compounds. This protocol identified BL-1743, a spirene-containing imidazole compound, as an inhibitor of M_2. BL-1743 exhibited a 50% inhibitory concentration of 250 nM in the yeast assay, whereas it exhibited a 50% inhibitory concentration of 2 μM in the virus plaque reduction assay. This disparity may result from the relative levels of M_2 protein produced in each system, differences in the solubility of BL-1743 in the two culture media, or inherent differences in the sensitivities of these assays. BL-1743 has a profile similar to that of amantadine in that it is active against an amantadine-susceptible strain (A/Udorn/72) but not against A/WSN/33, which is amantadine resistant. The same profile is observed in the yeast toxicity rescue assay. In the yeast assay, the simultaneous addition of amantadine and BL-1743 does not produce an additive effect on M_2 inhibition, suggesting that these two compounds interact with similar sites in the M_2 protein (data not shown). Also, the WSN (N31S) mutant protein, which is converted to amantadine susceptibility, is also susceptible to BL-1743. Thus, BL-1743 appears to represent a

novel structure with an antiviral profile similar to that of amantadine.

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