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Quantitative analysis of influenza M2 channel blockers

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ABSTRACT

The influenza M2 H^+ channel enables the concomitant acidification of the viral lumen upon endosomic internalization. This process is critical to the viral infectivity cycle, demonstrated by the fact that M2 is one of only two targets for anti-flu agents. However, aminoadamantyls that block the M2 channel are of limited therapeutic use due to the emergence of resistance mutations in the protein. Herein, using an assay that involves expression of the protein in *Escherichia coli* with resultant growth retardation, we present quantitative measurements of channel blocker interactions. Comparison of detailed K_s measurements of different drugs for several influenza channels, shows that the swine flu M2 exhibits the highest resistance to aminoadamantyls of any channel known to date. From the perspective of the blocker, we show that rimantadine is consistently a better blocker of M2 than amantadine. Taken together, such detailed and quantitative analyses provide insight into the mechanism of this important and pharmaceutically relevant channel blocker system.

1. Introduction

The function of M2 was the last step to be uncovered in the infectivity cycle of the influenza virus [1]. Viral attachment and entry into the cell are carried out through the activity of the hæmeagglutinin protein. Membrane fusion followed by viral genome release occurs after hæmeagglutinin undergoes a pH-triggered irreversible conformational change in the acidic endosomes. In parallel M2's role in the virus uncoating process is critical in that it enables the passage of H^+s from the acidic environment of the endosomal lumen into the virus [2]. This weakens the interactions between the matrix protein and the ribonucleoprotein core, enabling the release of the viral genome into the cytoplasm. Additionally, in some viral strains, it was not clear why hæmeagglutinin did not change its conformation in the exocytic pathway where the pH is sufficiently low to cause the conformational change. The answer to this question came upon identifying the pH-dependent H^+ channel activity of M2 [2], which negates the activity of the Golgi H^+ ATPase. We note that in many tissues hæmeagglutinin internal cleavage takes place extracellularly and hence a conformational change cannot take place in the exocytic pathway.

The homo-tetrameric structure of M2 has been investigated using several techniques: X-ray crystallography [3], solid-state NMR [4] and solution NMR [5]. The X-ray study, in detergent micelles [3], was of a peptide that encompasses the transmembrane domain of the protein (residues Ser22–Leu46). Furthermore, two structures were obtained: one at pH 7.3 and another with amantadine at pH 5.3. Both structures were shown to be highly similar to one another. The solid-state NMR derived structural model was also obtained for the same transmembrane peptide, but this time in lipid bilayers [4] Finally, a solution NMR study of a slightly longer peptide (residues Ser23–Lys60) with rimantadine was reported in detergent micelles, as well [5]. The peptide that was analyzed corresponded to residues Arg18–Lys60, however, the N-terminal five residues were shown to be disordered.

Perhaps the reason for the intense interest in the M2 protein described earlier, is that it is the molecular target of the anti-flu drugs, amantadine and rimantadine that block it H^+ channel activity [2,6]. Yet, as a therapeutic option aminoadamantyls are not particularly effective due to mutations that emerge in the M2 protein that render the channel insensitive to aminoadamantyls [7] (see [8] for a recent review). Therefore, it was our goal to provide a detailed analysis of the activity of aminoadamantyls on various M2 variants using a cell-based assay that is both rapid and accurate.

A cell-based assay is another method to measure channel activity aside from traditional electrophysiological approaches. In the assay the channel is heterologously expressed in a host cell. Subsequently, the host cell experiences growth retardation that is a direct function of the permeabilization of its membrane by the non-native channel.

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Hence, channels with high conductivity will be more detrimental to the host's growth relative to poorly conducting species.

Kurtz and co-workers have constructed such a system to analyze the activity of the influenza virus H^+ channel [9]. Specifically, the authors expressed the M2 channel in *Saccharomyces cerevisiae*, which resulted in growth inhibition. Specificity was demonstrated by showing that the growth inhibition could be relieved by the addition of the anti-viral-specific M2 channel blocker amantadine. Finally, the authors used this approach for a high-throughput screening effort that resulted in the identification of potentially new anti-viral agents.

Herein, we have extended the aforementioned approach, transitioning to a different host — *Escherichia coli*, in order to make it a quantitative tool that can be used for detailed analyses, as well as high-throughput screening. The ease of genetic manipulation in *E. coli*, compounded by the multitude of expression systems, enabled us to tune the system, such that it yields quantitative results. Using the aforementioned system, we were able to derive accurate Monod coefficients (half-saturation coefficient or K_s) values for different channel blockers, as well as to differentiate between channels from different viral strains. In doing so we were able to show that the swine flu channel is the most aminoadamantane-resistant channel known to date.

2. Materials and methods

2.1. Chemicals

Amantadine, Rimantadine and the HIS-Tag antibody were purchased from Sigma-Aldrich laboratories. Isopropyl-β-D-thiogalactopyranoside (IPTG) was purchased from Biochemika-Fluka (Buchs, Switzerland). The pMal-p2x vector was purchased from New England Biolabs (Ipswich, MA). pET-22b(+) plasmid and Tuner (DE3) cells were purchased from Novagen (Gibbstown, NJ). DH10B bacteria cells were purchased from Invitrogen. Western blot reagents were purchased from BioRad.

2.2. Plasmids and bacterial strains design

The Singapore M2 sequence was synthesized via a multistep PCR protocol. This wild-type construct was designed according to the Singapore H2N2 isolate, M2 sequence [7]. The gene was flanked by the NcoI and HindIII restriction sites, in the pUC57 plasmid. The sequence was transferred with the former 2 restriction sites into the pMal-p2x plasmid via XmnI and XbaI restriction sites, in frame to the carboxy terminus of the MalE protein — following a poly Asn site. Different bacterial strains were screened in this assay as hosts for the aforementioned plasmid. As described in the Results and discussion section, reproducible results were achieved with the DH10B and Tuner (DE3) cells.

All other forms of the M2 proteins were obtained from mutations of the Singapore wild-type strain with the Quick multi-Muatagenesis kit from Stratagene (La Jolla, CA) and are listed in [7]. The BM2 channel sequence was according to [10].

2.3. Cell growth

Cells bearing or lacking (as a reference) the ion channel genes were incubated overnight from glycerol stocks in LB containing 100 μg/ml Ampicillin. Thereafter, the culture was diluted 100 fold and the bacteria were grown until their O.D.600 reached 0.07–0.1, after which IPTG was added to the growth culture. Cells were than divided into 96 or 48 well flat bottomed plates containing the different treatments. The growth volume in the 48 or 96 well plates was 500 μl and 100 μl, respectively.

The plates were incubated for 16 h at 30 °C in a Synergy 2 multi-detection micro-plate reader from Biotek (Winooski, VT) at a constant high shaking rate. O.D.600 readings were recorded every 15 min.

2.4. Western blotting

Bacteria were grown as described earlier, with 50μ M IPTG induction, in the presence or absence of 100 μM Rimantadine for the indicated times. From every time point, 0.5 ml was taken from the growth culture and harvested by centrifugation. The pellet was washed in PBS buffer (10 mM $Na_2HPO_4 \cdot NaH_2PO_4$, 120 mM NaCl and 2.7 mM KCl pH = 7.4) and then resuspended in the SDS sample buffer (2% SDS) at pH = 6.8, containing 10% DTT, followed by heating to 60 °C for 20 min and intensive tip sonication (vibracell by sonics, Newtown, CT).

The sample was then loaded onto a 10% polyacrylamide gel and electrophoresed for 35 min under 30 mA. The gel was then blotted onto a nitrocellulose membrane and visualization of the Singapore wild-typeM2-MalE chimera was possible via blotting with an anti-His-Tag antibody kit from Sigma-Aldrich laboratories, Israel.

2.5. Inhibitory constant derivation

Monod coefficients (K_s) were derived by measuring the dose response effect of amantadine or rimantadine upon the maximal growth rate of the host bacteria. The maximal growth rates were obtained as the peaks in the graph indicating the change of the O.D.600 as a function of time. The resulting data were non-linearly fit according to the Monod equation relating the growth rate ($\mathcal{R}$) to the drug concentration:

$$\mathcal{R} = \frac{\mathcal{R}_{\max}[\text{drug}]}{K_s + [\text{drug}]}$$

Note the control data (i.e. data without any drug) were subtracted from the results in order to serve as a reference.

3. Results and discussion

The objective of this study was to quantitatively assess the interaction between the influenza M2 channel, a critical component of the viral life cycle [2], and its aminoadamantyl cognate blockers. We therefore developed a cell-based assay in which the channel protein is expressed in bacteria resulting in growth retardation. The effects of channel blockers were then assayed by their ability to relieve the aforementioned growth retardation.

3.1. Protein expression

The first step in the assay was to ensure proper heterologous expression and reconstitution of the channel in *E. coli*. In order to maintain successful incorporation into the bacterial inner membrane we fused the M2 protein to the C-terminus of the maltose binding protein. In Fig. 1, one can see the profile of channel expression in the bacteria as a function of time and the presence of an inhibitor. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of whole cell lysates exhibits a band at the calculated molecular weight of the chimeric protein (60 kDa). No protein is seen in the absence of induction, while two hours or more post induction, expression is clearly visible. Interestingly, the expression of the protein is enhanced when the bacteria are grown in the presence of the anti-flu drug rimantadine. The reasons for this finding are elaborated subsequently.

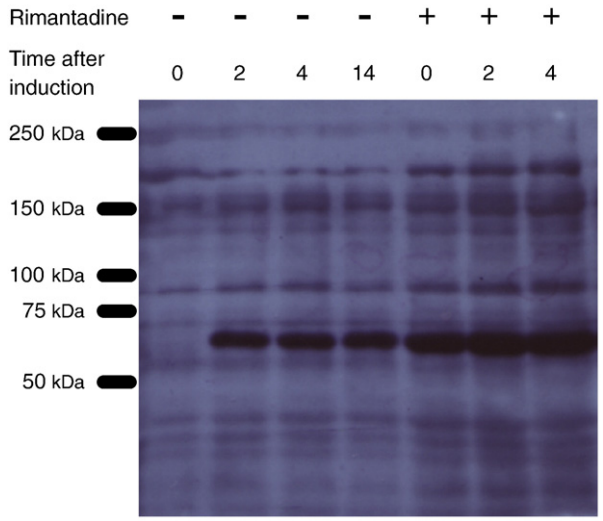

Fig. 1. Western blot analysis of the malE-M2 (Singapore wild-type strain) chimeric protein as a function of time post induction by 50 μM IPTG, and the presence of the anti-flu channel blocker rimantadine at 100 μM. Bacterial cells were examined by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted by an anti-His-Tag antibody. Molecular weights are shown on the left.

3.2. Bacterial growth

Following evidence that the M2 chimera is expressed in bacterial cells, we examined the protein's effect upon the growth rate of the bacteria. As seen in Fig. 2, bacteria that express the ion channel (labelled as induced) are virtually incapable of growth as compared to bacteria that do not harbor the M2 chimera (uninduced). However, it is essential to establish that the growth impairment is a specific function of the protein's channel activity and not just a general deleterious effect due to over-expression of a heterologous membrane protein [11]. This was achieved by demonstrating that a specific channel blocker of the protein — the anti-flu agent rimantadine [7], can appreciably alleviate the growth retardation (Fig. 2). Taken together, we can conclude that the viral ion channel permeabilizes the bacterial cell membrane, resulting in growth retardation, that can be reversed by the activity of a specific channel blocker. The effect of the drug to nullify the channel's deleterious impact upon cell growth might also be the reason why it promotes higher expression of the protein in its presence (see Fig. 1). In addition, the drug itself does not affect the growth rate of bacteria that do not harbor the M2 gene at all, as shown in Fig. 3. Finally, we note that while the M2 channel is activated in low pH [2], in neutral conditions under which the bacteria were grown, it maintains a non-zero open probability [6]. It is this low conductivity that hampers bacterial growth.

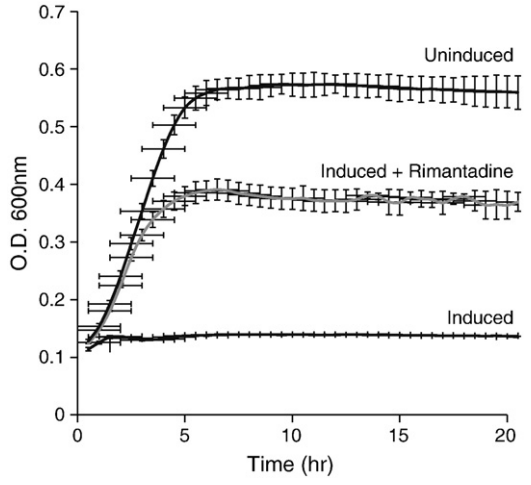

Fig. 2. Growth curves ($n=3$) of bacteria expressing the M2 (Singapore wild-type strain) channel (induced) and the influence of the anti-viral channel blocker rimantadine (in gray), thereupon. Bacteria that do not express the ion channel (uninduced) are shown as control. Induction takes place when the bacteria density reaches an O.D.600 of 0.1. The error bars reflect the plus/minus the standard deviation from three independent experiments. O.D.600 values were collected every 15 min. The rimantadine concentration was 70 μM in order to ensure saturation.

3.3. Channel strain effect

After demonstrating that the activity of the viral channel can be specifically studied in the bacterial membrane, we set forth to examine the effects of channels from various viral strains. Specifically, we expressed the following five different H^+ channels, each from a different influenza strain: (i) Singapore, (ii) Rostock, (iii) Singapore with a mutation of S31N, (iv) Swine flu and (v) BM2 from influenza B.

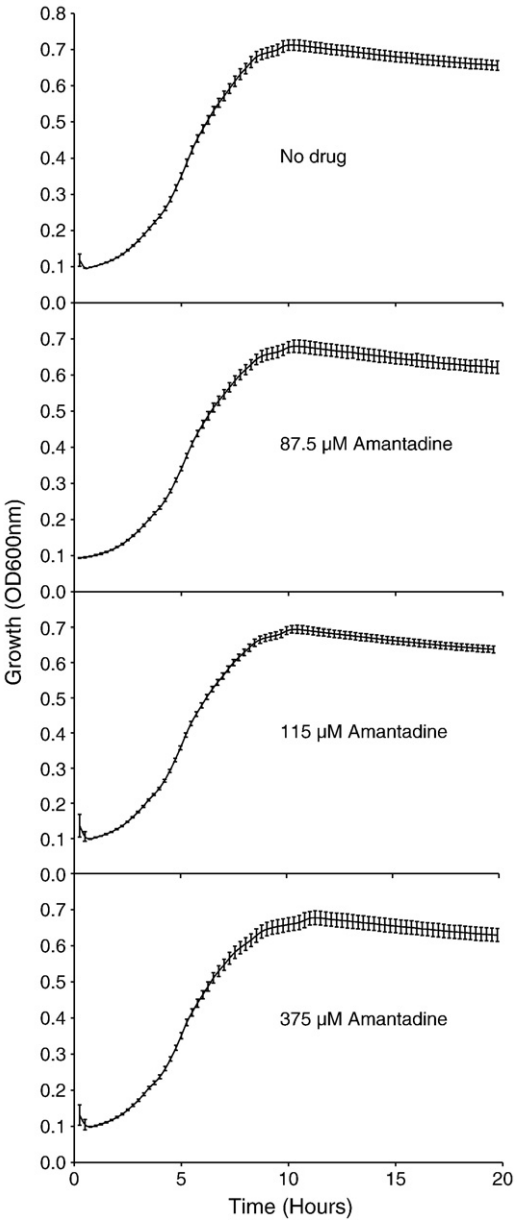

Fig. 3. Growth curves of bacteria that do not harbor the M2 gene and the influence of the anti-viral channel blocker amantadine, thereupon. The concentration of amantadine is as shown in the graph. The error bars reflect the plus/minus the standard deviation from eight independent experiments.

The mutation of Ser31 to Asn is known to render the channel insensitive to aminoadamantyl drugs [12]. Furthermore, swine flu is known to contain the S31N mutation and hence was assumed to be resistant to aminoadamantyl drugs [2]. Finally the BM2 channel from influenza B is known to be resistant to aminoadamantyl drugs as well [10].

As shown in Fig. 4, the different channels exhibit different sensitivities to the aminoadamantyl drugs. The growth inhibition by the channel from the Singapore strain is substantially relieved upon addition of either rimantadine or amantadine. In contrast, the effect of the drugs upon the growth retardation by channels from aminoadamantane-resistant viruses was significantly smaller. For example, only a single mutation - S31N in the expressed channel diminished the ability of aminoadamantyl drugs to relieve growth inhibition by more than 50% (compare solid black line versus dotted gray line in Fig. 4). Similarly, aminoadamantyl drugs exhibit poor growth retardation relief for bacteria that express the BM2 channel from influenza B that is known to be refractive to aminoadamantyl drugs [10].

However, it is important to note that the aforementioned assay is sufficiently sensitive to detect the reduced aminoadamantane sensitivity of the resistant channels such as the Singapore S31N mutant [12]. In other words, the assay was able to detect even the reduced levels of channel blocking which is important for identifying the full dynamic range of resistance towards aminoadamantyls. In addition this approach is suitable to high-throughput screening attempts, in that such screens would be able to identify even poorly blocking compounds, the activity of which can subsequently be enhanced by further optimization efforts.

3.3.1. Swine flu

One surprising outcome of our study was that the M2 channel from the swine flu virus was shown to be the most resistant to both aminoadamantyl drugs (dashed line in Fig. 4). Specifically, it was even more resistant than the Singapore S31N mutant [12] and the BM2 channel from influenza B [10]. Current efforts in our group are aimed at elucidating the mechanism of the enhanced resistance of the swine flu channel to aminoadamantyl drugs.

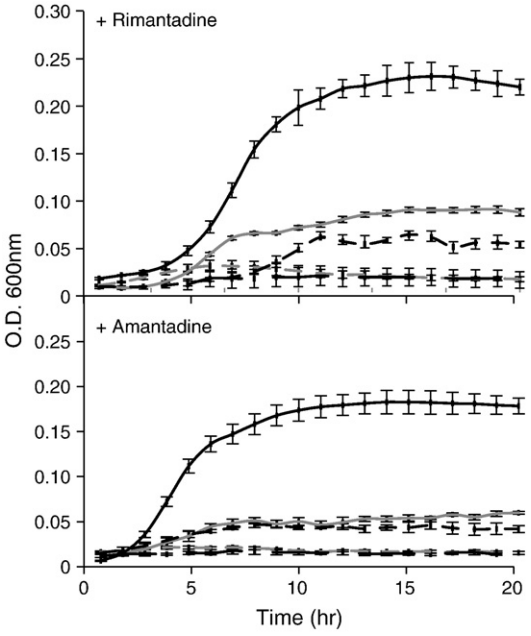

Fig. 4. Growth curves of bacteria expressing different M2 channel variants in the presence of rimantadine ($n=4$, top) or amantadine ($n=3$, bottom). Growth values without any drug treatment (e.g. Fig. 2) are subtracted as control from the aforementioned results. The different channel variants are: Singapore — black; Rostock wt — gray; BM2 — dotted black; Singapore S31N — dotted gray and swine flu — dashed. The concentration of both drugs was 70 μM. The error bars reflect the plus/minus the standard deviation from independent experiments.

3.4. Drug effect

Having established that the assay can quantitatively detect differences in sensitivity towards aminoadamantyl drugs between the different channels, it was interesting to examine if differences between various drugs can be observed as well. Here we compared the growth retardation relief effects of the only two aminoadamantyl drugs that are approved for prophylactic use: rimantadine and amantadine. Detailed comparisons between the top and bottom panels of Fig. 4 show that the activity of rimantadine is more pronounced than amantadine [13–15]. Specifically, one can see that regardless of the channel that is expressed, the bacteria were able to grow to higher O.D.600 when rimantadine was added to the media in comparison to amantadine. The different activities of the two drugs (see Fig. 5) reflect the known advantage of rimantadine over amantadine in channel blocking [13–18] that is mirrored in our assay.

3.5. K_s measurements

We then set forth to utilize the quantitative nature of the assay in order to derive *Ks* values for various drugs and channels. Here we calculated K_s values by measuring the dose response effect of amantadine and rimantadine upon the maximal growth rate of the host bacteria. As seen in Fig. 5, irrespective of the channel variant, or the drug, the measured data fit remarkably well to the Monod equation. The derived K_s for the Singapore wild-type strain for amantadine and rimantadine are 330 nM and 13 nM, respectively.

It is difficult to compare our results to the isochronic apparent inhibitory binding constants reported by Lamb and co-workers [6,15]. The reasons are that isochronic measurements by their very nature depend on the time when the measurements are taken. Hence, direct comparison to our procedure is not possible. In general one may note that our study yields significantly higher activities of aminoadamantyl drugs relative to the isochronic approach. For example the K_s of amantadine in the wild-type Singapore channel is 330 nM (Fig. 5) in comparison to 16 μM in the isochronic study [15]. Furthermore, the decrease in the S31N mutant sensitivity towards the drug in the isochronic study is an order of magnitude — 16 to 200 μM [15]. In our approach a much higher dynamic range is obtained (this time to rimantadine) 13 nM to 5.4 μM. Finally, the differences noted earlier may stem from the fact that the measurements in this study are of an indirect nature — the effect of the drugs upon bacterial growth rather than a direct measure of channel activity [6,15]. The advantage of conducting such a cell-based assay stems from its ease of operation and suitability to high-throughput screening. Herein, we have shown that it is nonetheless highly quantitative, as well.

Similarly, it is difficult to compare affinities of the drug that were measured to the M2 transmembrane peptide, either by ultracentrifugation [19] or surface plasmon resonance [20]. The reason being, that the affinity of the full length protein towards the drugs might differ from that of the transmembrane segment. Indeed, the affinities that are measured by both of the aforementioned studies are much lower than those measured in the current approach. Taken together, the results that are described in this study represent one of the most sensitive measurements of the activity of influenza channel blockers.

The last issue to consider is the dynamic range of our assay. As seen in Fig. 5, our cell-based assay yields detailed K_s measurements in a span of three orders of magnitude — from micro to nano-molar. This aspect is critical to allow the assay to be used in high-throughput screening since it ensures that one would be able to detect blockers that are both highly efficacious along-side marginally active compounds. Finally, this assay is applicable to any channel as long as it can

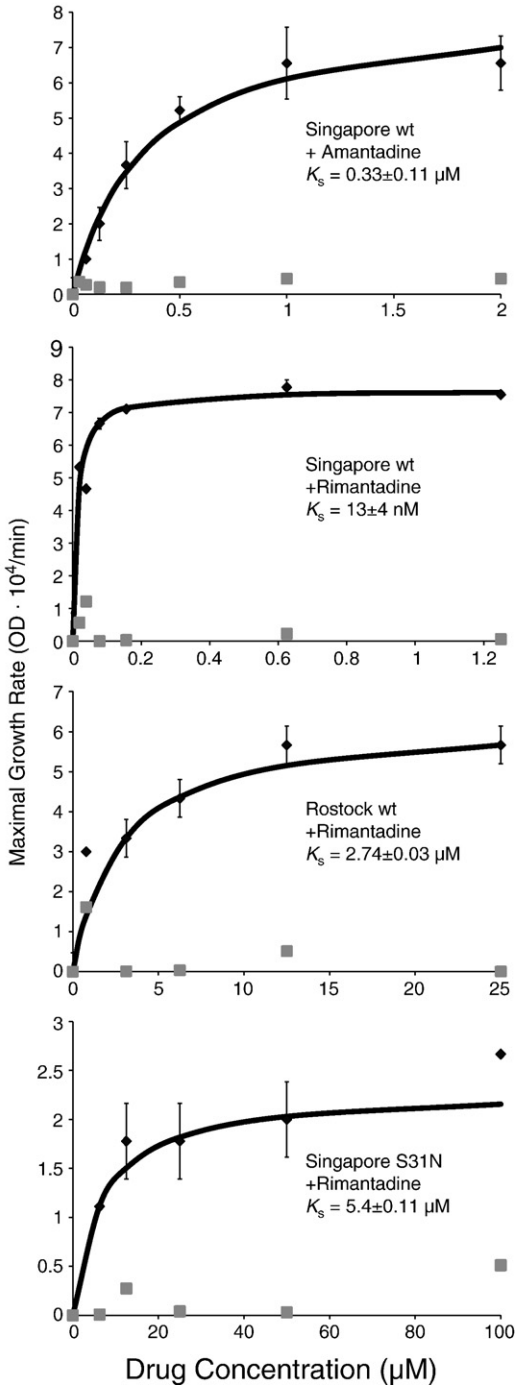

Fig. 5. Dose response curves of amantadine and rimantadine for various M2 channels (as indicated) upon the growth rate of the host bacteria. Note different drug concentrations for each panel. Experimental values (black diamonds) were fit according to the Monod equation (black lines) yielding the K_s values as indicated. The residuals are shown in gray squares. The error bars reflect the plus/minus the standard deviation from three independent experiments.

be expressed in functional form in bacteria and has deleterious effects on the host.

4. Conclusion

In this study we report a simple system that can be used to assay channel activity and inhibition thereof. In common with a previous cell-based assay in *S. cerevisiae* [9], the assay involves expression of the channel in a microorganism host and channel activity is measured by growth retardation. Herein, we have transitioned the system into a simpler host — *E. coli*, that allowed us to screen a large number of expression systems and host strains. This optimization resulted in an assay that is quantitative with a large dynamic range on the one hand and rapid and economic on the other. For example we were able to detect marginal activities of aminoadamantyl drugs even in channels that are clinically resistant towards them. In doing so we were able to show that the channel for the swine flu virus is the most resistant of all known channels.

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