Novel assay for the influenza virus M2 channel activity

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Received 15 November 1994; revised version received 1 December 1994

Abstract The influenza A M2 ion channel was expressed and activity characterized in *Xenopus* oocytes. Based on the activation properties of the channels, a high throughput, non-electrophysiological screening assay was developed in order to identify novel inhibitors of the channel. This will facilitate discovery of novel agents to treat influenza viral infections.

Key words: Influenza A; M2; Whole-cell recording; Amantadine; *Xenopus* oocyte; Natural product screening

1. Introduction

A novel, acid activated ion channel, M2, was identified as a component of the influenza A virus [1]. Influenza A virus is a negative strand RNA virus, and is the primary viral agent for influenza [2]. The M2 channel is a viral encoded, single membrane spanning, protein of 97 amino acids with the amino terminus present on the extracellular side and a cytoplasmic tail [3–6]. The native protein is thought to form a functional homotetramer of, either, pairs of disulfide-linked dimers, or four disulfide linked monomers [7–10]. While potential N-linked glycosylation sites are present, they are not thought to be of functional significance [6]. In addition, the protein is acylated and phosphorylated, however, these modifications are not essential for activity [9,11–14]. The M2 protein is abundantly synthesized on the surface of the host cell, although only 5–10 copies of the channel are present in the membrane of each virion [5,6,15,16].

The M2 channel is thought to be activated by low pH (~6.2) while the viral particle is present in the secondary endosome of the host cell [9,17–19]. Once the channel is activated, it is thought to pass protons to the interior of the viral particle allowing for the acid-induced changes which mediate viral uncoating resulting in the release of the viral genome [20]. A second physiological function has been indicated in the avian form of the virus where the M2 channel dissipates the pH gradient across the golgi membrane allowing the hemagglutinin protein, necessary for receptor binding, to emerge on the cell/virion surface in the high pH conformation essential for infectivity in the next cycle [19,21–25]. Therefore, one target for pharmacological intervention of influenza infections would be the M2 channel. Indeed, the only clinical anti-influenza A agents available, amantadine and rimantadine, specifically block the M2 channel [1,26]. This channel is an optimal target since M2-like channels have not been identified in mammalian systems, nor does the M2 sequence share significant homology to known mammalian proteins [1]. Amantadine, while currently used prophylactically, does show CNS side effects in 5–10 percent of the population [27–30]. A second generation analog, rimantadine, reportedly has reduced CNS side effects, however, it does not appear to have an improved potency [31,32]. Aside from the CNS effects of these agents, it is also clear that viral resistance to these drugs can readily develop, and the resistance maps to the M2 channel [33,34]. Therefore, an improved agent would also seek to lower the probability of developing resistance. Since influenza/pneumonia is the sixth leading cause of death in the adult population of the US with influenza A being the main viral agent [2], there is a clear need for an antiviral agent which would improve upon the clinical profile of amantadine/rimantadine.

One means to identify new chemical entities in the treatment of any disease is to undertake a screening effort of chemical and natural product libraries. Screening of a large number of samples would be facilitated by a rapid and high throughput assay. This provides a unique challenge when looking for specific inhibitors of functional ion channels since whole cell recording is not amenable to high throughput, and in many cases, binding assays do not utilize channels in their functional state. In addition, the unique pH properties of the M2 channel make creating a stably transformed mammalian cell line extremely difficult. Since the *Xenopus* oocyte can tolerate extreme pH ranges, we have utilized the system in the expression and study of the M2 channel. Additionally, under the appropriate recording conditions, the M2 channel can pass sodium ions making the study of the channel feasible. We, therefore, have characterized the properties of the M2 channel when expressed in *Xenopus* oocytes and have developed a high throughput, functional screen.

2. Materials and methods

2.1. Expression of the M2 channel

The M2 gene from influenza A/Udorn/72 was kindly supplied by Dr. Robert Lamb (Northwestern University) [1]. The vector was linearized with *Xba*I and runoff cRNA transcripts were made via Ambion's T7 mMessage mMachine transcription kit per manufacturer's directions. Adult female *Xenopus laevis* (Xenopus I or Nasco) were maintained and oocytes harvested by published methods [35]. The oocytes were defolliculated with 2 mg/ml collagenase (type 1A; Sigma) in Ca^{2+} free ND96 [36] 1–2 h at room temperature (~20°C) and then washed with multiple buffer changes for up to 2 h in sND96. Healthy defolliculated stage IV/V/VI oocytes were collected, stored overnight in sND96 with 1% dialyzed fetal bovine serum (3 kDa cutoff; Sigma). The next day cells were injected with ~50 nl of a 1 mg/ml cRNA solution in RNAse-free

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Abbreviations: M2, matrix protein 2; RNA, ribonucleic acid; cRNA, cDNA derived mRNA; CNS, central nervous system; NMDA, *N*-methyl-D-aspartate; MES, 2-(4-morpholino)-ethane sulfonic acid; HEPES, *N*-[hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]; sND96, ND96 supplemented with 2.5 mM sodium pyruvate (Gibco), 100 U/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco)

SSDI 0014-5793(94)01369-1

H_2O and incubated 1–7 days at 18–19°C in sND96 with 1% serum at either pH 7.5 or 8.5, as indicated, prior to experimentation.

2.2. Electrophysiology

Macroscopic currents were recorded using the two-microelectrode voltage clamp technique. Bath solution was ND96 with reduced (0.18 mM) Ca^{2+} at the appropriate pH, and as indicated amantadine (Research Biochemicals Inc.) was added. Microelectrodes from KG-33 glass (Garner Glass Co.) were pulled on a Narishige PB-7 pipet puller and then back filled with 3 M KCl with resulting resistances between 0.2–2.0 MΩ. Membrane currents were recorded using either an Axoclamp2 (Axon) or a TEV 200 (Dagan) voltage clamp. Experiments were controlled and data collected on a CompuAdd 386 computer equipped with an analog interface (Labmaster) to the electrophysiological equipment. Experimental variables were manipulated, data analyzed, and graphically reproduced using pClamp software (Axon Instruments), Excel (Microsoft), Deltagraph Pro (Delta Point) and CorelDraw (Corel).

2.3. Cell assay methods

M2 expressing cells, 48 h post injection, were incubated in the appropriate test conditions, as indicated, for ~14 h at 18–19°C. In order to have sufficient buffering at pH 8.5 and 5.5 when test compounds were added, 10 mM MES (Fisher) was added to ND96 solutions in addition to the 5 mM HEPES. After the incubation, cells were scored visually under a dissecting microscope.

2.4. Statistical methods

Analysis of Variance (ANOVA) type I was used to test statistical differences among the means of the different treatment groups. We assume that the differences among the group means are due to the fixed treatment effects determined by the experimenter. Multiple unplanned comparisons among the means from 16 replicates of cell assay data were made using the T-Method [37]. This a posteriori method allows for all pairwise comparisons of the group means to be performed.

3. Results

3.1. Expression of influenza A virus M2 protein in Xenopus *oocytes*

Cells were initially perfused in oocyte recording buffer, ND96 pH 7.5. The cells were clamped to a holding potential of −40 mV, and stepped for 3 s to test potentials between −60 and −130 mV, allowing background current waveforms to be recorded. The M2 channels were activated by changing to ND96 at pH 5.5, and the voltage paradigm described above was repeated. Once steady state was achieved (30–60 s) the M2 current was inhibited by changing the bath solution to ND96 (pH 5.5) with 100 μM amantadine. Control injected oocytes (injected with cRNA for the cardiac Kv1.5 channel [38]) were studied in an identical manner.

M2 current records obtained at a test potential of −120 mV are presented in Fig. 1. As shown, cells injected with M2 cRNA have robust inward currents at pH 5.5 which are blocked by 100 μM amantadine (Fig. 1A). Although not shown, the M2 currents activated at pH 5.5 are induced very rapidly upon switching the buffers and can be quickly deactivated by returning to the control ND96 at pH 7.5. The amantadine block is not rapidly reversible (data not shown). The cells injected with control Kv1.5 cRNA do not elicit a low-pH activatable current (Fig. 1B).

The current–voltage relationship for the activated M2 channel presented as the mean (±S.E.M., $n = 6$) is shown in Fig. 2. Current amplitudes at each potential for each cell were measured as the low pH induced current (the current amplitude recorded at pH 5.5 minus the current amplitude recorded at pH 7.5). As shown, a low pH-activated current is only observed in the cells injected with M2 cRNA, and the M2 current is inhibited beyond pH 7.5 baseline by the clinical agent amantadine presumably due to a small number of open channels at pH 7.5. In addition, the current–voltage relationship appears to be ohmic in this voltage region.

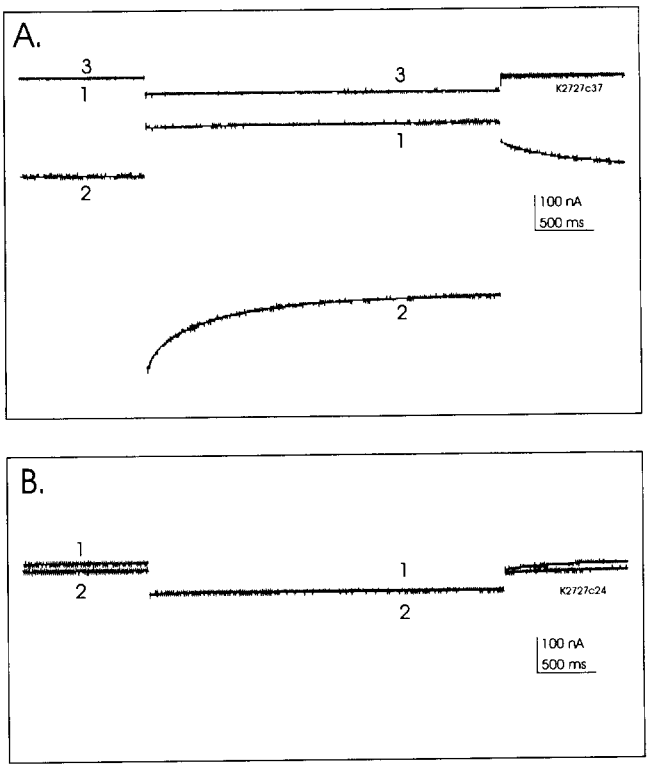

Fig. 1. Typical waveforms of M2 currents expressed in *Xenopus* oocytes. Cells were initially perfused in ND96, pH 7.5. Cells were clamped to a holding potential of −40 mV and current waveforms were evoked at a test potential of −120 mV for 3 s. (A) (1) Current waveform elicited from the oocyte in ND96 pH 7.5. (2) Current elicited from the same cell at pH 5.5. (3) Demonstrates the M2 current is inhibited by 100 μM Amantadine in ND96 pH 5.5. (B) Xenopus oocytes injected with 50 ng of Kv1.5 cRNA were treated as above. (1) Current elicited from the oocyte at pH 7.5. (2) Current elicited from the same cell at pH 5.5.

3.2. pH activation profile of the M2 channel

In order to reduce the number of open channels, cells were initially perfused in ND96 pH 8.5. The cells were clamped to a holding potential of −40 mV and stepped for 3 s to a test potential of −110 mV, and current waveforms were obtained. The recording media was then switched to ND96 at different pHs (8.5–5.5 in 1.0 increments) and the voltage paradigm described above was repeated. Current amplitudes at each potential for each cell were measured as the test pH induced current (the current amplitude recorded at variable pH minus the current amplitude recorded at pH 8.5). The mean (±S.E.M.; $n = 3$) are presented as a normalized current versus pH relationship in Fig. 3.

3.3. M2 cell assay

It was noted that survival of oocytes expressing M2 channels was dramatically reduced (1–3 days) as compared to the oocytes expressing Kv1.5 channels (7–10 days). Also, as indi-

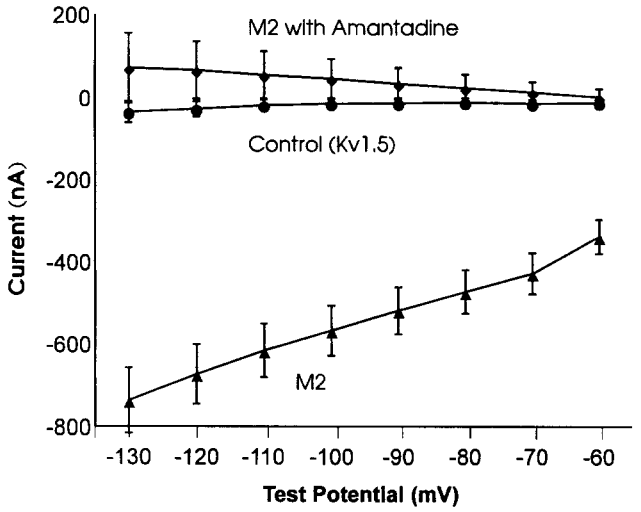

Fig. 2. Current–Voltage properties of M2 Channels. Cells were initially perfused in ND96, pH 7.5 and clamped to a holding potential of −40 mV. Current waveforms were obtained from cells stepped for 3 s to test potentials between −60 and −130 mV in 10 mV increments at 10 s intervals. The recording media was switched to ND96 at low pH (5.5) and the voltage paradigm described above was repeated. The low pH-induced currents were then measured as described above in the presence of ND96, pH 5.5 with 100 μM Amantadine. In addition, control injected oocytes (with Kv1.5 cRNA) were examined following the same pH-voltage paradigm. Current amplitudes at each potential for each cell were measured at the end of the test pulse as the low pH induced current (the current amplitude recorded at pH 5.5 minus the current amplitude recorded at pH 7.5). The mean (±S.E.M., $n = 6$) are presented.

cated in the preceding Figures, the pH profile of the channel suggested basal activity at pH 7.5. The next experiment, therefore, investigated the survival of the cells at various buffered pHs. M2 channel expressing and control, non-injected cells ($n = 12$ for each condition), were incubated in ND96 at the following pHs: 8.5, 7.5, 6.5, and 5.5. After ~14 h, the cells were examined visually and scored, and the data is presented in Table 1. There was a dramatic rearrangement (condensation or loss) of the oocyte animal pole which correlated with the activation of the M2 channel (see Fig. 4 below). When visually scoring, this rearrangement was termed cell 'death'.

The next set of experiments illustrated that the M2 specific cell 'death' can be prevented in a reproducible, dose-dependent manner by the presence of amantadine. Fig. 4 presents representative examples of M2 expressing oocytes in which the oocyte animal pole undergoes a condensation and eventual elimination via the chronic activation of the M2 channel; this rearrangement was blocked by the presence of amantadine.

Table 1
pH dependence of cell survival

pH	Survival control	Survival M2
8.5	12	11
7.5	12	11
6.5	12	2
5.5	12	0

Assay performed as described in section 2. The time course of the experiment was 14 h. 12 cells, either non-injected or M2 injected, were used for each condition.

Similar results were obtained with rimantadine (data not shown). Data for a total of 16 assays is presented in Fig. 5, and shows that cells are protected in a dose-dependent fashion by amantadine. No significant difference ($P < 0.01$) was seen between the permissive (pH 8.5) control and the highest drug (pH 5.5, 10 μM amantadine) treatment groups. Also, the negative control group (pH 5.5, 0 μM amantadine) and the lowest drug (pH 5.5, 1 μM amantadine) treatment were not significantly different ($P < 0.01$) from each other. Whereas, the intermediate (pH 5.5, 5 μM amantadine) treatment is significantly different ($P < 0.01$) from all other treatments.

4. Discussion

The M2 ion channel provides an amenable target to develop new classes of inhibitors for pharmaceutical intervention of influenza A infections. This channel appears to be unique to the viral genome, as low stringency Southern analysis of the human genome was negative ([1] R. Forgey, unpublished observations]. The channel is readily activated at low pH when ex-

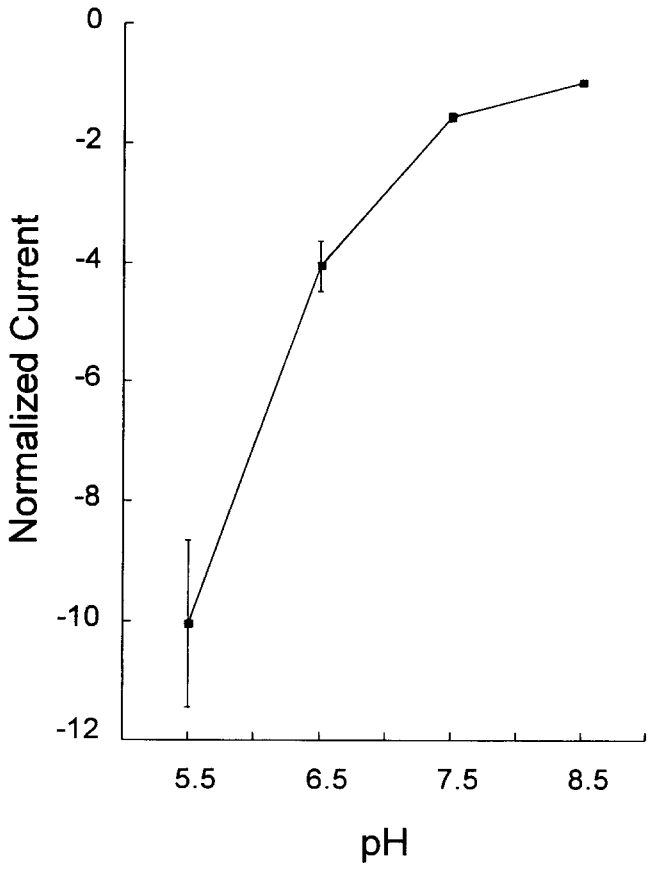

Fig. 3. pH Induction of the M2 Ion Channel. Cells were perfused in ND96, pH 8.5. The cells were clamped to a holding potential of −40 mV and were stepped for 3 s to a test potential of −110 mV, and current waveforms were obtained. The recording media was then switched to ND96 at different pHs (5.5–7.5 in 1.0 increments) and the voltage paradigm described above was repeated. Current amplitudes at the test potential for each cell were measured as the test pH induced current at the end of the test pulse (the current amplitude recorded at variable pH minus the current amplitude recorded at pH 8.5). Current amplitudes for each cell were normalized to the current evoked at the test potential at pH 8.5. The mean normalized current (±S.E.M.; $n = 3$) are presented as a function of pH.

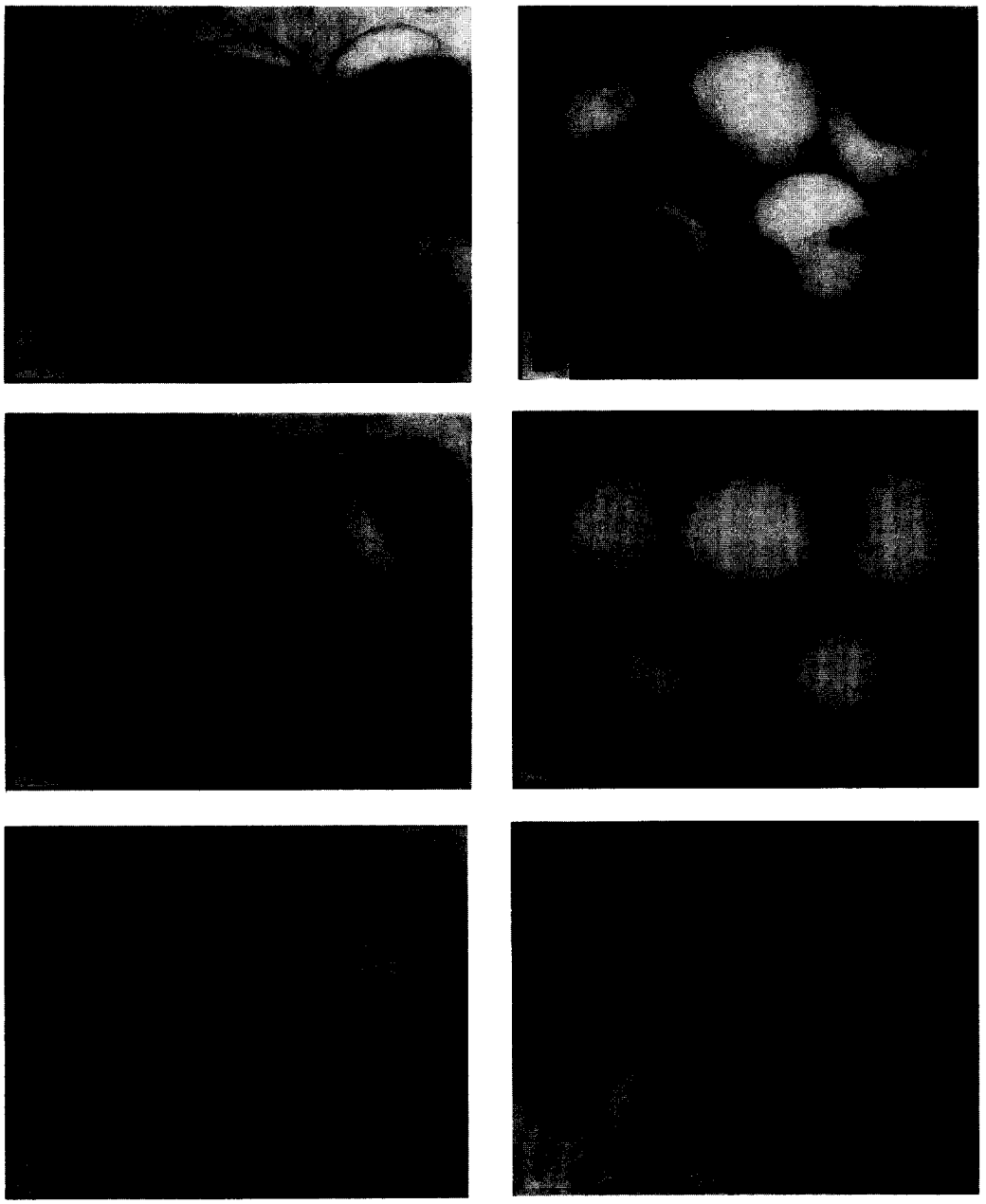

Fig. 4. M2 cell assay. Cells were incubated for 14 h as described in section 2. (A) M2 injected cells at pH 8.5; (B) M2 injected cells at pH 5.5; (C) Non-injected cells at pH 5.5; (D) M2 injected cells at pH 5.5 with 1 μM amantadine; (E) M2 injected cells at pH 5.5 with 5 μM amantadine; (F) M2 injected cells at pH 5.5 with 10 μM amantadine. As presented, cells shown in panels A, C, and F are 'viable'. While preliminary rearrangement of the animal pole is evident in panel E, the cells are scored 'viable'. Cells in panels B and D are scored 'dead'.

pressed in *Xenopus* oocytes, and pharmacologically, can be inhibited by the clinical agents amantadine and its closely related analog, rimantadine. While these agents are used clinically, both show some level of CNS side effects, likely due at least in part to the interaction of the adamantine class of chemistry with NMDA receptors [39–41]. Therefore, a different class of inhibitors could result in an ion channel blocker with greater specificity and a reduced side effect profile.

The *Xenopus* oocyte expression system is widely used for the expression and study of ion channels. Data presented here characterizing the electrophysiological properties of the M2 channel indicate the channel is activated as pH is lowered, and in addition, the cellular assay also indicates that the pH induced cell 'death' directly correlates with the pH activation properties of the M2 channel. We believe that oocyte 'death' in the assay is likely due to the continual influx of Na^+ ions into the cell. Increasing the sodium concentration from 96 mM to 150 mM decreases the time course of 'death' (K. Giffin, unpublished observation). The ability of amantadine/rimantadine to prevent cell 'death' correlates with the ability of amantadine to block

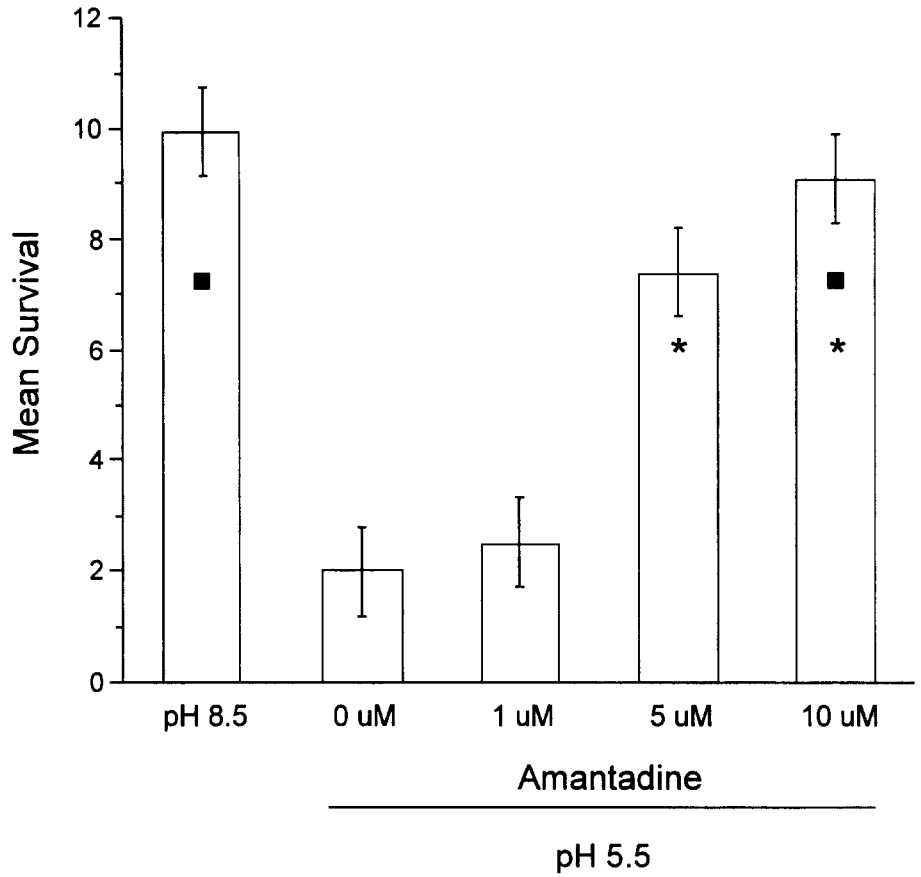

Fig. 5. Reproducibility of cell survival in the assay with Tuckey's 99% confidence intervals. Ten cells, either non-injected, or M2 injected were used for each condition in each replicate. Cells were cultured as described in section 2. Data were collected for 16 assays. Multiple unplanned comparisons among means were made by the T-Method ($a = 5$, $n = 16$. No significant difference ($p \leq 0.01$) was seen between the pH 8.5 and pH 5.5, 10 μM amantadine treatment groups. Also, the pH 5.5, 0 μM amantadine and pH 5.5, 1 μM amantadine treatments were not significantly different ($p \leq 0.01$) from each other. Whereas, the pH 5.5, 5 μM amantadine treatment is significantly different ($P \leq 0.01$) from all other treatments. *Significantly different at $p \leq .01$; (■) not significantly different $p \leq 0.01$).

the channel. As reported by Wang et. al. [26], the calculated IC_{50} of amantadine for the Udorn M2 channel is ~0.3 μM, and the block has a very slow onset. The data presented here suggests that 1 μM is not protective, however, it appears likely that most, if not all, M2 channels need to be blocked in order for cells to 'survive'. This correlates with a calculated IC_{90} of ~5 μM (R. Forgey, unpublished observation).

Electrophysiological recordings are not practical in a screen involving millions of compounds, however, a screen based upon rapid visual inspection of cells is amenable to such an endeavor. Indeed, we have screened greater than ten thousand test substances a week against the M2 channel, and also screened against the clinically relevant amantadine resistant channels (results will be reported in the future). Since each oocyte requires manual injection of the cRNA, it likely would have been more time efficient to screen this number of compounds in an automated system with an M2 expressing mammalian cell line. This has not, however, been achievable. The M2 protein is basally activated at physiological pH, 7.0–7.5, and therefore, it appears that the production of a stable M2, high level expressing mammalian cell line is improbable. With the present assay, we can be assured that the channels are functional and inhibition of cell 'death', is thereby, a consequence of block the M2 channel.

Acknowledgements: We thank Donna Turner for excellent technical skills. The authors are very grateful to the M2 discovery team for lively interactions. In addition, we thank Drs. Catherine Ifune and Les Bell for critical comments on the manuscript. Lastly, we thank Drs. Robert Lamb and Larry Pinto for sharing their unpublished data.

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