# INHIBITION OF PROTON CONDUCTANCE OF THE INFLUENZA A M2 CHANNEL BY AMINOADAMANTANES

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*The photos show Dr. A. Hay and a student of P.G. Kostyuk, Dr. S. Tokar.*

## Introduction

The M2 protein of influenza A virus is the smallest known protein which exhibits the basic properties of an ion channel. It is characterized by high proton selectivity and a proton regulated gating mechanism (Chizhmakov et al., 1996a). The proton conductivity of the M2 protein plays important roles in two stages of virus replication. As virus particles enter cells by endocytosis, the M2 channel transfers protons from the acidic endosomal environment to the virus interior, triggering dissociation of the viral matrix protein from the viral ribonucleoprotein (RNP) and release of free viral RNP. The M2 channel reduces the acidity within trans

Golgi vesicles, to preserve the structural integrity of the haemagglutinin and infectivity of progeny virus particles (Sugrue et al., 1990).

The active M2 channel is a homotetramer; each monomer is 97 amino acids long, divided into three domains of 24, 19, and 54 amino acid residues: an extracellular N-terminal domain, a transmembrane domain, which forms the channel pore, and a C-terminal domain, internal to the virus or infected cell, respectively. The transmembrane domain forms a 4-helix bundle with very low proton conductance, in the range from ten to a few hundred attosiemens. Truncated channels formed by peptides corresponding to amino acids 22-46 or 21-61, which possess the essential functional characteristics of the native protein, have been used to obtain X-ray crystal and NMR structures of the channel (Stouffer et al., 2008; Schnell and Chou, 2008).

The M2 protein is the target of the drugs amantadine and rimantadine, which cause irreversible inhibition of proton conduction and prevent virus infection (Hay et al., 1985). The recent emergence of drug-resistant epidemic influenza A(H3N2) viruses and the pandemic A(H1N1) viruses has, however, limited their use. The resistance is due to single amino acid substitutions within the transmembrane domain, the most common occurring in positions 26, 27, 30 or 31 (Hay et al., 1985). Despite the available structural data, the mechanism of inhibition is still not known. This paper describes some investigations of the physicochemical conditions which influence rimantadine inhibition of M2 proton conduction.

## Materials and methods

Expression of the M2 proteins of influenza viruses A/Chicken/Germany/27 (H7N7, Weybridge strain; W-M2), A/Beijing/262/95 (H1N1; BJ-M2) and A/Port Chalmers/1/72 (H3N2; PC-M2) in mouse erythroleukaemia (MEL) cells was as previously described (Ogden et al., 1999). Cells were voltage-clamped in the whole cell patch-clamp configuration 3-4 days after induction with 2% DMSO. pH control was optimized by the use of high concentrations of buffered solutions (Chizhmakov et al., 1996a; Ogden et al., 1999) containing: in the pipette 130 mM NMDG, 10 mM EGTA, 125 mM HEPES pH 8.0; in the bath, 2 mM CaCl2 replaced EGTA; external test solutions (also 280 mOsm) contained NMDG, 2 mM $CaCl_2$ and MES or HEPES, pH 4.0 to 7.3. Fast perfusion with different pH used the U-tube quick application system (Krishtal and Pidoplichko, 1980), and cells were re-equilibrated between test pulses by slow perfusion with bath solution (pH 8.0). Time of current increase was between 300 and 600 ms. Experimental protocol: quick application of the test pH solution for 1.6 s; rewash with control solution for 30 s; apply some test pH solution containing rimantadine (100 μM) for 25-200 s; rewash for 30 s before 20 s application of test solution to estimate leak current.

## Results and discussion

Susceptibility to inhibition depends both on the structure of the channel and the characteristics of the aminoadamantane inhibitor. Thus, whereas the inhibitor sensitivities of the M2 channels of the Weybridge (W-M2) and Rostok (R-M2) strains of avian H7 viruses were similar, the M2s of H3N2 (A/Port Chalmers/1/72; PC-M2) and H1N1 (A/Beijing/262/95; BJ-M2) subtype human viruses were more sensitive. The time course of inhibition of proton current by rimantadine was dependent on extracellular pH, as illustrated in Fig. 1, *b*. The time constant for inhibition of the W-M2 was almost insensitive in the range 7.3 to 6.5, but increased 3-fold between pH 6.5 and 5.0 (Fig. 1, *c*). The inhibition was 'irreversible' and fitted *by* a single exponential curve over the whole pH range and was insensitive to changes in membrane potential or intracellular pH. The rate of BJ-M2 inhibition showed a similar dependence on $pH_o$, 3.8-fold between 7.0 and 5.0, but the time constants were 3-4-fold lower than for the WM2. Thus, together with the results of Chizhmakov et al. (1996b), which demonstrate that the rate constant for PC-M2 inhibition is 10-fold higher than that for R-M2 and W-M2, the M2s of the human viruses are more sensitive to inhibition than those of the avian viruses.

The amino acid sequences of the M2 proteins of the human and avian viruses differ in the number of residues in the N- and C- terminal domains; the only common difference in the transmembrane domain between the 2 avian and 2 human virus proteins is I28V in the region with which the drug is perceived to interact. These differences were not observed to alter the general characteristics of the channel, in terms of the pH dependence of conductance or activation. An interesting difference between the BJ-M2 and the other three was replacement of cysteine 50 by a serine, with the consequent loss of the Cys-associated palmitate. Although replacement of cysteine 50 by phenylalanine in the M2 proteins of equine H3N8 viruses did not affect the properties of M2, mutation of residue 50 to serine in the W-M2 was observed to shift the pH dependence of conductance to higher pH (I. Chizhmakov, personal communication).

However, no difference was observed in the BJ-M2 properties, and proximal substitutions, F48S, L43I and L54I, in the M2 of A/Beijing/262/95(H1N1)-like viruses may complement any effect of the single C50S substitution. Furthermore, the differences between BJ-M2 and PC-M2 did not significantly affect the time constant of rimantadine inhibition.

Since the pKa of rimantadine is 10.4 and 99.9% of rimantadine is already protonated at neutral pH, the influence of extracellular pH is not due to change in the molecule charge but is mediated by changes in channel properties. Proton conductance showed a pH dependence similar to that of rimantadine inhibition (Fig. 1, *c, d*). Although in general the influence of external pH on conductance of

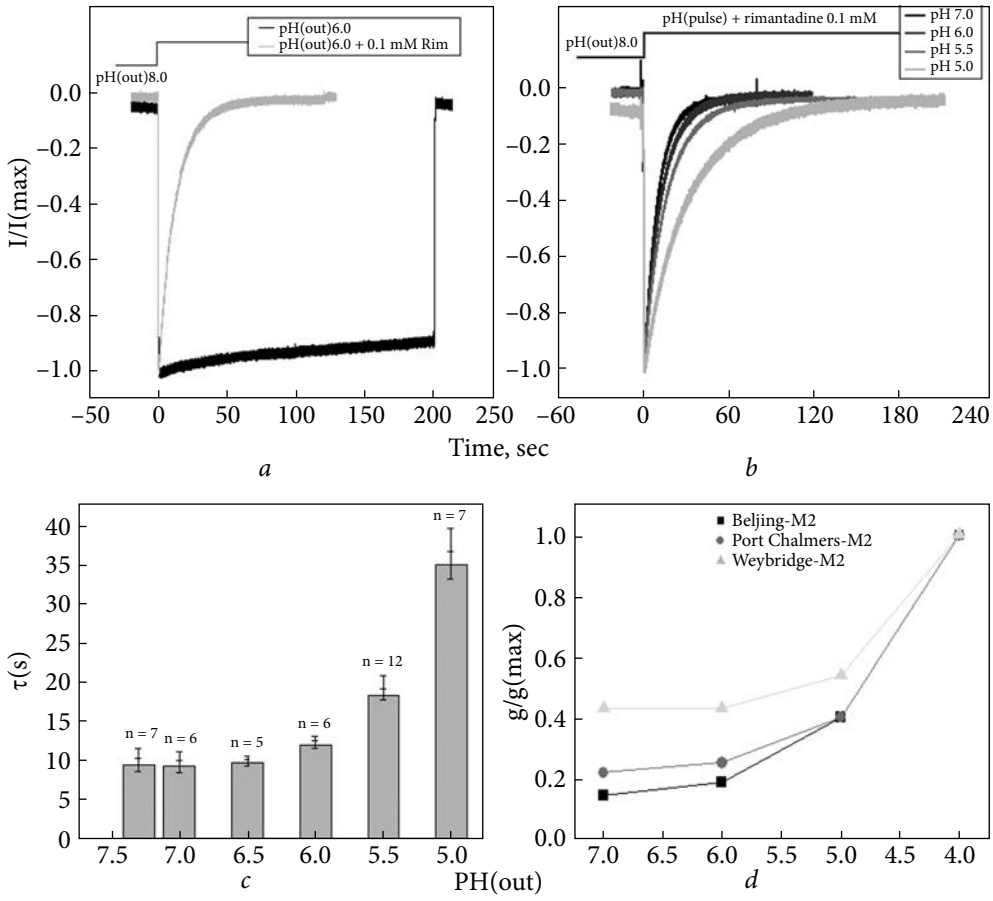


***Fig. 1.*** Influence of external pH on the proton conductance and the rate of rimantadine inhibition: *a* — Comparison of proton currents obtained after application of pH 6.0 solution with or without rimantadine, $pH_i$ 8.0 (current traces were normalized to the maximum amplitude); *b* — Time course of rimantadine inhibition at different external pH; *c* — Time constants for rimantadine inhibition at different extracellular pH, $pH_i$ 8.0; *d* — pH dependence of M2 proton conductance, normalized to conductance at pH 4.0. Membrane conductance was calculated as g = I/(V-Vrev), where I is current (pA); V is membrane potential (mV); Vrev is reversal potential (mV) calculated for the available proton gradient by the Nernst equation. $pH_i$ = 8.0, T = 21 °C, V = 0 mV

the three M2s were similar, the conductance at $pH_o$ 7.0 relative to that at $pH_o$ 4.0 was lower for BJ-M2 and PC-M2 than for W-M2 (Fig. 1, *d*) suggesting that the W-M2 has a higher open probability at neutral pH. The correlation between a higher rate of inhibition and lower open probability is consistent with the drug interaction with a closed form of the channel at high pH.

A wealth of data from electrophysiological, biophysical, and structural studies have resulted in a model of M2 proton transport in which protonation/depro-

tonation of His 37, responsible both for proton activation and proton selectivity of M2 (Pinto and Lamb, 2006), is linked to alternating access of His37 to outer and inner environments, respectively (Khurana et al., 2009). In particular, the narrow aperture of the 'Val27 gate', closed when the 'His37–W41 pore' is open, can appear to account for the relatively slow rate of voltage-independent inhibition of M2, rate constant of the order of $10^2$-$10^3$ $M^{-1}s^{-1}$ compared with greater than $10^8$ $M^{-1}s^{-1}$, typical of open channel blockers, and the more restricted access to the drug binding site of the open than of the closed form of the channel. Consistent with these considerations is the reversibility of inhibition by the smaller planar cyclooctylamine in contrast to the essentially irreversible inhibitition by larger inhibitors. Furthermore, the reciprocal pH dependence of conductance and rimantadine inhibition corresponds to the pH dependence of interaction between His37 (pKa 5.7) and Trp41 involved in channel opening, which is inhibited by rimantadine (Okada et al., 2001; Czabotar et al., 2004; Salom et al., 2000). This may also account in part for differences between the relative rates of inhibition and relative dissociation constants for amantadine and rimantadine. Their similar sizes corresponding to a less than 2-fold difference in the rates of inhibition, whereas the 20-fold lower dissociation constant indicates a stronger interaction of rimantadine with a site within the channel pore in the region of Val27, Ala30, and Ser31 (P. Spearpoint, personal communication). Further study of different features of inhibitors and their interaction with mutant channels should provide a better understanding of the mechanism of inhibition and the potential for development of alternative inhibitors, also effective against rimantadine-resistant viruses.

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