On the mechanism of 4-aminopyridine action on the cloned mouse brain potassium channel mKv1.1

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1. This study used the whole-cell patch clamp technique to investigate the mechanism of action of the K^+ channel blocker 4-aminopyridine (4-AP) on the cloned K^+ channel mouse Kv1.1 (mKv1.1) expressed in Chinese hamster ovary cells.
2. Cells transfected with mKv1.1 expressed a non-inactivating, delayed rectifier-type K^+ current. 4-AP induced a dose-, voltage- and use-dependent block of mKv1.1.
3. 4-AP blockade of mKv1.1 was similar whether 4-AP was administered extracellularly ($IC_{50} = 147$ μM) or intracellularly ($IC_{50} = 117$ μM).
4. Inclusion of the first twenty amino acids of the *N*-terminus sequence of the *Shaker* B K^+ channel ('inactivation peptide') in the patch electrode transformed mKv1.1 into a rapidly inactivating current. The time constant of decay for the modified current was dependent on the concentration of inactivation peptide, and under these conditions extracellular 4-AP had a reduced potency (IC_{50} values of 471 and 537 μM for 0·5 and 2 mg ml^{-1} inactivation peptide, respectively).
5. A permanently charged analogue of 4-AP, 4-aminopyridine methiodide (4-APMI), was found to block mKv1.1 when applied inside the cell, but was without effect when administered externally.
6. Decreasing the *intracellular* pH (pH_i) to 6·4 caused an increase in 4-AP potency ($IC_{50} = 76$ μM), whereas at pH_i 9·0, the 4-AP potency fell ($IC_{50} = 295$ μM). Conversely, increasing *extracellular* pH (pH_o) to 9·0 caused an increase in 4-AP potency ($IC_{50} = 93$ μM), whereas at pH_o 6·4, 4-AP potency decreased ($IC_{50} = 398$ μM).
7. Taken together, these findings support the hypotheses that the uncharged form of 4-AP crosses the membrane, and that it is predominantly the cationic form which acts on mKv1.1 channels intracellularly, possibly at or near to the binding site for the inactivation peptide.

4-Aminopyridine (4-AP) has been shown to block a range of voltage-activated K^+ channels from non-inactivating delayed rectifiers (Meves & Pichon, 1977) to rapidly inactivating 'A'-type channels (Thompson, 1977). Overall, 4-AP appears to be a more potent blocker of non-inactivating than rapidly inactivating currents (e.g. Stühmer *et al.* 1989); this selectivity difference, combined with structural information on K^+ channels, may provide clues as to the site of action of 4-AP. We have recently reported the stable transfection of cloned mKv1.1 channels in Chinese hamster ovary (CHO) cells, which resulted in the expression of a non-inactivating, delayed rectifier-type current (Robertson & Owen, 1993). The mKv1.1 channel and its homologues are sensitive to 4-AP (Christie, Adelman, Douglass & North, 1989; Klumpp, Farber, Bowes, Song & Pinto, 1991). It is possible to transform the mKv1.1 current with a twenty amino acid peptide (MAAVAGLYGLGEDRQHRKKQ) from the *N*-terminus sequence of the *Shaker* B K^+ channel ('inactivation peptide') into a rapidly inactivating current (Robertson & Owen, 1993). The effects of 4-AP were therefore compared on both the non-inactivating mKv1.1 current and the same current modified by inactivation peptide.

A number of anomalies have arisen in the study of 4-AP on native K^+ channels and the location of the 4-AP binding site(s) remains to be fully elucidated (Pongs, 1989). The binding of 4-AP appears to be dependent on the kinetic state of the channel. It has been proposed that 4-AP acts predominantly on K^+ channels in the closed state in rat melanotrophs (Kehl, 1990) and ventricular myocytes (Castle & Slawsky, 1992). In contrast, an open channel blocking mechanism has been suggested in lymphocytes (Choquet & Korn, 1992) and in the GH_3 pituitary cell line

(Wagoner & Oxford, 1990). A thorough understanding of the mechanism of 4-AP is complicated by the fact that it is a weak organic base ($pK_a = 9 \cdot 17$) which can exist in cationic and uncharged forms at physiological pH (see Howe & Ritchie, 1991). Studies on the effect of pH on 4-AP action have led to the proposal that it is the uncharged form which penetrates the lipid membrane, and subsequently the cationic form which acts intracellularly to block K^+ channels (Howe & Ritchie, 1991; Choquet & Korn, 1992). In contrast, 4-AP blockade of K^+ conductance in squid giant axons had no pH dependency (Meves & Pichon, 1977). These findings indicate the complexity of the mechanism of action of 4-AP and the variation between K^+ channels.

This study examined the effect of 4-AP on a single, cloned K^+ channel in order to elucidate the mechanism of action of this archetypal blocker on a mammalian K^+ channel. A preliminary account of some of these findings has been presented (Stephens, Garratt, Owen & Robertson, 1993).

METHODS

Cell culture

CHO mKv1.1 cell lines were supplied by Dr Bruce Tempel (University of Washington, Seattle, WA, USA). The cell line contained the plasmid (pZMK228R) with the cDNA insert for the mouse brain K^+ channel gene mKv1.1 (pZMK6.2SS; Tempel, Jan & Jan, 1988). Cells were grown in tissue culture in 75 ml flasks in a medium containing 92 % RPMI 1640 medium (Sigma), 5 % fetal bovine serum, 1 % L-glutamine (ICN Flow, High Wycombe, UK), 1 % antibiotic solution (Sigma) and 1 % geneticin solution (0·3 mg ml^{-1}, geneticin sulphate in 10 % Hepes buffered distilled water solution). Flasks of cells were maintained at 37 °C in a humidified atmosphere containing 5 % CO_2 in air. Cells were subsequently plated onto 35 mm Petri dishes (Gibco, UK) at least 2 h prior to electrophysiological recording.

Electrophysiology

CHO cells were investigated by the whole-cell patch clamp technique. Patch electrodes were pulled from thin-walled borosilicate glass (GC150TF-10, Clark Electromedical Instruments, Reading, UK) using a Brown & Flaming horizontal puller (Sutter Instruments, San Rafael, CA, USA) and had a resistance of 5–10 MΩ after heat polishing on a Narashige microforge. Voltage-gated currents were acquired and stored on a ATARI-ST computer using an EPC9 amplifier (HEKA, Lambrecht, Germany). Currents were elicited by 250 ms pulses from a holding potential of −90 mV and sampled at a rate of 500 μs per point filtered by a low-pass Bessel filter set at 2·3 kHz. Currents were leak- and capacity-subtracted on-line with a p/4 subtraction protocol. Steady-state block by 4-AP was taken as the time after which no further block of current occurred; this was substantiated by frequency–dependence curves.

Recording solutions

Electrodes were back-filled with an 'intracellular solution' which comprised (mM): potassium aspartate, 120; KCl, 20; $MgCl_2$, 1; MgATP, 2; EGTA (ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid), 10; Hepes (*N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulphonic acid]), 10; pH 7·4, adjusted with NaOH. In some experiments, the pH of intracellular solution (pH_i) was altered. For pH_i 6·4, Hepes in the intracellular solution was replaced with 10 mM Pipes (piperazine-*N,N′*-bis[2-ethanesulphonic acid]); for pH_i 9·0, Hepes was replaced by 10 mM bis-tris-propane. Intracellular solutions were stored frozen in 1 ml aliquots and defrosted immediately prior to use. Cells were mounted in a recording chamber and continuously perfused via a micropipette at 3 ml min^{-1} with an 'extracellular solution' which comprised (mM): NaCl, 124; $MgCl_2$, 4; KCl, 2·5; $CaCl_2$, 2; glucose, 10; sucrose, 20; Hepes, 10; pH 7·4 adjusted with NaOH. The pH of extracellular solution (pH_o) was altered in some experiments. For pH_o 6·4, Hepes was substituted with 10 mM Pipes; for pH_o 9·0, Hepes was replaced by 10 mM bis-tris-propane. Drugs were dissolved in the extracellular solution and applied directly to cells via a microperfusion system. All experiments were conducted at room temperature (21–24 °C). 4-AP was obtained from Sigma, UK. 4-Aminopyridine methiodide (4-APMI) was made by Medicinal Chemistry at Wyeth Research, Taplow, UK. The *Shaker* B 'inactivation peptide' (of sequence MAAVAGLYGLGEDRQHRKKQ) was synthesized by Peptide Products Ltd (Salisbury, UK), and was confirmed by NMR as > 95 % pure.

Data analysis

The mKv1.1 charge transfer during the voltage step (Q_{step}) was calculated as the integral of current over the duration of the voltage pulse. Conductance was derived from the peak current divided by the driving force ($E_M - E_K$), where E_M is the membrane voltage and E_K is the potassium null potential (this was −100 mV for the solutions used). Conductance– membrane potential curves were fitted with a Boltzmann function of the form:

$$G = G_{max}/[1 + \exp((E_{1/2} + E_M)/k)], \qquad (1)$$

where G_{max} is the maximum limiting conductance, $E_{1/2}$ is the mid-point for voltage dependence and k is the slope factor. The effect of 4-AP on mKv1.1 was quantified using dose–response data which were fitted with a sigmoidal logistic function (De Lean, Munson & Robard, 1978) of the form:

$$Y = [(a - d)/(1 + (X/c)^b)] + d, \qquad (2)$$

where Y is the response, X is the concentration of 4-AP, a is the response when concentration of 4-AP is zero, d is the response of the 'infinite' concentration of 4-AP, c is the IC_{50} concentration, and b is the 'slope factor' of the curve. Time constants of decay were derived from a single exponential fitted to currents using the REVIEW program (Instrutech Corp., New York, USA) on the ATARI-ST. All data are expressed as means ± S.E.M, with statistical analysis performed using two sample t tests.

RESULTS

Externally applied 4-AP caused a rapid, fully reversible, dose-dependent blockade of peak mKv1.1 current and charge transfer during the voltage step (Q_{step}) (Fig. 1*A*). In agreement with the study of Robertson & Owen (1993), mKv1.1 K^+ currents in CHO cells activated from potentials positive to −40 mV and were essentially non-inactivating, even at large positive voltages. Conductance–membrane potential curves for mKv1.1 were fitted with a Boltzmann function; this analysis gave $E_{1/2} = -6 \pm 2$ mV and

$k = 15 \pm 1$ mV, $n = 10$. The dose–response relationship for inhibition of Q_{step} by 4-AP (Fig. 1*B*) gave an IC_{50} of 147 μM. An examination of the effects of 4-AP over a range of membrane potentials revealed a moderate voltage dependence (Fig. 1*C* and *D*) with block by 4-AP being relieved by depolarization. The slope of the linear regression for percentage block of the Q_{step}–membrane potential relation was greater at higher concentrations of 4-AP (-22 ± 4 % (100 mV)$^{-1}$ at 150 μM ($n = 3$) and -34 ± 4 % (100 mV)$^{-1}$ ($n = 3$) at 1 mM).

4-AP block of mKv1.1 did not reach steady state immediately, but initially showed time-dependent effects (Fig. 2*A*); steady state was reached by 30 s. This is consistent with an action of 4-AP on open channels during the voltage pulse and on closed channels between voltage pulses (see also Robertson & Owen, 1993). Closed channel block was determined as the percentage of 4-AP block in the absence of current pulses; pulses were then applied and open channel block determined (as detailed by Castle & Slawsky, 1992); after a 3 min application of 1 mM 4-AP this analysis gave a closed channel block of 41 ± 6 % and an open channel block of 35 ± 5 % ($n = 4$). The effect of 4-AP was use dependent (Fig. 2*B* and *C*); hence, increasing the frequency of voltage pulses (thereby increasing the average amount of time channels spent in the open state) clearly reduced the time taken for 4-AP to block mKv1.1. At 0·2 Hz (the pulse frequency used throughout this study) steady state was attained between 20 and 25 s.

Some other studies have reported that 4-AP blocks K^+ channels at least as effectively when applied to the cytoplasmic site of the membrane (Yeh, Oxford, Wu & Narahashi, 1976; Thompson, 1982). The inhibition of mKv1.1 current in the presence of different concentrations of 4-AP in the patch electrode was tested on individual cells; by this route of administration, 4-AP again caused a dose-dependent inhibition of Q_{step} with an IC_{50} of 117 μM (Fig. 3*A*). The block of the current by internally applied 4-AP was always faster in comparison to that induced by external 4-AP. Block by 4-AP began as soon as the whole-cell configuration was established; in some cases a clear decay phase was captured prior to the steady-state blockade (Fig. 3*B*), but full steady state was typically reached within 5 s. However, the recording of this phase in some cells did indicate that a similar time-dependent blockade of mKv1.1 occurred with internally as well as externally applied 4-AP.

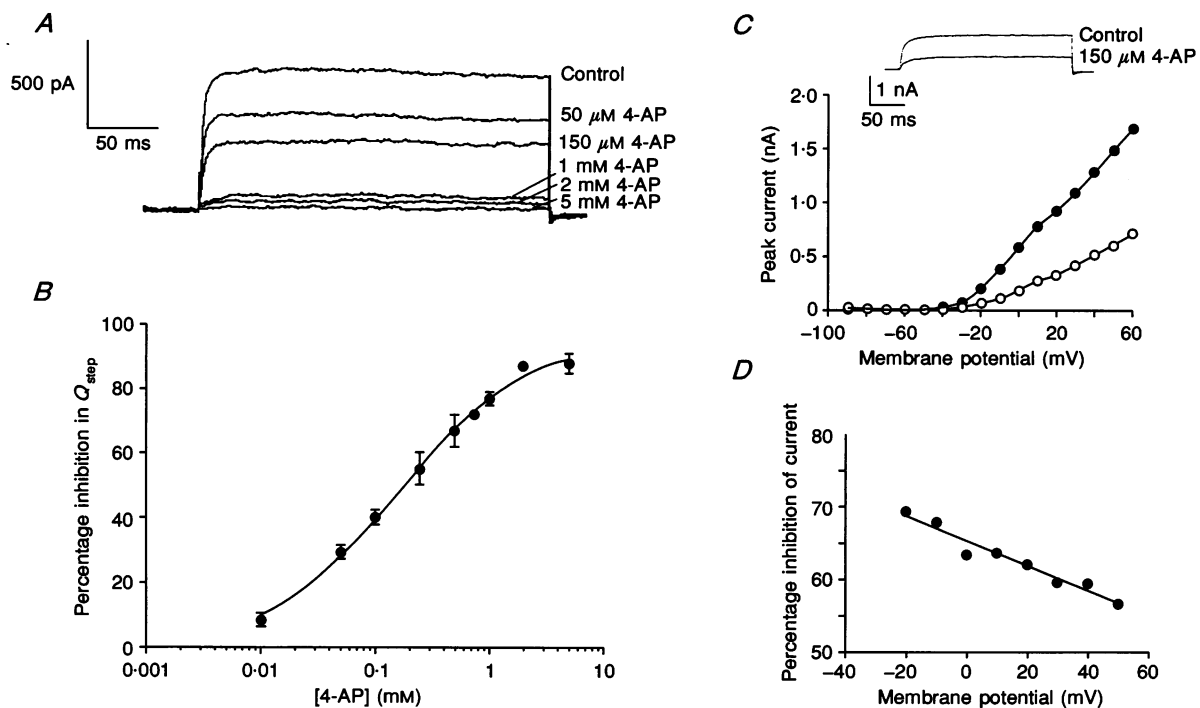

Figure 1. Block of mKv1.1 by external 4-AP is dose and voltage dependent

A, 4-AP dose-dependent block of mKv1.1. Currents were activated by stepping from a holding potential of −90 mV to +40 mV using 250 ms pulses applied every 5 s. *B*, dose–response relationship for 4-AP inhibition of Q_{step}. Data fitted with eqn (2) gave an IC_{50} of 147 μM ($r = 0·997$). Points represent means ± S.E.M. for a minimum of 5 cells. *C*, current–voltage relationship of mKv1.1 in the presence and absence of 150 μM 4-AP. ●, control; ○, 150 μM 4-AP. Inset shows mKv1.1 current inhibition by 150 μM 4-AP at a voltage step to +40 mV. *D*, effect of 150 μM 4-AP on percentage inhibition of mKv1.1 current. The blockade of mKv1.1 was voltage dependent, being relieved by depolarization. Slope of linear regression was −17 % (100 mV)$^{-1}$ ($r = 0·976$) for this cell.

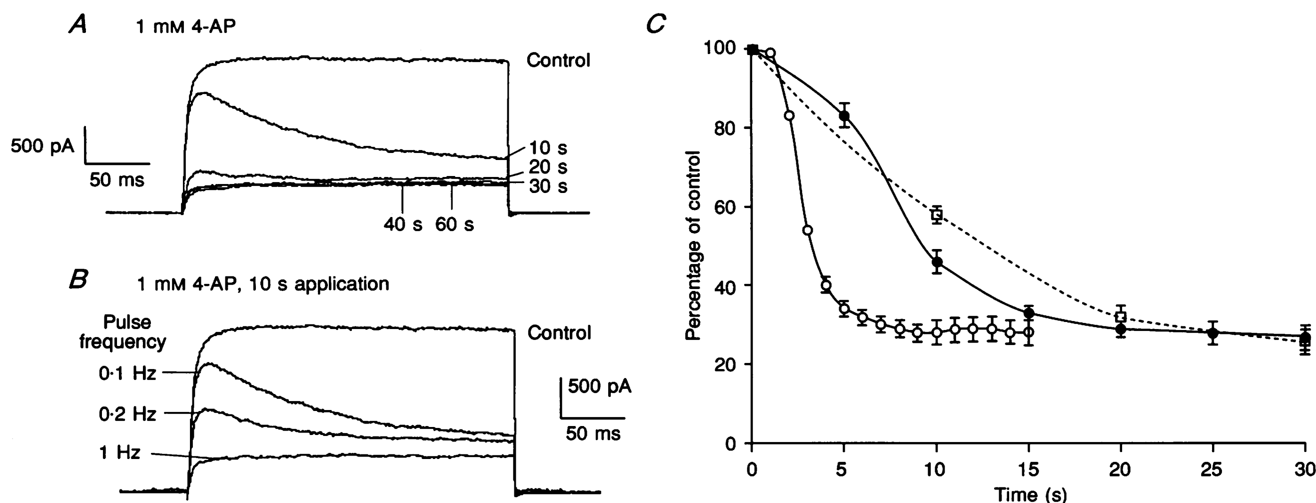

Figure 2. Action of 4-AP is time and use dependent

A, time-dependent block of mKv1.1 by 4-AP. At 1 mM 4-AP initially blocked open mKv1.1 channels resulting in a clear decay phase until a steady-state block was achieved. This was taken as the time after which no further block of current occurred (by 30 s). Currents were activated by stepping from a holding potential of −90 mV to +40 mV using 250 ms pulses applied every 10 s. *B*, effect of pulse frequency on 4-AP block of mKv1.1 channels. 4-AP was applied at 1 mM for 10 s with a pulse frequency of 0·1, 0·2 and 1 Hz, 4-AP inhibition of mKv1.1 was markedly accelerated by increasing the frequency of voltage pulses. Cells were stepped from a holding potential of −90 mV to +40 mV using 250 ms pulses. *C*, quantification of use dependence of 4-AP block of mKv1.1. For data pooled from 5 individual cells, the times for 1 mM 4-AP to reach 50 % block for different pulse frequencies were 3·3 s (1 Hz), 9·3 s (0·2 Hz) and 12·6 s (0·1 Hz). 250 ms pulses were applied from a holding potential of −90 mV to +40 mV. ○, 1 Hz; ●, 0·2 Hz; □, 0·1 Hz.

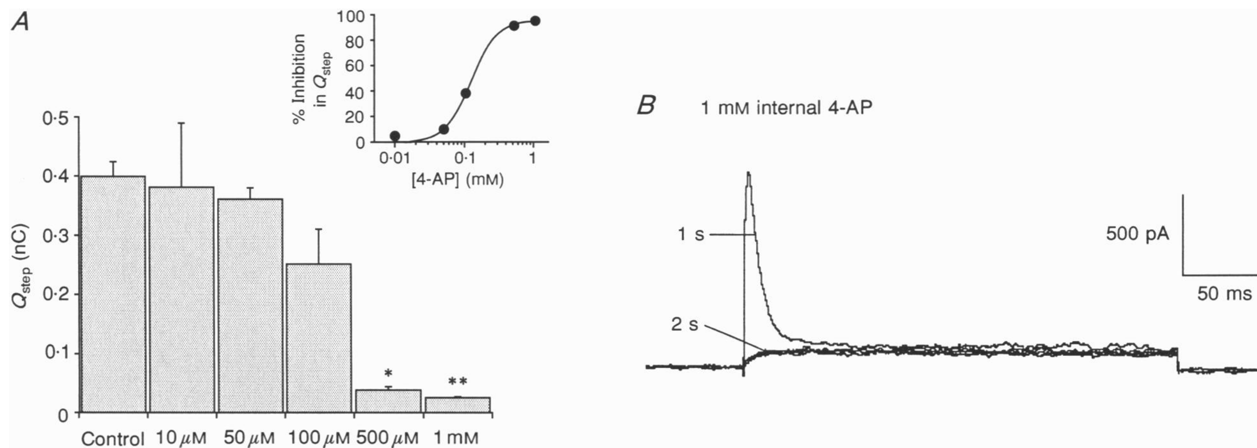

Figure 3. mKv1.1 is blocked by internal 4-AP

A, quantitative dose-dependent inhibition of Q_{step} by internal 4-AP. Each concentration was applied to an individual cell; points represent means ± S.E.M. for a minimum of 3 cells. A significant decrease in charge was seen at 500 µM and 1 mM 4-AP; * $P \leqslant 0·05$; ** $P \leqslant 0·001$. Inset shows data expressed as a percentage of the control group fitted with eqn (2); this gave an IC_{50} of 117 µM ($r = 0·998$). *B*, time-dependent block of mKv1.1 by internal 1 mM 4-AP. In this example, a time-dependent block occurred prior to a steady-state block; in most cases, however, the initial decay phase was too rapid to record. Currents were activated by stepping from a holding potential of −90 mV to +40 mV using 250 ms pulses applied every 1 s.

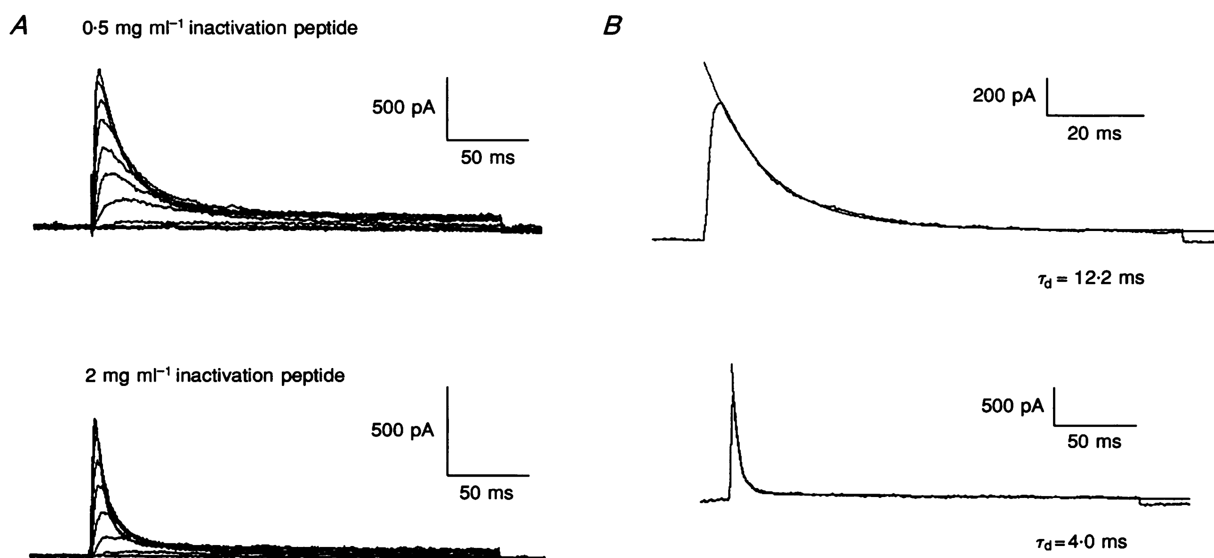

Figure 4. *Shaker* B inactivation peptide transforms mKv1.1 into a rapidly inactivating current
A, current responses of mKv1.1 in the presence of inactivation peptide at 0·5 mg ml^{-1} (upper trace) and 2 mg ml^{-1} (lower trace). 250 ms voltage pulses were applied in 10 mV increments in cells held at −90 mV. Currents activated with voltage steps from −40 to +60 mV. Inactivation peptide was applied intracellularly via the patch electrode. *B*, time constants of decay (τ_d) for mKv1.1 in the presence of inactivation peptide at 0·5 mg ml^{-1} (upper trace) and 2 mg ml^{-1} (lower trace). Currents were fitted with a single exponential; for these records, τ_d = 12·2 ms (0·5 mg ml^{-1} inactivation peptide) and τ_d = 4·0 ms (2 mg ml^{-1} inactivation peptide). Currents were activated by stepping from a holding potential of −90 mV to +40 mV.

Effect of inactivation peptide on 4-AP action

Inclusion of part of the *N*-terminus of the *Shaker* B K^+ channel (inactivation peptide) in the patch pipette solution (0·5 and 2 mg ml^{-1}) modified mKv1.1 into a rapidly inactivating current (Fig. 4*A*). Inactivating currents were fitted with a single exponential function and time constants for decay (τ_d) were obtained for the two different concentrations of inactivation peptide (Fig. 4*B*). The fast component of decay was faster with higher concentration of inactivation peptide; $\tau_d = 13 \pm 2$ ms ($n = 8$) at 0·5 mg ml^{-1} inactivation peptide, and $\tau_d = 4·1 \pm 0·5$ ms ($n = 17$) at 2 mg ml^{-1} inactivation peptide at +40 mV.

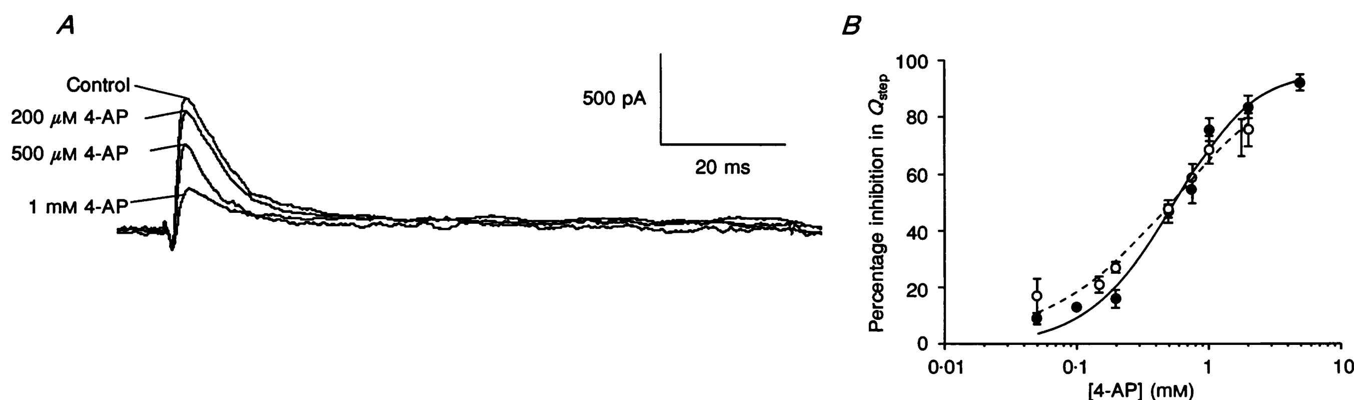

Figure 5. 4-AP blocks mKv1.1 modified by inactivation peptide
A, dose-dependent 4-AP block of mKv1.1 modified by 2 mg ml^{-1} inactivation peptide. 4-AP was applied for 30 s by which time steady-state block was achieved. *B*, dose–response relationship for 4-AP inhibition of Q_{step} in the presence of 0·5 and 2 mg ml^{-1} inactivation peptide. ●, 2 mg ml^{-1} inactivation peptide; ○, 0·5 mg ml^{-1} inactivation peptide. Data fitted with eqn (2) gave IC_{50} values of 471 μM ($r = 0·986$) for 0·5 mg ml^{-1} inactivation peptide, and 537 μM ($r = 0·989$) for 2 mg ml^{-1} inactivation peptide. Points represent means ± S.E.M. for a minimum of 3 cells.

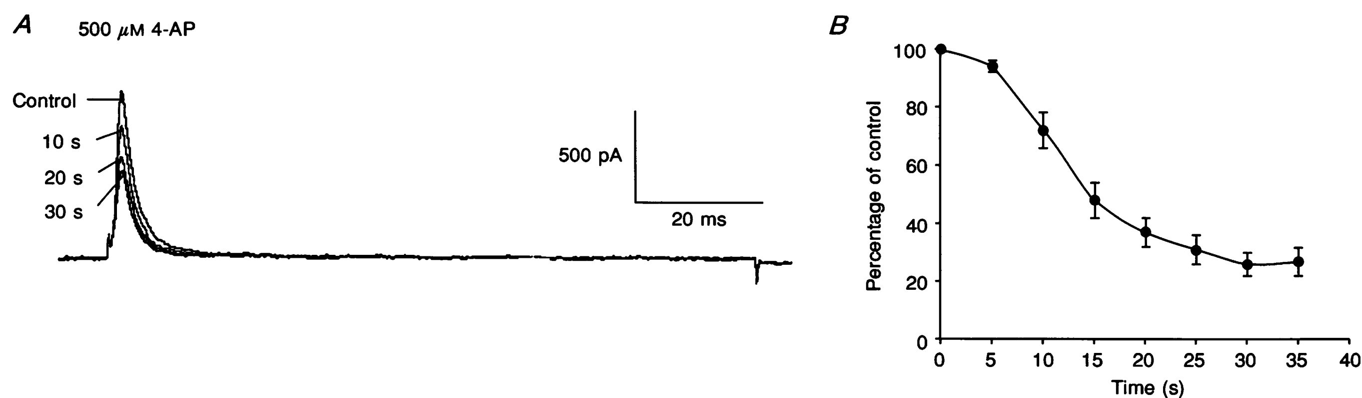

Figure 6. Inactivation peptide affects 4-AP block
A, time-dependent 4-AP block of mKv1.1 modified by 2 mg ml^{-1} inactivation peptide. 500 μM 4-AP was applied for 30 s by which time steady state was achieved. Currents were activated by stepping from a holding potential of −90 mV to +40 mV. *B*, time for 1 mM 4-AP to reach steady-state block in the presence of 2 mg ml^{-1} inactivation peptide. 4-AP took longer to reach steady state than for unmodified mKv1.1 current (approximately 30 s). For data pooled from 5 individual cells, the times for 1 mM 4-AP to reach 50 % block for 0·2 Hz was 14·5 s. 250 ms pulses were applied from a holding potential of −90 mV to +40 mV.

4-AP produced a dose-dependent block of the transformed mKv1.1 (Fig. 5*A*). Dose–response relationships for 4-AP inhibition of mKv1.1 in the presence of the inactivation peptide (Fig. 5*B*) revealed that the IC_{50} for 4-AP block of the transformed current increased approximately threefold (471 μM for 0·5 mg ml^{-1} inactivation peptide and 537 μM for 2 mg ml^{-1} inactivation peptide) compared to the unmodified current. 4-AP also caused a time-dependent block of the modified current (Fig. 6*A*). The presence of 2 mg ml^{-1} inactivation peptide slowed the action of 4-AP (Fig. 6*B*); the time taken for 4-AP to attain steady-state block of the inactivating current at a pulse frequency of 0·2 Hz was slightly longer than that found for the unmodified current at approximately 30 s.

Effect of 4-APMI

The results obtained in the presence of inactivation peptide were consistent with an intracellular site of action of 4-AP; therefore we tested the effects of 4-APMI applied externally and internally via the patch electrode on mKv1.1. External 4-APMI (up to 10 mM) was ineffective in blocking mKv1.1 in cells normally responsive to 4-AP (Fig. 7*A*). In contrast, internal 4-APMI was able to block mKv1.1 (Fig. 7*B*): 1 mM 4-APMI caused an inhibition in Q_{step} from a control value of 0·59 ± 0·08 ($n = 10$) to 0·15 ± 0·02 nC ($n = 8$); in comparison, equimolar 4-AP reduced Q_{step} to 0·023 ± 0·004 nC ($n = 11$). With intracellular concentrations of 4-APMI above 1 mM it was not possible to obtain reliable recordings due to

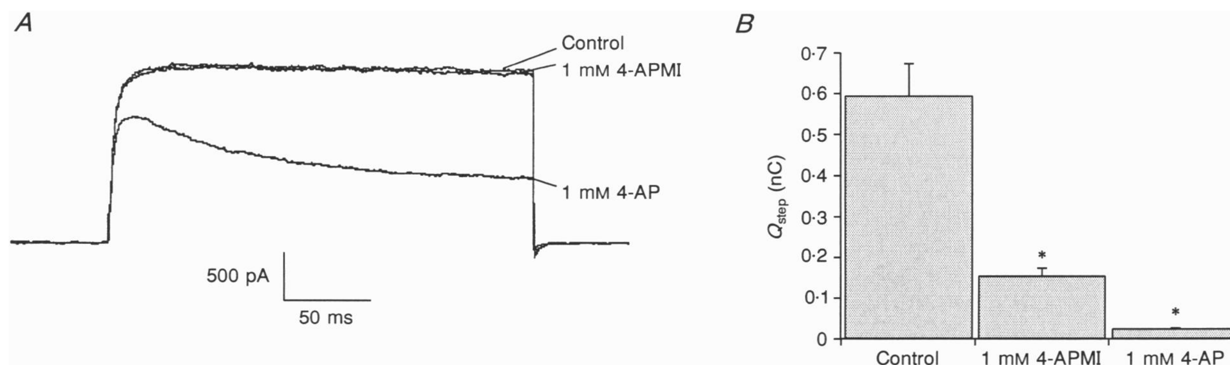

Figure 7. 4-APMI only blocks mKv1.1 internally
A, external application of 1 mM 4-APMI. 4-APMI was ineffective on mKv1.1, whilst 1 mM 4-AP still blocked current in the same cell. Currents were activated by stepping from a holding potential of −90 mV to +40 mV. *B*, inhibition of Q_{step} by internal 1 mM 4-APMI and 1 mM 4-AP. These analogues caused a significant decrease in Q_{step} of 75 % (4-APMI) and 96 % (4-AP). Control values were obtained from 10 cells and other points represent means ± S.E.M. for a minimum of 5 cells; * $P \leq 0.001$.

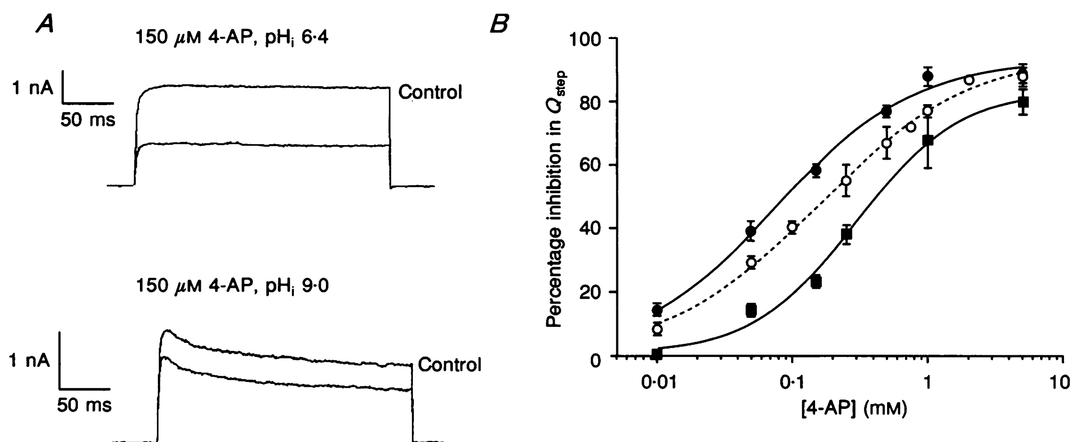

Figure 8. Reduction of pH_i increases the potency of 4-AP block of mKv1.1
A, steady-state block (reached by 30 s) of mKv1.1 by 150 μM 4-AP at pH_i 6·4 (upper trace) and 9·0 (lower trace). At pH_i 6·4, 4-AP was more potent, causing a 57 % inhibition of Q_{step}, in comparison to a 29 % reduction seen at pH_i 9·0 for these cells. Currents were activated by stepping from a holding potential of −90 mV to +40 mV using 250 ms pulses applied every 5 s. Note the change in degree of current inactivation for pH_i 9·0. *B*, dose–response relationship for the effect of pH_i on 4-AP inhibition of Q_{step}. ○, 4-AP inhibition at physiological pH (pH_i 7·4); ●, pH_i 6·4; ■, pH_i 9·0. Data fitted with eqn (2) gave an IC_{50} of 76 μM ($r = 0·998$) for pH_i 6·4 and an IC_{50} of 295 μM ($r = 0·997$) for pH_i 9·0. Points represent means ± S.E.M. for a minimum of 3 cells.

unstable electrode potentials; we believe this was caused by an interaction between the drug and the Ag–AgCl wire in the patch pipette.

Effect of pH on 4-AP action

The results above suggest that it is predominantly the charged form of 4-AP which acts intracellularly to block mKv1.1. The effect of altering pH_i was examined in detail using 150 μM 4-AP, the half-maximal inhibitory concentration of 4-AP at physiological pH (Fig. 8*A*). At pH_i 6·4, extracellular 150 μM 4-AP caused an inhibition in Q_{step} of 58 ± 2 % ($n = 5$). In comparison, 150 μM 4-AP only induced a reduction in Q_{step} of 23 ± 2 % ($n = 5$) at pH_i 9·0. When the intracellular environment was made alkaline, a marked increase in the degree of mKv1.1 current inactivation was consistently seen (see Fig. 8*A*); this may reflect a modification of C-type inactivation by pH. Dose–response relationships for 4-AP at different pH_i values showed a

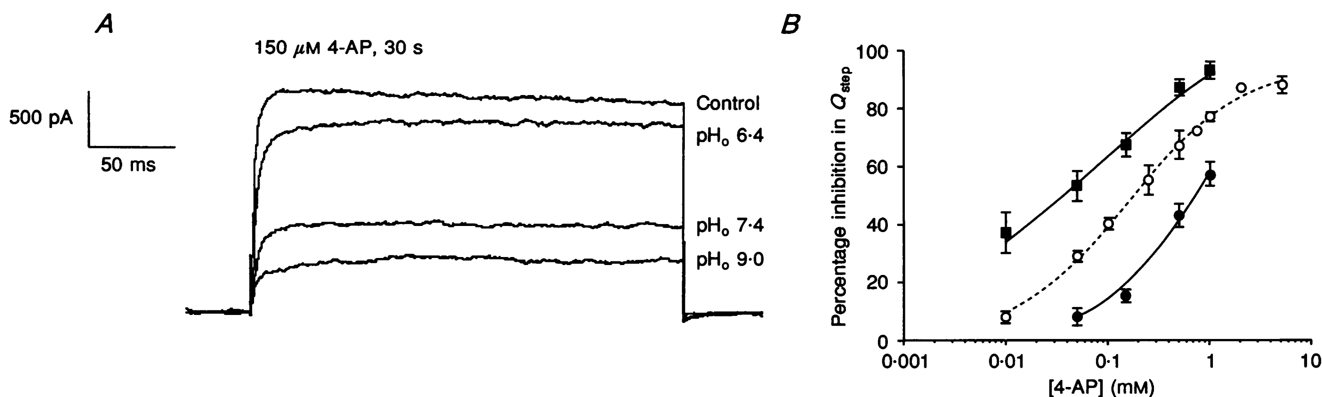

Figure 9. Increasing pH_o increases the potency of 4-AP block of mKv1.1
A, effect of pH_o on block of mKv1.1 by 150 μM 4-AP. 4-AP was more potent as pH_o was increased, causing an inhibition of Q_{step} of 14 % (pH_o 6·4), 54 % (pH_o 7·4) and 75 % (pH_o 9·0) at steady state (reached by 30 s) for this cell. Currents were activated by stepping from a holding potential of −90 mV to +40 mV using 250 ms pulses. *B*, dose–response relationship for the effect of pH_o on 4-AP inhibition of Q_{step}. ○, 4-AP inhibition of mKv1.1 at physiological pH (pH_o 7·4); ●, pH_o 6·4; ■, pH_o 9·0. Data fitted with eqn (2) gave an IC_{50} of 93 μM ($r = 0·998$) for pH_o 9·0 and an IC_{50} of 398 μM ($r = 0·997$) for pH_o 6·4. Points represent means ± S.E.M. for a minimum of 3 cells.

clear shift in the IC_{50} for 4-AP (Fig. 8*B*). As the intracellular environment became more acidic, the potency of 4-AP increased, with an IC_{50} of 295 μM at pH_i 9·0 and 76 μM at pH_i 6·4.

A shift in potency of 4-AP was also seen when the extracellular pH was altered. Figure 9*A* shows the effects of application of 150 μM 4-AP to a single cell at different pH_o values; this is an advantage over experiments investigating the effects of pH_i where it was necessary to compare results from a population of cells. At 150 μM 4-AP, the degree of blockade of mKv1.1 increased as pH_o was made more alkaline; at pH_o 9·0, 150 μM 4-AP caused an inhibition in Q_{step} of 67 ± 4 % ($n = 4$), but inhibited only 16 ± 2 % ($n = 5$) at pH_o 6·4. This effect was reflected in the dose–response relationships for 4-AP at different pH_o values (Fig. 9*B*). A clear increase in 4-AP efficacy was seen at alkaline pH, with an IC_{50} of 93 μM at pH_o 9·0 in comparison to 398 μM at pH_o 6·4. The activation kinetics of K^+ channels have been reported to be affected by pH_o in Schwann cells (Howe & Ritchie, 1991) and lymphocytes (Deutsch & Lee, 1989), with an increase in the threshold for activation at higher pH_o values. In the present study, no such shift in activation was seen; currents activated at potentials more positive than −40 mV, independently of pH_o. Furthermore, when Boltzmann functions were fitted to conductance–membrane potential curves obtained at pH_o 9·0 according to eqn (1), fitted parameters showed no difference to those obtained at physiological pH ($E_{1/2} = -7 \pm 3$ mV; $k = 16 \pm 1$ mV; $n = 3$).

DISCUSSION

The expression of a single, isolated ion channel in a mammalian cell affords us a unique opportunity to investigate the site of action and the mechanism by which drugs produce their effects at specific ion channels. The present study sought to characterize the effects of 4-AP on the cloned mouse brain Kv1.1 channel, a highly conserved mammalian K^+ channel found also in rat (Stühmer *et al.* 1988; Wang, Castle & Wang, 1992) and human brain (Kamb, Weir, Rudy, Varmus & Kenyon, 1989). The results presented are consistent with an intracellular site of action by the cationic form of 4-AP. Furthermore, the presence of inactivation peptide from the *Shaker* B *N*-terminus may go some way to accounting for the reported differences in 4-AP potency between non-inactivating and rapid 'A'-type K^+ currents (Stühmer *et al.* 1989; Stocker *et al.* 1990).

Characteristics of 4-AP blockade of mKv1.1

Expression of mKv1.1 in CHO cells gave a non-inactivating, delayed rectifier-type K^+ current (Robertson & Owen, 1993). 4-AP induced a dose- and use-dependent block of mKv1.1 conductance. Block of mKv1.1 current was also voltage dependent, with a relief of block at depolarizing voltages. The anomaly that a cationic species acting intracellularly has an efficacy which decreases with depolarization (see Woodhull, 1973) has been discussed for 4-AP action on rabbit Schwann cells by Baker, Howe & Ritchie (1993); these authors suggest that the presence of two different currents might account for the apparent voltage dependence. In the present study, similar voltage dependence was found on a single, cloned channel, indicating that this phenomenon may hold for 4-AP on a range of K^+ channels. This is one of the advantages of studying the effects of 4-AP on an isolated channel, namely that the preparation contains a single, uncontaminated current. Similar voltage dependence of 4-AP block has also been reported for K^+ currents in squid giant axon (Meves & Pichon, 1977) and rat ventricular myocytes (Castle & Slawsky, 1992).

The use dependence of 4-AP blockade is in agreement with other studies on K^+ channels in different preparations, including mouse lymphocytes (Choquet & Korn, 1992), GH_3 cells (Wagoner & Oxford, 1990) and rat melanotrophs (Kehl, 1990). Increasing the stimulation frequency facilitated 4-AP block of open channels. Our results also showed that 4-AP did not bind exclusively to open channels, but that there was an appreciable amount of blockade of closed channels between stimulating voltage steps (see also Robertson & Owen, 1993).

Blockade of mKv1.1 was similar whether 4-AP was administered extracellularly ($IC_{50} = 147$ μM) or intracellularly ($IC_{50} = 117$ μM). This suggests that 4-AP is able to cross the cell membrane to its site of action. Similar findings have been reported previously for a variety of preparations, including squid giant axons (Yeh *et al.* 1976; Meves & Pichon, 1977), molluscan neurones (Thompson, 1982) and rat melanotrophs (Kehl, 1990). However, an important difference in the route of 4-AP application was in the time course of action, as internal 4-AP acted far more rapidly, with block typically occurring within seconds. Internal 4-AP has also been reported to produce a more rapid blockade of K^+ channels in comparison to externally applied drug in molluscan neurones (Hermann & Gorman, 1981) and squid giant axon (Kirsch & Narahashi, 1983). These findings are also concomitant with an internal site of action for 4-AP.

Modification of mKv1.1 by inactivation peptide

The transformation of mKv1.1 from a delayed rectifier-type current to a rapidly inactivating current by the inactivation peptide was similar to its effect on *Shaker* mutant K^+ currents in oocytes (Zagotta, Hoshi & Aldrich, 1990). This implies some homology in the inactivation peptide binding sites between *Shaker* B and mKv1.1 K^+ channels. The peptide 20-mer sequence has been shown to be located at the *Shaker* B *N*-terminus on the cytoplasmic side of the membrane (Hoshi, Zagotta, & Aldrich, 1990). Point mutations in the proposed membrane-spanning S4 and S5 segments in *Shaker* B and DRK1 K^+ channels (Isacoff, Jan & Jan, 1991) influence the normal inactivation process, and it is suggested that this region (which is also conserved in mKv1.1) may be part of the receptor site for

the inactivation peptide. In the RCK potassium channel family, RCK1, RCK3 and RCK5 express delayed rectifier-type channels which are blocked by 4-AP with IC_{50} values close to 1 mM (Stühmer *et al.* 1989); in contrast, RCK4, which is a rapidly inactivating current, requires tenfold higher concentrations of 4-AP for blockade. Sequence homology studies showed that RCK4 had an extended amino terminus which confers a rapid inactivation of K^+ current. In the non-inactivating *Shaker* D2, and the rapidly inactivating *Shaker* A2, which differ only in the amino terminus, 4-AP is tenfold less potent in its effect on the inactivating current (Stocker *et al.* 1990). In the present study, the presence of inactivation peptide reduced 4-AP potency and also increased the time for 4-AP block to reach steady state, suggesting that inactivation peptide hinders 4-AP's blocking action, as has been reported in *Shaker* (Hice, Swanson, Folander & Nelson, 1992) and RHK1 (Yao & Tseng, 1993) K^+ channels. This effect may be due to a direct or indirect occlusion of the 4-AP binding site. Interestingly, the slope factor of the dose–response relationship for 4-AP is greater in the presence of inactivation peptide, which suggests that site(s) available to 4-AP in mKv1.1 channels are occupied by the peptide; moreover, at higher concentration of inactivation peptide this effect is more pronounced. Choi, Mossman, Aubé & Yellen, (1993) have reported that intracellular tetraethylammonium (TEA) causes a slowing of fast inactivation in *Shaker* K^+ channels, and they suggest a competition for binding between TEA and the inactivation particle. In contrast, in the present study τ_d values for 4-AP in the presence of the inactivation peptide showed no such slowing. If anything there was a slight increase in inactivation rate, as has also been reported for 4-AP action on *Shaker* K^+ channels by Hice *et al.* (1992). These findings indicate that the interaction between 4-AP and the inactivation peptide are clearly more complex than those seen for TEA.

Site of action and active species of 4-AP

The action of 4-APMI, a permanently charged analogue of 4-AP, is confined to that side of the membrane to which it is applied (see Yeh, 1982). External administration of 4-APMI was ineffective even at concentrations as high as 10 mM, in agreement with other studies (Kirsch & Narahashi, 1983; Howe & Ritchie, 1991; Choquet & Korn, 1992). These results suggest that the cationic form of 4-AP exerts no extracellular blockade of mKv1.1. In contrast, inclusion of 4-APMI in the patch electrode produced a significant block of the mKv1.1 current. These results are in agreement with findings in squid giant axon (Kirsch & Narahashi, 1983), but differ from those in GH_3 cells (Wagoner & Oxford, 1990) and Schwann cells (Howe & Ritchie, 1991) where internal administration of 4-APMI did not block K^+ currents. Intracellular 4-APMI was found to be less effective than 4-AP in blocking mKv1.1. This may indicate that the uncharged as well as the cationic form of 4-AP contributes to the intracellular block; although we cannot discount this possibility, 4-APMI has been reported to be 100 times less potent than 4-AP (Kirsch & Narahashi, 1983) and this may account for the residual Q_{step} not blocked by 4-APMI.

The finding that mKv1.1 was blocked by internal but not external 4-APMI indicates an intracellular site of action for the cationic form of 4-AP. The effects of altering pH_o and pH_i on 4-AP block of mKv1.1 confirmed this idea. At physiological pH, 4-AP exists predominantly (98·4 %) in the charged form. Increasing pH_o caused an increase in potency of 4-AP block of mKv1.1; conversely, making the extracellular environment more acidic decreased 4-AP efficacy. This is consistent with an increase in the proportion of 4-AP in the uncharged, lipid-soluble form at alkaline pH which is available to cross the membrane, and would argue against an extracellular site of action for the cationic form of 4-AP. Similar effects of pH_o on 4-AP potency have been reported for skeletal muscle (Gillespie & Hutter, 1975) and Schwann cells (Howe & Ritchie, 1991). When the cytoplasm was made more acidic, the potency of 4-AP on mKv1.1 was increased. This is in accord with the hypothesis that the cation is the active species of 4-AP since driving the equilibrium of 4-AP toward the charged form inside the cell increases its potency.

In order to establish if changes in 4-AP potency were solely due to the percentage of 4-AP in the cationic form, data were normalized to the concentration of the intracellular charged form. It was found that the effects of pH on 4-AP equilibrium did not wholly account for the changes in potency, suggesting that the 4-AP binding site is also influenced by pH. Howe & Ritchie (1991) proposed a model for K^+ channels in rabbit Schwann cells whereby charged 4-AP binds to an unprotonated site which has an apparent pK_a of 7·0. Following their methods, we have calculated a pK_a (−log of the dissociation constant) of 7·7 for mKv1.1 channels, consistent with protons competing with 4-AP for the binding site. Therefore, changes in pH_i will not only affect the intracellular 4-AP charge equilibrium, but can also change the affinity of the receptor site on the channel for 4-AP.

4-AP is a potent blocker of non-inactivating mKv1.1 current. Modification of mKv1.1 to a rapidly inactivating current by the inactivation peptide decreased 4-AP potency, suggesting that 4-AP binds at or in close proximity to the site of action of the inactivation particle. Other evidence supports an internal site of action for 4-AP; namely, internally applied 4-AP has a faster time course of action than external 4-AP, and 4-APMI is only effective when applied intracellularly. The latter also suggests that it is the cationic form of 4-AP which blocks mKv1.1. This was confirmed by the pH dependence of 4-AP. Taken together, these results indicate that it is predominantly the cationic form of 4-AP which acts intracellularly to block the cloned mKv1.1 channel.

REFERENCES

BAKER, M., HOWE, J. R. & RITCHIE, J. M. (1993). Two types of 4-aminopyridine-sensitive potassium current in rabbit Schwann cells. *Journal of Physiology* **464**, 321–342.

CASTLE, N. A. & SLAWSKY, M. T. (1992). Characterization of 4-aminopyridine block of the transient outward K^+ current in adult rat ventricular myocytes. *Journal of Pharmacology and Experimental Therapeutics* **264**, 1450–1459.

CHOI, K. L., MOSSMAN, C., AUBÉ, J. & YELLEN, G. (1993). The internal quaternary ammonium receptor site of *Shaker* potassium channels. *Neuron* **10**, 533–541.

CHOQUET, D. & KORN, H. (1992). Mechanism of 4-aminopyridine action on voltage-gated potassium channels in lymphocytes. *Journal of General Physiology* **99**, 217–240.

CHRISTIE, M. J., ADELMAN, J. P., DOUGLASS, J. & NORTH, R. A. (1989). Expression of a cloned rat brain potassium channel in *Xenopus* oocytes. *Science* **244**, 221–224.

DE LEAN, A. P. J., MUNSON, P. J. & ROBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose–response curves. *American Journal of Physiology* **235**, E97–102.

DEUTSCH, C. & LEE, S. C. (1989). Modulation of K^+ currents in human lymphocytes. *Journal of Physiology* **413**, 399–413.

GILLESPIE, J. I. & HUTTER, O. F. (1975). The actions of 4-aminopyridine on the delayed potassium current in skeletal muscle fibres. *Journal of Physiology* **252**, 70*P*.

HERMANN, A. & GORMAN, A. L. F. (1981). Effects of 4-aminopyridine on potassium currents in a molluscan neuron. *Journal of General Physiology* **78**, 63–86.

HICE, R. E., SWANSON, R., FOLANDER, K. & NELSON, D. J. (1992). Aminopyridines alter inactivation rates of transient potassium channels. *Biophysics Journal* **61**, A376.

HOSHI, T., ZAGOTTA, W. N. & ALDRICH, R. W. (1990). Biophysical and molecular mechanisms of *Shaker* potassium channel inactivation. *Science* **250**, 533–538.

HOWE, J. R. & RITCHIE, J. M. (1991). On the active form of 4-aminopyridine: block of K^+ currents in rabbit Schwann cells. *Journal of Physiology* **433**, 183–205.

ISACOFF, E. Y., JAN, Y. N. & JAN, L. Y. (1991). Putative receptor for the cytoplasmic inactivation gate in the *Shaker* K^+ channel. *Nature* **353**, 86–90.

KAMB, C. A., WEIR, M., RUDY, B., VARMUS, H. & KENYON, C. (1989). Identification of genes from pattern formation, tyrosine kinase, and potassium channel families by DNA amplification. *Proceedings of the National Academy of Sciences of the USA* **86**, 4372–4376.

KEHL, S. J. (1990). 4-Aminopyridine causes a voltage-dependent block of the transient outward potassium current in rat melanotrophs. *Journal of Physiology* **431**, 515–528.

KIRSCH, G. E. & NARAHASHI, T. (1983). Site of action and active form of aminopyridines in squid axon membranes. *Journal of Pharmacology and Experimental Therapeutics* **226**, 174–179.

KLUMPP, D. J., FARBER, D. B., BOWES, C., SONG, E.-J. & PINTO, L. H. (1991). The potassium channel MBK1 (Kv1.1) is expressed in the mouse retina. *Cellular and Molecular Neurobiology* **11**, 611–622.

MEVES, H. & PICHON, Y. (1977). The effect of internal and external 4-aminopyridine on the potassium current in intracellularly perfused squid giant axons. *Journal of Physiology* **268**, 511–532.

PONGS, O. (1989). Molecular basis of potassium channel diversity. *Pflügers Archiv* **414**, suppl., 71–75.

ROBERTSON, B. & OWEN, D. G. (1993). Pharmacology of a cloned potassium channel from mouse brain (MK-1) expressed in CHO cells: effects of blockers and an 'inactivation peptide'. *British Journal of Pharmacology* **109**, 725–735.

STEPHENS, G. J., GARRATT, J. C., OWEN, D. G. & ROBERTSON, B. (1993). Site of action of 4-aminopyridine on mouse brain mKv1.1 cloned potassium channels expressed in Chinese hamster ovary cells. *Journal of Physiology* **473**, 179*P*.

STOCKER, M., STÜHMER, W., WITTKA, R., WANG, X., MÜLLER, R., FERRUS, A. & PONGS, O. (1990). Alternative *Shaker* transcripts express either rapidly inactivating or noninactivating K^+ channels. *Proceedings of the National Academy of Sciences of the USA* **87**, 8903–8907.

STÜHMER, W., RUPPERSBERG, J. P., SCHRÖTER, K. H., SAKMANN, B., STOCKER, M., GIESE, K. P., PERSCHKE, A., BAUMANN, A. & PONGS, O. (1989). Molecular basis of functional diversity of voltage-gated potassium channels in mammalian brain. *EMBO Journal* **8**, 3235–3244.

STÜHMER, W., STOCKER, M., SAKMANN, B., SEEBURG, P., BAUMANN, A., GRUPE, A. & PONGS, O. (1988). Potassium channels expressed from rat brain cDNA have delayed rectifier properties. *FEBS Letters* **242**, 199–206.

TEMPEL, B. L., JAN, Y. N. & JAN, L. Y. (1988). Cloning of a probable potassium channel gene from mouse brain. *Nature* **332**, 837–839.

THOMPSON, S. H. (1977). Three pharmacologically distinct potassium channels in molluscan neurones. *Journal of Physiology* **265**, 465–488.

THOMPSON, S. H. (1982). Aminopyridine block of potassium current. *Journal of General Physiology* **80**, 1–18.

WAGONER, P. K. & OXFORD, G. S. (1990). Aminopyridines block an inactivating potassium current having slow recovery kinetics. *Biophysical Journal* **58**, 1481–1489.

WANG, S.-Y., CASTLE, N. A. & WANG, G. K. (1992). Identification of RBK-1 potassium current having slow recovery kinetics. *Glia* **5**, 146–153.

WOODHULL, A. M. (1973). Ionic blockage of sodium channels in nerve. *Journal of General Physiology* **61**, 687–708.

YAO, J.-A. & TSENG, G.-N. (1993). Removing inactivation of an A-type channel (RHK1) potentiates block by 4-aminopyridine (4-AP). *Biophysical Journal* **64**, A313.

YEH, J. Z. (1982). A pharmacological approach to the structure of the Na channel in squid axon. In *Proteins in the Nervous System: Structure and Function*, ed. HABER, B., PEREZ-POLO, J. R. & COULTER, J. D., pp. 17–49. Alan R. Liss, New York.

YEH, J. Z., OXFORD, G. S., WU, C. H. & NARAHASHI, T. (1976). Dynamics of aminopyridine block of potassium channels in squid axon membrane. *Journal of General Physiology* **68**, 519–535.

ZAGOTTA, W. N., HOSHI, T. & ALDRICH, R. W. (1990). Restoration of inactivation in mutants of *Shaker* potassium channels by a preparation derived from ShB. *Science* **250**, 568–571.

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