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The Impermeant Ion Methylammonium Blocks K^+ and NH_4^+ Currents through KAT1 Channel Differently: Evidence for Ion Interaction in Channel Permeation

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Abstract. The permeation properties of KAT1, an inward rectifying potassium channel from plant cells, were investigated with different ions in the external medium. With either K^+, NH_4^+ or methylammonium (MA) in the external solution, the channel, expressed in *Xenopus* oocytes, appeared permeable to K^+ and, to a lesser extent, to NH_4^+ but not to the slightly bigger, methylated analogue of NH_4^+, MA. Substituting NH_4^+ for K^+ shifted the voltage dependency of channel activation further negative and hastened activation kinetics. This suggests that channel operation depends on the transported substrate. In mixed solution (50 mM K^+, 50 mM MA) MA inhibited K^+ current in a voltage-independent manner. The maximum block did not exceed 50% of the K^+ current. In contrast, when NH_4^+ was the permeant ion (50 mM NH_4^+, 50 mM MA) MA caused a voltage-dependent, slowly developing open channel block, achieving complete inhibition at very negative voltages. The latter block could be partially overcome by the addition of K^+ in the external solution. The data support a model in which ions, after entering the channel pore, compete with different affinities for binding sites on their permeation pathway.

Key words: KAT1 — Potassium channel — Permeation — Block — Ammonium (NH_4^+) — Methylammonium (MA)

Introduction

Inward rectifying channels provide plant cells with the dominant pathway for passive uptake of cations. Selectivity of these channels to K^+ supports uptake of this cation, which is required both as a major nutrient and for turgor-driven growth (Schroeder, Ward & Gassmann, 1994). Generally, these plant inward rectifiers exhibit also considerable permeability to NH_4^+ (Schroeder et al., 1994), suggesting that at least a fraction of this essential nutrient also enters cells *via* these channels (Venegoni et al., 1996). On the other hand ions such as Cs and the often abundant Na^+, both toxic in plants (Greenway & Munns, 1980; Shenhan, Ribeiro-Neto & Sussman, 1993), are effectively excluded from the cells due to the low permeability of the channels to these ions (Schroeder et al., 1994).

Understanding of the molecular mechanisms underlying channel selectivity has now been advanced by cloning and subsequent mutations of channel proteins (Jan & Jan, 1992; Pongs, 1992). KAT1, an inward rectifying K^+ channel cloned from *Arabidopsis thaliana* (Anderson et al., 1992) shares with K^+-selective channels from animal cells a highly conserved amino acid motive in the postulated pore region (H5) (Uozumi et al., 1995). The latter motive is believed to be crucial in determining the selectivity of the channel (Jan & Jan, 1992; Pongs, 1992), since point mutations in the pore region of KAT1 can profoundly alter the channel selectivity (Becker et al., 1996; Nakamura, Anderson & Gaber, 1997).

While these data highlight the importance of specific amino acid sequences in the pore region for selectivity, they give no direct information about the mechanisms which limit permeation. Thus, for example, it is not clear if the amino acid sequence determines a steric barrier for the permeant ion or if interaction of ions with specific binding sites in the channel pore limits ion permeation. These aspects of ion permeation have in the past been extensively investigated in channels from ani-

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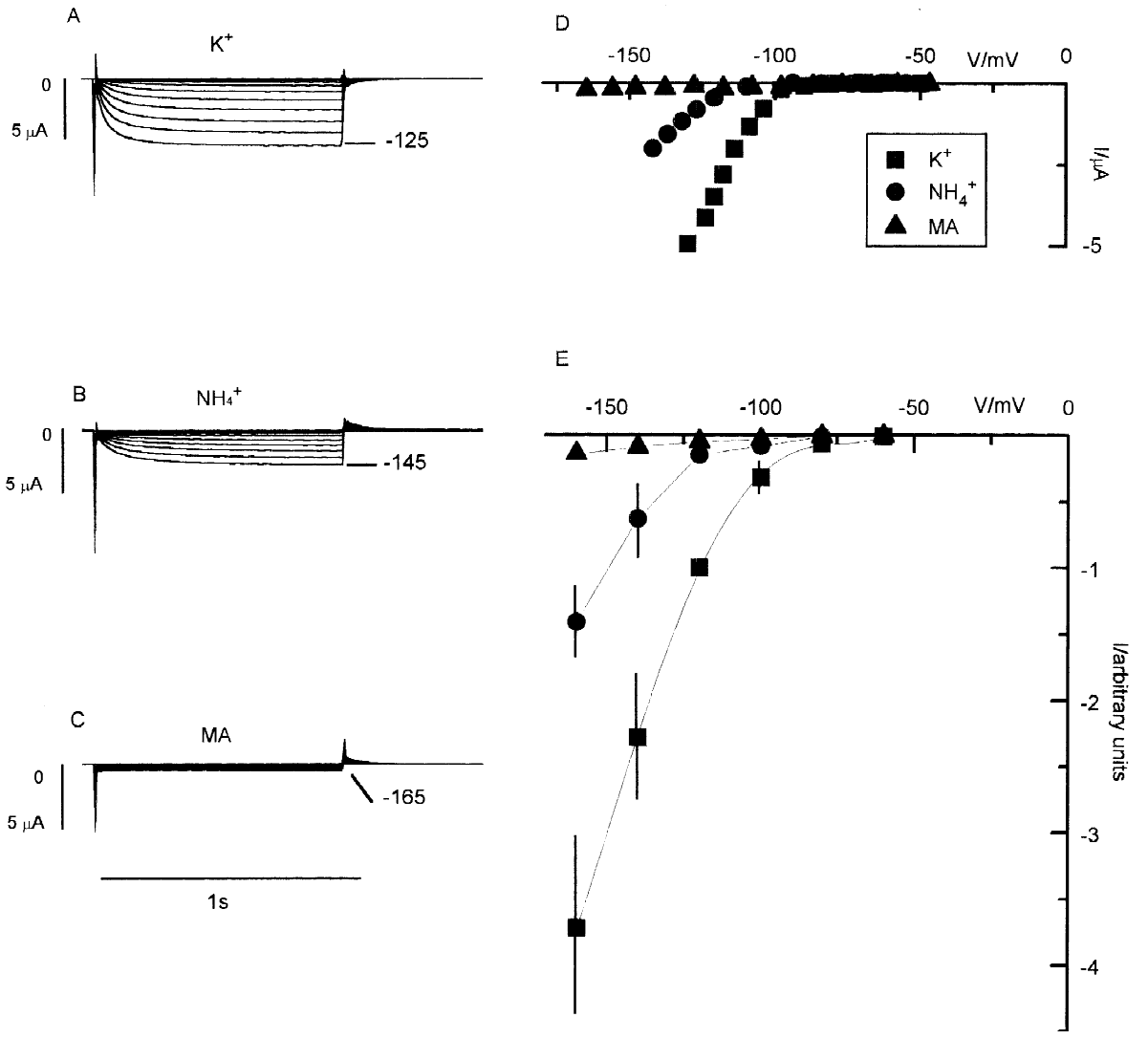

Fig. 1. Ion selectivity of inward currents in an oocyte injected with KAT1 RNA. Currents were evoked in response to hyperpolarizing pulses from a holding voltage of −50 mV. The solution bathing the oocytes obtained (*A*) 50 mM KCl, (*B*) 50 mM NH_4Cl, (*C*) 50 mM CH_3NH_3Cl (MA). (*D*) Steady-state currents shown in (*A*), (*B*) and (*C*) are plotted as a function of applied voltages. (*E*) Mean steady-state current-voltage relation obtained from 7 KAT1-expressing oocytes. The current measured in K^+ at −120 mV was scaled to (−) unity and all the other currents compared to the latter.

mal cells by analyzing the flow of permeant ions in combination with other permeant or nonpermeant ions (Hagiwara et al., 1977; French & Shoukimas, 1985; Wu, 1992; Hille, 1992). Following this approach, in the present study permeation of K^+ and NH_4^+ through KAT1 was investigated in the presence of the ammonium analogue methylammonium ($CH_3NH_3^+$), an ion slightly bigger than NH_4^+ but generally impermeant to animal K^+ channels (Hille, 1975; French & Shoukimas, 1985). The steep voltage dependence of methylammonium (MA) block in KAT1, only found when ammonium is the permeant ion, reveals the existence of binding site(s) within the permeation pathway for which NH_4^+ competes less effectively than K^+.

Materials and Methods

Channel Expression

KAT1 cDNA was kindly provided by Prof. Gaber, Northwestern University (Evanston, IL). RNA was transcribed using T7 polymerase and injected into *Xenopus* oocytes (46 nl per cell, 1 μg/μl) as described by Véry et al., 1995.

Electrophysiology

Electrophysiological measurements were performed 2–7 days after the oocyte injection. The whole-cell KAT1 currents were recorded using a custom-made two-electrode voltage-clamp amplifier. Current-passing

and voltage-recording electrodes were filled with 3 M KCl and had, respectively, 0.5–1MΩ and 1.5–2 MΩ tip resistance in 100 mM KCl. Voltage-pulse protocols, data acquisition and data analysis were performed using ''pClamp'' programme (Axon Instruments, Foster City, CA). Both membrane voltage and current were recorded. The oocyte was continuously superfused (1 ml min^{-1}) during the experiment. All experiments were performed at room temperature (20–25°C).

SOLUTIONS

The standard extracellular solution contained (in millimolar): 50 KC1 (or NH_4Cl), 50 LiCl, 1.8 $CaCl_2$, 1 $MgCl_2$ 5 Hepes/TRIS pH 7.4. Additions of different MA concentrations were osmotically balanced by correspondingly removing LiCl. LiCl has been chosen as a background cation because it does not carry any current through KAT1 channel (Véry et al., 1995).

Results

KAT1 PASSES K^+, NH_4^+ BUT NOT METHYLAMMONIUM

Figure 1 illustrates the current recorded from one *Xenopus* oocyte, expressing KAT1, during steps from a holding voltage of −50 mV to increasingly negative voltages. When the oocyte was bathed in 50 mM K^+ (Fig. 1*A*) stepping to levels more negative than approximately −90 mV evoked a typical current response, characterized by a slowly activating, inward current carried by KAT1 channels (Schachtman et al., 1992) superimposed on a smaller background instantaneous current. Although reduced in amplitude, the same slowly activating current was observed when external K^+ was replaced by NH_4^+ (Fig. 1*B*). However, with MA in the bath the time-dependent component of the current was absent (Fig. 1*C*), indicating that KAT1 is impermeant to MA. Steady state current-voltage relations from the oocyte shown in Fig. 1*A, B* and *C* are plotted in Fig. 1*D.* Similar current records were obtained in 7 other oocytes expressing KAT1; the data are summarized as normalized mean current-voltage relations in Fig. 1*E.*

KAT1 SHOWS A REDUCED PERMEABILITY TO NH_4^+

With ammonium as a permeant cation the following differences from control currents in K^+ were observed: (i) a decrease in the steady state current value over the entire voltage range; (ii) an apparent negative shift of the *I/V* relation and of the current reversal potential of about 20 mV and (iii) apparent slower kinetics of activation of NH_4^+ current, as compared to the current in K^+.

Figure 2 illustrates that steady-state current reduction (expressed as $1 - (I_{NH4+}/I_{K^+})$) depends on voltage: at

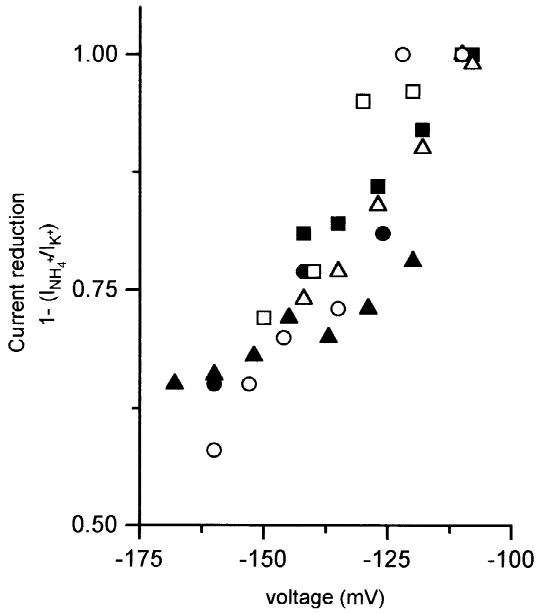

Fig. 2. Relative decrease in current obtained after replacing K^+ with NH_4^+ as a permeant ion. Different symbols correspond to $n = 6$ experiments from 5 injected oocytes. The reduction in current is maximal at moderate negative voltages and decreases with hyperpolarization.

−120 mV, for example, K^+ substitution by NH_4^+ reduced the current by 95 ± 8% ($n = 6$), whereas, at −160 mV, the reduction was 63 ± 4% ($n = 4$).

Changes in the *I/V* relation induced by replacement of K^+ with NH_4^+ were analyzed from the fully activated current-voltage relations obtained in the presence of both cations (*not shown*). With 50 mM K^+ as a permeant ion, the reversal potential was −17.1 ± 1.6 mV ($n = 8$), a value close to the expected equilibrium potential for potassium (−18 mV) calculated assuming the intracellular K^+ concentration 100 mM (Kubo et al., 1993); replacing K^+ with 50 mM NH_4^+, shifted the reversal potential to −44 ± 1.5 mV ($n = 3$). This indicates a lower permeability of KAT1 to NH_4^+ in comparison to K^+ (*compare* Schachtmann et al., 1992; Uozumi et al., 1995; Becker et al., 1996) and suggests that the shift observed in the *I/V* relation is related to the different permeability of the two ions. To further analyze this effect, we have compared the activation curves obtained under both ionic conditions. Figure 3 shows mean values from 6 experiments. Fitting the Boltzmann equation to experimental data, yielded a negative shift of the mid-activation voltage (E_h) of 11 mV.

Analysis of activation kinetics, obtained from single exponential fitting to current traces in the voltage range of activation, also indicated a difference in channel gating when NH_4^+ replaced K^+. Fig. 4 gives mean values of time-constants from 4 experiments and shows that re-

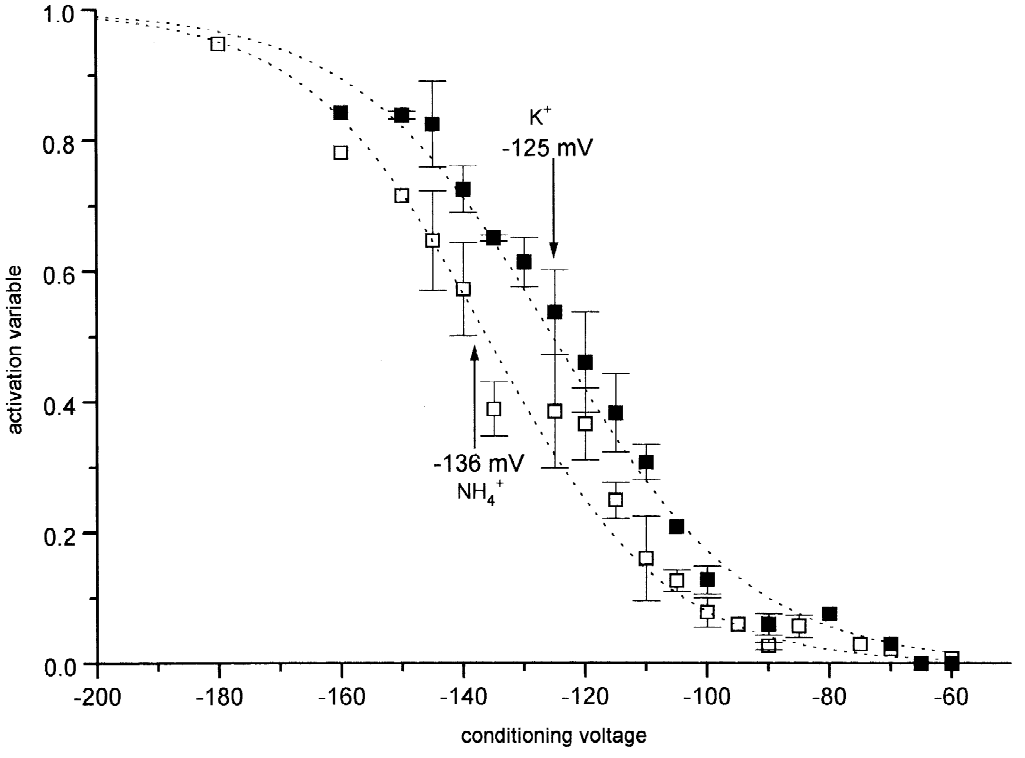

Fig. 3. KAT1 activation curves ($y_\infty(E)$) in (■) 50 mM K^+- and (□) 50 mM NH_4^+-containing bath solutions, measured from tail current amplitudes. Tail currents were recorded by stepping to −65 mV from different conditioning voltages. We were unable to obtain full activation of KAT1 channels as this is likely to occur at voltages more negative than −180 mV, where an endogenous inward current activates randomly. Therefore data (from 6 experiments) were normalized to a I_{max} value that was obtained by fitting a Boltzmann equation to each set of data. Mean values ± SD are plotted. Best fits of the Boltzmann equation $y_\infty = 1/\{1 + exp[(E - E_h)/S]\}$ (dotted lines) to the data yielded values of $E_h = -125$ and −136 mV for the half-maximum activation voltage in K^+ and NH_4^+, respectively.

placing NH_4^+ for K^+ leads to a slowing of activation kinetics, shifting the time-constant-voltage curve by about 20 mV in the negative direction.

K^+ and NH_4^+ Permeate the Channel Independently

As shown by Hagiwara (1977) in echinoderm egg, the inward rectifier membrane conductance can depend on the mole fraction of two permeant ions in a peculiar way, such that the conductivity decreases at intermediate concentrations of either permeant ions (anomalous mole fraction behavior).

To test whether K^+ permeation through the KAT1 channel exhibits an anomalous mole-fraction dependence, we measured the inward currents through KAT1 in the presence of either one of the two permeant ions or in the presence of both. Figure 5 illustrates that perfusion with K^+/NH_4^+ equimolar mixtures, results in a conductance which is intermediate between the conductance values in the presence of the two ions alone. This argues against an anomalous mole fraction behavior, suggesting that K^+ and NH_4^+ do not interact significantly in their channel permeation.

Methylammonium Inhibits Differently K^+ and NH_4^+ Current

Block of MA on K^+ Current

The experiment illustrated in Fig. 6*A* shows that addition of 50 mM MA to a 50 mM K^+ solution inhibited the current as compared to that recorded in 50 mM K^+ alone. The decrease of steady-state K^+ current was observed in 6 oocytes (and the measured ratio I_{MA}/I_{K^+} ranged, at −140 mV, between 0.20 and 0.72). The mean values (±SE) obtained from 6 experiments in 5 oocytes, were 0.64 ± 0.16, 0.57 ± 0.07, 0.41 ± 0.10 and 0.56 ± 0.11 at −120, −135, −140 and −165 mV, respectively. These values were not significantly different ($P > 0.05$, ANOVA), indicating no voltage-dependence of the inhibition. The inhibition of K^+ current by MA was fully reversible on washing out of the solution (*data not shown*).

The time course of current activation was not changed by the addition of MA. Fitting a double exponential to the current traces (*data not shown*) we obtained values for the fast component of activation, τ_1, of 106 ±

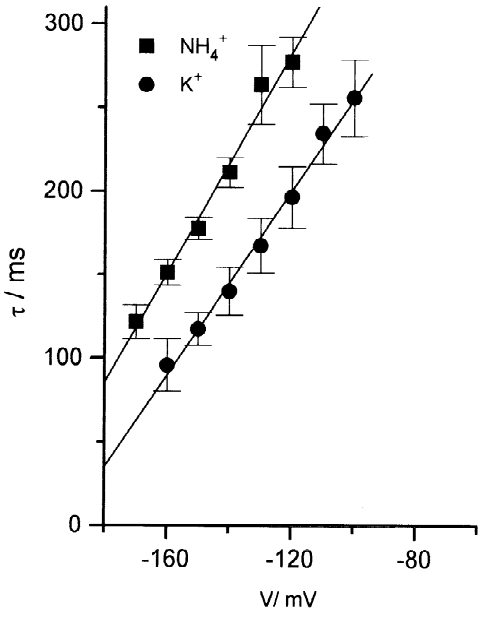

Fig. 4. Time constants (τ) of KAT1 activating currents in K^+ and in NH_4^+. Time constants approximated by a single exponential fit (least-squares fitting) to activating currents. Data are means ± SD of 4 experiments in three oocytes. Inset: the data are current responses for a voltage step from a holding potential of −50 mV to −160 mV in the same oocyte with 50 mM K^+ or 50 mM NH_4^+ in the bath. The currents are scaled to the same ordinate and clearly show that the NH_4^+ current is slower.

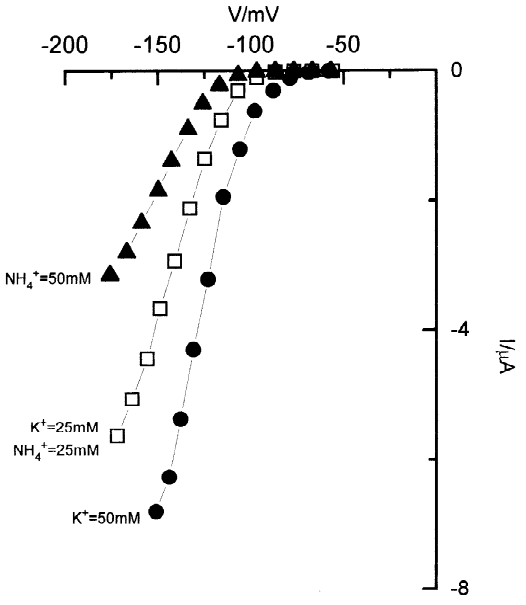

Fig. 5. Steady-state current-voltage relations from one oocyte injected with KAT1 obtained at different molar fractions of K^+ (indicated at the end of each curve). The total concentration $C_K^+ + C_{NH4^+}$ was 50 mM.

10.7, 67 ± 4.9, 37 ± 5.7 msec in control and 112 ± 8.4, 74.6 ± 4.58, 40.5 ± 4.9 msec in the presence of MA, at the voltages of −120, −140, and −165 respectively (n = 5, ±SE). These values were not significantly different when a paired t-test was performed at the significance level of 0.05.

Block of MA on NH_4^+ Current

As shown in Fig. 6*B*, when MA (50 mM) was added to a 50 mM NH_4^+ solution the current passing through KAT1 was markedly inhibited in a characteristic time- and voltage-dependent manner.

In the presence of MA, the initial current activation was followed by a slower decay which became faster and more pronounced at more negative voltages. Block of the current at steady-state appeared strongly voltage-dependent increasing with hyperpolarization, as shown in Fig. 6*B*. Comparative time- and voltage-dependent block of NH_4^+ current by MA was observed in 12 other experiments in 9 oocytes. The inhibition of MA on NH_4^+ current was fully reversible on MA washout (*data not shown*).

Concentration Dependence of Block

Figure 7 compares the concentration dependence of the block of MA on K^+ and NH_4^+ current, estimated by plotting the amount of steady-state current inhibition, measured at −160 mV, as a function of MA concentration. The block of NH_4^+ current, as measured in 7 oocytes, was nearly complete at 25 mM MA. On the other hand the mean block induced by MA on K^+ current did not exceed 50% even at the highest concentration tested (100 mM MA, *data not shown*).

The Nature of the MA Block of NH_4^+ Current

The MA-dependent block of NH_4^+ current was further analyzed with respect to time-, concentration- and voltage-dependence.

Open Channel Block

The current traces obtained with NH_4^+ as a permeant ion were fitted with the sum of two exponential equations both in the absence and in the presence of MA. Analysis of activation and block kinetics was based on the following assumptions: (i) channel activation is described by a fast (τ_1, < 150 msec) and a slow (τ_2, > 400 msec) process; (ii) block occurs according to a single exponential time-course superimposed on the slow activation and (iii) block does not affect the fast channel activation process (τ_1). The resulting time constants τ_1 of the fast activation, in control and in the presence of MA, and τ_2, sum of slow activation plus block, are plotted in Fig. 8 as a function of membrane voltage. From the analysis it appears that the fast time course of channel activation

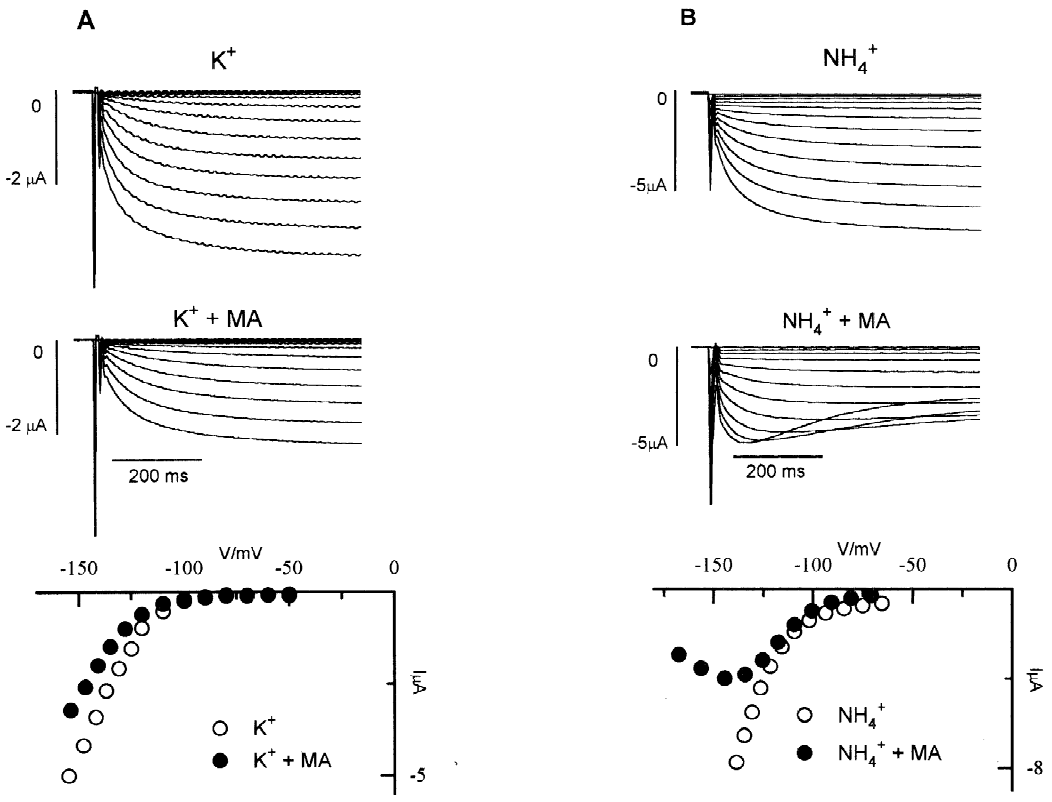

Fig. 6. Effect of the addition of MA on the currents carried by K^+ and NH_4^+ through KAT1. Holding potential −50 mV. (*A*) Current measured in 50 mM K^+ before and after addition of 50 mM MA; lower panel shows *I/V* relation obtained from steady-state current values reported above. (*B*) current measured in 50 mM NH_4^+ before and after addition of 50 mM MA; lower panel shows *I/V* relation obtained from steady-state current values reported above.

was not affected by the addition of MA. Values for τ_1 in the absence of MA were similar to those obtained in the presence of MA (Fig. 8*A*, values for τ_1 from the experiment of Fig. 6*B*). In $n = 4$ similar experiments we obtained mean ± SE values for τ_1 of 122.75 ± 18.98, 84.25 ± 12.28, 69.67 ± 10.11 msec in control and 113 ± 20.3, 79.25 ± 12.67, 47 ± 4.87 msec in the presence of MA, at the voltages of −120, −135, −155 mV, respectively. These values were not significantly different ($P > 0.05$) when a paired *t*-Test was performed. The time constants for activation of NH_4^+ current, both in the absence and in the presence of MA, were voltage-dependent and become faster at more negative voltages. This observation suggests that KAT1 channels are not blocked by MA in the closed state. As proposed by Armstrong (1971) in the case of the block of quaternary ammonium ions of squid axon potassium channel, the data in Fig. 8 agree with the assumption that the channel has to open first before the blocking molecule can reach its binding site (open channel block).

Figure 8*B* reports τ_2 (sum of slow activation and block), as a function of voltage ($n = 4$). The slow time constant obtained in the presence of MA reveals a steep voltage-dependence. Assuming constant slow activation ± MA, the MA-generated block appears 10 to 20 times slower than the channel activation process, and it becomes faster in an exponential fashion at more negative voltages.

The concentration- and voltage-dependence of MA block of NH_4^+ current are shown in Fig. 9. In Fig. 9*A* steady state *I/V* relations are plotted for various concentrations of the blocking ion; the plots show that at each MA concentration, the blocked fraction of current strongly depends on voltage, and increases at more negative voltages. Figure 9*B* shows dose-response curves obtained from the data of Fig. 9*A*, where the normalized current values ($I_{MA}/I_{control}$) are expressed as a function of the MA concentration for different membrane voltages. Fitting the Hill equation to the data yielded a Hill coefficient always higher than 1.6 (*see* figure legend). The plots show that, particularly at the most negative voltages used, MA is effectively blocking the current flow through KAT1 channels at millimolar concentrations ($EC_{50} = 5.8$ mM at −165 mV and 18.9 at −137 mV).

Position of the MA Blocking Site Inside the Channel

The finding that the early rate of activation of NH_4^+ current is unaffected by the addition of MA (shown in Fig.

8) suggests that the channels need to open before they are blocked. The strong voltage-dependence of MA block is indeed consistent with the idea that block proceeds during channel opening and needs the blocking MA molecule to enter the channel. This can be described by the Woodhull model (1973), assuming that the blocking ion enters the channel and runs a fraction of the electrical field before reaching its blocking site. As a result the block efficiency increases at more negative voltages because of the augmented probability that the inner blocking site is occupied by the blocking ion.

To perform a quantitative analysis of the block we have fitted the data with the Woodhull block model. According to this model, a simple relation exists between degree and blockade (measured as the ratio between residual current after block, I_{MA}, and blocked current I-I_{MA}, I being control current) and membrane voltage, E (DiFrancesco, 1982):

$$1n\, r = 1n\,(I_{MA}/(I - I_{MA})) = \delta zE/(RT/F) - 1n\,(MA)/K_0$$

where δ is the fraction of the electrical field crossed by MA ions before reaching the blocking site (''electrical'' distance), K_0 the dissociation constant of the binding reaction at $E = 0$ mV and R, T and F have their usual thermodynamic meanings.

Figure 10 shows semilog plots of the reactive blocking ratio $r = I_{MA}/(I - I_{MA})$ as a function of membrane voltage (E) for an experiment in which MA was added at concentrations of 10, 25 and 50 mM to a solution containing 50 mM NH_4^+. At least in the far negative voltage range, 1n r and E are linearly related and the slopes of the fitting lines do not substantially depend on the MA concentration. The mean value of δ was 1.96 ± 0.16 ($n = 5$) (*see* Discussion).

Potassium Removes the Block of MA on NH_4^+ Current

The unexpected finding that MA blocks NH_4^+ currents with higher efficiency and with different modalities than K^+ currents may suggest that K^+ competes more effectively than NH_4^+ with MA for binding site(s) inside the channel pore. Therefore, the differential blocking of K^+ and NH_4^+ currents may be due to the fact that K^+ can more effectively remove or overcome the MA imposed block. To test this hypothesis, oocytes were first exposed to NH_4^+ plus MA to block the channel. Then K^+ was added at different concentrations. Figure 11 illustrates that increasing concentrations of K^+ were able to progressively remove the block induced by MA, as shown by the increase in current at negative voltages. This was already apparent with the lowest K^+ concentration added (3 mM), and became more pronounced by further increasing the K^+ concentration. The insert of Fig. 11 shows the amount of current measured at −140 mV plotted *vs.* K^+ concentration. However, comparison of the current levels measured in the same oocyte with 50 mM K^+ alone and with 50 mM K^+ plus NH_4^+ and MA, showed that K^+ was unable to fully override the MA-induced current block. In the presence of MA and NH_4^+, addition of 50 mM K^+ led to a current which was, at −140 mV, only about 50% of the current in 50 mM K^+ alone. It should be noted that this is close to the fractional block induced by 50 mM MA on the KAT1 current in the presence of 50 mM K^+ (*see* Fig. 6*A*). This confirms that K^+, but not NH_4^+, is able to flow through KAT1 channels in the presence of MA.

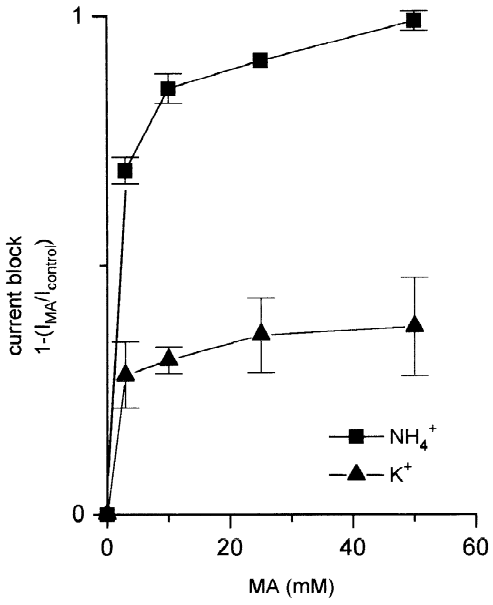

Fig. 7. Concentration dependence of MA block on K^+ and NH_4^+ currents in KAT1-expressing oocytes. 1-($I_{MA}/I_{control}$) represents the amount of current inhibited at −160 mV. Open symbols (□) 50 mM K^+, closed symbols (■) 50 mM NH_4^+. Data are mean values ± SD ($n = 7$). The data were jointly fitted with the velocity equation (*see* Discussion):

$$v = \frac{[MA]}{k_S\,(1 + [K^+]/k_i) + [MA](1 + [K^+]/\alpha k_i)}$$

and yielded a value for α of 7.99 and equilibrium constants of 1.45 and 7.88 for k_s and k_i respectively.

Discussion

We have found that KAT1 channels, expressed in *Xenopus* oocytes, conduct K^+ ions and to a lesser extent NH_4^+ ions, but are impermeant to MA ions. At first glance this may suggest that the ionic permeability through the channel is simply related to the ionic size, since K^+ is the smallest and MA the largest ion (Hille, 1973). However, the action of the impermeant ion MA on K^+ and NH_4^+ currents demonstrates an interaction of the cations with

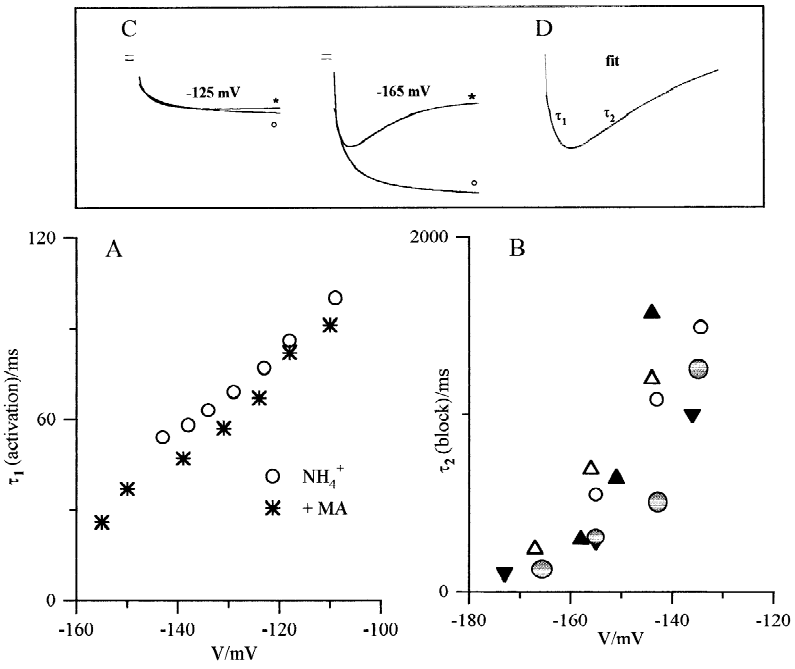

Fig. 8. Kinetics of KAT1 activation/block with NH_4^+ as a permeant ion. KAT1 currents measured in NH_4^+ in the absence and in the presence of 50 mM MA were fitted with the sum of two exponential equations. The resulting time constants were considered to reflect: τ_1 time constant of rapid activation and τ_2 time constant of slow activation plus MA-generated block, when MA was present. Figure 8*A:* time constants of rapid current activation in the absence and in the presence of MA (same data as in Fig. 6*B*). The presence of MA does not affect τ_1. Figure 8*B:* time constants (τ_2) obtained from NH_4^+ currents in the presence of 50 mM MA ($n = 5$, including data from (*A*)). Figure 8*C:* Current traces recorded in the same oocyte before and after the addition of MA, at two different voltages (−125 and −165 mV) are superimposed to illustrate the similarity in activation kinetics. Figure 8*D:* NH_4^+ current with MA block including best fit (dotted line) with the sum of two exponentials.

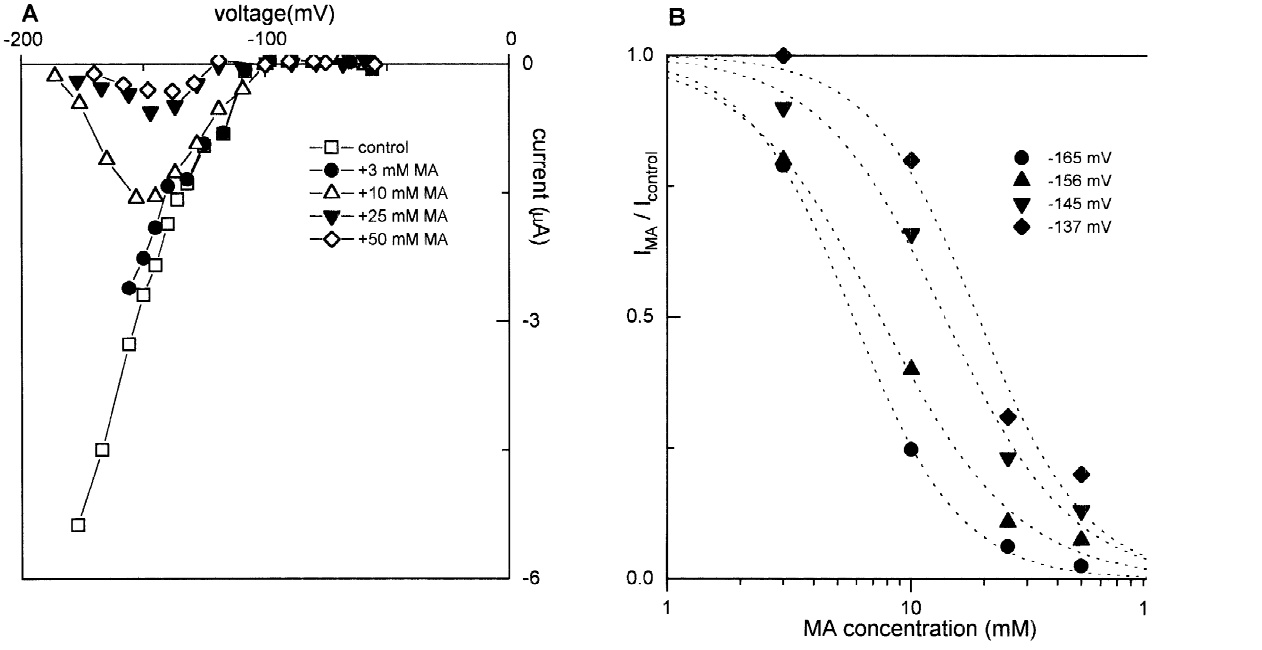

Fig. 9. Concentration- and voltage-dependence of block by MA on NH_4^+ current. (*A*) Steady-state *I/V* relation of 50 mM NH_4^+ current (□) in the presence of 3 (●), 10 (△), 25 (▽) and 50 (◇) mM MA. (*B*) Dose-response curves obtained from (*A*) plotting the normalized value $I_{MA}/I_{control}$ as a function of [MA] at four different voltages (−165, −156, −145, and −137 mV). Fitting the Hill binding equation ($I_{drug}/I_{control} = 1/(1 + X/K_d)^n$ yielded Hill factors, *n* of: 1.98, 1.61, 1.7, 1.95 and K_d (mM): 5.8, 7.48, 17.8, 18.86 for −165, −156, −145 and −137 mV, respectively.

specific sites inside the electrical field of the channel pore. Thus size-exclusion is not the only determinant for ion permeation through KAT1.

The first line of evidence supporting the view of specific ion interaction with the channel protein stems from the different kinetic properties of the KAT1 currents carried by K^+ and NH_4^+ ions. The most likely interpretation for this is that the gating process of the channel depends upon the permeating ion species.

This interpretation could be questioned and the

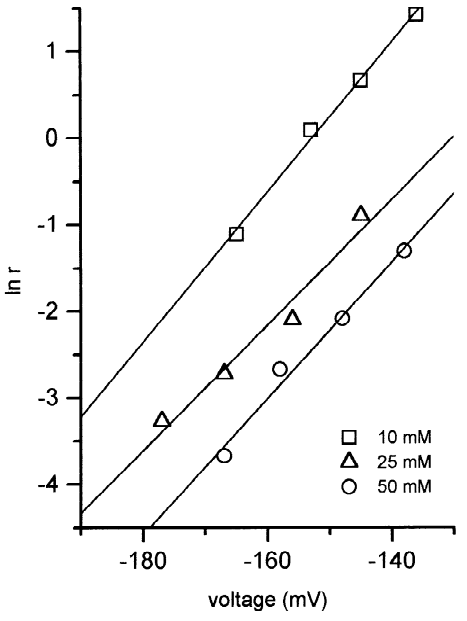

Fig. 10. Semilog plots of the relative blocking ratio $r = I_{MA}/(I - I_{MA})$ as a function of membrane voltage for an experiment where MA was added at concentrations of 10, 25 and 50 mM to a solution containing 50 mM NH_4^+. The slope of the fitted lines was: 0.0864, 0.0724 and 0.0788 for 10, 25 and 50 mM MA, respectively. The calculated δ were: 2.18, 1.83 and 1.99. Mean δ was: 1.96 ± 0.16.

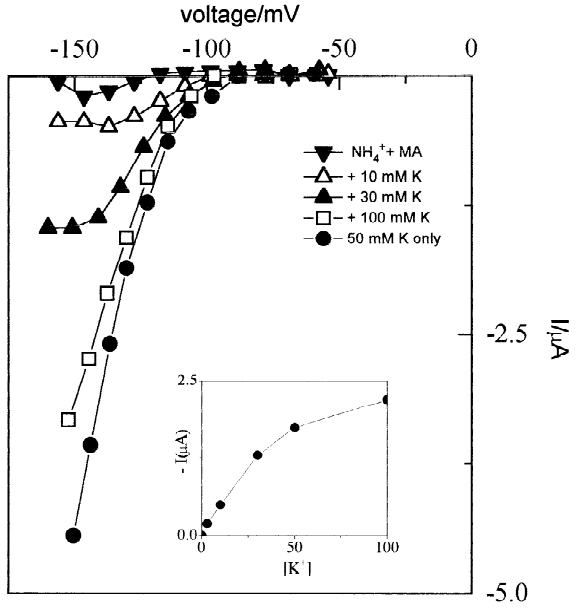

Fig. 11. Addition of K^+ restores inward current when added to an oocyte in which NH_4^+ current was previously blocked by MA. *I/V* relation of currents measured in 50 mM NH_4^+ and 50 mM MA (blocked current, ▽) and after sequential addition of 10 (△), 30 (△) and 100 (□) mM K^+. Also shown, for comparison, is the current measured in the same oocyte in a solution containing 50 mM K^+ (○). The insert shows the increase of inward current as a function of $[K^+]$.

modification of current kinetics in the presence of NH_4^+ might be explained in the context of NH_4^+-generated intracellular acidification (Cao et al., 1995; Burchardt & Thelen, 1995). However, this scenario is unlikely for two reasons: firstly, as with NH_4^+, we observed that addition of MA led to an increase in leak current and membrane depolarization (*data not shown*), two features commonly attributed to cytoplasmic acidification induced by ammonium compounds in *Xenopus* oocytes (Burchardt & Thelen, 1995). Although MA addition is likely to also cause some internal acidification, no apparent shift of the current activation threshold or of activation kinetics is observed in its presence. This suggests that the NH_4^+-evoked acidification in oocytes is not relevant for KAT1 gating. This observation agrees with the evidence that, in a native K^+ inward rectifier from *Vicia faba* guard cells, cytoplasmic acidification amplifies the inward current but does not modify the voltage-dependence of activation kinetics (Blatt, 1992; Blatt & Armstrong, 1993). Secondly, in single channel measurements of the KAT1 channel, it has been reported by Hoshi (1995) that acidification of the cytoplasmic side causes a positive shift in the channel activation curve which is opposite to what was observed here in the presence of NH_4^+. Thus, the changes induced in current activation kinetics through KAT1 channels by NH_4^+ are likely to reflect primary interactions of the permeant ion with the channel protein. A dependence of gating kinetics on the permeant ion species is not unique to KAT1, but has been reported for another plant cation channel (White, 1996).

The absence of a significant anomalous mole-fraction behavior in K^+ and NH_4^+ mixed solutions suggests that K^+ and NH_4^+ do not interfere with each other when passing through KAT1, at least in the negative voltage range investigated.

Further support to the view that channel permeation involves interactions of the permeant ions with sites inside the channel pore derives from the block of K^+ and NH_4^+ currents by MA. An important finding of the present work is that the MA-dependent reduction of the NH_4^+ current appears in many respects different from the block observed when K^+ is the permeant ion. For example, with K^+ as permeant ion, the block reveals no apparent time- and voltage-dependence. Furthermore, the dose-response relation of K^+ current reduction against MA concentration yields a lower affinity and a significantly smaller maximum block compared to that for NH_4^+ currents. This implies that MA does not cause a steric hindrance for ion permeation through KAT1, since in the latter case MA should inhibit K^+ currents as well as NH_4^+ currents.

The MA-induced inhibition of NH_4^+ current can be explained in terms of an open channel block (Armstrong, 1971). The time course of current activation remains unaltered upon addition of MA, indicating that MA ions

need to enter open channels before reaching the blocking site inside the electrical field of the channel pore. This requires that the external mouth of the channel pore has a minimum diameter $\geq$3.6 Å (Hille, 1975) to allow the entrance of the large methylated ammonium ions. The separation between the channel activation time course and block development is simplified by the fact that the block kinetics are about ten times slower than the channel activation kinetics. This indicates that the voltage-dependent block inside the pore is characterized by a slow association process.

Voltage-dependent open channel block of KAT1 has already been described for Cs^+ as a blocking agent (Schachtman et al., 1992; Véry et al., 1994; Ichida & Schroeder, 1996). In contrast to block by MA, Cs^+ block develops orders of magnitude faster and its kinetics have not yet been resolved. The voltage-dependent Cs^+ block of KAT1 channels can be explained by a single ion block, with δ (*see below*) = 0.38 (Ichida & Schroeder, 1996). The substantial difference between the voltage dependence of Cs^+ and MA block of KAT1 channels indicates two different ion block mechanisms and blocking sites.

We characterized the block properties of MA on NH_4^+ current by use of the Woodhull (1973) model. As expected from the apparent voltage-independence. MA block of K^+ was not successfully fitted by this model (*data not shown*). On the other hand, MA block of NH_4^+ showed the predicted relation between degree of blockage and membrane voltage but yielded a calculated value of δ bigger than 1 (1.96), a physical impossibility.

A block with similar properties in which MA (from the cytoplasmic side) generated a steep voltage-dependent block of NH_4^+ currents but a shallow block of K^+ currents was previously reported for animal K^+ outward rectifiers (Hille, 1975; French & Shoukimas, 1985). Also in this case, fitting the Woodhull model to NH_4^+ current block, yielded a value for δ bigger than 1 (1.6). It is worth noting in this context that the plant inward rectifier not only shares structural similarities with the animal outward rectifier (Anderson et al., 1992) but also exhibits similar permeation/blocking features but with an inversed sideness.

The finding that the block involves the equivalent of moving a charge across more than 100 percent of the membrane voltage, has been tentatively interpreted by Hille (1975) as the moving of several charges across the membrane, suggesting also the possibility that more than one MA ion is involved in the block. The contemporary presence of more than one ion inside the pore cannot thus be explained by single-site theories, such as the Woodhull model, and calls for more complex models of the pore.

Another aspect which emerges is the aforementioned fact that MA blocks K^+ and NH_4^+ currents differently. This differential block of MA can be explained by assuming that NH_4^+ competes less effectively than K^+ for the MA binding site(s) inside the channel. Indeed, our data clearly show that, as expected for competitive binding to sites within the channel, addition of K^+ is able to restore a current through KAT1 channel after it has been blocked by MA ions. Hence, the role played by K^+ in releasing the block could be interpreted as that of an antagonist (inhibitor) of the MA-dependent -block of the NH_4^+ current. The differences between the MA-generated blocks of K^+ and NH_4^+ currents can thus be described by analogy with the inhibition of enzyme reactions. A simple model able to account for the results obtained involves a mixed type inhibition with a partial competitive and pure noncompetitive inhibition (Segel, 1975). In this model, MA ions bind to their binding sites inside the pore and K^+ ions are viewed as inhibitors of this reaction, according to the following scheme:

$$\begin{array}{ccccc} & & \overset{k_s}{} & & \\ C + MA & \rightleftharpoons & CMA & \rightarrow & I \\ + & & + & & \\ K^+ & & K^+ & & \\ k_i \updownarrow & & \alpha k_i \updownarrow & & \\ & & \alpha k_s & & \\ O \leftarrow CK^+ + MA & \rightleftharpoons & CMAK^+ & \rightarrow & O \end{array}$$

where C denotes the channel binding site, MA the substrate and K^+ the inhibitor, k_s and K_i the respective equilibrium constants. The factor α is the factor by which k_s changes when K^+ occupies the channel binding site. The conductive channel is denoted by O and the inactive channel by I.

The velocity equation for this type of reaction is:

$$v = \frac{[\mathrm{MA}]}{k_S\,(1 + [K^+]/k_i) + [\mathrm{MA}](1 + [K^+]/\alpha k_i)}$$

The velocity equation was jointly fitted to the data sets for MA block of K^+ and NH_4^+ currents (Fig. 7) yielding a value for α of 7.99, and equilibrium constants of 1.45 and 7.88 for k_s and k_i, respectively.

The successful description of the data suggest that the MA block of KAT1 is generated inside the channel pore by a mixed type inhibition with partial competitive and pure non competitive inhibition.

According to this scheme, KAT1 has two binding sites which can be occupied by MA or K^+. The C-K^+ complex will have a lower affinity for MA than the unoccupied binding site. Furthermore, the channel conducts current when the site is occupied by MA and K^+ or by K^+ only. Therefore as long as K^+ is present, the channel will always be in a conductive state, which explains why the inhibition in the presence of K^+ is never complete. Furthermore, at any K^+ concentration, a portion of

the binding sites available for combination with MA will exist in the lower affinity form C-K^+, so that the presence of K^+ lowers the affinity for block by MA. In the absence of any blocker both binding sites may be occupied by K^+.

Conclusions

The present data show that ions do not diffuse freely through the KAT1 channel but they interact apparently with more than one site within the channel. Ion binding to these sites has profound effects on the gating of the channel but also on the flow of other ions. Such a demonstration of ion-specific binding sites in the process of ion permeation agrees with the evidence derived from single point mutations of KAT1 (Uozumi et al., 1995) where it has been argued that the increased conductance to NH_4^+ in mutated KAT1 proteins was unlikely to arise from a simple widening of the channel pore.

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