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Ionic effects on amiloride block of the mechanosensitive channel in *Xenopus* oocytes

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1 Patch clamp techniques were used to measure the ionic dependence of amiloride block of single mechanosensitive (MS) channels in frog (*Xenopus laevis*) oocytes.

2 The primary aim was to determine whether the difference in potency of amiloride block of MS channels in frog oocytes ($IC_{50} = 0.5$ mM) and chick auditory hair cells ($IC_{50} = 50$ μM) was due to the different ionic recording solutions.

3 Amiloride block of the oocyte MS channel does not vary significantly with complete substitution of external Na^+ (i.e. 100 mM) with K^+ in Ca^{2+}-free recording solution (in both Na^+ and K^+ the $IC_{50} = 0.5$ mM).

4 A physiological concentration (1.8 mM) of external Ca^{2+} blocks the oocyte MS channel and reduces the potency of amiloride block ($IC_{50} = 1.1$ mM) without altering the voltage-dependence or the Hill coefficient ($n = 1.8$) of amiloride block. The reduction in potency can be explained by surface charge screening by Ca^{2+} which reduces the effective amiloride surface concentration.

5 The present results indicate that factors other than ionic recording conditions must underlie the difference in potency of amiloride block of MS channels in oocytes and auditory hair cells.

Keywords: Ionic dependence; amiloride block; mechanosensitive channels; *Xenopus* oocytes; patch clamp

Introduction

Amiloride blocks mechanosensitivity in a variety of sensory and nonsensory cell types (Jorgensen & Ohmori, 1988; Lane *et al.*, 1991). Recently, it has been demonstrated that a series of amiloride analogues display the same order of potency in blocking mechanosensitive (MS) channels in frog oocytes (Lane *et al.*, 1992) and auditory hair cells (Ruesch *et al.*, 1991). This common pharmacology may indicate that mechanosensitive (MS) channels expressed in these functionally distinct cell types share a common 'amiloride receptor' and are structurally related molecules. Such a relationship could be important for biochemical studies since the oocyte MS channel is expressed with a much higher copy number ($> 10^6$ per oocyte) compared with that in the hair cell (10–100 per hair cell see Howard *et al.*, 1988). For this reason the oocyte may prove a more convenient preparation in attempts to purify the MS channel protein.

Although the amiloride receptor on MS channels in oocytes and hair cells appears qualitatively similar there is a quantitative difference in that amiloride and amiloride analogues are approximately 10 times less potent in blocking oocyte compared with hair cell MS channels (c.f., Lane *et al.*, 1991; 1992; Jorgensen & Ohmori, 1988; Ruesch *et al.*, 1991). However, this difference in absolute potency may be due to differences in the ionic recording conditions rather than structural differences in the receptor. Specifically, amiloride block in the oocyte was characterized in the absence of extracellular Ca^{2+} (Lane *et al.*, 1991; 1992), while the block in the hair cell was measured in the presence of normal (2 mM) Ca^{2+} (Jorgensen & Ohmori, 1988; Ruesch *et al.*, 1991). Since it has been demonstrated previously for other amiloride-sensitive ion pathways that extracellular Ca^{2+} may be an essential requirement for amiloride block (Cuthbert & Wong, 1972; Benos *et al.*, 1979; but see Desmedt *et al.*, 1991), we have examined the effect of extracellular Ca^{2+} on the amiloride block of the oocyte MS channel. Our study indicates that a physiological concentration of Ca^{2+} (1.8 mM) reduces rather than increases amiloride blocking potency. We also demonstrate that amiloride block of the MS channel is relatively insensitive to whether K^+ or Na^+ is the predominant charge carrier through the MS channel.

Methods

The methods used here to study amiloride block of MS channels in *Xenopus* oocytes were similar to those described previously (Lane *et al.*, 1991; 1992). Frogs were anaesthetized by being placed for approximately 20 min in a beaker containing 300 mg ethyl 3-aminobenzoate methanesulphonic acid (Aldrich) in 200 ml of distilled water. Sterile surgical procedures were used to remove the oocytes. Standard patch clamp techniques (Hamill *et al.*, 1981) were used to record single MS channel currents from *Xenopus laevis* oocytes on cell-attached patches (Methfessel *et al.*, 1986). Single MS channels were activated by brief mouth applied suction (10–50 mmHg). Different external recording (i.e. pipette) solutions were used. The control solution (Lane *et al.*, 1992) we refer to as '100 mM K^+, 0 mM Ca^{2+}' contained (in mM in all cases): KCl 100, EGTA 10 (KOH) and HEPES 10 (KOH), pH 7.2. The solution referred to as '100 mM Na^+, 0 mM Ca^{2+}' contained (mM): NaCl 100, EGTA 10 (NaOH) and HEPES 10 (NaOH). The solution referred to as '100 mM Na^+, 1.8 mM Ca^{2+}' contained: NaCl 100, $CaCl_2$ 1.8 and HEPES 10 (NaOH). Amiloride was dissolved in the pipette solution in concentrations ranging from 0.25–2 mM. The bath solution for all recordings contained (in mM): NaCl 115, $CaCl_2$ 1.8, KCl 1.5, HEPES 10 (NaOH), pH 7.2. All recordings were carried out at room temperature 19–22°C. Single MS channel currents were initially recorded on a digital video recorder (Neuro-corder, DR484, Neuro Data Instrument Corp. NY). Amplitudes were determined during subsequent playback directly from a storage oscilloscope screen, by centering the baseline current (i.e. closed channel state) on a grid line and reading the open channel current amplitude from a line judged to pass through the average of the open

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channel noise. A minimum of 5 channel events per patch and 2–4 patches were used for any one condition (i.e. as defined by patch potential, amiloride concentration and ionic composition). This convenient technique is sufficiently accurate to discriminate amplitude changes as small as 0.1 pA (e.g. see Figure 2c).

Results

In our initial characterization of amiloride block of the oocyte MS channel (Lane *et al.*, 1991) we used zero Ca^{2+} and substituted Na^+ with 100 mM K^+ in the external recording solution in order to (1) remove the complication of Ca^{2+} block of the MS channel (Taglietti & Toselli, 1988; Yang & Sachs, 1989; Crawford *et al.*, 1991) and (2) measure MS channel current-voltage relations under near symmetrical transmembrane cationic conditions. In contrast, Jorgensen & Ohmori (1988) measured amiloride block with normal avian Ringer containing (in mM): NaCl 155, $CaCl_2$ 2.5, $MgCl_2$ 1.0, glucose 17, HEPES 10 (KOH). Since in other amiloride-sensitive pathways both Na^+ and Ca^{2+} have been shown to influence amiloride blocking potency, we tested their effects on amiloride block of the oocyte MS channel.

Figure 1 compares single MS channel current-voltage (I–V) relations measured in 100 mM external Na^+ with either zero or 1.8 mM Ca^{2+}. The inclusion of 1.8 mM Ca^{2+} in Na^+ pipette solution caused a significant reduction in inward MS channel currents (see single channel currents in Figure 1 insert) and gave a slope conductance measured at − 100 mV of 26 pS compared with 86 pS measured in Ca^{2+}-free Na^+ solution (Figure 1). This blocking effect of Ca^{2+} on the MS channel has been modelled previously (Taglietti & Toselli, 1988; Yang & Sachs, 1989), in terms of a 2 barrier single site permeation model in which Ca^{2+} inhibits current because of its higher affinity for a channel ion binding site compared with Na^+ and K^+.

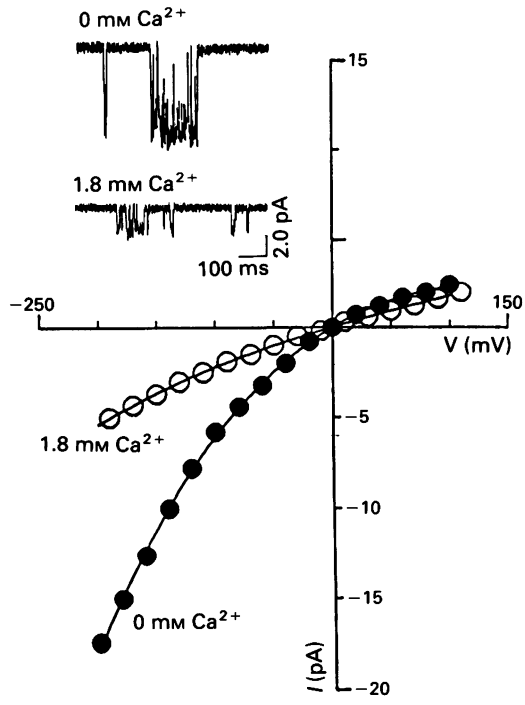

Figure 1 External Ca^{2+} block of the mechanosensitive (MS) channel. Single MS channel current-voltage relations measured from two different cell-attached patches in which the pipette solution contained either (in mM): NaCl 100, $CaCl_2$ 1.8, HEPES 10 (NaOH) (○) or NaCl 100, EGTA 10 (NaOH), HEPES 10 (NaOH) (●). The solid curves were fitted to the data by eye. The single MS channel current recordings shown in the insert were recorded at a patch potential of − 100 mV in each solution.

Figure 2 shows the I–V relations as a function of amiloride concentration and the corresponding Hill plots at − 100 mV for three different ionic solutions. The general character of the amiloride block was not changed in that the voltage-dependence was unaltered. However, the presence of Ca^{2+} caused a two fold reduction in amiloride potency. One possible explanation for this reduction is that Ca^{2+} screens surface negative charges and in this way reduces the effective amiloride surface concentration. For example, according to Hille *et al.* (1975), solutions with no divalent ions give a shift of − 18 mV in the surface potential of frog myelinated nerve relative to solutions with 2 mM Ca^{2+}. Figure 2a represents data from the initial characterization (Lane *et al.*, 1991) and are presented to facilitate comparison. Figure 2b shows data recorded when Na^+ was substituted for K^+. Consistent with previous studies of MS channels in both oocytes (Taglietti & Toselli, 1988) and hair cells (Corey & Hudspeth, 1979), we found that the MS channel discriminates weakly between Na^+ and K^+. The slope conductance measured at − 100 mV in the Na^+ solution was 86 pS compared with 91 pS K^+ solution. Figure 2c shows I–V relations obtained when 1.8 mM Ca^{2+} was included in the Na^+ solution. Although Ca^{2+} itself blocks the MS inward current by a factor of 4 or more at hyperpolarized potentials, amiloride causes an additional block which is qualitatively similar to that seen in Figure 2a and b. The solid curves fitted to the I–Vs in Figure 2a–c are the predicted relationships based on an extension of a model introduced previously (Lane *et al.*, 1991; 1992). The original model assumed a voltage-dependent conformational change of the MS channel followed by co-operative, voltage-independent binding of two blocking molecules. We have found that the reduction in amiloride potency caused by Ca^{2+} can be adequately explained by surface charge screening effects. In the extended model both the amiloride concentration and channel conformation are weighted by a Boltzmann term containing the shift in surface potential and this extended model is represented mathematically by Equation 1.

$$I_B = I_O \frac{1}{1 + ([B]/K_1 + [B]^2/(K_1K_2))(K_C/(1 + K_C))}$$

$$[B] = [B]_O f \qquad (1)$$

$$K_C = K_{OC} \exp\left\{-\frac{zF}{RT}\delta V\right\}/f$$

$$f = \exp\left\{-\frac{zF}{RT}\psi\right\}$$

In this equation, I_B and I_O are the MS currents in the presence and absence blocker, respectively. $[B]_O$ is the blocker concentration in the bulk aqueous phase, $[B]$ is the surface concentration of the blocker, K_1 and K_2 are the voltage independent dissociation constants for the first and second ligand binding, respectively. K_C and K_{OC} are the equilibrium constants at V and at V = 0, respectively, for the conformational change and δ describes the voltage-dependence of this change. f is the Boltzmann factor for the surface potential effect. ψ represents the change in the surface potential due to Ca^{2+} screening (see Hille, 1992) and was considered to be zero in the absence of Ca^{2+} and allowed to vary to give the best fit in the presence of Ca^{2+}. z, F, R, T and V have their usual meaning. By use of this model the difference in amiloride blocking potency measured in the presence and absence of Ca^{2+} could be explained by assuming the surface potential was increased by 23 mV when Ca^{2+} was present with no accompanying change in the rate constants K_1 (1.25 mM) and K_2 (160 μM). Analysis of the Hill plots for the K^+, Na^+ and Na^+ plus Ca^{2+} solutions gave IC_{50}s of 0.5 mM, 0.5 mM and 1.1 mM, respectively, and Hill coefficients of 2.0, 1.8 and 1.8, respectively.

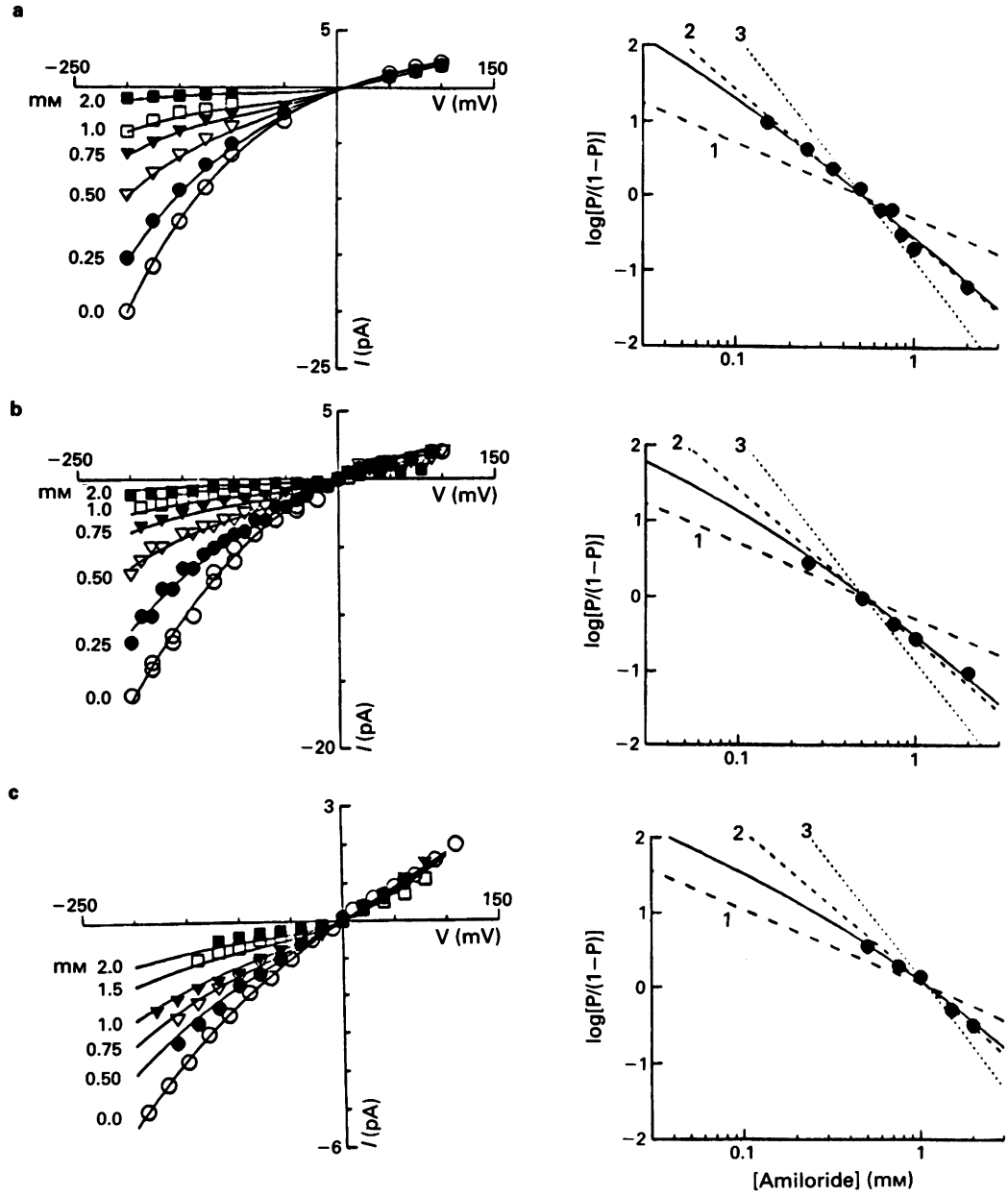

Figure 2 Single mechanosensitive (MS) channel current-voltage relations and Hill plots indicating the voltage- and concentration-dependence of amiloride block recorded under various ionic conditions. All measurements were from cell-attached patches and represent pooled data for each drug concentration from 2–4 patches. In all panels the solid lines fitted to the data were derived from the model described by equation 1. For all fits, $\delta = 0.65$ and $K_{OC} = 0.1$. The amiloride block in (a) (in 100 mM K^+ and 0 mM Ca^{2+}) produced values of $K_1 = 5$ mM (the binding of the first molecule) and $K_2 = 50$ μM (the binding of the second molecule) and for (b) (100 mM Na^+ and 0 mM Ca^{2+}), K_1 and $K_2 = 1.25$ mM and 160 μM, respectively, and (c) (100 mM Na^+ and 1.8 mM Ca^{2+}) K_1 and $K_2 = 1.25$ mM and 160 μM, respectively. For (a) and (b) the surface potential change was assumed to be zero. While for (c), K_1 and K_2 were set at the same values as in (b), and the best fit for the surface potential change gave a value of −23 mV. Hill plots calculated from MS channel currents measured at − 100 mV gave Hill coefficients of 2 (a), 1.8 (b) and 1.8 (c). $P = I/I_O$ where I and I_O are the single MS channel currents in the presence and absence of blocker, respectively. The broken lines show the slopes predicted for Hill coefficients (n) of 1, 2 and 3.

The above experiments indicate that the presence of Na^+ or Ca^{2+} does not potentiate the amiloride block of the oocyte MS channel. Furthermore, we found no significant change in blocking potency (i.e. $IC_{50} = 1.2$ mM) when 1 mM Mg^{2+} was included with Ca^{2+} in the Na^+ Ringer (i.e. 1.8 Ca^{2+}, 100 Na^+, 1 Mg^{2+}).

Discussion

This study indicates that differences in external ionic recording conditions are unable to explain the 10 fold difference in potency obtained for amiloride block of the oocyte (Lane *et al.*, 1991) compared with the hair cell MS channel (Jorgensen & Ohmori, 1988). Neither the presence of high (100 mM) external K^+nor the absence of Na^+, Ca^{2+} and Mg^{2+} in the original oocyte recording solution (Lane *et al.*, 1991) can explain the lower potency of amiloride block in this preparation. In fact the presence of a physiological Ca^{2+} concentration reduces the potency of amiloride block of the oocyte MS channel. If Ca^{2+} has a similar effect on amiloride block of the hair cell, then this drug may be an even more potent blocker *in situ* than previously found (i.e. $IC_{50} < 50$ μM). Ca^{2+} concentration in the endolymph that normally baths

the hair cell transducing apparatus has been measured as 65 μM (see Crawford *et al.*, 1991), which is much lower than the 2 mM used in artificial endolymph solution (Jorgensen & Ohmori, 1988; Ruesch *et al.*, 1991).

Although Ca^{2+} and amiloride block MS channels they do so by apparently different mechanisms. Ca^{2+} block can be modelled in terms of a permeation mechanism in which Ca^{2+} displays a higher affinity for an ion binding site compared with Na^+ and K^+ and in this way inhibits ion conduction through the channel (Taglietti & Toselli, 1988; Yang & Sachs, 1989). In contrast, the voltage- and concentration-dependence of amiloride block is more consistent with a voltage-dependent conformational change of the MS channel followed by co-operative, voltage-independent binding of two amiloride molecules (Lane *et al.*, 1991). We have shown that the effect of Ca^{2+} of reducing amiloride blocking potency can be modelled as surface charge screening effect. For example, it was possible to fit data obtained in the presence of Ca^{2+} by use of identical amiloride binding constants to those used in the absence of Ca^{2+} by including a surface potential Boltzmann coefficient (see Figure 2b and c). Despite the adequate fit we cannot exclude alternative explanations such as direct effects of Ca^{2+} on either amiloride binding reactions or on the proposed voltage-dependent conformational change of the channel.

The higher potency of amiloride block of hair cell compared with oocyte MS channels may reflect structural differences in the 'amiloride receptors' on the MS channels. However, the finding that the two MS channels display the same order of sensitivity to block by a series of amiloride analogues (Ruesch *et al.*, 1991; Lane *et al.*, 1992) indicates they are more similar to one another than to other non-MS amiloride-sensitive ion pathways, such as the epithelial Na^+ channel, the Na^+-H^+exchanger and the Na^+-Ca^{2+} exchanger (see Lane *et al.*, 1992; Hamill *et al.*, 1992). It may be that physical or structural features, not directly associated with the amiloride binding site, limit amiloride's access to an otherwise identical amiloride receptor on the two MS channel molecules.

This study indicates further differences between amiloride block of the epithelial Na^+ and the MS cation channel. In the case of the epithelial Na^+ channel the absolute potency of amiloride block has been shown to vary with changes in either extracellular Na^+ and Ca^{2+} ions (Cuthbert & Wong, 1972; Benos *et al.*, 1979; Garty & Benos, 1988, but see Desmedt *et al.*, 1991). In contrast, this study indicates that amiloride block of the oocyte MS channel is relatively insensitive to whether Na^+ or Ca^{2+} is present or absent in the extracellular solution.

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