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Structure-activity relations of amiloride and its analogues in blocking the mechanosensitive channel in *Xenopus* oocytes

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1 Patch clamp recording techniques have been used to compare the block caused by amiloride and some of its structural analogues of the mechanosensitive (MS) cation selective channel in frog (*Xenopus laevis*) oocytes.

2 Like amiloride, the amiloride analogues dimethylamiloride (DMA), benzamil and bromohexamethyleneamiloride (BrHMA) block the MS channel in a highly voltage-dependent manner.

3 All analogues tested were more potent blockers than amiloride with IC_{50}'s of 500 μM (amiloride), 370 μM (DMA), 95 μM (benzamil) and 34 μM (BrHMA).

4 Hill plots gave Hill coefficients of 2 (amiloride), 1.8 (DMA), 1 (benzamil) and 1.2 (BrHMA) indicating that the binding of two ligand molecules may be necessary for the block caused by amiloride, DMA and possibly BrHMA whereas only a single ligand molecule may be required for the block by benzamil.

5 The potential use of BrHMA as a light-activated, covalent label of the MS channel protein is discussed.

6 The amiloride analogue 'fingerprinting' of the blocking site on the MS channel indicates it is structurally different from previously described amiloride-sensitive ion transport pathways but may be related to the amiloride binding site on outer hair cells of the ear.

Keywords: Mechanosensitive channel; amiloride analogues; structure-activity; *Xenopus* oocytes

Introduction

The mechanosensitive (MS) channel remains the only major class of membrane ion channel molecule for which there is no structural information. It has been proposed that due to its critical role in cell volume regulation (Hamill, 1983; Christensen, 1987), the MS channel was the first channel molecule to have evolved (Hille, 1989). Furthermore, since the MS channel is the only channel class shared by prokaryotes and eukaryotes (Martinac *et al.*, 1987), Hille (1989) has speculated that it may be the ancestor or 'channel prototype' for other classes of membrane ion channels, namely the ligand-gated and voltage-gated channels. For these reasons, there is major interest in obtaining structural/sequence information on the MS channel molecule. However, a major problem for both protein purification and physiological studies is the absence of specific, high affinity ligands for this molecule. Such ligands proved crucial in the isolation and identification of other membrane ion channels, such as tetrodotoxin for the voltage-gated Na^+ channel (Agnew *et al.*, 1978), α-bungarotoxin for the acetylcholine receptor channel (Changeux *et al.*, 1970) and a light-activated strychnine analogue for the glycine receptor channel (Graham *et al.*, 1981).

Recently, we have demonstrated that amiloride blocks a MS cation selective channel in *Xenopus* oocytes (Lane *et al.*, 1991). Our original interest in this compound was based on previous reports that amiloride blocked mechanotransduction in a variety of systems including the lateral line organ of *Necturus*, the frog skin and the chick ear hair cell (reviewed in Jorgensen & Ohmori, 1988). Our studies indicated that amiloride blocks the MS channel in a cooperative, voltage-dependent manner with an IC_{50} of 500 μM. Although amiloride does block the channel, the IC_{50} is too high for its use in purification studies. In an attempt to find higher affinity blockers we have screened a number of structurally related amiloride analogues (Kleyman & Cragoe, 1988; 1990) for their effectiveness in blocking the MS channel.

Amiloride is known to block at least three other distinct ion transport pathways, namely, the epithelial Na^+ channel, the Na^+-Ca^{2+} exchanger and the Na^+-H^+ exchanger (Kleyman & Cragoe, 1988; 1990). Therefore, at the same time as screening for a high affinity blocker of the MS channel we were also interested in 'fingerprinting' this channel with respect to block by different amiloride analogues and thus determine to which, if any, of the three amiloride blockable pathways the MS channel is most related. For this reason we initially chose benzamil, dimethylamiloride (DMA) and hexamethyleneamiloride (HMA) because these compounds are known to be potent blockers of either one or more of the above ion transport pathways.

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). 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). The pipette solution contained (in mM): KF 80, KCl 20, EGTA (KOH), 10 and HEPES (KOH), 10; pH 7.2. The amiloride analogues were dissolved in the pipette solution in concentrations ranging from 10 μM to 1 mM. Previous control experiments (Lane *et al.*, 1991) in which KCl was used instead of KF gave identical results. The bath solution for all recordings contained (in mM): NaCl 115, $CaCl_2$ 1.8,

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KCl 1.5, HEPES (NaOH) 10, 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) and amplitudes were determined during subsequent playback directly from the oscilloscope screen.

Figure 1 illustrates the structure of amiloride and the three analogues that were studied. Amiloride was obtained from Sigma and the three analogues were generous gifts provided by Dr Thomas Kleyman. Due to availability, bromohexamethyleneamiloride (BrHMA) was used in these studies instead of HMA. Kleyman & Cragoe (1990) have shown bromo- substitution does not significantly alter potency of HMA at sites previously tested.

Results

Figure 2 describes the single MS channel current-voltage relationship at several analogue concentrations for each of the analogues tested. The data shown in Figures 2a and 3a are taken from our previous study (Lane *et al.*, 1991) and are presented for comparison. As a general observation all three analogues were more potent than amiloride in blocking the MS channel and showed the same voltage-dependence in that the block was relieved at positive potentials. The solid lines in Figure 2a, b and d are the predicted relationship based on a previously described model which assumes a voltage-dependent conformational change of the MS channel followed by cooperative, voltage-independent binding of two blocking molecules (Lane *et al.*, 1991). However, the data in Figure 2c were adequately described by assuming a single binding site. Equation 1 represents these models mathematically (see also equation A3 in Lane *et al.*, 1991. However, note that a minus sign in the voltage term of equations A2 and A3 was inadvertently omitted).

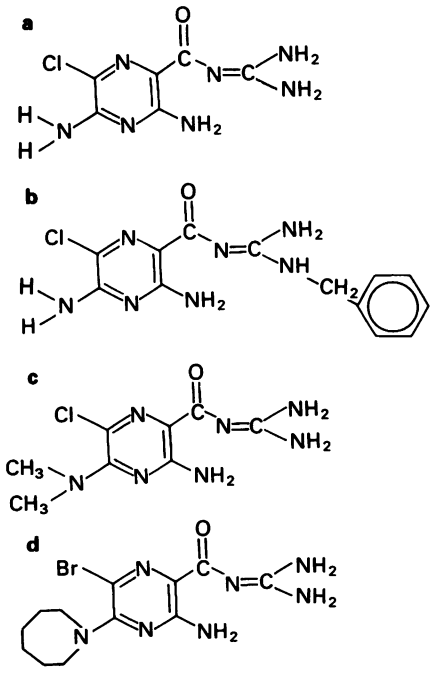

Figure 1 The structure of amiloride (a) and three amiloride analogues: (b) benzamil; (c) dimethylamiloride and (d) bromohexamethyleneamiloride. Amiloride is a pyrazinoylguandine bearing amino groups in the 3- and 5- positions and a chloro group in the 6-position of the pyrazine ring.

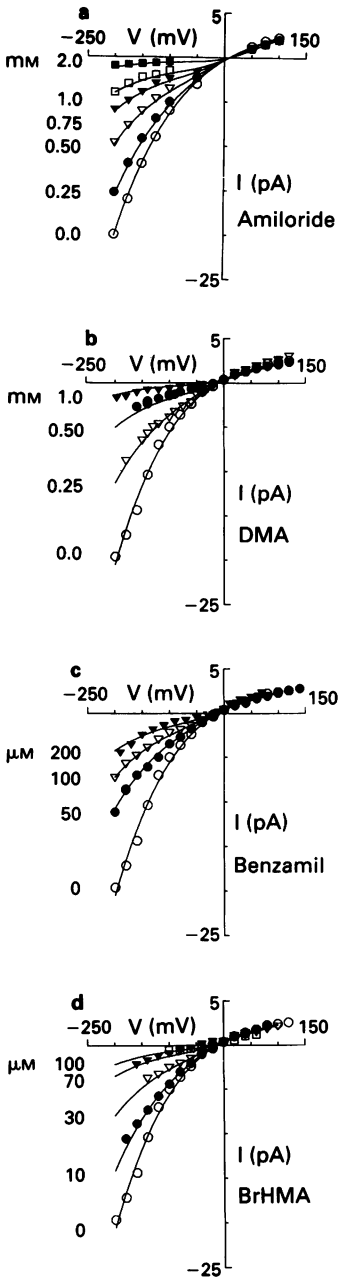

Figure 2 Single channel current-voltage relationship for amiloride and the three analogues. All measurements were from cell-attached patches and represent pooled data for each drug concentration from 2–4 patches. The solid lines fitted to the data were derived from the model described in equation 1. The model assumes a voltage-dependent conformational change with two voltage-independent, cooperative binding sites for amiloride, dimethylamiloride (DMA) and bromohexamethyleneamiloride (BrHMA) and a single voltage-independent site for benzamil and predicts the voltage- and concentration-dependent block of single MS channel currents by extracellular blocker. For all fits, $K_{oc} = 0.1$ and $\delta = 0.65$. (a) For amiloride $K_1 = 5$ mM (the binding of the first molecule); and $K_2 = 50$ μM (the binding of the second molecule). (b) DMA, K_1 and $K_2 = 1.6$ mM and 57 μM, respectively. (c) Benzamil, $K_1 = 56$ μM. (d) BrHMA, K_1 and $K_2 = 23$ μM and 186 μM, respectively.

$$I_B = I_0 \frac{1}{1 + \left(\frac{[B]}{K_1} + \frac{[B]^2}{K_1 K_2}\right)\left(\frac{K_c}{1 + K_c}\right)} \qquad \text{Eqn.(1)}$$

$$K_c = K_{oc} \exp\left\{\frac{-zF}{RT}\,\delta\,V\right\}$$

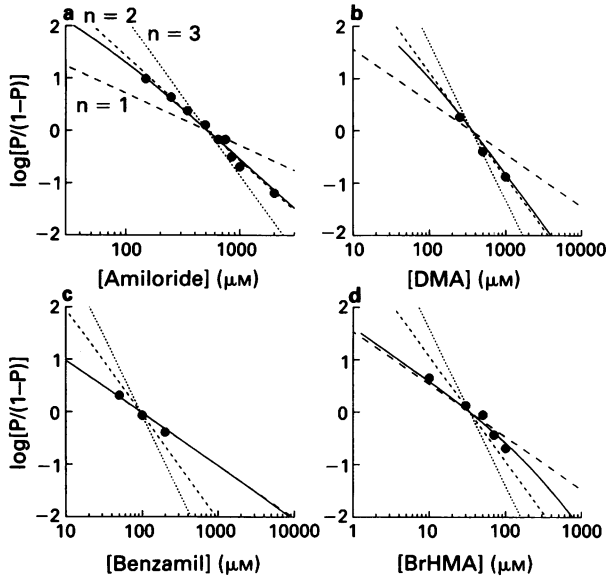

Figure 3 Hill plots calculated from MS channel currents measured at −100 mV with Hill coefficients of 2 (amiloride), 1.8 (dimethylamiloride, DMA), 1 (benzamil) and 1.2 (bromohexamethyleneamiloride, BrHMA). $P = I/I_0$, where I and I_0 are the single MS channel currents in the presence and absence of blocker, respectively. The solid line is the prediction of the conformational model drawn using eqn (1), parameters listed in Figure 2 legend. The interrupted lines show the slopes predicted for Hill coefficients (n) of 1, 2 and 3.

In this equation, I_B and I_0 are the MS currents in the presence and absence of blocker, respectively. [B] is the blocker concentration, 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. z, F, R, T and V have their usual meaning. When applying the model to benzamil, the term in the denominator containing K_2 is omitted. In cases where two sites are necessary, they are not assumed to be identical. The model does not distinguish between the possibilities that (a) the second binding site is always accessible but with negligible binding until the first site is occupied and (b) the second site is inaccessible or nonexistent until the first site is occupied at which time the second site becomes available.

Figure 3 shows the Hill plots for amiloride and the three analogues. The Hill coefficients were 2.0 (amiloride), 1.8 (DMA), 1.0 (benzamil) and 1.2 (BrHMA). The Hill coefficients indicate that there are probably two binding sites for amiloride and DMA and possibly BrHMA while a single binding site is sufficient for the benzamil block. From the Hill plots the IC_{50} of the block was determined for each compound. Based on these IC_{50}'s (included in parentheses) the relative potency is BrHMA (34 μM) > benzamil (95 μM) > DMA (370 μM) > alimoride (500 μM).

Discussion

The main goal of this study was to find a MS channel blocking ligand of higher affinity than amiloride. Our results demonstrate that the photoactivatable amiloride analogue, BrHMA, blocks the channel with an IC_{50} of 34 μM which is an order of magnitude more potent that amiloride. This IC_{50} value is comparable to or better than other compounds such as streptomycin and quinidine, which have been shown to block mechanotransduction in other systems (Martinac, 1992). Although the lanthanide gadolinium is a more potent blocker of the oocyte MS channel (Yang & Sachs, 1989), BrHMA may prove more useful in isolation and purification procedures because of its potential application, when photoactivated and radiolabelled, as a covalently bound label. Photolysis of 6-bromo amiloride analogues has been used previously to identify putative subunits of the epithelial Na^+ channel (Kleyman *et al.*, 1986; Benos *et al.*, 1987) and the Na^+/H^+ exchanger (Friedrich *et al.*, 1986). The relative high affinity of BrHMA for the MS channel may make it a good candidate for this technique.

Comparison of the order of blocking potency of a series of analogues can serve to classify the binding site of the receptor and enable comparison with the binding sites on other amiloride-sensitive ion transport pathways. Table 1 summarizes the results of the analogues we tested and gives their relative potencies in blocking three other amiloride-sensitive ion transport pathways (Kleyman & Cragoe, 1990). Also included in the table are recent results, reported in abstract form, of the analogue block of the transducer currents recorded from mouse outer hair cells (Rüsch *et al.*, 1991). In contrast to the qualitative differences observed between MS channels and other transport pathways, which would suggest structurally different binding sites, the MS channels in the oocyte and the outer hair cell display an identical order of potencies and differ only in their IC_{50}s. A similar quantitative difference in amiloride block at the two sites has been noted previously (cf, Jorgensen & Ohmori, 1988; Lane *et al.*, 1991). Our ability to 'fingerprint' (i.e., determine the relative potency of specific analogues) the amiloride receptor of the MS channel should prove particularly useful in identifying the role of MS channels in different physiological functions. For example, amiloride blockable pathways have been implicated in a variety of physiological functions including volume regulation, fertilization and cell proliferation (Benos, 1982). However, the exact pathway or step which amiloride blocks in these processes is not known. By fingerprinting a particular function with respect to its block by amiloride analogues and comparing it to the MS channel fingerprint it may be possible to determine the involvement of mechanosensitivity in that particular function.

Table 1 Relative potency of block by amiloride and its analogues of various ion transport pathways

Transport pathway	*Amiloride* [μM]	*Benzamil*	*DMA*	*BrHMA or HMA**
MS channel *Xenopus* oocyte	1 [500.0]	5.3	1.4	14.7
MS channel hair cell	1 [53.0]	6.0	1.3	11.0*
Epithelial Na^+ channel	1 [0.34]	9.0	< 0.04	<0.04*
Na^+-Ca^{2+} exchanger	1 [1100.0]	11.0	2.0	11.0*
Na^+-H^+ exchanger	1 [84.0]	<0.08	12	524.0* 566.0

The relative IC_{50} (IC_{50} amiloride/IC_{50} analogue) for amiloride and analogue block of MS channel and other ion transport pathways. The actual IC_{50} of the amiloride block for each pathway is listed in parentheses (in μM). The oocyte MS channel data are from this study, the outer hair cell data from Rüsch *et al.* (1991) and the other data from Kleyman & Cragoe, 1988; 1990. The data with an * are for HMA, otherwise the data are for BrHMA. In the case of the Na^+/H^+ exchanger, data for both analogues were available.
DMA: dimethylamoride; BrHMA: bromohexamethyleneamiloride; HMA: hexamethyleneamiloride.

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