Extracellular amiloride inhibits all known DEG/ENaC ion channels, including BNC1, a proton-activated human neuronal cation channel. Earlier studies showed that protons cause a conformational change that activates BNC1 and exposes residue 430 to the extracellular solution. Here we demonstrate that, in addition to blocking BNC1, amiloride also exposes residue 430. This result suggested that, like protons, amiloride might be capable of activating the channel. To test this hypothesis, we introduced a mutation in the BNC1 pore that reduces amiloride block, and found that amiloride stimulated these channels. Amiloride inhibition was voltage-dependent, suggesting block within the pore, whereas stimulation was not, suggesting binding to an extracellular site. These data show that amiloride can have two distinct effects on BNC1, and they suggest two different interaction sites. The results suggest that extracellular amiloride binding may have a stimulatory effect similar to that of protons in BNC1 or extracellular ligands in other DEG/ENaC channels.

A characteristic shared by DEG/ENaC cation channels is that extracellular amiloride inhibits their current. The ability of amiloride to inhibit the human epithelial Na⁺ channel (ENaC) makes it clinically useful as a diuretic (1). Amiloride-sensitivity is also a valuable marker of currents generated by other DEG/ENaC channels from vertebrates, insects, mollusks, and nematodes (2, 3). However, the mechanism of amiloride’s action is not understood. For example, some data suggest that amiloride blocks current by occluding the ion-conducting pore (4), whereas other evidence suggests that amiloride binding may have a stimulatory effect similar to that of protons in BNC1 or extracellular ligands in other DEG/ENaC channels.

Paradoxical Stimulation of a DEG/ENaC Channel by Amiloride*

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Christopher M. Adams‡, Peter M. Snyder, and Michael J. Welsh§

From the Howard Hughes Medical Institute and Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242

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§ Investigator of the Howard Hughes Medical Institute. To whom correspondence should be addressed: Howard Hughes Medical Institute, University of Iowa College of Medicine, 500 EMBR, Iowa City, IA 52242. Tel.: 319-335-7618; Fax: 319-335-7623; E-mail: mjwelsh@blue.weeg.uiowa.edu.

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The abbreviations used are: MTS, methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate bromide; MTSET, 2-(trimethylammonium)ethyl methanethiosulfonate bromide (MTS(ethyl)); and NHEPS, sodium 2-sulfonatoethyl methanethiosulfonate (MTSES).
Amiloride Activation of BNC1

RESULTS

Zn$^{2+}$ Reversibly Activates BNC1-G430C—In earlier work (15) we showed that when Gly$^{430}$ was mutated to cysteine, covalent modification of residue 430 by MTS reagents irreversibly locked BNC1-G430C in an activated state. Therefore we hypothesized that a noncovalent interaction between Zn$^{2+}$ and Cys$^{430}$ might also activate the channel, but in a reversible way. Fig. 1A shows that extracellular Zn$^{2+}$ had no effect on current in oocytes expressing wild-type BNC1. However, at neutral extracellular pH, Zn$^{2+}$ reversibly activated current in oocytes expressing BNC1-G430C (Fig. 1B). The EC$_{50}$ for activation of BNC1-G430C by Zn$^{2+}$ was approximately 300 $\mu$M (Fig. 1C).

Previous studies (15) showed that pH-dependent activation of BNC1-G430C allowed MTS compounds to modify Cys$^{430}$. Fig. 1D shows that activation by Zn$^{2+}$ also allowed the MTS compound, MTSES, to modify Cys$^{430}$ and irreversibly activate the channel. How might both Zn$^{2+}$ and MTSES interact with Cys$^{430}$? Because the BNC1 channel is a homomultimer composed of multiple BNC1 subunits (17), it is likely that within one channel there are multiple cysteines with which these agents may interact. Taken together, these data indicate that Zn$^{2+}$ binds to Cys$^{430}$ to reversibly activate BNC1-G430C.

Amiloride Increases the Accessibility of Cys$^{430}$ to Zn$^{2+}$ and MTS Reagents—Fig. 2A shows that 100 $\mu$M Zn$^{2+}$ activated BNC1-G430C and that current was blocked by 1 mM amiloride. However, as shown in Fig. 2B and C, Zn-activated currents were not smaller but larger if a submaximal concentration of amiloride (100 $\mu$M) was present. In the absence of Zn$^{2+}$, amiloride either had no effect (Fig. 2B) or blocked a small amount of current (Fig. 2C), depending on the amount of basal current generated by BNC1-G430C. The combined effects of amiloride and Zn$^{2+}$ concentrations are shown in Fig. 2D; at low concentrations, amiloride enhanced Zn$^{2+}$ stimulation of current, and amiloride blocked at high concentrations. Enhanced stimulation in the presence of intermediate amiloride concentrations may also explain the transient current stimulation observed on washing out high concentrations of amiloride and Zn$^{2+}$ (Fig. 2A).

These data show that amiloride alone does not stimulate current, but when both amiloride (intermediate concentrations) and Zn$^{2+}$ are present, current is greater than with Zn$^{2+}$ alone. One interpretation of these results is that amiloride binds to the channel, and in so doing makes Cys$^{430}$ more accessible for interaction with Zn$^{2+}$. In this respect, amiloride might be similar to protons; a decrease in extracellular pH causes a conformational change that exposes Cys$^{430}$ to the extracellular solution where it can be irreversibly modified by MTS reagents (15).

Therefore, we hypothesized that amiloride causes a conformational change that exposes Cys$^{430}$ to the extracellular solution. To test this hypothesis, we asked if amiloride would allow MTS reagents to irreversibly modify and activate the channel at pH 7.4. Fig. 3A shows that in the absence of amiloride, MTSEA had little or no effect at pH 7.4. Current remained low when amiloride and MTSEA were simultaneously present in the extracellular solution. However, once amiloride and MTSEA were removed, it was apparent that a large current had been activated; this current was blocked by subsequent treatment with amiloride. We obtained similar results with two other MTS reagents, MTSET and MTSES (Fig. 3, B and C). These results indicate that in the presence of amiloride, MTS reagents irreversibly modified Cys$^{430}$, locking the channel open. Because the activated channel remained sensitive to amiloride block, current was only apparent after amiloride was removed from the extracellular solution.

The G437C Mutation Reduces Sensitivity to Amiloride Block—Extracellular amiloride and protons shared the ability to expose Cys$^{430}$ to MTS compounds. However, an important difference between the two interventions was that protons activated current (15), whereas amiloride alone did not. We hypothesized that amiloride might have two effects; like protons, amiloride might bind to one site to stimulate current, but in addition, amiloride might bind to a second site to block the channel. Thus, any stimulatory effect would be obscured by the

FIG. 1. Zn$^{2+}$ reversibly activates BNC1-G430C. A, effect of extracellular Zn$^{2+}$ on wild-type BNC1; B, BNC1-G430C; C, effect of Zn$^{2+}$ concentration on current in BNC1-G430C. Data are mean ± S.E. of percent maximal activation, n = 7. D, Zn$^{2+}$ allows MTSES (abbreviated ES) to irreversibly activate BNC1-G430C. Zn$^{2+}$ (100 $\mu$M), MTSES (5 mM), and amiloride (1 mM) were present during times indicated by bars. We previously showed that MTSES alone has no effect on BNC1-G430C when extracellular pH is 7.4 (see Ref. 15 and Fig. 3C). In all figures, the 0 indicates zero current and membrane voltage was -60 mV.

FIG. 2. Amiloride enhances stimulation by extracellular Zn$^{2+}$. A, effect of 1 mM amiloride on current activated by 100 $\mu$M Zn$^{2+}$; B, effect of 100 $\mu$M amiloride and 100 $\mu$M Zn$^{2+}$; C, effect of 100 $\mu$M amiloride and 300 $\mu$M Zn$^{2+}$; D, effect of Zn$^{2+}$ concentration in the presence of indicated concentrations of amiloride. Data are mean ± S.E. from at least six oocytes. All experiments were performed with BNC1-G430C.
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ability of amiloride to simultaneously block the channel. To test this hypothesis, we asked whether the inhibitory effect of amiloride could be selectively abolished by mutation. We tested a mutation equivalent to G437C had several effects; if present in the β or γ subunits of the channel, the mutations reduced amiloride block and Na^+ conductance. Also, the introduced cysteine could be modified by MTSET to inhibit current (17, 18). However, if present in all ENaC subunits, the mutations abolished channel activity (18).

To determine whether the G437C mutation reduced amiloride inhibition, we studied it in the context of BNC1-G430V, which generates large constitutive currents (9, 15). BNC1-G430V generated large currents (904 ± 84 nA; n = 9) that were blocked by amiloride (Fig. 4B). Expression of the double mutant BNC1-G430V/G437C failed to generate current; this was consistent with the observation that mutations abolish ENaC function if present in all subunits of that channel (18). Therefore, we coexpressed BNC1-G430V/G437C with a lesser amount of BNC1-G430V (5:1 ratio). This combination generated no current on its own; basal current (133 ± 13 nA; n = 10) was not different from that in uninjected cells (not shown). However, addition of amiloride stimulated current (Fig. 4D). In contrast, amiloride did not stimulate wild-type BNC1 (Fig. 4E). Simulation was reversible and evident only at concentrations greater than 100 μM, an increase in current at 1 mM, followed by inhibition at 3 mM. However, because the G430V Deg mutation was present in every subunit, a stimulatory effect of amiloride would be minimized.

Amiloride Stimulates Current in Cells Expressing BNC1-G430V/G437C with Wild-type BNC1—To test whether amiloride might stimulate BNC1-G430V/G437C, we coexpressed it with a small amount of wild-type BNC1 (5:1 ratio). This combination generated no current on its own; basal current (133 ± 13 nA; n = 10) was not different from that in uninjected cells (not shown). However, addition of amiloride stimulated current (Fig. 4D). In contrast, amiloride did not stimulate wild-type BNC1 (Fig. 4E). Simulation was reversible and evident only at concentrations greater than 100 μM, an increase in current at 1 mM, followed by inhibition at 3 mM. However, because the G430V Deg mutation was present in every subunit, a stimulatory effect of amiloride would be minimized.

Amiloride May Interact with Two Different Sites in BNC1—Because amiloride had two effects (stimulation and inhibition), we hypothesized it might interact with two separate sites in BNC1. To test this idea, we determined the effect of voltage on inhibition and stimulation. Because amiloride is positively charged, effects should be voltage-dependent if the binding site lies within the membrane electrical field. To test inhibition, we studied BNC1-G430V. Block was steeply voltage-dependent, suggesting that amiloride blocked within the membrane electrical field (Fig. 5, A–D). Fig. 5, B and C, shows that block depends on the reversal potential of BNC1 current, suggesting that outward flow of cations prevented amiloride from

Fig. 3. Amiloride exposes Cys^430 to extracellular MTS compounds in BNC1-G430C. Panels A–C, effect of amiloride on modification by 100 μM MTSEA (EA), 100 μM MTSET (ET), or 3 mM MTSES (ES) Amiloride concentration was 300 μM. Panel A, effect of amiloride on modification of Cys^430. Panel B, effect of amiloride on modification of Cys^437. Panel C, effect of amiloride on modification of Cys^430 and Cys^437.
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reaching a blocking site. These results suggest that when amiloride blocks, it binds within the channel pore.

To test stimulation, we studied oocytes expressing BNC1-G430V/G437C with wild-type BNC1. Panel E, current-voltage relationship of amiloride-stimulated current. Amiloride concentration was 1 mM. Data are average ± S.E. from three oocytes. Panel F, families of currents at voltages ranging from −120 mV to +40 mV. Amiloride concentration is indicated.

DISCUSSION

Our data show that amiloride can have two distinct effects on the BNC1 channel and suggest that there may be two different sites of interaction. One site appears to lie within the channel pore; when amiloride interacts with this site it blocks the channel. Three pieces of evidence suggest that amiloride blocks within the channel pore: the voltage dependence suggested block within the membrane electrical field; alteration of the reversal potential and the outward flow of cations reduced block by extracellular amiloride; and block was attenuated by mutation of residue 437, which other data suggest lies within the second membrane-spanning helix (19, 20).

The other site appears to be different from the blocking site; when amiloride interacts there it can cause a conformational change that activates the channel and exposes residue 430 to extracellular MTS reagents. Our data make no predictions about the location of the stimulatory site, except that it is not likely deep within the pore because the transmembrane electrical field has little effect on amiloride-dependent stimulation.

We considered the possibility that amiloride-dependent stimulation might represent the interaction of amiloride at a single site producing open channel block. However, this mechanism does not explain stimulation because net current increased in the presence of amiloride, and amiloride stimulated current in channels that were largely insensitive to amiloride block. Moreover, membrane voltage had an effect on block, but not on stimulation. We also considered the possibility that amiloride might interact with a single binding site that might move during channel gating to give both voltage-dependent and -independent effects. This also seems unlikely, because the G437C mutation selectively attenuated block. Nevertheless, we cannot exclude an interaction at a single site that somehow both blocks and activates the channel; the multimeric nature of BNC1 might allow for such an effect.

Our data suggesting that amiloride may interact with two sites may help explain earlier studies of other DEG/ENaC channels. Studies of ENaC suggest that amiloride might block the pore. First, amiloride block is voltage-dependent (4). Second, amiloride is a more potent blocker in reduced extracellular Na⁺, suggesting that amiloride and Na⁺ may compete for a binding site in the channel pore (4). Third, mutations that alter ion conduction or selectivity also alter amiloride block (4, 18, 20, 21). On the other hand, studies of ENaC identified residues in the predicted extracellular domain that bind an amiloride anti-idiotypic antibody; mutation of those sites altered amiloride sensitivity (5). In addition, nonfunctional ENaC splice variants that lack the pore-forming second transmembrane domain but retain much of the extracellular domain retain binding to phenamil (an amiloride derivative) with high specificity (6). These findings are consistent with an extracellular amiloride-binding site on ENaC. The suggestion of two interaction sites in BNC1 raises the question of whether amiloride will have two effects on all DEG/ENaC family members. Alternatively, some family members may lack one or both sites and may not be inhibited by amiloride.

The large extracellular domain may regulate the function of DEG/ENaC channels. In the FMRFamide-activated Na⁺ channel (FaNaCh), this region may form a ligand-binding site for FMRFamide. In BNC1, ASIC, and DRASIC, this region may form a ligand-binding site for a variety of ligands (22). Consistent with this possibility, mutations in the predicted extracellular domain reduce sensitivity to FMRFamide. In BNC1, ASIC, and DRASIC, this region may be responsible for proton activation (2). In C. elegans DEG/ENaC proteins, genetic studies suggest that this region may interact with extracellular collagen to affect channel gating (23, 24). Our data suggest that there is also an amiloride-binding site in BNC1, perhaps located in the extracellular domain, that can modulate channel activity. Future studies that identify this site may help our understanding of the function of the extracellular domain and could possibly identify agents that activate or inhibit the channels for therapeutic purposes.

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FIG. 5. Voltage dependence of amiloride block and amiloride stimulation. Panels A–D, amiloride block of BNC1-G430V. Panel A, families of BNC1-G430V currents at voltages ranging from −120 to +60 mV. Holding voltage (Vh) was −20 mV. Amiloride concentration is indicated. Panel B, current-voltage relationship from an oocyte expressing large BNC1-G430V currents. For several minutes before current measurements were made, holding voltage was clamped to 0, −40, or −80 mV, as indicated. When holding voltage was −40 or −80 mV, intracellular Na⁺ accumulation and the reversal potential became more negative (27). In oocytes expressing ENaC, a similar increase in intracellular [Na⁺] occurs (26). Once the reversal potential no longer drifted, current was measured during brief 1 s voltage steps in the absence (open symbols) or presence (closed symbols) of 30 µM amiloride. Panel C, current values from panel B normalized to the reversal potential in each condition. Panel D, current-voltage relationship of amiloride-inhibited current. Amiloride concentration was 1 µM (squares), 10 µM (triangles), 100 µM (circles), or 1 mM (triangles). Data are average ± S.E. from six oocytes where Vh = −20 mV. Panels E and F, amiloride stimulation of current in oocytes expressing BNC1-G430V/G437C with wild-type BNC1. Panel E, current-voltage relationship of amiloride-stimulated current. Amiloride concentration was 1 mM (■) or 3 mM (○). Data are average ± S.E. from three oocytes. Panel F, families of currents at voltages ranging from −120 mV to +40 mV. Amiloride concentration is indicated.

2 J. Rogers and M. J. Welsh, unpublished observations.
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