A Single Amino Acid Tunes Ca^{2+} Inhibition of Brain Liver Intestine Na^{+} Channel (BLINaC)

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Ion channels of the degenerin/epithelial Na^{+} channel gene family are Na^{+} channels that are blocked by the diuretic amiloride and are implicated in several human diseases. The brain liver intestine Na^{+} channel (BLINaC) is an ion channel of the degenerin/epithelial Na^{+} channel gene family with unknown function. In rodents, it is expressed mainly in brain, liver, and intestine, and to a lesser extent in kidney and lung. Expression of rat BLINaC (rBLINaC) in Xenopus oocytes leads to small unspecific currents that are only weakly sensitive to amiloride. Here, we show that rBLINaCs is inhibited by micromolar concentrations of extracellular Ca^{2+}. Removal of Ca^{2+} leads to robust currents and increases Na^{+} selectivity of the ion pore. Strikingly, the species ortholog from mouse (mBLINaC) has an almost 250-fold lower Ca^{2+} affinity than rBLINaC, rendering mBLINaC constitutively active at physiological concentrations of extracellular Ca^{2+}. In addition, mBLINaC is more selective for Na^{+} and has a 700-fold higher amiloride affinity than rBLINaC. We show that a single amino acid in the extracellular domain determines these profound species differences. Collectively, our results suggest that rBLINaC is opened by an unknown ligand whereas mBLINaC is a constitutively open epithelial Na^{+} channel.

EXPERIMENTAL PROCEDURES

Molecular Biology—Mouse BLINaC (GenBank accession no. NM_021370) was cloned by PCR from liver and rat BLINaC (GenBank accession no. NM_022227) from brain. Both cDNAs were subcloned into the expression vector pR SSP, which is optimized for functional expression in Xenopus oocytes, containing the 5’-untranslated region from Xenopus β-globin and a poly(A) tail (19). rBLINaC contained a constitutively active channel such as ENaC (2), mechanosensitive ASICs (acid-sensing ion channel) (4) and peptide-gated FaNaC (FMRFamide-gated peptide-gated ion channel) (6, 7).

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FLAG epitope in the extracellular domain (ECD) between amino acids Asp156 and Phe157. Chimeras and single amino acid substitutions were generated by site-directed mutagenesis with KAPA HiFi polymerase (Epicenter) using standard protocols. PCR inserts were completely sequenced. Using the mMessage mMACHINE kit (Ambion, Austin, TX), capped cRNA was generated by SP6 RNA polymerase from linearized plasmids.

Electrophysiology—Oocytes were surgically removed under anesthesia (2.5 g/liter tricainemethanesulfonate for 20–30 min) from adult X. laevis females. Anesthetized frogs were killed after the final oocyte collection by decapitation. Animal care and experiments followed approved institutional guidelines at RWTH Aachen University.

Between 0.08 and 8 ng of cRNA was injected into stage V or VI oocytes of X. laevis. Oocytes were kept in low Na+ OR-2 medium (5 mM NaCl, 77.5 mM N-methyl-d-glucamine, 2.5 mM KCl, 1.0 mM Na₂HPO₄, 5.0 mM HEPES, 1.0 mM MgCl₂, 1.0 CaCl₂, and 0.5 g/liter polyvinylpyrrolidone) at 19 °C and studied 24–48 h after injection. Whole cell currents were recorded with a TurboTec 03X amplifier (npi electronic, Tamm, Germany) using an automated, pump-driven solution exchange system together with the oocyte-testing carousel controlled by the interface OTC-20 (npi electronic) (20). With this system, 80% of the bath solution (10–90%) is exchanged within 300 ms (21). Data acquisition and solution exchange were managed using CellWorks version 5.1.1 (npi electronic). Data were filtered at 20 Hz and acquired at 1 kHz. Holding potential was −70 mV if not stated otherwise. All experiments were performed at room temperature (20–25 °C). The bath solution for two-electrode voltage clamp contained 140 mM NaCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 10 mM HEPES. Low Ca²⁺ bath solutions contained 140 mM NaCl, 10 mM HEPES, 2 mM EDTA or H-EDTA and adequate amounts of CaCl₂ calculated with the program CaBuf (22). Solutions with Ca²⁺ concentrations <1.8 mM were supplemented with 0.1 mM flufenamic acid to block the large conductance induced in Xenopus oocytes by divalent-free extracellular solutions.

Data Analysis—Data were collected and pooled from at least two preparations of oocytes isolated on different days from different animals, if not stated otherwise. Data were analyzed with Student’s unpaired t test.

The permeability ratio P_H/P_Na = Pᵣ was calculated from the shift in reversal potential when proton concentration was raised from 100 nM (pH 7) to 100 μM (pH 4) using the following equation derived from the Goldman-Hodgkin-Katz equation,

$$
\Delta E_{rev} = E_{rev, pH 4} - E_{rev, pH 3} = (RT/F) \ln \left( \frac{[Na^+]_o}{[Ca^2+]_o} \right) + P[[H^+]_o]/([Na^+]_o + P[[H^+]_o])
$$

where R = ideal gas constant, T = absolute temperature, F = Faraday constant, [Na⁺]₀ = [Ca^2+], [Na⁺]₀ = 10⁻³ M, [H⁺]₀ = 10⁻⁴ M, and [H⁺]₀ = 10⁻⁷ M. The effect of Ca²⁺, Mg²⁺, and N-methyl-d-glucamine (NMGD) in the extracellular solution was considered negligible. The intracellular concentration of K⁺ was unknown but equal under both conditions and therefore did not affect the change in reversal potential ΔE_{rev}.

RESULTS

Rat BLINaC Is Strongly Inhibited by Extracellular Ca²⁺—Similar to previous findings (11), Xenopus oocytes expressing rBLINaC had only a slightly increased conductance compared with un.injected oocytes. The amplitudes of the small constitutive currents were in the range of 0.3–1.5 μA and only blocked by high concentrations (4 mM) of amiloride (Fig. 1A). Because the related ASICs are inhibited by extracellular Ca²⁺ (16–18), we wondered whether Ca²⁺ might also inhibit BLINaC. Reducing [Ca²⁺] in the bath solution to 10 nM indeed dramatically increased the amplitude of the amiloride-sensitive (4 mM) current to 8.1 μA (Fig. 1B, upper, representative current trace recorded in the presence of decreasing [Ca²⁺]EMPLATE). Lower, concentration-dependent inhibition of rBLINaC by [Ca²⁺] was recorded. Currents were normalized to the current in the presence of 10 nM [Ca²⁺], n = 10. The dashed line highlights a Ca²⁺ concentration of 1.8 mM. Error bars, S.E. C, upper, representative current trace recorded in the presence of increasing concentrations of amiloride (Amilor); [Ca²⁺] was 1.8 mM, C, lower, concentration-dependent inhibition of rBLINaC by amiloride in physiological [Ca²⁺], (open squares, n = 8); currents were normalized to the current in the absence of amiloride.

To determine the current amplitude of rBLINaC-expressing oocytes with different concentrations of Ca²⁺, we measured the current at 70 mV if not stated otherwise. All experiments were performed at room temperature (20–25 °C). The bath solution for two-electrode voltage clamp contained 140 mM NaCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 10 mM HEPES. Low Ca²⁺ bath solutions contained 140 mM NaCl, 10 mM HEPES, 2 mM EDTA or H-EDTA and adequate amounts of CaCl₂ calculated with the program CaBuf (22). Solutions with Ca²⁺ concentrations <1.8 mM were supplemented with 0.1 mM flufenamic acid to block the large conductance induced in Xenopus oocytes by divalent-free extracellular solutions.

Data Analysis—Data were collected and pooled from at least two preparations of oocytes isolated on different days from different animals, if not stated otherwise. Data were analyzed with the software IgorPro (WaveMetrics, Lake Oswego, OR) and are presented as means ± S.E. Statistical significance was calculated using Student’s unpaired t test.

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where R = ideal gas constant, T = absolute temperature, F = Faraday constant, [Na⁺]₀ = [Ca^2+], [Na⁺]₀ = 10⁻³ M, [H⁺]₀ = 10⁻⁴ M, and [H⁺]₀ = 10⁻⁷ M. The effect of Ca²⁺, Mg²⁺, and N-methyl-d-glucamine (NMGD) in the extracellular solution was considered negligible. The intracellular concentration of K⁺ was unknown but equal under both conditions and therefore did not affect the change in reversal potential ΔE_{rev}.

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Amiloride is an open channel blocker that binds to the outer mouth of the DEG/ENaC pore (23). In ASICs, Ca\(^2+\) also binds to the outer mouth of the ion pore and competes there with amiloride, decreasing its apparent affinity (18). This finding suggested that the low amiloride sensitivity of rBLINaC (11) might be due to strong Ca\(^2+\) block at physiological concentrations of Ca\(^2+\). To test this hypothesis, we determined the amiloride-sensitive currents of oocytes expressing rBLINaC (undiluted cRNA) or mBLINaC (25-fold diluted cRNA), \(n = 8, \star, p < 0.005\). Representative current trace from an oocyte injected with 100-fold diluted mBLINaC. Amiloride-sensitive (100 \(\mu\)M) current was recorded in 1.8 mM and 10 mM [Ca\(^{2+}\)]_e, respectively. Holding potential was \(-70\) mV. C, concentration-dependent inhibition of mBLINaC by [Ca\(^{2+}\)]_e. Currents were normalized to the current in the presence of 10 \(\mu\)M [Ca\(^{2+}\)]_e; \(n = 16\). D, concentration-dependent inhibition of mBLINaC by amiloride; \(n = 10\). Currents were normalized to the current in the absence of amiloride.

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**Application of high concentrations of amiloride (>100 \(\mu\)M; Fig. 2B) completely blocked mBLINaC, and block was half-maximal at ~700-fold lower concentrations of amiloride than for rBLINaC (IC\(_{50}\) = 7.1 ± 0.9 \(\mu\)M, \(n = 16, p < 0.005\); Fig. 2D). In summary, the comparative analysis of rat and mouse BLINaC revealed dramatic differences in the apparent affinity for Ca\(^2+\) and amiloride. Whereas rBLINaC is inhibited by physiological [Ca\(^{2+}\)]_e and is active only in solutions with very low [Ca\(^{2+}\)]_e, mBLINaC is constitutively active at physiological [Ca\(^{2+}\)]_e.

A Single Amino Acid Determines the Different Apparent Affinities for Ca\(^2+\) and Amiloride of Rat and Mouse BLINaC—The functional differences between rat and mouse BLINaC were all the more surprising because their amino acid sequences are 97% identical. To identify the region responsible for the different Ca\(^2+\) affinities of rat and mouse BLINaC, we generated several chimeras and determined the amplitude of the amiloride-sensitive currents (4 mM) in 1.8 mM extracellular Ca\(^{2+}\) as a first indication for the apparent Ca\(^2+\) affinity of the chimeras. First, we exchanged the cytosolic N- and C-terminal domains of rBLINaC by the corresponding domains of mBLINaC, either individually (chimeras “N-term” and “C-term”) or together (“N/C-term”). The amiloride-sensitive current amplitude of these three chimeras was not increased compared with rBLINaC (Fig. 3A), suggesting that the cytosolic domains of rBLINaC do not determine the small current amplitude of these chimeras. In the next step, we exchanged the ECD of rBLINaC in two portions (chimeras “loop1” and “loop2”). Exchange of the first part of the ECD (loop1) did not increase the amplitude of the amiloride-sensitive current (Fig. 3A), whereas exchange of the second part of the ECD (loop2) strongly increased the amplitude of the amiloride-sensitive current to values comparable with mBLINaC (Fig. 3A). Moreover, although we did not systematically investigate amiloride sensitivity of these chimeras, the highly active chimera loop2 was almost completely blocked by 100 \(\mu\)M amiloride, showing that this chimera also had a lower IC\(_{50}\) for amiloride than rBLINaC.

To evaluate apparent Ca\(^2+\) affinity of the chimeras further, we measured current amplitude with 10 mM Ca\(^{2+}\) in the bath solution. This reduction in [Ca\(^{2+}\)]_e strongly increased (3–8-fold; \(n = 5, p < 0.005\)) the current amplitude of all chimeras, except the highly active chimera loop2, for which the current amplitude was only modestly (1.1-fold, \(n = 6\)) increased (results not shown). This response to a reduction in [Ca\(^{2+}\)]_e is in agreement with the idea that all chimeras, except loop2, had a high apparent Ca\(^2+\) affinity and were almost completely blocked by standard [Ca\(^{2+}\)]_e (1.8 mM) and that the loop2 chimera had a
lower apparent Ca\(^{2+}\) affinity, leading to a modest block of this chimera by standard [Ca\(^{2+}\)]\(_{o}\). Together, these results strongly suggest that the second portion of the ECD determines apparent Ca\(^{2+}\) affinity of BLINaC.

Within the second portion of the ECD, mouse and rat BLINaC differ in nine amino acids. We individually substituted these nine amino acids in rBLINaC by those of mBLINaC and determined the amplitude of the amiloride-sensitive (4 mM) currents in 1.8 mM extracellular Ca\(^{2+}\). Eight of the nine substitutions did not significantly increase the current amplitude compared with wt rBLINaC (Fig. 3B). One substitution, however, A387S, dramatically increased the current amplitude of rBLINaC to levels not significantly different from mBLINaC (Fig. 3B). In fact, like mBLINaC, rBLINaC-A387S had to be injected in a 1:25 dilution to get current amplitudes <50 μA. This robust current in the presence of physiological [Ca\(^{2+}\)]\(_{o}\) suggested that the apparent Ca\(^{2+}\) affinity of rBLINaC was strongly decreased by the A387S substitution. This was indeed the case: the apparent Ca\(^{2+}\) affinity of rBLINaC-A387S was 500-fold lower (IC\(_{50}\): 5.0 ± 0.3 mM, n = 11; Fig. 4A) than that of wild-type (WT) rBLINaC and in a millimolar range similar to that of mBLINaC.

Similar to chimera loop2, the rBLINaC-A387S substitution was strongly blocked by 100 μM amiloride (Fig. 3B), and amiloride sensitivity of rBLINaC-A387S was dramatically increased (IC\(_{50}\): 2.9 ± 0.3 μM, n = 14; Fig. 4B) compared with WT rBLINaC and similar to mBLINaC. Thus, the single Ala to Ser substitution at position 387 explains both the different apparent Ca\(^{2+}\) and amiloride affinities of rat and mouse BLINaC.

**Rat and Mouse BLINaC Have Different Ion Selectivities, Which Are Determined by Amino Acid 387**—Besides small current amplitude and low apparent amiloride affinity, WT rBLINaC is characterized by unselectivity for monovalent cations (11), a feature that is rather uncommon for DEG/ENaC channels (1). To investigate ion selectivity of BLINaC, we first determined reversal potentials of rBLINaC, mBLINaC, and rBLINaC-A387S in physiological [Ca\(^{2+}\)]\(_{o}\). The reversal potential of rBLINaC was −9.6 ± 2.0 mV (n = 8; Fig. 5A), similar to previous results (11), whereas it was significantly more positive for mBLINaC (14.6 ± 1.8 mV, n = 10; p < 0.001; Fig. 5A), indicating an increased selectivity for Na\(^{+}\). For rBLINaC-A387S, the reversal potential was similar to mBLINaC (15.4 ± 1.6 mV, n = 8; Fig. 5A), indicating that this amino acid substitution also converted ion selectivity. Oocytes expressing mBLINaC and rBLINaC-A387S had a more positive membrane potential than oocytes expressing rBLINaC (data not shown), suggesting higher intracellular Na\(^{+}\) concentrations in these oocytes due to the much stronger activity of mBLINaC and rBLINaC-A387S. Considering that higher intracellular Na\(^{+}\) concentrations will reduce the Na\(^{+}\) equilibrium potential, a reversal potential of ~15 mV indicates that mBLINaC is a Na\(^{+}\)-selective ion channel.
FIGURE 5. Amino acid 387 determines ion selectivity of BLINaC. A, normalized mean current-voltage relationships of amiloride-sensitive currents of rBLINaC (black circles), and rBLINaC-A387S (gray triangles), determined in 1.8 mM [Ca\(^{2+}\)](e). For rBLINaC, I-V relationships determined in 10 nM [Ca\(^{2+}\)](e) are also shown (gray squares). The holding potential was increased stepwise from −120 to +60 mV (20- or 30-mV steps) in the absence and the presence of amiloride (100 μM for mBLINaC and rBLINaC-A387S, 4 mM for rBLINaC). Currents in the presence of amiloride were subtracted from currents in the absence of amiloride to yield the amiloride-sensitive currents at each holding potential. B, left, representative current traces for rBLINaC, mBLINaC, and rBLINaC-A387S. Currents were recorded at alternating holding potentials of −70 and +50 mV, in 1.8 mM [Ca\(^{2+}\)](e), and 140 mM varying extracellular monovalent cations. B, right, conductances of mBLINaC, rBLINaC, and rBLINaC-A387S. Conductance was calculated according to the following equation: $g = \frac{I}{V_\text{Holding Potential} - V_\text{measured}}/120$ mV. Conductances in the presence of Li\(^+\) and K\(^+\) were normalized to the conductance in the presence of Na\(^+\). C, left, representative current traces for rBLINaC in 1.8 mM and 10 mM [Ca\(^{2+}\)](e). Holding potential was alternated between −70 and +50 mV. C, right, normalized conductances in 1.8 mM and 10 mM [Ca\(^{2+}\)](e); n = 9; **, p < 0.005. D, left, representative current trace for rBLINaC in 1.8 mM and 10 mM [Ca\(^{2+}\)](e). Holding potential was alternated between −70 and +50 mV. C, right, normalized conductances in 1.8 mM and 10 mM [Ca\(^{2+}\)](e); n = 9; **, p < 0.005.

We then determined reversal potentials in low [Ca\(^{2+}\)](e) (0.1 mM). The reversal potential of rBLINaC was significantly more positive (9.7 ± 0.8 mV, n = 9; Fig. 5A) than in physiological [Ca\(^{2+}\)](e), indicating that Ca\(^{2+}\) block of rBLINaC is at least partly responsible for the different ion selectivities of rat and mouse BLINaC. In contrast, reversal potential of mBLINaC in low [Ca\(^{2+}\)](e) (10 nM) was not significantly different (14.4 ± 1.8 mV, n = 9; data not shown) from reversal potential in physiological [Ca\(^{2+}\)](e), as expected from the modest block by Ca\(^{2+}\).

We further investigated ion selectivity by ion substitution experiments; whole oocyte currents were measured in physiological [Ca\(^{2+}\)](e) with varying monovalent cations at a holding potential of −70 mV that was stepped to +50 mV for 5 s every 5 s. This protocol allowed simultaneous monitoring of current amplitude, membrane conductance, and degree of rectification. In these experiments, rBLINaC revealed a significantly larger conductance in the presence of K\(^+\) than in the presence of Na\(^+\) and Li\(^+\) (Fig. 5B). In contrast, mBLINaC had a significantly larger conductance in the presence of Na\(^+\) than in the presence of Li\(^+\) and K\(^+\) (Fig. 5B). These results agree with the more positive reversal potential and a higher Na\(^+\) selectivity of mBLINaC (Fig. 5A). Similarly, for rBLINaC-A387S, ion substitution experiments revealed a significantly larger conductance in the presence of Na\(^+\) than in the presence of Li\(^+\) and K\(^+\) (Fig. 5B).

No strong rectification was observed, except for currents through mBLINaC and rBLINaC-A387S in the presence of K\(^+\), similar to mBLINaC (Fig. 5B). In contrast to mBLINaC, also in low [Ca\(^{2+}\)](e) currents through rBLINaC did not rectify, which is expected if the rectification reflected higher intracellular Na\(^+\) concentrations in oocytes expressing constitutively active mBLINaC. In summary, ion substitution experiments confirmed that mBLINaC is a Na\(^+\)-selective ion channel and that Na\(^+\) selectivity of rBLINaC is higher in low [Ca\(^{2+}\)](e) than in high [Ca\(^{2+}\)](e).

BLINaC Is Permeable for H\(^+\)—The related channels ENaC and ASIC1a also conduct H\(^+\), in addition to Na\(^+\) (4, 24, 25). We therefore investigated H\(^+\) permeability of BLINaC. Unlike ASICs, neither rat nor mouse BLINaC was activated by H\(^+\). In contrast, stepwise reduction of the pH of the bath solution from 7.8 to 4.0 reduced current amplitude of mBLINaC (Fig. 6A), suggesting blockage of the inward (Na\(^+\)) current by H\(^+\). Such a block does, however, not exclude permeation of H\(^+\). Therefore, we completely replaced Na\(^+\) by NMDG\(^+\), which abolished inward currents at −70 mV, indicating that the large NMDG\(^+\) cation does not permeate through mBLINaC. When we increased the H\(^+\) concentration (pH 6.0, 5.0 and 4.0) in these Na\(^+\)-free solutions, we observed a weak inward current that was abolished when the pH was stepped back to 7.4 (Fig. 6A), suggesting that this current was carried by H\(^+\) and that mBLINaC is permeable for H\(^+\). The smaller currents of rBLINaC-expressing oocytes rendered characterization of H\(^+\) permeability of rBLINaC more difficult, but, qualitatively, rBLINaC behaved similarly to mBLINaC.
We further investigated H\(^+\) permeability of mBLINaC by strongly reducing the extracellular Na\(^+\) concentration (1 mM) and looking for a dependence of the reversal potential on the H\(^+\) concentration. Raising the H\(^+\) concentration 1,000-fold from 100 nM to 100 \(\mu\)M (pH 7 to pH 4; Fig. 6B) significantly shifted the reversal potential by 19.4 mV ± 2.2 mV (from -64.6 ± -5.6 mV to -45.2 ± 8.2 mV, \(n = 8; p < 0.001\)). This corresponds to a relative permeability ratio \(P_{H+}/P_{Na^+}\) of 7.4 ± 0.6 (see “Experimental Procedures”). Hence, mBLINaC is highly permeable for H\(^+\), similar to ASIC1a (4, 25).

For rBLINaC, we investigated the dependence of the reversal potential on the H\(^+\) concentration in 100 \(\mu\)M [Ca\(^{2+}\)]\(_{ex}\), a Ca\(^{2+}\) concentration at which the channel is partially activated (Fig. 1). Raising the H\(^+\) concentration from 100 nM to 100 \(\mu\)M (pH 7 to pH 4), significantly shifted the reversal potential by 13.1 mV ± 2 mV (from -31.5 ± -2.3 mV to -18.4 ± 1.5 mV, \(n = 10; p << 0.001\); results not shown). This corresponds to a relative permeability ratio \(P_{H+}/P_{Na^+}\) of 4.5 ± 0.6. To test whether [Ca\(^{2+}\)]\(_{ex}\) affects the permeability for H\(^+\) we repeated the experiment in the presence of a 100-fold lower Ca\(^{2+}\) concentration (1 \(\mu\)M), at which the channel is almost fully activated (Fig. 1). Under these conditions, the reversal potential shifted by 9 mV ± 0.3 mV (from -31.3 ± -0.6 mV to -22.3 ± 0.7 mV, \(n = 10; p << 0.001\); results not shown), which is not significantly different from the shift in 100 \(\mu\)M [Ca\(^{2+}\)]\(_{ex}\) (\(p = 0.8\)) and which corresponds to a relative permeability ratio \(P_{H+}/P_{Na^+}\) of 2.9 ± 0.3. Taken together, these results show that also rBLINaC is permeable for H\(^+\) and that, in contrast to its relative Na\(^+\) and K\(^+\) permeabilities, its H\(^+\) permeability is not significantly affected by [Ca\(^{2+}\)]\(_{ex}\).

**DISCUSSION**

Our study has two key findings: (i) rBLINaC is strongly inhibited by extracellular Ca\(^{2+}\) and (ii) mBLINaC is constitutively open, due to a much lower apparent Ca\(^{2+}\) affinity. In a previous study (11), rBLINaC was characterized by small current amplitudes but could be activated by introduction of a gain-of-function mutation. This mutation replaces an amino acid with a small side chain at the beginning of TMD2 (Ala\(^{443}\) in BLINaC) by an amino acid with a large side chain. Immediately after this so-called degenerin site (26), in BLINaC there is an Asp residue (Asp\(^{444}\)) that is conserved in most ASICs and that has been shown to be crucial for open channel Ca\(^{2+}\) block of ASIC1a (18). We now show that rBLINaC is strongly inhibited by physiological concentrations of extracellular Ca\(^{2+}\). The gain-of-function mutation probably relieves Ca\(^{2+}\) inhibition. rBLINaC was half-maximally blocked by 10 \(\mu\)M extracellular Ca\(^{2+}\) (Fig. 1B). Because it is unlikely that such a low concentration will be reached physiologically, rBLINaC presumably requires an as yet unknown ligand for robust activation. Peptide-gated HyNaCs (6) are also inhibited by extracellular Ca\(^{2+}\), showing that the gating of BLINaC shares fundamental features with the gating of ASICs and HyNaCs. Therefore, we speculate that BLINaC is activated by an extracellular ligand, possibly a peptide.

Similar to rBLINaC, mBLINaC activity may be enhanced by an extracellular ligand, but clearly mBLINaC is constitutively open at physiological concentrations of extracellular Ca\(^{2+}\). The large amplitudes of mBLINaC currents were comparable with amplitudes of ENaC currents in oocytes (23, 27), suggesting that, similar to ENaC, mBLINaC is physiologically a constitutively open channel. Furthermore, considering the rather high Na\(^+\) selectivity of mBLINaC and the predominant expression of BLINaC in nonneuronal tissues (11), this finding suggests that BLINaC is an epithelial Na\(^+\) channel. Further studies revealing the cellular expression pattern of the BLINaC protein in different tissues are needed to clarify this issue.

We identified a single amino acid substitution (Ala in rBLINaC, Ser in mBLINaC) that explains (i) the different apparent Ca\(^{2+}\) affinities, (ii) the different amiloride sensitivities, and (iii) the different ion selectivities of rBLINaC and mBLINaC. Given that the side chains of Ala and Ser residues differ only in one hydroxyl group, the profound effects of this amino acid substitution were surprising. A serine is invariably conserved at the corresponding position in all ASICs and HyNaCs, suggesting an important function for these ligand-gated channels. Because BLINaC and ASICs are closely related (6, 11), the crystal structure of chicken ASIC1 (cASIC1) (28) gives a first indication of the position of the critical residue in the ECD of BLINaC. The ECD of cASIC1 is composed of five subdomains, the palm, finger, thumb, knuckle, and β-ball domains, which assemble in a structure that resembles a clenched hand (28). Like mBLINaC, cASIC1 has a serine at the critical position (Ser\(^{370}\)), which local-

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5 S. Dürrnagel and S. Gründer, unpublished data.
izes to β-sheet 10 within the palm domain (28). Ser376 of cASIC1 is far (~50 Å) away from the ion pore, excluding a direct contribution to the pore. Moreover, because it is unlikely that A387 of rBLINaC directly binds Ca²⁺ or amiloride, it is highly likely that it affects apparent Ca²⁺ and amiloride affinities as well as ion selectivity by an allosteric mechanism.

Previously, it was found that rBLINaC is an unselective cation channel and that the DEG mutation increases Na⁺ selectivity of the channel (11). Our results confirm that in the presence of physiological [Ca²⁺]₀ rBLINaC does not select between monovalent cations. In addition, we found that in the presence of low [Ca²⁺]₀ (0.1 mM) selectivity for Na⁺ increases (Fig. 5). If Ca²⁺ inhibition were due to a simple open channel block, a change in ion selectivity of the pore would be rather surprising. Therefore, it is likely that removal of Ca²⁺ not only unblocks the ion pore but in addition induces a conformational change of the protein accompanied by open gating of the channel. A similar allosteric mechanism is also likely for Ca²⁺ inhibition of ASICs (18, 29) and HyNaCs. In cASIC1, the side chain of Ser376 points toward β-sheet 8 in the palm domain of an adjacent subunit. We speculate that gating of BLINaC involves conformational changes at subunit-subunit interfaces, invoking β-sheets 10 and 8 with residue Ser376 occupying a critical position. In this model, the presence of a serine at position 376 in mBLINaC would strongly stabilize the open state of the channel, opening the channel in the absence of a putative ligand, whereas an alanine at position 376 would stabilize the closed state of the channel, rendering rBLINaC inactive in the absence of this putative ligand.

In summary, we propose that there are at least two states of BLINaC: one state of low activity with Ca²⁺ tightly bound and an unselective ion pore and one state of high activity with no Ca²⁺ bound and a Na⁺ selective ion pore. At rest, rBLINaC would be predominantly in the first, low activity state whereas mBLINaC would be predominantly in the second, high activity state. A putative extracellular ligand would shift the equilibrium distribution between these two states for rBLINaC (and perhaps mBLINaC).

At present, it is a complete puzzle why rBLINaC is closed at rest whereas mBLINaC is constitutively open. Perhaps these differences correlate with species differences in the abundance of the putative ligand and/or with species differences in the required regulation of the ion transport pathway that is mediated by BLINaC.

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