Members of the degenerin/epithelial Na\textsuperscript{+} channel superfamily of ion channels subserve many functions, ranging from whole body sodium handling to mechanoelectrical transduction. We studied brain Na\textsuperscript{+} channel 2 (BNaC-2) in planar lipid bilayers to examine its single channel properties and regulation by Ca\textsuperscript{2+}. Upon incorporation of vesicles made from membranes of oocytes expressing either wild-type (WT) BNaC-2 or BNaC-2 with a gain-of-function (GF) point mutation (G433F), functional channels with different properties were obtained. WT BNaC-2 resided in a closed state with short openings, whereas GF BNaC-2 was constitutively activated; a decrease in the pH in the trans compartment of the bilayer activated WT BNaC-2 and decreased its permeability for Na\textsuperscript{+} over K\textsuperscript{+}. Moreover, these maneuvers made the WT channel more resistant to amiloride. In contrast, GF BNaC-2 did not respond to a decrease in pH, and its amiloride sensitivity and selectivity for Na\textsuperscript{+} over K\textsuperscript{+} were unaffected by this pH change. Buffering the bathing solutions with EGTA to reduce the free [Ca\textsuperscript{2+}] to <10 nM increased WT single channel open probability 10-fold, but not that of GF BNaC-2. Ca\textsuperscript{2+} blocked both WT and GF BNaC-2 in a dose- and voltage-dependent fashion; single channel conductances were unchanged. A drop in pH reduced the ability of Ca\textsuperscript{2+} to inhibit these channels. These results show that BNaC-2 is an amiloride-sensitive sodium channel and suggest that pH activation of these channels could be, in part, a consequence of H\textsuperscript{+} “interference” with channel regulation by Ca\textsuperscript{2+}.

Amiloride-sensitive sodium channels were initially thought to be restricted to sodium-reabsorbing epithelia such as urinary bladder, renal collecting tubules, and gastric mucosa (1, 2). However, following the cloning of the first subunit of the epithelial Na\textsuperscript{+} channel (ENaC) in 1993 (3, 4) and the subsequent development of specific immunological and molecular biological reagents, a new gene superfamily of ion channels was quickly defined (5, 6). Members of this degenerin/ENaC superfamily include the mammalian, avian, and amphibian orthologs of ENaC; subfamily members in snails and insects; and the genes and coding proteins involved in touch sensation and neurodegeneration in the nematode Caenorhabditis elegans (7). Over 60 members of this gene superfamily have been identified to date (8). Thus, these channels participate in a myriad of biological processes and are found not only in epithelia, but also in lymphocytes, muscle, endothelia, testes, oocytes, arterial baroreceptors, neurons, and astrocytes. Dysfunction of these channels has been implicated in several human diseases, including Liddle’s disease (9–12), pseudohypoaldosteronism type 1 (13–15), cystic fibrosis (16, 17), respiratory distress syndrome (18), and influenza (19).

Acid-sensing ion channels (ASICs; otherwise known as brain Na\textsuperscript{+} channels (BNaCs)) compose one branch of the degenerin/ENaC superfamily (20). The polypeptide is predicted to have two hydrophobic domains, a large extracellular loop, and intracellularly located amino and carboxyl termini. These channels are found throughout the nervous system and were first discovered in sensory neurons (21, 22). However, their localization is not restricted to the nervous system, but extends to tissues such as the small intestine, lung, and pituitary gland (23–25). The fact that lowering extracellular pH activates the majority of these channels led to the hypothesis that they are involved in nociception (8, 26). Moreover, evidence exists suggesting that these channels may serve as mechanosensors (27, 28).

BNaC-1 (MDEG, BNC1, or ASIC-2a) and BNaC-2 (ASIC-1a), or brain sodium channels, have been cloned from mammalian brain and studied in heterologous expression systems (20–22). The major goal of this work was to characterize the single channel properties of BNaC-2 in planar lipid bilayers so that the regulatory properties of these channels could be studied in a controlled environment. Calcium caused a voltage-dependent block of both wild-type (WT) BNaC-2 and gain-of-function (GF) BNaC-2 in dose-dependent manner. At lower pH, these channels became less sensitive to the inhibitory effects of Ca\textsuperscript{2+}. This “shift” in Ca\textsuperscript{2+} sensitivity at lower pH might be responsible for pH activation of BNaCs.

**EXPERIMENTAL PROCEDURES**

Planar Lipid Bilayer Experiments—Planar lipid bilayers were formed from a solution containing a 2:1 mixture of diphytanoylphosphatidylethanolamine and diphytanoylphosphatidylserine dissolved in n-octanol at a concentration of 25 mg/ml. Membranes were painted onto a 200-μm diameter hole in a polystyrene cup. Membrane capacitance in all of the experiments reported herein averaged 250–350 picofarads. The standard bathing solution contained in both compartments of the bilayer system was composed of 100 mM sodium chloride plus 10 mM MOPS-Tris buffer (pH 7.4). Lipids were purchased from Avanti Polar Lipids (Alabaster, AL). All solutions were made using Milli-Q water and were filtered-sterilized using 0.22-μm Sterivex-GS filters (Millipore Corp., Bedford, MA). Free Ca\textsuperscript{2+} concentrations were adjusted using
RESULTS

Single channel traces of WT BNaC-2 incorporated into planar lipid bilayers at neutral or acidic extracellular pH are shown in Fig. 1a. When bathed in 100 mM NaCl, these channels displayed a conductance of 20 pS; and at neutral pH, they were only open an average 8% of the time. However, lowering the trans solution pH to 6.2 caused the channel to remain open >90% of the time ($P_{\text{o}} = 0.90 \pm 0.08$, $n = 10$). Fig. 1b shows the effects of amiloride on WT BNaC-2 activity at pH 6.2. Amiloride produced a flickery block of the channel, consistent with its effects on other members of the degenerin/ENaC family (35–37). Fig. 1c summarizes amiloride dose-response curves of the channel at these two pH values. At pH 7.4, the apparent equilibrium inhibitory dissociation constant ($K_i$) for amiloride was 0.82 ± 0.09 μM ($n = 7$). At pH 6.2, the curve was slightly right-shifted, with $K_i = 2.0 \pm 0.23$ μM ($n = 6$). There was no indication that lowering the extracellular pH changed the conductance or produced a time-dependent ion activation or inactivation of channel activity (see “Discussion”). Under asymmetric solutions of NaCl, WT BNaC-2 displayed a permeability ratio ($P_{\text{Na}}/P_{\text{K}}$) of 9.1 ($n = 5$) (data not shown). This cation/anion ratio was not altered by pH changes.

Fig. 1d presents a summary of the results of experiments in which WT BNaC-2 was examined for its ability to discriminate between Na⁺ and K⁺ at neutral and acid extracellular pH values. The open and closed circles represent the current-voltage curves obtained under symmetrical conditions of NaCl and different pH values. The cis solutions of the same bilayer containing the channel were then replaced with 100 mM KCl either at pH 7.4 (open squares) or with the external solution reduced to pH 6.2 (closed squares), and bi-ionic reversal potentials were measured. The reversal potential at pH 7.4 under bi-ionic conditions was ~50 mV; using the Goldman-Hodgkin-Katz formulation, this translated into a $P_{\text{Na}}/P_{\text{K}}$ of 7.1. However, this ratio was slightly right-shifted, with $K_i = 2.0 \pm 0.23$ μM ($n = 6$). There was no indication that lowering the extracellular pH changed the conductance or produced a time-dependent ion activation or inactivation of channel activity (see “Discussion”). Under asymmetric solutions of NaCl, WT BNaC-2 displayed a permeability ratio ($P_{\text{Na}}/P_{\text{K}}$) of 9.1 ($n = 5$) (data not shown). This cation/anion ratio was not altered by pH changes.

Fig. 1e presents a summary of the results of experiments in which WT BNaC-2 was examined for its ability to discriminate between Na⁺ and K⁺ at neutral and acid extracellular pH values. The open and closed circles represent the current-voltage curves obtained under symmetrical conditions of NaCl and different pH values. The cis solutions of the same bilayer containing the channel were then replaced with 100 mM KCl either at pH 7.4 (open squares) or with the external solution reduced to pH 6.2 (closed squares), and bi-ionic reversal potentials were measured. The reversal potential at pH 7.4 under bi-ionic conditions was ~50 mV; using the Goldman-Hodgkin-Katz formulation, this translated into a $P_{\text{Na}}/P_{\text{K}}$ of 7.1. However, this ratio was slightly right-shifted, with $K_i = 2.0 \pm 0.23$ μM ($n = 6$). There was no indication that lowering the extracellular pH changed the conductance or produced a time-dependent ion activation or inactivation of channel activity (see “Discussion”). Under asymmetric solutions of NaCl, WT BNaC-2 displayed a permeability ratio ($P_{\text{Na}}/P_{\text{K}}$) of 9.1 ($n = 5$) (data not shown). This cation/anion ratio was not altered by pH changes.

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Experimental data points to the Michaelis-Menten equation rewritten as:

channel

produce neurodegeneration (38).

tions in proteins homologous to the mammalian BNaCs that

produce neurodegeneration in C. elegans have identified specific mutations that cause neurodegeneration in C. elegans (38–44). Because these same mutations that cause neurodegeneration in C. elegans have been shown to activate BNaCs in heterologous expression systems (22, 45–47), we tested whether or not the same mutation could produce a constitutive activation of BNaC-2 in bilayers. Fig. 2 presents the results of these experiments. Substituting a phenylalanine for glycine at position 433 of BNaC-2 resulted in constitutive activation of the channel, increasing $P_o$ from 0.08 ± 0.03 in the WT channel to 0.89 ± 0.09 (n = 5). The channel's sensitivity to amiloride (Fig. 2b and c) was slightly right-shifted compared with the WT channel ($-2.5 \, \text{uM versus } 0.8 \, \text{uM at pH 7.4}$). However, this constitutively activated channel was insensitive to reduction of extracellular pH (Fig. 2a, lower trace). Also, acidification did not change the $K_i$ for amiloride ($K_i = 2.55 \pm 0.20 \, \text{uM at pH 7.4}$ (n = 8) versus $K_i = 2.45 \pm 0.24 \, \text{uM at pH 6.2}$) of this gain-of-function mutation, in contrast to the WT channel. Another difference between G433F BNaC-2 and the WT channel was the poor ability of the mutant to discriminate between Na$^+$ and K$^+$. $P_N/P_K = 2.5:1$. $P_N/P_K$ was determined from reversal potential measurements under bi-ionic conditions, which were not affected by acidification (Fig. 2d).

To determine the relationship between the pH and $P_o$ of BNaC-2, we performed additional pH clamp experiments. The single channel activities of both WT BNaC-2 and G433F BNaC-2 were measured under conditions in which the pH of the trans bathing compartment was varied. These results are summarized in Fig. 3. Acidification of the bathing solution increased the activity of WT BNaC-2. In contrast, the $P_o$ of constitutively activated G433F BNaC-2 was unaffected by alterations in pH, at least over the pH range 6.2–7.4.

All of the aforementioned sets of experiments were performed with $-10 \, \text{uM Ca}^{2+}$ in the bilayer bathing solution. Because the role of Ca$^{2+}$ in modulating native and cloned epithelial Na$^+$ channels is well established (48–51), we next tested the hypothesis that Ca$^{2+}$ may influence the activity of BNaC-2.

Fig. 4 displays current traces of WT BNaC-2 at pH 7.4 in a planar lipid bilayer. In Fig. 4a, the upper trace was obtained under control conditions in which the channel was bathed with standard 100 mM NaCl solution containing $-10 \, \text{uM Ca}^{2+}$ as determined by Fura-2 measurements. Buffering the solutions bathing both sides of the bilayer with EGTA to be nominally free of Ca$^{2+}$ (lower trace) increased the open probability of BNaC-2 (from 0.08 ± 0.02 to 0.91 ± 0.08 (n = 8)). Subsequent
The concentration of Ca\(^{2+}\) in either compartment of the bilayer chamber resulted in a dose-dependent decrease in \(P_o\), but not in the unitary conductance of the channel. In Fig. 4a, the effects of increasing the cis Ca\(^{2+}\) concentration (either 5, 10, or 25 \(\mu M\)) on the single channel activity are shown. A comparable outcome was observed when addition of Ca\(^{2+}\) was made to the trans compartment of the bilayer chamber, albeit with a different dose dependence (Fig. 4, b and c). Also, the efficacy of both cis and trans Ca\(^{2+}\) as blockers of BNaC-2 was dependent upon membrane voltage (Fig. 4d).

trans and cis Ca\(^{2+}\) dose-response curves at holding potentials of −100 and +100 mV are shown in Fig. 4 (b and c, respectively). Fitting the experimental data to the Michaelis-Menten equation transformed for open probabilities allowed the calculation of [Ca\(^{2+}\)] required for half-maximal inhibition of the channels (\(K_{i(Ca)}\)). The trans \(K_{i(Ca)}\) was 51.3 ± 6.9 \(\mu M\) (n = 7) at +100 mV and 2.4 ± 0.36 \(\mu M\) (n = 7) at −100 mV; the cis \(K_{i(Ca)}\) was 1.4 ± 0.21 \(\mu M\) (n = 9) at +100 mV and 35.6 ± 5.4 \(\mu M\) (n = 7) at −100 mV. Fig. 4d presents a semilogarithmic plot of cis and trans \(K_{i(Ca)}\) as a function of applied membrane voltage. The lines through the data points were computed from the Woodhull formulation (52) using a best fit approach and varying \(\delta\) in the Woodhull equation, \(K_i(V) = K_i(0) \exp(\delta z F V / R T)\), where \(K_i(0)\) is \(K_{iCa}\) inhibitory dissociation constants at given and zero holding potentials, respectively; \(\delta\) is the fraction of the electric distance between the surface of the channel and the binding site; \(z\) is the valence of the blocker; and \(R\), \(F\), and \(T\) have their usual meanings.

An assumption utilizing this equation is that the binding sites for Ca\(^{2+}\) are located within the electric field. From these plots, there was an inverse voltage dependence between cis and trans inhibition of BNaC-2 by Ca\(^{2+}\), suggesting a minimum of two binding sites within the electric field. The locations of these sites computed by this equation are at 18 ± 1.9 and 20 ± 2.1% of the electric field from the cis and trans surfaces of the channel, respectively. Thus, we conclude that our results are consistent with the hypothesis that Ca\(^{2+}\) directly blocks BNaC-2 in a manner similar to that of the rat epithelial sodium channel and in a manner similar to that of amiloride-sensitive channels recorded in native epithelial cells (48–50).

As described above, a decrease in pH resulted in some changes in the basic biophysical profile (e.g., ion selectivity and amiloride inhibition) of BNaC-2 activity. These changes prompted us to test the possibility that the effects of trans and cis Ca\(^{2+}\) could be different on BNaC-2 activity at decreased pH. Fig. 5 presents a similar analysis done for the WT channel at pH 6.2. Buffering the bilayer bathing solution to be nominally free (<10 \(\mu M\)) of Ca\(^{2+}\) with EGTA had little effect on the open probability of the channel (Fig. 5a). However, increasing the concentrations of Ca\(^{2+}\) in either the cis or trans compartment reduced the single channel open probability in a dose-dependent manner. Fig. 5 (b and c) presents the trans and cis Ca\(^{2+}\) dependence at −100 and +100 mV. Compared with pH 7.4, the effectiveness of either cis or trans Ca\(^{2+}\) as an inhibitor of channel activity was diminished by nearly an order of magnitude at either +100 or −100 mV at lower pH. The voltage dependence at pH 6.2 of the Ca\(^{2+}\) block of BNaC-2 is shown in Fig. 5d. The apparent electrical distances for Ca\(^{2+}\) from both the cis and trans solutions were the same as determined at pH 7.4, viz. 18 and 20%, respectively. The simplest explanation for these observations is that hydrogen ions compete for Ca\(^{2+}\) by binding to the same sites.

The effects of cis and trans Ca\(^{2+}\) on the activity of G343F BNaC-2 at pH 7.4 and 6.2 were also examined. These experiments are summarized in Table I. Because this mutant channel was constitutively activated, buffering the bathing solution to <10 \(\mu M\) free Ca\(^{2+}\) with EGTA had no significant effect on \(P_o\) at either pH 7.4 or 6.2 (data not shown). Addition of Ca\(^{2+}\) to either the cis or trans compartment produced a voltage- and concentration-dependent decrease in \(P_o\). Table I summarizes these data and provides a comparison with the WT channel. The major effect of this GF mutation was to reduce the effectiveness of Ca\(^{2+}\) in blocking the channel at all applied voltages and at both pH values. The locations within
the electric field of both the cis and trans Ca\(^{2+}\)-binding sites were unaffected by the G433F mutation.

**DISCUSSION**

**BNaC-2 Forms a Functional Channel in Bilayers**—We have examined BNaC-2, a member of the degenerin/ENaC superfamily of ion channels, using a model planar lipid bilayer approach. Both WT BNaC-2 and GF BNaC-2 formed functional amiloride-sensitive Na\(^+\) channels. Decreasing the pH had a differential effect on their biophysical properties. A decrease in pH activated a predominantly closed WT channel, but had little or no influence on constitutively activated GF BNaC-2. Also, a decrease in pH resulted in slight rightward shift of amiloride sensitivity (−0.8 μM at pH 7.4 versus −2 μM at pH 6.2) and decreased the ability of WT BNaC-2 to select between Na\(^+\) and K\(^+\) (from P\(_{Na}/P_{K}\) = 7.1 at pH 7.4 to P\(_{Na}/P_{K}\) = 2.1 at pH 6.2). These maneuvers with pH had no effect on the amiloride sensitivity (−2.5 μM) and cation selectivity of GF BNaC-2. The fact that the introduction of the GF point mutation in the pre-M2 region of BNaC-2 resulted in changes in channel activity and selectivity may be indicative of involvement of this residue in pore and/or selectivity filter formation. In fact, mutation at a similar position in MDEG (BNaC-1) led to a change in ion selectivity and conductance (22) and pH sensitivity (47). Lazdunski and co-workers (22, 47) consider this residue to be part of an inhibitory domain rather than part of the pore lining. They proposed that steric constraints or activation by yet unidentified mechanisms open the channel. In another report, acidification made this residue susceptible to chemical modification (45). It was proposed that a lowered pH induces a conformational change, “locking” the channel in the open state. Our observation of changes in GF BNaC-2 activity and selectivity favors the possibility of participation of this residue in the lining of the pore, perhaps as part of a selectivity loop, by analogy to the region preceding the first transmembrane domain of ASIC-2 (53). Alternatively, either low pH or the GF mutation alters gating of BNaC-2 to create a high-\(P_{o}\) state, which obligatorily diminishes Na\(^+\) selectivity.

Some differences in channel characteristics between the bilayer studies and those reported using heterologous expression systems are noted. First, in the bilayer system, even under basal conditions, a low level of WT channel activity is seen. This may result from the fact that the surface pH in negatively charged bilayers of the type used in these experiments would produce a more acidic pH at the membrane-solution interface. Based on the surface charge distribution of phosphatidylethanolamine/phosphatidylserine bilayers, we calculate (using a charge density of one charge/120 Å) from the Gouy-Chapman theory (54) that the surface pH should be ~6.6, giving a channel open probability of 0.4–0.5. Recently, de Weille et al. (55) reported spontaneous channel activity at pH ~7.3 in outside-out membrane patches excised from COS-7 cells transfected with human ASIC-3. Second, once a WT channel is activated by pH, the channel stays activated as long as the pH is maintained at acidic levels. In heterologous expression systems, the current activation is transient (8). These observations suggest that there may be modifier protein(s) associated with the channels.
in native or expressed cell systems that inactivate the channels. This possibility is also underscored by the fact that members of this branch of the degenerin/ENaC superfamily can associate with each other (56–58) and ENaC (21) to form a functional channel. This observation further complicates the physical picture of the channel and its potential interaction with any cellular binding partners. Needless to say, the cell-free bilayer system is devoid of such potential modifier proteins. Third, GF mutations of MDEG-1 are more sensitive to amiloride and pH than GF BNaC-2 incorporated in the bilayer (47). Correspondingly, mutation of the Drosophila Ripped Pocket (RPK(A523V)) protein also significantly increases the sensitivity of the current to amiloride (59). These results are in contrast to what was observed in the bilayer, viz. a reduced sensitivity to amiloride. The reason for these discrepancies is not known, but probably relates to more complex effects of amiloride on BNaCs (60). Alternatively, the introduction of particular amino acids could affect amiloride sensitivity because substitution of valine for glycine at position 430 of BNC-1 does indeed reduce sensitivity to amiloride (46). In addition to GF mutations, pH modification of the channel activity could, at least in part, contribute to an uncontrollable influx of cations, leading to osmotic disturbances that ultimately can cause degeneration of the cell.

Decreases in pH Desensitize BNaC-2 to Ca\(^{2+}\) —Ca\(^{2+}\) can influence a wide variety of cellular events, and adequate operation of ion channels is not an exception. ASIC (also called ASIC-1a, ASIC-\(\alpha\), and BNaC-2) is permeable to calcium ions, but increasing extracellular calcium concentrations become inhibitory (61, 62). This is not the case for its splice variant, ASIC-\(\beta\) (63), although Sutherland et al. (62) reported inhibition of ASIC-1b (also called ASIC-\(\beta\) in Ref. 63) by Ca\(^{2+}\). This discrepancy is explained by different pH measurements and/or single amino acid differences in the clones used (62). Additionally, different concentrations of Ca\(^{2+}\) were used in these studies (500 \(\mu\)M versus 10 mM). Ca\(^{2+}\) is also able to block currents generated by coexpression of ASIC-2 and ASIC-3 subunits without being permeant (64). ASIC-1a currents are potentiated if extracellular Ca\(^{2+}\) is raised from 2 to 10 mM, whereas both ASIC-2a and ASIC-1a plus ASIC-2a currents are partially blocked; Na\(^{+}\) remained the principal conducting ion (65). Also, Ca\(^{2+}\) induced a decrease in unitary currents of both ASIC-1a (BNaC-2) and ASIC-2a (BNaC-1). It was suggested that open channel probability increases due to recruitment of rundown channels by Ca\(^{2+}\). Interpretation of the data in these studies was confounded by the natural complexity of the cell machinery. Using the planar lipid bilayer approach allowed us to minimize changes in these variables such as cellular pH, membrane voltage, and number of channels.

Our results indicate a direct modulation of BNaC-2 activity by cis and trans Ca\(^{2+}\), although we cannot rule out the possibility that other physiological constituents can take part in this process (49, 66, 67). These possibilities are not necessarily mutually exclusive; different modes of regulation may be operational under given circumstances.

This study also establishes the exact range of concentrations at which either cis or trans Ca\(^{2+}\) blocks brain sodium channels. The \(K_c\), for intracellular (i.e., cis) Ca\(^{2+}\) at \(-100\) mV was \(-36\) \(\mu\)M at pH 7.4. For the constitutively activated single point mutant BNaC-2 channel (i.e., G433F BNaC-2), the \(K_c\) for intracellular Ca\(^{2+}\) increased to \(122\) \(\mu\)M. For both the WT and mutant channels, decreasing the pH to 6.2 made Ca\(^{2+}\) a less effective blocker; the \(K_c\) values for cis Ca\(^{2+}\) were increased to 469 \(\mu\)M and 1.45 mM for WT BNaC-2 and G433F BNaC-2, respectively. These results suggest a competition between protons and Ca\(^{2+}\) ions. A decrease in pH is a well established feature of ischemia (68). Even though mild acidosis has been considered neuroprotective (69), its role in cell survival or death is not fully understood. Also, intracellular Ca\(^{2+}\) overload has been implicated as a possible mechanism of neuronal injury in ischemia (70, 71). Moreover, a role of ASICs in mediating the cellular response to an ischemic insult was suggested (72). Our findings of increased inhibitory constants for Ca\(^{2+}\) under acidic pH support this possibility. Acidic pH can override the inhibitory influence of Ca\(^{2+}\) on BNaCs, converting the channel to be less selective and more resistant to Ca\(^{2+}\). If the neuroprotective role of acidosis is due to N-methyl-d-aspartate receptor inhibition (73), its adverse impact may arise from relief of the inhibitory influence of Ca\(^{2+}\) on BNaCs.

Because Ca\(^{2+}\) is such an effective blocker of BNaC-2, reducing cis or intracellular [Ca\(^{2+}\)] to values <1 \(\mu\)M (typical [Ca\(^{2+}\)] found within mammalian cells) would result in BNaC-2 being constitutively activated in the absence of other extrinsic or intrinsic regulators of channel function. This observation has important implications for the physiological role of this channel, particularly for high-grade glioma cells, where BNaCs appear to be open (74). Interestingly, when WT BNaC-2 cRNA was injected into and heterogeneously expressed in Xenopus oocytes, channel activity could be activated only by extracellular acid. Intracellular [Ca\(^{2+}\)] is known to exceed 10 \(\mu\)M and to be as high as 30 \(\mu\)M in oocytes (75), which, extrapolating from the present results, would result in a channel that is constitutively closed. Our bilayer data suggest that the channel can be activated by low pH just as in the case of oocytes. As the actual molecular composition of these ion channels is unknown, this hypothesis awaits further experimentation.

The absence of cytoskeletal elements in our bilayer experiments can account for the lack of effect of Ca\(^{2+}\) on unitary currents of BNaC-2. Indeed, we did observe an almost 2-fold reduction of BNaC-2 single channel conductance in the presence of actin (76, 77). Moreover, the effects of actin on the single channel conductance of ENaC were evident only in the presence of Ca\(^{2+}\) (78).

In summary, we have successfully reconstituted amiloride-sensitive brain sodium channels in planar lipid bilayer membranes. Our results show that these channels can be activated by low pH, that amiloride blocks these channels with a relatively low affinity, and that the activity of both WT BNaC-2 and constitutively activated BNaC-2 can be modulated by both cis and trans Ca\(^{2+}\). Lowering the pH decreased the effectiveness of Ca\(^{2+}\) in blocking these channels. We suggest that relief of the Ca\(^{2+}\) block of BNaCs by acidic pH in vivo may result in a cellular osmotic imbalance, ultimately leading to degeneration of the cell.

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REFERENCES

1. Benos, D. J. (1982) Am. J. Physiol. 242, C131–C145
2. Garty, H., and Benos, D. J. (1988) Physiol. Rev. 68, 309–373
3. Canessa, C. M., Horisberger, J. D., and Rossier, B. C. (1993) Nature 361, 467–470
4. Lingueglia, E., Voilley, N., Waldmann, R., Lazdunski, M., and Barpy, P. (1993) FEBS Lett. 318, 95–99
5. Corey, D. P., and Garcia-Anoveros, J. (1996) Science 273, 323–324
6. Garcia-Anoveros, J., and Corey, D. P. (1997) Annu. Rev. Neurosci. 20, 567–594
7. Alvarez de la Rosa, D., Canessa, C. M., Frye, G. K., and Zhang, P. (2000) Annu. Rev. Physiol. 62, 573–594
