Protons Activate Brain Na\(^{+}\) Channel 1 by Inducing a Conformational Change That Exposes a Residue Associated with Neurodegeneration

(Received for publication, August 7, 1998, and in revised form, August 25, 1998)

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BNC1 is a mammalian neuronal cation channel in the novel DEG/ENaC ion channel family. BNC1 channels are transiently activated by extracellular protons and are constitutively activated by insertion of large residues, such as valine, in place of Gly-430; residue 430 is a site where analogous mutations in some Caenorhabditis elegans family members cause a swelling neurodegeneration. Mutation of Gly-430 to a small amino acid, cysteine, neither generated constitutive currents nor allowed modification of this residue by sulfhydryl-reactive methanethiosulfonate (MTS) compounds. However, when protons activated the channel, Cys-430 became accessible to extracellular MTS reagents, which modified Cys-430 to generate constitutive currents. These data indicate that protons induce a reversible conformational change that activates BNC1 thereby exposing residue 430 to the extracellular solution. Once Cys-430 is modified with a large chemical group, the channel is prevented from relaxing back to the inactive state. These results link ligand-dependent activation and activation by mutations that cause neurodegeneration.

Proteins in the DEG/ENaC superfamily form multimeric cation channels that are blocked by amiloride (1–3). Several neuronal members of this family require extracellular ligands to open the channel. For example, the FMRFamide-activated Na\(^{+}\) channel (FaNaCh) from neurons of the snail Helix aspersa requires application of neuropeptide to the extracellular surface for channel activation (4). Likewise, mammalian DEG/ENaC family members, BNC1 (also called MDEG and BNaC-l) (5–7), BNaC-2 (also called ASIC) (7, 8), and DRASIC (9) require application of neuropeptide to the extracellular surface for channel activation (4). Likewise, mammalian DEG/ENaC channels are gated and how Deg mutations cause persistent channel activation, thereby disrupting control of cell volume. Deg mutations affect a residue thought to lie just external to the second membrane-spanning sequence (M2) in or near the pore. The neurodegenerative phenotype required that Deg mutations substitute a Val or other large residue for the wild-type Ala residue, leading to the speculation that bulky side chains might interfere with the closing of a channel gate (1). Subsequent electrophysiologic studies showed that analogous Deg mutations in BNC1 (6), the Drosophila Na\(^{+}\) channel Ripped Pocket (16), and UNC-105 (17) caused channel activation even in the absence of any ligand. To understand how DEG/ENaC channels are gated and how Deg mutations cause persistent channel activation, we studied BNC1, a proton-activated Na\(^{+}\) channel expressed in human neurons (5, 6, 18).

EXPERIMENTAL PROCEDURES

cDNA Constructs—BNC1 mutants were constructed by single-stranded mutagenesis of BNC1 (5) in pBluescript. The validity of constructs was confirmed by DNA sequencing. Constructs were cloned into pMT3 (19) for expression.

Expression and Electrophysiological Analysis in Xenopus Oocytes—cDNA constructs were expressed in defolliculated albino Xenopus laevis oocytes (Nasco, Port Atkinson, WI) by nuclear injection of plasmid DNA at concentrations ranging from 5 to 10 ng/µl. Following injection, oocytes were incubated at 18 °C in modified Barth’s solution. 12–24 h after injection, whole cell currents were measured using a two-electrode voltage clamp. During recording, oocytes were bathed in frog Ringer’s solution (116 mM NaCl, 0.4 mM CaCl\(_2\), 1 mM MgCl\(_2\)). pH 7.4 and pH 6 solutions were buffered with 5 mM HEPES; more acidic solutions were buffered with 5 mM MES.\(^{1}\) Membrane voltage was held
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**RESULTS AND DISCUSSION**

When wild-type BNC1 is expressed in *Xenopus* oocytes, it generates only a very small basal Na⁺ current (5). However, acidification of the extracellular solution produces a transient activation (21). We first examined the effect of Deg mutations on basal channel activity at pH 7.4. In BNC1, the Deg residue activates BNC1 in the presence of a proton stimulus (21). We first examined the effect of Deg mutations with activation of BNC1-G430C at pH 5 and pH 7.4 (6); like BNC1-G430V, BNC1-G430C activated with MTSEA also showed a significant K⁺ conductance as evidenced by the reversal potential and data not shown. D, effect of amiloride concentration on current generated by BNC1-G430V (squares) and BNC1-G430C following modification by MTSEA (circles). n = 5–9 oocytes for each point; in some cases error bars are hidden by symbols.

**FIG. 1.** Current induced by mutation of residues at the Deg position. A–C, current traces showing constitutive amiloride-sensitive current in oocytes expressing wild-type BNC1 (A), BNC1-G430C (B), or BNC1-G430V (C). Amiloride was present in the extracellular solution during times indicated by the solid bars. Membrane voltage was −60 mV, pH was 7.4. D, current inhibited by 1 mM amiloride at −60 mV in wild-type BNC1 and BNC1 Gly-430 mutants. Data are mean ± S.E. from at least 6 oocytes. E and F, effect of extracellular protons on BNC1-G430C at −60 mV. Unless indicated, extracellular pH was 7.4. In F, data are mean ± S.E. from 7 oocytes; error bars are hidden by symbols. The EC₅₀ for protons was pH 5.3.

**FIG. 2.** Although protons activate BNC1-G430C, MTSEA locks channels in an open state (A and B). Representative experiments showing effect of MTSEA (labeled as EA) and protons (pH 5) on BNC1-G430C. MTSEA (100 μM) and amiloride (labeled Amil, 1 mM) were present in the extracellular solution during times indicated by bars. Membrane voltage was −60 mV; C, current–voltage relationship of BNC1-G430V (●), BNC1-G430C (○), and BNC1-G430C after modification with MTSEA at pH 5 (■). The reversal potential for BNC1-G430V in oocytes is close to 0 mV, consistent with a cation-selective channel that shows poor discrimination between Na⁺ and K⁺ (6); like BNC1-G430V, BNC1-G430C activated with MTSEA also showed a significant K⁺ conductance as evidenced by the reversal potential and data not shown. D, effect of amiloride concentration on current generated by BNC1-G430C (squares) and BNC1-G430C following modification by MTSEA (circles). n = 5–9 oocytes for each point; in some cases error bars are hidden by symbols.

**FIG. 3.** Modification of BNC1-G430C by MTS compounds. At times indicated by bars, 300 μM MTSET (ET), 5 mM MTSES (ES), 300 μM MTS-biotin (biotin), 1 mM amiloride, or pH 5 solution was applied to extracellular surface. Total number of experiments was, two (ET), four (ES), and four (biotin). Membrane voltage was −60 mV.

The finding that in MEC-4, mutation of the Deg residue to Cys did not cause neurodegeneration (13). Because Thr, Val, and Phe are larger than Gly or Cys, these results suggested that large amino acids at the Deg position activate BNC1 in the absence of a proton stimulus.
Because a Cys at the Deg position may have been too small to activate BNC1, we tested the hypothesis that BNC1-Cys-430 might be activated if its side chain was chemically modified to attach a larger group. To test this, we used water-soluble sulfhydryl-reactive MTS compounds. When we applied MTSEA (100 µM) to the extracellular solution at pH 7.4, it did not activate BNC1-G430C, nor did it alter subsequent activation by protons (Fig. 2A). We also tested three other MTS compounds, (2-(trimethylammonium)ethyl) methanethiosulfonate bromide (MTSET), sodium 2-sulfonatoethyl methanethiosulfonate (MTSES), and N-biotinylaminomemiothyl methanethiosulfonate (MTSEA-biotin), and found that they also failed to activate BNC1-G430C at pH 7.4 (Fig. 3A-C). These results suggest that MTS compounds did not modify Cys-430, perhaps because the residue was not accessible to the extracellular solution under basal conditions at pH 7.4.

To test the hypothesis that Cys-430 might become accessible once the channel is open, we simultaneously applied both protons (extracellular pH 5, to open the channel) and MTSEA (to modify Cys-430). This generated a large nonactivating current (Fig. 2B) that persisted even after MTSEA and protons were removed. The current showed a cation selectivity and amiloride-sensitivity similar to that of BNC1-G430V (Fig. 2, C and D). The effect of MTSEA at pH 5 was likely due to covalent modification of Cys-430 because the effect was irreversible, and because MTSEA did not stimulate BNC1-G430C or the wild-type channel under basal or activated conditions (not shown). Persistent activation of the channel after covalent modification of Cys-430 is consistent with the constitutive currents observed when Gly-430 is mutated to a larger residue (Fig. 1). The ability of MTSEA to modify Cys-430 at an acidic pH cannot be explained by a change in the protonation state of Cys-430, because MTS compounds react much more slowly with protonated cysteine side chains.

Rather, the acidic pH altered the channel conformation, activating the channel and exposing Cys-430 to extracellular MTSEA.

MTSEA is a relatively small, cationic compound. To examine size and charge constraints for access to Cys-430, we tested slightly larger cationic (MTSET) and anionic (MTSES) compounds, as well as a much larger compound consisting of MTSEA linked to biotin. Like MTSEA, each of these compounds irreversibly activated BNC1-G430C, but only when the channel was activated by protons (Fig. 3, A-C). The MTS compounds did not appear to modify Cys-430 in the basal state because a drop in pH did not produce sustained activation after pretreatment with MTS. Moreover, application of MTS alone did not prevent subsequent activation by MTS when pH was reduced.

We noticed that in some experiments a single application of an MTS compound with protons did not produce a maximal effect on current. For example, as shown in Fig. 3C, repeated proton activation of BNC1-G430C in the presence of MTSES progressively increased current. This suggested that accessibility of Cys-430 might be limited to the time the channel was open, and once the channel had inactivated, Cys-430 was no longer accessible to MTS reagents. To test this further, we slowed the rate of inactivation by reducing the temperature from 23° to 10 °C (Fig. 4B). Of note, the kinetics of inactivation were complex, perhaps consistent with the multimeric nature of the channel. Importantly, when inactivation was slowed and the duration of channel activity increased, a single application of protons with MTSET produced a greater sustained increase in current (Fig. 4, A and C).

These results suggest that Cys-430 is accessible to MTS.
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Fig. 6. Model showing activation of BNC1 by protons and large side chains on the Deg residue. A, cross-section of the BNC1 pore viewed from the side; note the constriction that prevents ion permeation and the location of the Deg residue, Cys-430. B, BNC1 viewed from the extracellular side; the Deg residue is shown as a notch in the side of each subunit. In this model, activation occurs when each subunit rotates counter-clockwise to dilate the pore. When the subunits rotate, the Deg residue faces the pore and is accessible to modification from the extracellular side. Modification prevents relaxation into the closed conformation and causes irreversible activation. The number of subunits modified is not known; three are shown as modified in this schematic. Additional states that would be required to more completely model gating are not shown, and inactivation is shown for simplicity as a return to the closed state.

Acknowledgments—We thank Dan Bucher, Dawn Melsen, Ellen Tarr, and Theresa Mayhew for excellent assistance. We especially appreciate the discussions and help of Joseph Cotten, John Rogers, and Tom Moninger. We thank the University of Iowa DNA Core Facility for assistance with sequencing and oligonucleotide synthesis.

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Compartments only during the time that the channel is open. To confirm this and to directly label the channels, we examined the accessibility of Cys-430 to labeling by MTS-4-fluorescein, a fluorescent MTS reagent. As with the other MTS reagents, MTS-4-fluorescein irreversibly activated BNC1-G430C at pH 5 (Fig. 5A). After labeling, confocal microscopy showed patchy areas of cell-surface fluorescence (Fig. 5, B and E) that may suggest organization and localization by association with cytosolic or membrane structures. In contrast, uninjected oocytes or oocytes expressing wild-type BNC1 had only a small amount of diffuse background fluorescence (Fig. 5, C and E). In addition, oocytes expressing BNC1-G430C that were labeled at pH 7.4 had only a small amount of diffuse fluorescence (Fig. 5, D and E), indicating that labeling required activation by protons. These results are consistent with the electrophysiologic data showing that Cys-430 was accessible to MTS compounds only when the channel was activated. In addition, the ability to selectively modify open channels with a fluorescent MTS reagent provides a novel reporter of the cell surface localization and functional status of these channels.

These data indicate that protons induce a reversible conformational change to activate BNC1 and expose the Deg residue. Fig. 6 shows a schematic of a potential mechanism suggested by the results; we model BNC1 activation as a synchronous rotation of channel subunits based on studies of another ligand-gated channel, the nicotinic acetylcholine receptor (23). In this model, protons bind a regulatory site to reversibly rotate the channel subunits. The rotation would have two effects. It would reversibly open the pore to allow current flow. As a consequence, it would also move residue 430 from a buried position to one facing the extracellular environment where it can be irreversibly modified by MTS compounds. Modification of residue 430 with a bulky side chain would sterically hinder rotation of the subunits back to a closed state that buries residue 430. Thus, following modification, the channel would be locked in an open state. This model can also explain the finding that introduction of large amino acids at the Deg position constitutively activates several DEG/ENaC channels (12–14) and causes a swelling degeneration in C. elegans neurons; large side chains at residue 430 prevent the conformational changes required for the channel to close.