Tetraethylammonium Block of the BNC1 Channel

Christopher M. Adams, Margaret P. Price, Peter M. Snyder, and Michael J. Welsh
Howard Hughes Medical Institute and Departments of Internal Medicine and Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242 USA

ABSTRACT The brain Na^+ channel-1 (BNC1, also known as MDEG1 or ASIC2) is a member of the DEG/ENaC cation channel family. Mutation of a specific residue (Gly430) that lies N-terminal to the second membrane-spanning domain activates BNC1 and converts it from a Na^+-selective channel to one permeable to both Na^+ and K^+. Because all K^+ channels are blocked by tetraethylammonium (TEA), we asked if TEA would inhibit BNC1 with a mutation at residue 430. External TEA blocked BNC1 when residue 430 was a Val or a Thr. Block was steeply voltage-dependent and was reduced when current was outward, suggesting multi-ion block within the channel pore. Block was dependent on the size of the quaternary ammonium; the smaller tetramethylammonium blocked with similar properties, whereas the larger tetrapropylammonium had little effect. When residue 430 was Phe, the effects of tetramethylammonium and tetrapropylammonium were not altered. In contrast, block by TEA was much less voltage-dependent, suggesting that the Phe mutation introduced a new TEA binding site located ~30% of the way across the electric field. These results provide insight into the structure and function of BNC1 and suggest that TEA may be a useful tool to probe function of this channel family.

INTRODUCTION

The DEG/ENaC superfamily is a diverse group of nonvoltage-gated cation channel proteins that includes neuronal channels in mammals, *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Helix aspersa* and channels in mammalian and *Drosophila* epithelia (Tavernarakis and Driscoll, 1997; Fyfe et al., 1998; Waldmann and Lazdunski, 1998). Individual DEG/ENaC proteins are subunits that associate as homomultimers or heteromultimers to form amiloride-sensitive cation channels. Each subunit has two transmembrane domains (M1 and M2), cytoplasmic N- and C-termini, and a large extracellular loop (Snyder et al., 1994; Renard et al., 1994; Canessa et al., 1994; Lai et al., 1996).

The primary structure and the function of DEG/ENaC channels clearly indicate that they represent a distinct channel superfamily. However, with their two transmembrane domains, they have at least a gross similarity to other types of cation channels such as inward-rectifier K^+ channels and the ATP-gated P2X cation channels (North, 1996). Furthermore, it has been speculated that DEG/ENaC proteins may contain a region N-terminal to M2 that extends partially into the plasma membrane, perhaps in a manner analogous to the pore (P) loops in other types of ion channels (Canessa et al., 1993; Jan and Jan, 1994). In support of this hypothesis, the region N-terminal to M2 in an ENaC subunit was protected from protease (Renard et al., 1994).

We investigated the functional architecture of DEG/ENaC ion channels by studying BNCl. We focused on the effect of mutations at a specific residue, the "Deg" residue (Gly430 in BNC1) for several reasons. First, the Deg residue lies just N-terminal to M2, perhaps within a region that shows similarity to a P-loop, but upstream of residues known to contribute to DEG/ENaC pores. Second, Deg mutations have a striking ability to constitutively activate several DEG/ENaC channels, including BNC1, and to cause neurodegeneration in vivo (Chalfie and Wolinsky, 1990; Driscoll and Chalfie, 1991; Huang and Chalfie, 1994; Waldmann et al., 1996; Adams et al., 1998b; Garcia-Anoveros et al., 1998), suggesting that the Deg residue plays a key role in channel function. Third, consistent with an earlier report, we found that BNCl containing a Deg mutation (BNCl-G430V) conducted K^+, as well as Na^+ (see below and Waldmann et al., 1996). Because wild-type BNCl is Na^+-selective (Price et al., 1996), this result indicated that the Deg mutation altered ion selectivity and that the residue may lie near the conduction pathway. Because BNCl-G430V conducts K^+, we hypothesized that it might be inhibited by a K^+ channel blocker, external tetraethylammonium (TEA), and that we might be able to use this property to study the pore of BNC1.

EXPERIMENTAL PROCEDURES

cDNA constructs

BNC1 mutants were constructed by single-stranded mutagenesis of human BNC1 (Price et al., 1996) in pBluescript. In each construct, a single residue (valine, threonine, or phenylalanine) was substituted for the glycine at position 430. Human DRASIC (Waldmann et al., 1997a) was cloned from mouse embryonic RNA and residue 424 was altered from glycine to valine using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA). The validity of constructs was confirmed by DNA sequencing. Constructs were cloned into pMT3 (Swick et al., 1992) for expression.

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Address reprint requests to Dr. Michael J. Welsh, Howard Hughes Medical Institute, University of Iowa College of Medicine, 500 EMRB, Iowa City, IA 52242. Tel.: 319-335-7619; Fax: 319-335-7623; e-mail: mjwelsh@blue.weeg.uiowa.edu.

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Expression and electrophysiological analysis in *Xenopus* oocytes

cDNA constructs were expressed in albino *Xenopus* laevis oocytes (Nasco, Fort Atkinson, WI) by nuclear injection of plasmid DNA as previously described (Adams et al., 1998b). BNC1 DNA was injected at concentrations ranging from 5–10 ng/μl. RPK-A524V (Adams et al., 1998a) and DRASIC-G424V cDNAs were injected at 80 and 40 ng/μl, respectively. hENaC (McDonald et al., 1994; McDonald et al., 1995) was expressed by coinjecting cDNAs encoding the α, β, and γ hENaC subunits (20 ng/μl each). Following injection, oocytes were incubated at 18°C in modified Barth's solution, then studied 8–24 h later. Whole-cell currents were measured using a two-electrode voltage-clamp. During recording, oocytes were bathed in frog Ringer's solution containing 116 mM NaCl or 116 mM KCl, 0.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.4.

RESULTS

BNC1 containing a Deg mutation is permeable to K^+

Expression of wild-type BNC1 in *Xenopus* oocytes generates a small whole-cell current that is Na^+ selective and inhibited by submicromolar doses of amiloride (Price et al., 1996). However, as previously reported (Waldmann et al., 1996) substitution of Val for Gly430 (BNC1-G430V) altered current in several ways. First, BNC1-G430V currents were ~50-fold larger than wild-type currents (Adams et al., 1998b). Second, BNC1-G430V conducted both Na^+ and K^+ (Fig. 1). Third, BNC1-G430V had a reduced sensitivity to amiloride; at −60 mV, 147 nM amiloride inhibited half the current of wild-type BNC1 (Price et al., 1996), whereas 11.5 μM was required to inhibit half the current of BNC1-G430V (not shown).

External TEA blocks the pore of BNC1 containing a Deg mutation

In all K^+ channels, TEA blocks the channel pore (Heginbotham and MacKinnon, 1992). TEA applied to the extracellular solution inhibited BNC1-G430V K^+ and Na^+ currents (Fig. 2 *A* and data not shown). TEA block was both time- and voltage-dependent (Fig. 2, *B-D*). At negative voltages, inward Na^+ current was inhibited at TEA concentrations similar to those required for inhibition of K^+ channels (reviewed in Kavanaugh et al., 1991). For example, at −120 mV the Kd_{TEA} was 4.5 ± 0.4 mM. However, TEA did not inhibit outward current, even at a concentration of 50 mM; the marked voltage dependence suggested that TEA may block BNCl by occluding the pore.

If TEA blocks the BNC1 pore, block may be influenced by cations moving through the pore. To test this hypothesis, we altered the reversal potential of BNC1-G430V current by two different maneuvers. First, we altered membrane-holding voltage (V_H). Because cells expressing BNC1-G430V have a large whole-cell cation conductance, changes in V_H are expected to alter the intracellular Na^+ concentration, as reflected by a change in the membrane reversal potential. With 116 mM extracellular Na^+, the resting membrane potential of oocytes expressing BNC1-G430V averaged +2.9 ± 0.7 mV (n = 23). When oocytes expressing BNC1-G430V were clamped to more negative values of V_H, the reversal potential became more negative (Fig. 3 *A*). The shift in reversal potential occurred over several minutes and was consistent with accumulation of intracellular Na^+. Accumulation of intracellular Na^+ was previously observed

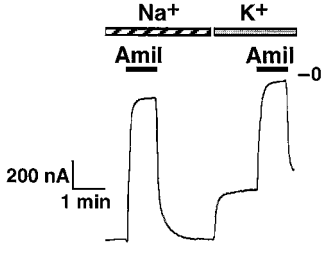

FIGURE 1 Deg mutations produce a K^+ permeable channel. Data are current from an oocyte expressing BNCl-G430V bathed in either Na^+ or K^+ Ringer's solution as indicated by bars. Membrane voltage was clamped at −80 mV. Amiloride (100 μm) was present in bath during times indicated by the bars. Zero current is indicated; current and time scales are shown below trace.

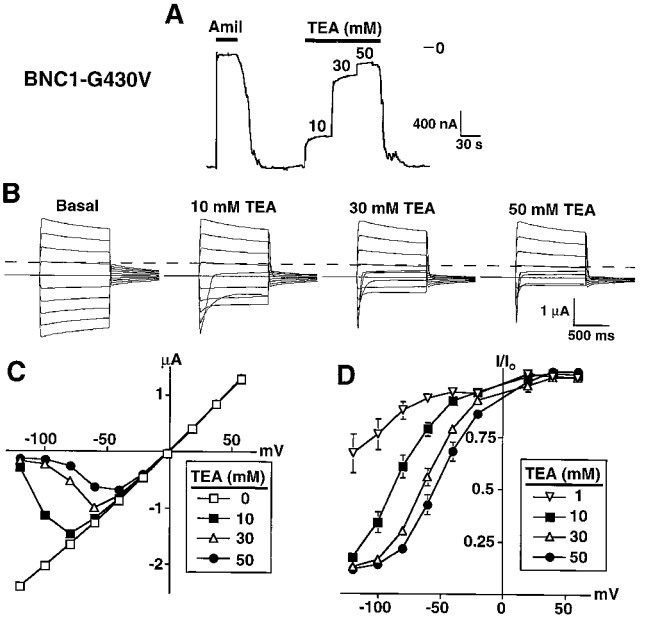

FIGURE 2 BNCl-G430V Na^+ current is blocked by extracellular TEA. (*A*) Current from an oocyte expressing BNCl-G430V. Membrane voltage was clamped at −80 mV and oocyte was bathed in Na^+ Ringer's solution. Amiloride (100 μM) or TEA (10, 30, or 50 mM) was present in bath during times indicated by bars. (*B*) Families of BNCl-G430V currents at voltages ranging from −120 to +60 mV. Holding voltage was −20 mV. Extracellular TEA concentrations are indicated above each current family. (*C*) Data from *B* plotted as current-voltage relationships. Current values were obtained at the end of each 1-s voltage step. (*D*) Fractional BNC1-G430V current (I/Io) in 1, 10, 30, or 50 mM TEA, plotted against voltage. Data are mean ± SE from at least 11 oocytes.

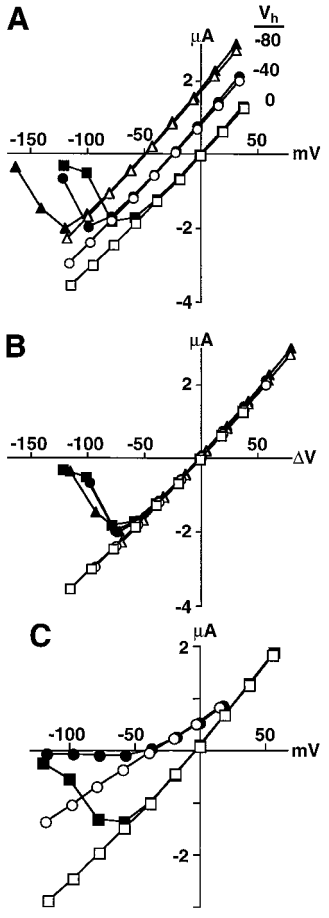

FIGURE 3 TEA block is influenced by membrane reversal potential. (*A*) Current-voltage relationship of BNC1-G430V in the absence (*open symbols*) or presence (*solid symbols*) of 50 mM TEA. Holding voltage (V_h) was 0, −40 or −80 mV and extracellular Na^+ concentration ($[Na]_{out}$) was 116 mM, as indicated. (*B*) Current values from *A* normalized to the reversal potential in each condition. (*C*) Current-voltage relationships from an oocyte expressing BNC1-G430V and bathed in 116 mM $[Na]_{out}$ (*squares*) or 12 mM $[Na]_{out}$ (*circles*). $[Na]_{out}$ was reduced by substitution with *N*-methyl D-glucamine. V_h was 0 mV. Current was measured under control conditions (*open symbols*) or with 10 mM TEA (*closed symbols*).

under similar conditions in oocytes expressing ENaC (McDonald et al., 1995). Fig. 3 *A* shows that TEA block was dependent on reversal potential. When the I-V relationship was normalized to the reversal potential (Fig. 3 *B*), the effect of TEA was similar. We also altered the reversal potential of BNC1-G430V current by reducing extracellular Na^+ from 116 to 12 mM. With this maneuver, the reversal potential shifted to a more negative value. Again, TEA did not appreciably block outward current (Fig. 3 *C*). These data suggest that TEA block was dependent on the transmembrane gradient of permeant ions; that is, outward cation flow prevented block. Thus, the results suggest that TEA blocked BNC1-G430V by occluding the channel pore.

Effect of TEA on other DEG/ENaC channels

We asked if TEA sensitivity is a general characteristic of DEG/ENaC channels. BNC1 is most closely related to the neuronal DEG/ENaC channels ASIC and DRASIC (Waldmann et al., 1997a; Waldmann et al., 1997b). To test whether TEA blocked other members of this subgroup, we studied DRASIC that contained a Deg mutation (substitution of Val for Gly424). Like wild-type BNC1, wild-type DRASIC is Na^+ selective and generates little if any basal current (Waldmann et al., 1997a). Fig. 4 *A* shows that DRASIC-G424V generated large amiloride-sensitive currents and was permeable to both Na^+ and K^+. Thus, Deg mutations had similar effects on BNC1 and DRASIC. Also like BNC1-G430V, DRASIC-G424 was blocked by TEA in a voltage-dependent manner (Fig. 4, *B*, *C*, and *E*).

We also examined two Na^+-selective family members, hENaC and RPK, containing a Deg mutation (RPK-A524V). Both of these channels were largely insensitive to TEA (Fig. 4, *D* and *E* and not shown). These results indicate that TEA-sensitivity is not a general property of all DEG/

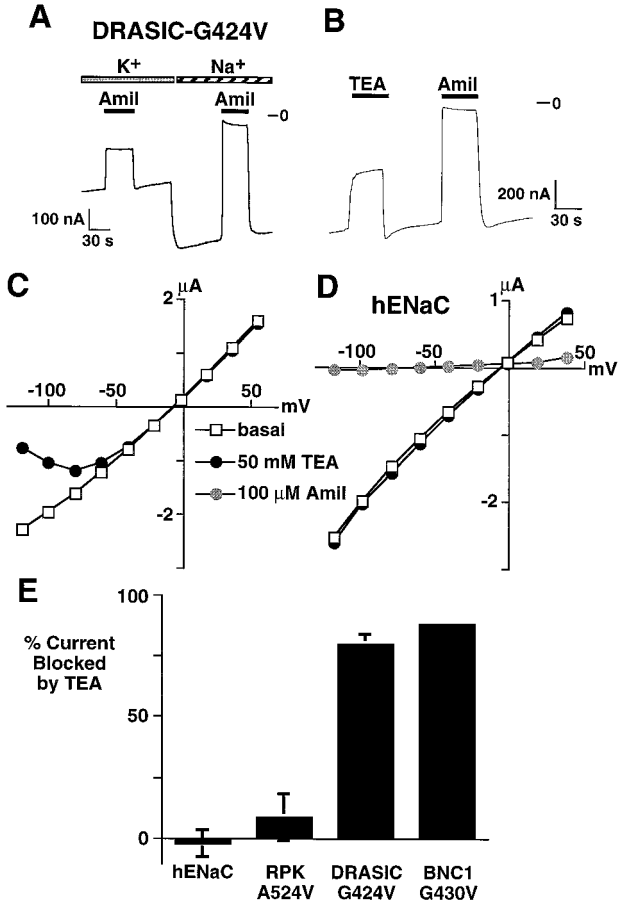

FIGURE 4 Effect of TEA on other DEG/ENaC family members. (*A*) Current from an oocyte expressing DRASIC-G424V bathed in either K^+ or Na^+ Ringer's solution, as indicated. (*B*) Effect of 50 mM TEA on DRASIC-G424V current. In *A* and *B*, membrane voltage was −80 mV and amiloride concentration was 1 mM. (*C* and *D*) Current-voltage relationship of DRASIC-G424V (*C*) or human ENaC (*D*) in presence and absence of 50 mM TEA, as indicated. In *D*, amiloride concentration was 100 μM. (*E*) Current inhibited by 50 mM TEA at −120 mV. Data are mean ± SE from at least five oocytes; in the BNC1-G430V group error bars are too small to see. In (*B-E*), oocytes were bathed in Na^+ Ringer's solution.

ENaC channels but may be observed in family members that show a significant K^+ conductance.

Residue at the Deg position alters TEA block of BNC1

Because the Deg mutation in BNC1 altered conductive properties (Na^+ to K^+ selectivity) (Waldmann et al., 1996; Fig. 1) and because TEA appeared to block the pore, the side chain of the amino acid at the Deg position might influence TEA block. To test this possibility, we examined TEA block of BNC1 when Gly430 was mutated to Thr or Phe. Notably, BNC1-G430V, BNC1-G430T, and BNC1-G430F are similar in single channel conductance, cation selectivity, and amount of whole-cell current generated (Waldmann et al., 1996; Adams et al., 1998b). Figs. 5, *A-C* show that TEA blocked BNC1-G430T as it blocked BNC1-G430V, with steep voltage-dependence and no appreciable block of outward current. In contrast, TEA block of BNC1-G430F was less voltage-dependent; TEA blocked both inward and outward current (Fig. 5, *D-F*). These results indicated that the side chain of residue 430 influenced TEA block.

The voltage-dependence allowed us to calculate the fractional distance in the membrane electrical field ($z\delta$) of the site of TEA block (Woodhull, 1973; Hille, 1992). For BNC1-G430V, $z\delta$ was greater than 1 ($z\delta = 1.40$; Fig. 6). Because TEA is a monovalent cation, this result suggested that TEA block involved more than one blocking ion in the channel pore (Hille, 1992). In this respect, TEA block of BNC1-G430V resembles multi-ion block of other ion channels, such as block of some K^+ channels by extracellular Cs^+ (Hille, 1992). Similarly, $z\delta$ was greater than 1 (1.38) for TEA block of BNC1-G430T (not shown). Multi-ion block also indicates that TEA blocks in the pore and not at a superficial site. In contrast, $z\delta$ was only 0.3 when residue was Phe. This suggested that TEA block of BNC1-G430F might involve the binding of one TEA molecule to a site 30% across the electrical field from the extracellular surface.

TEA binding site in BNCl-G430F is size selective

In K^+ channels, sensitivity to extracellular TEA is greatly enhanced when an aromatic residue such as Phe lies at the extracellular mouth of the channel pore (Kavanaugh et al., 1991; MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992). The aromatic side chain of Phe is thought to bind TEA via a π-cation interaction (Heginbotham and MacKinnon, 1992). Therefore, we hypothesized that Phe430 might be a new binding site for TEA in BNC1. In K^+ channels, the aromatic residues generate a size-selective site in which one TEA molecule can bind and block the channel. Larger or smaller quaternary ammonium ions (such as tetrapropylammonium (TPA) or tetramethylammonium (TMA)) are unable to stably interact at this site and are therefore much weaker inhibitors (Heginbotham and MacKinnon, 1992). Therefore, we tested the hypothesis that TPA and TMA might also be unable to bind an aromatic binding site in BNC1-G430F. In oocytes expressing either BNC1-G430V or BNC1-G430F, TPA blocked only a small fraction (<15%) of current and only at the most negative voltages (<−100 mV; not shown). This result suggested

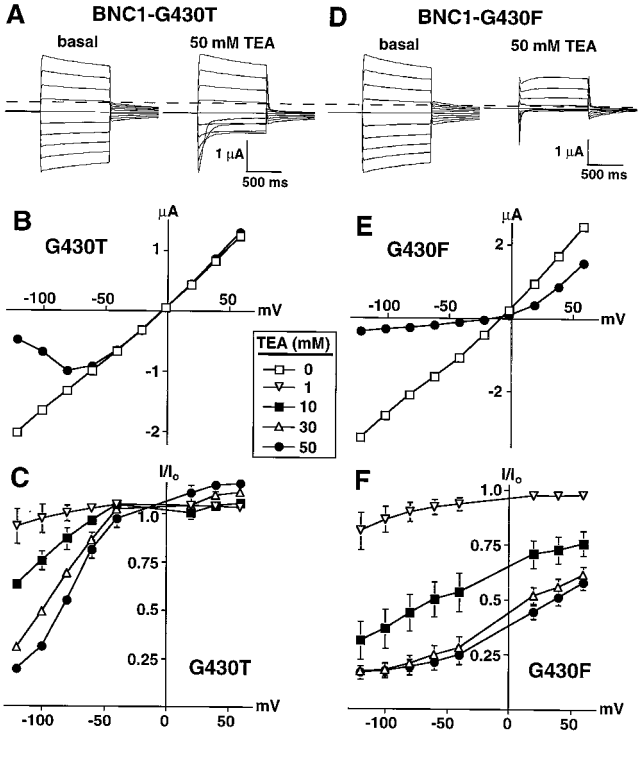

FIGURE 5 The Deg residue influences TEA blockade. Data are from oocytes expressed BNCl-G430T (*A-C*) or BNCl-G430F (*D-F*) bathed in Na^+ Ringer's. (*A* and *D*) Current in the presence and absence of 50 mM TEA at voltages ranging from −120 to +60 mV. Holding voltage was −20 mV. (*B* and *E*) Current-voltage relationships of data from (*A* and *D*). (*C* and *F*) Fractional current (I/Io) in 1, 10, 30, or 50 mM TEA, plotted against voltage. Data are mean ± SE from at least five oocytes.

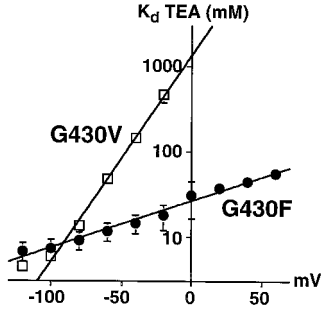

FIGURE 6 Voltage-dependence of TEA blockade. The K_d for TEA block of BNCl-G430V or BNCl-G430F was calculated using the formula: $K_d = (\text{TEA} \times I)/(I_o - I)$, in which TEA = 30 mM, I = current in the presence of 30 mM TEA, and I_o = basal current. K_d values were plotted on a logarithmic scale against voltage. Data are mean ± SE from at least eight oocytes.

that TPA may be too large to enter the pore of either BNC1 mutant.

TMA had similar effects when the Deg residue was Val or Phe (Fig. 7, *A-F*); both mutants showed steep voltage-dependent block with a similar dose-dependence. The $z\delta$ was 1.20 and 1.10 for TMA block of BNC1-G430V and BNC1-G430F, respectively. These data indicated that TMA interacted with the same site(s) when residue 430 was either Val or Phe. In addition, the results suggest that TMA and TEA interact with the same site(s) when 430 is Val. Conversely, the data suggested that TMA did not interact with Phe430. Stated differently, when residue 430 was Phe, the TEA binding site was size selective.

DISCUSSION

Our results indicate that extracellular TEA and TMA block BNC1 current, and block depends on the amino acid at the Deg position (residue 430). When residue 430 is a Val or a Thr, TEA and TMA but not TPA block the channel in a similar manner. This suggests similar or identical sites of interaction for TMA and TEA. The interaction site lies within the pore based on the multi-ion nature of the block with a steep voltage-dependence and high $z\delta$ value and on the finding that outward flow of ions reduced block by extracellular TEA (Hille, 1992). Although our data do not allow us to approximate the location of the blocking site(s), they indicate that the pore of BNC1-G430V can likely accommodate more than one ion simultaneously.

In contrast, when residue 430 is a Phe, TEA shows a very different pattern of block, suggesting a new site of interaction. The voltage-dependence of block no longer shows multi-ion behavior and instead suggests that the new TEA-binding site lies ≤30% across the membrane electrical field from the outside. The data suggest that Phe430 contributes to this new site. The accessibility of residue 430 is supported by the finding that sulfhydral-reactive agents modify the site when it contains a Cys (Adams et al., 1998b). Interestingly, changing residue 430 from a Val to a Phe did not alter the characteristics of TMA block. This suggests that TMA interacted with the same site(s) in the two channels.

A simple model to explain these findings is shown in Fig. 8. In BNCl-G430V (*left*), TEA (and TMA) block at a site within the pore. In BNCl-G430F (*right*), TMA blocks at the same site as in BNCl-G430V. However, the Phe creates a new binding site for TEA. Neither the smaller molecule, TMA, nor the larger molecule, TPA, block at this site, suggesting that the site discriminates between ions partly on the basis of size. Furthermore, binding of TEA to this new site seems to preclude an interaction of TEA with the site of block in BNCl-G430V and BNCl-G430T. The suggestion that Phe430 is the new TEA binding site is consistent with previous work on K^+ channels suggesting that TEA blocks by participating in π-cation interactions with aromatic residues such as Phe (Heginbotham and MacKinnon, 1992).

There are other alternatives to the model shown above. First, instead of adding a binding site, mutation of residue 430 to Phe could eliminate a binding site. Second, Phe430 might cause a conformational change that generates a new TEA binding site some distance away from residue 430. We cannot exclude these possibilities, but they seem less likely for several reasons. 1) The effect was specific to Phe and

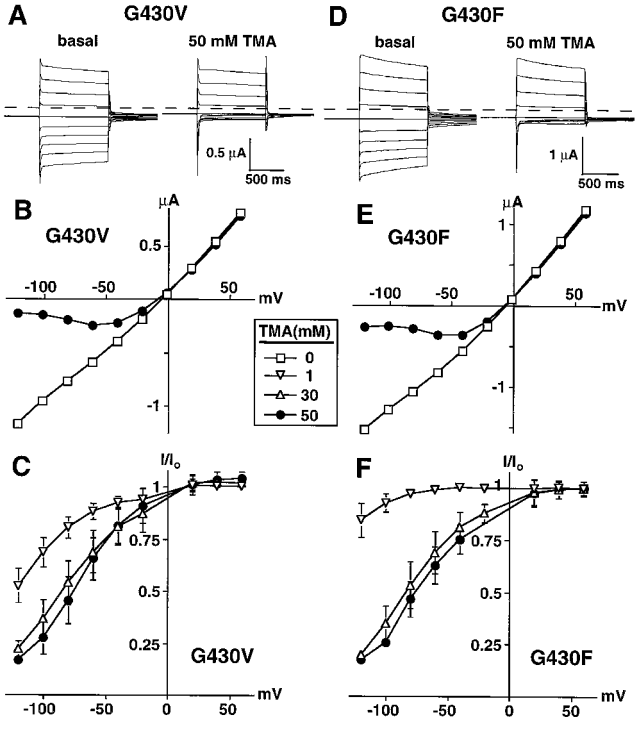

FIGURE 7 TMA block of BNCl-G430V and BNCl-G430F. Oocytes expressed BNCl-G430V (*A-C*) or BNCl-G430F (*D-F*) and were bathed in Na^+ Ringer's. (*A* and *D*) Current in the presence and absence of 50 mM TMA at voltages ranging from −120 to +60 mV. Holding voltage was −20 mV. (*B* and *E*) Current-voltage relationships of data from *A* and *D*. (*C* and *F*) Fractional current (I/Io) in 1, 30, or 50 mM TMA plotted against voltage. Data are mean ± SE from at least five oocytes.

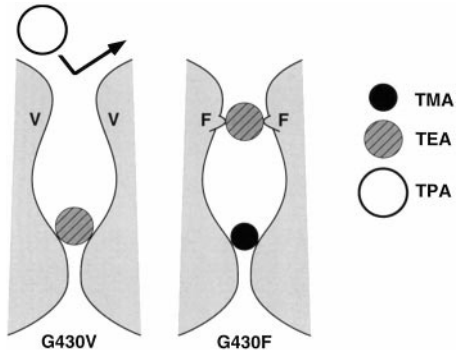

FIGURE 8 Model describing block by TEA and TMA. In BNCl-G430V (*left*) either TEA or TMA can block. However because of its large size, TPA does not block. In BNCl-G430F (*right*), the introduced phenylalanine forms a new binding site for TEA. Size constraints prevent TMA from binding at this site. However, TMA continues to block at the same site as in BNCl-G430V.

was not seen with Val or Thr at residue 430. 2) The effects of TMA and TPA were not altered by the Phe mutation. 3) Channels with mutations to Val or Phe had several other properties that were not different from each other, including single channel conductance, cation selectivity, amount of whole-cell current, and sensitivity to amiloride (Waldmann et al., 1996; Adams et al., 1998b; and data not shown).

It is interesting that TEA, which is usually recognized as a K^+ channel blocker, also blocks proteins in a Na^+ channel family. Recent work indicates that channels in the BNC1/ASIC/DRASIC subfamily are activated by an acidic extracellular pH (Waldmann et al., 1997a,b; Bassilana et al., 1997; Lingueglia et al., 1997), suggesting that these channels may represent amiloride-sensitive proton-activated channels observed in vivo. Consistent with this hypothesis, TEA produces a voltage-dependent block of amiloride-sensitive proton-activated Na^+ channels in rat trigeminal neurons (Korkushko and Krishtal, 1984). However, some DEG/ENaC channels, such as ENaC and RPK, appear to be TEA-insensitive. Perhaps TEA may be a useful pharmacological tool for discriminating between DEG/ENaC channels and for probing their structure.

Our findings suggest that residue 430 lies within the pore of BNC1 and affects ionic selectivity, as well as TEA and amiloride block. The ability of pore-lining residues to affect channel activity has a precedent in the *weaver* mutation of the inward rectifier K^+ channel GIRK2. Both the Deg and the *weaver* mutations greatly enhance constitutive channel activity (Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996). Both mutations alter ion selectivity; whereas BNC1 is normally Na^+ selective and GIRK2 is K^+ selective, and channels with the Deg or *weaver* mutations select poorly between Na^+ and K^+ (Price et al., 1996; Waldmann et al., 1996; Slesinger et al., 1996; Kofuji et al., 1996; Navarro et al., 1996). Both mutations produce channels that are toxic to cells; heterologous expression of either BNC1 Deg mutants (Waldmann et al., 1996; and not shown) or GIRK2 *weaver* mutants (Navarro et al., 1996) cause cells to swell. Finally, cerebellar granule neurons of the *weaver* mouse exhibit unusual ultrastructural changes that are also seen in *C. elegans* neurons that express DEG/ENaC proteins bearing Deg mutations (Hall et al., 1997).

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