Interaction of the BK<sub>Ca</sub> channel gating ring with dendrotoxins

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**Keywords:** Ca<sup>2+</sup>-activated K<sup>+</sup> channel, dendrotoxin, gating, ion channels, K<sup>+</sup> channel, subconductance

**Abbreviations:** BK<sub>Ca</sub> (KCNMA1, KCa1.1, Slo1) large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel alpha subunit; DTX, dendrotoxin; BPTI, bovine pancreatic trypsin inhibitor; TEA<sup>+</sup>, tetraethylammonium

Two classes of small homologous basic proteins, mamba snake dendrotoxins (DTX) and bovine pancreatic trypsin inhibitor (BPTI), block the large conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel (BK<sub>Ca</sub>, KCa1.1) by production of discrete subconductance events when added to the intracellular side of the membrane. This toxin-channel interaction is unlikely to be pharmacologically relevant to the action of mamba venom, but as a fortuitous ligand-protein interaction, it has certain biophysical implications for the mechanism of BK<sub>Ca</sub> channel gating. In this work we examined the subconductance behavior of 9 natural dendrotoxin homologs and 6 charge neutralization mutants of δ-dendrotoxin in the context of current structural information on the intracellular gating ring domain of the BK<sub>Ca</sub> channel. Calculation of an electrostatic surface map of the BK<sub>Ca</sub> gating ring based on the Poisson-Boltzmann equation reveals a predominantly electronegative surface due to an abundance of solvent-accessible side chains of negatively charged amino acids. Available structure-activity information suggests that cationic DTX/BPTI molecules bind by electrostatic attraction to site(s) on the gating ring located in or near the cytoplasmic side portals where the inactivation ball peptide of the β2 subunit enters to block the channel. Such an interaction may decrease the apparent unitary conductance by altering the dynamic balance of open versus closed states of BK<sub>Ca</sub> channel activation gating.

**Introduction**

The large-conductance Ca<sup>2+</sup>-activated K<sup>+</sup> channel (also known as MaxiK, BK<sub>Ca</sub>, Slo1, KCa1.1) is a tetrameric membrane protein encoded by a single human α-subunit gene (KNCMA1) expressed in multiple forms in different tissues depending on the pattern of alternatively spliced exons. BK<sub>Ca</sub> channels also exist as complexes of α subunits with β1–β4 or γ1–γ4 regulatory subunits as determined by co-expression of 2 different families of genes, KCNMB1-4 and LRRC26, 52, 55 and 38, respectively. BK<sub>Ca</sub> channels are dually activated by intracellular Ca<sup>2+</sup> and depolarizing voltage. Given their robust ability to hyperpolarize membrane potential, their function involves physiological feedback regulation of electrical excitation and Ca<sup>2+</sup> entry in various cells and organelles such as neurons, smooth muscle, and mitochondria of cardiac myocytes.

Many natural peptide toxins such as the scorpion toxins, char-ybdotoxin (ChTX) and iberiotoxin (IbTX), physically block K<sup>+</sup> flux through BK<sub>Ca</sub> channels and various other K<sup>+</sup> channels by binding to a site at the extracellular pore entrance of the tetrameric channel-forming α-subunit. Similarly, venom of African mamba snakes contain a class of peptide toxins called dendrotoxins that externally block certain voltage-gated K<sup>+</sup> channels and the inward rectifier K<sup>+</sup> channel, Kir1.1 (ROMK1, gene KCNJ1). Dendrotoxins such as DTX-I (or Toxin I) of the black mamba snake (Dendroaspis polylepis) are ~60-residue mini-proteins stabilized by 3 disulfide bonds that are also structurally homologous to bovine pancreatic trypsin inhibitor (BPTI) and related Kunitz inhibitors of serine proteinases. Studies of such toxin interactions have yielded much information on molecular mechanisms of K<sup>+</sup> channels and their physiological functions. However, a puzzling and still unexplained toxin phenomenon involves rectifying subconductance events in single-channel current records upon addition of DTX-I to the intracellular side of the BK<sub>Ca</sub> channel. In pursuing the molecular mechanism of this unusual toxin-channel interaction, we found that the trypsin inhibitor BPTI produced similar subconductance events in BK<sub>Ca</sub> channels with kinetics consistent with reversible binding to a single intracellular site. Amino acid substitution of a critical residue of BPTI...
(Lys15) that normally binds in the substrate specificity pocket of serine proteases such as trypsin was found to dramatically alter the current-voltage (I-V) behavior of the subconductance (or substate) events. The irreversible complex of BPTI and trypsin was also found to be inactive in substate production, another finding implying that the inhibitory loop of BPTI containing Lys-15 made contact with the channel. Such observations led to the proposal that DTX and BPTI bind to an intracellular site on the BKCa channel that is structurally similar to a serine protease domain. Based on a tentative sequence alignment, we suggested that a ~250-residue region near the C-terminus of the intracellular domain of the BKCa channel protein may correspond to a hypothetical serine protease-like domain. This hypothesis was later challenged on the basis of the weak statistical significance of the alignment and was recently disproven when X-ray crystal structures of the whole C-terminal intracellular domain of BKCa showed that this region forms a unique “gating ring” structure that contains 2 tandem RCK (regulator of conductance for K+) domains.

Recent atomic-level structures of the BKCa gating ring have provided new insights on the mechanism by which binding of Ca2+ to a loop of acidic amino acids called the “calcium bowl” is coupled to conformational changes of the gating ring that open the intracellular gate of the channel. Since the mechanism of substate production by DTX and BPTI is still unresolved, we revisited this issue in the context of new structure-activity data on a collection of natural DTX homologs and mutants, the 3-dimensional structure of the gating ring, and relevant studies on the mechanism of rapid inactivation of BKCa channel mediated by the unstructured N-terminal peptide of the β2 accessory subunit. We also examined the role of surface electrostatics of the BKCa gating ring. The results suggest several new ideas for the mechanism of subconductance interaction of dendrotoxins and BPTI with the BKCa channel that may ultimately help to understand the function of the gating ring in activation and inactivation gating.

**Results**

**Subconductance-activity of various dendrotoxin homologs and mutants**

Four species of mamba snakes (*Dendroaspis polylepis, D. angusticeps, D. jamesoni, and D. viridis*) native to Africa comprise a genus of the Elapidae family of venomous snakes. Two classes of peptide components isolated from mamba venom include 57- to 60-residue dendrotoxins and an unrelated group of 59- to 60-residue toxins called calciseptines. Dendrotoxins contain 3 conserved disulfide bonds and are structural homologs of the Kunitz-type trypsin inhibitor known as aprotinin or BPTI (bovine pancreatic trypsin inhibitor). Calciseptines contain 4 conserved disulfide bonds and are structural homologs of the family of 3-finger α-neurotoxins (e.g., α-bungarotoxin from the Taiwanese banded krait *Bungarus multicinctus*) that are classically known as competitive antagonists of nicotinic acetylcholine receptors. Many dendrotoxins such as DTX-I, DTX-K, and DpL1 from *D. polylepis; α-DTX and δ-DTX from D. angusticeps*; and, DjR2 from *D. jamesoni* block certain voltage-gated K+ channels from the extracellular side. Other dendrotoxins such as DaP1 from *D. angusticeps* and DaE4 from *D. polylepis* have low activity as neurotoxins and are active inhibitors of serine proteinase enzymes. A particular subgroup of dendrotoxin homologs called calcicludines (e.g., DjT2 from *D. jamesoni*) block certain isoforms of voltage-gated Ca2+ channels.

**Figure 1** shows a sequence comparison of various purified mamba toxins used in the present study. We tested these toxins on single BKCa from rat brain channel and compared their activity with BPTI. The sequences are aligned using ClustalW, and conserved residues are marked in yellow. The figures also show the activity of the toxins with respect to control (90% activity). The toxins were tested at 10 μM concentration, and the activity was measured in terms of voltage-gated K+ channel activity. The results show that DTX-I, DTX-K, and DpL1 have high activity, while DTX-I, DTX-K, and DpL1 have low activity.

**Table 1**

| Inhibitor | Sequence | Activity | Charge |
|-----------|----------|----------|--------|
| DTX-I     | KQQLRKILHRNPGRQYKIPAFYNYKHQCQEGFTWGCQGNSNRFKITEEBRTCIRK | KV      | +10    |
| DjR2      | KQPRKLILHRNPGRQYKIPAFYNYKHQCQEGFTWGCQGNSNRFKITEEBRTCIRK | KV      | +10    |
| α-DTX     | WQPPPPKQPRKLILHRNPGRQYKIPAFYNYKHQCQEGFTWGCQGNSNRFKITEEBRTCIRK | KV      | +8     |
| DTX-K     | AAYKQKLPRILGCKRKISPITYKWKACCLPPFDSGCQGNSNRFKITEEBRTCIRK | KV      | +10    |
| DpL1      | AAYKQKLPRILGCKRKISPITYKWKACCLPPFDSGCQGNSNRFKITEEBRTCIRK | KV      | +10    |
| δ-DTX     | QWQPWPPPQPRKLILHRNPGRQYKIPAFYNYKHQCQEGFTWGCQGNSNRFKITEEBRTCIRK | KV      | +10    |
| DjT2      | WQPPPPKQPRKLILHRNPGRQYKIPAFYNYKHQCQEGFTWGCQGNSNRFKITEEBRTCIRK | CaV     | +10    |
| DaP1      | RHTFTKLPAEGPGKASIPAFLYNYNAAKQLFLYGGKKGPLNFTEKCRACVG | SerP    | +8     |
| DaE4      | RPYCPHELAAVAPGMMFPAMFYYSGSNKCFPPYTCSSGNSNRFKITEEBRTCIRK | SerP    | +5     |
| BPTI      | RPYCPHELAAVAPGMMFPAMFYYSGSNKCFPPYTCSSGNSNRFKITEEBRTCIRK | SerP    | +6     |
| S-S pairs: | RIYHKASLPRATKDEYKMFIRTYQMSKQREMCSQCGTAMWQPTECQKGDRCNK | CaV     | +6     |

**Figure 1.** Sequences of dendrotoxin homologs, BPTI, and calciseptine. Dendrotoxin inhibitors which are venom components of mamba snakes *Dendroaspis polylepis* (DTX-I, DTX-K, DpL1, DpE4, calciseptine), *D. angusticeps* (α-DTX, δ-DTX, DaP1), and *D. jamesoni* (DjR2, DjT2) are labeled according to Schweitz and Moigner. Activity refers to known inhibitory activity on voltage-gated K+ channels (Kv), voltage-gated Ca2+ channels (Cav), or serine proteinases (SerP). Cys residues are highlighted in yellow and disulfide bonds (S-S pairs) are identified by paired numerals. Charge is calculated as the number of basic residues (R, K in blue) minus acidic residues (D, E in magenta). Residue positions of the alignment identical to DTX-I are highlighted in gray.
muscle incorporated into planar lipid bilayers to further investigate the structure-activity basis of the subconductance effect previously described for DTX-I and BPTI.\textsuperscript{17-19}

All 9 dendrotoxins listed in Fig. 1 are active in the production of discrete substate events when tested at 2–6 μM concentration on the intracellular side of single BK\textsubscript{Ca} channels (Fig. 2). Each dendrotoxin causes the appearance of discrete current interruptions that give the appearance of a major sublevel between the zero-current closed state and the fully open conductance level. Control single-channel behavior of a BK\textsubscript{Ca} channel under these conditions is similar to that of the bottom record in Fig. 1 which was taken in the presence of 2 μM calci-septine, an α-neurotoxin homolog that does not induce substates.

The average duration of substate events induced by dendrotoxins varies considerably from 28.8 ± 3.1 s (±SE, n = 118) for DjR2 to 1.2 ± 0.1 s (±SE, n = 99) for DpE4. In general, a shorter substate dwell time is exhibited by dendrotoxins less similar in sequence to DTX-I and DjR2 such as SerP inhibitors, DaP1 and DpE4; and, DjT2 previously classified as a calci-cludine.\textsuperscript{29} Since dendrotoxins are highly basic small proteins with net charges ranging from +5 to +10, we also studied a collection of δ-DTX mutants\textsuperscript{12} corresponding to Ala substitution of 6 different basic residues (Lys or Arg). All of the tested charge-neutralization mutants of δ-DTX (K6A, R10A, K16A, K17A, R44A, and R53A) were also active in the production of substate events. However, they exhibited a shorter mean substate dwell time relative to δ-DTX that ranged from a 1.8-fold reduction for K17A to 5.5-fold reduction for R44A (Fig. 3).

We studied the dendrotoxin-BK\textsubscript{Ca} channel interaction in more detail by measuring kinetic parameters of the substate events which correspond to residence times of the toxin on the channel. Sample durations of substate/blocked and open/unblocked dwell time events (n ≥ 100) were collected for each toxin and plotted as probability density histograms in a linear-log format. Fits of the dwell-time histogram to single-exponential functions were used to estimate the first order dissociation rate, k\textsubscript{off} = τ\textsubscript{substate}^{-1}, and the bimolecular association rate, k\textsubscript{on} = ([toxin] τ\textsubscript{unblocked})^{-1}, for each toxin as previously described\textsuperscript{18}. Fig. 4 shows examples of dwell time event histograms for toxins DpL1 (Fig. 4A) and DpE4 (Fig. 4B) that are fit by an exponential distribution.

Measured rate constants, k\textsubscript{off} and k\textsubscript{on}, for each toxin are summarized in Figs. 5A and 5B, respectively, along with the equilibrium dissociation constant for each toxin, K\textsubscript{D} (Fig. 5C), calculated from the ratio of k\textsubscript{off}/k\textsubscript{on}. This comparison reveals a rather modest effect of amino acid changes on the kinetics of the.
toxin-channel interaction. For example, there is a 29-fold difference in $k_{off}$ between the slowest (DjR2) and fastest (DbE4) toxin dissociation rate and a 92-fold difference in $k_{on}$ between the slowest ($\alpha$-DTX) and fastest (DjT2) toxin association rate. The overall kinetic effects correspond to a modest 50-fold difference in equilibrium $K_D$ between the highest affinity (DjT2) and lowest affinity (DpE4) toxins. This weak structure-activity dependence contrasts with substantial dissimilarity between 2 sequences such as DjT2 and DpE4; i.e., ~60% of the residues are different. Similarly, the $\delta$-DTX set of mutations of single Lys/Arg residues to Ala exhibit a maximum 18-fold difference in $K_D$ between the highest affinity toxin ($\delta$-DTX) and lowest affinity mutant (R10A) (Fig. 4C).

Structure-activity behavior of the dendrotoxins was also examined by comparing the fractional mean current level of the substate to that of the open state, $I_{\text{substate}}/I_{\text{open}}$ at $+30\,\text{mV}$. The results summarized in Fig. 6 show subtle variations for the different dendrotoxins that range from a low value of $I_{\text{substate}}/I_{\text{open}} = 0.38 \pm 0.01$ for DTK-K to a high value of $0.80 \pm 0.01$ for DaP1. Neutralization of single Lys/Arg residues to Ala resulted in a small, but consistent reduction in the fractional substate current ranging from a decrease of 2% to 29% in $I_{\text{substate}}/I_{\text{open}}$ for the 6 tested Ala mutations of $\delta$-DTX in comparison to the native toxin (Fig. 6). The latter result demonstrates that charged residues of the toxin affect the subconductance level, but comparison of the 9 different dendrotoxin homologs does not reveal an absolute requirement for particular structural determinants.

**Surface electrostatics of the BK$_{\alpha}$ channel gating ring**

DTX and BPTI induce substates exclusively from the intracellular side of the *Drosophila* or mammalian BK$_{\alpha}$ channel in transfected cells expressing the cloned $\alpha$-subunit.33 Thus, it is likely that the cytoplasmic portion of the BK$_{\alpha}$ $\alpha$-subunit contains the site(s) of interaction with these small, soluble toxin proteins. The BK$_{\alpha}$ $\alpha$-subunit polypeptide has an N-terminal pore domain similar to that of other voltage-gated K$^+$ channels, which consists of 7 membrane-spanning $\alpha$-helices denoted S0-S6.34 At the C-terminal end of the S6 transmembrane helix, the BK$_{\alpha}$/Slo1 channel has a unique ~800 residue cytoplasmic tail similar to that present in 3 gene paralogs (Slo2.1, Slo2.2, Slo3) which comprise the mammalian Slo family of K$^+$ channels.35 In BK$_{\alpha}$/Slo1, the large cytoplasmic tail contains 2 tandem, structurally similar, RCK sub-domains denoted N-terminal RCK1 and C-terminal RCK2, consisting of ~270 and ~370 residues, respectively.23,24 When truncated from the channel-forming domain and separately expressed as a soluble protein, the cytoplasmic tail of BK$_{\alpha}$ $\alpha$-subunit forms a tetrameric “gating ring” that contains one or more binding sites for Ca$^{2+}$ and other divalent metal cations such as Mg$^{2+}$ per $\alpha$-subunit. The major Ca$^{2+}$-selective site located within the RCK2 domain is known as the “calcium bowl” and contains a high density of negative charges (7 Asp residues within a 9 residue sequence, DQDDDDDPD).23,26,36,37

The atomic structure of the gating ring has been determined in the absence and presence of Ca$^{2+}$ by X-ray crystallography of truncated, soluble C-terminal domains of human and zebrafish BK$_{\alpha}$ $\alpha$-subunit, at a resolution of 3.1 Å and 3.6 Å, respectively.23-25 Comparison of the ‘closed’ (Ca$^{2+}$-free) vs. the ‘open,’ (Ca$^{2+}$-bound) structures of the BK$_{\alpha}$ gating ring shows that Ca$^{2+}$ binding causes a large conformational change described as...
a circular widening of the upper layer of the gating ring closest to the membrane. This conformational change is characterized by a \( \sim 12 \) Å increase in distance between Lys343 residues of 2 diagonally apposed subunits.\(^{25}\) Lys343 is located at the N-terminus of RCK1 and is connected to the C-terminal end of the S6 helix of the pore domain by a \( \sim 16 \)-residue linker that couples this \( \text{Ca}^{2+} \)-dependent widening of the gating ring to channel opening. Evidence suggests that the widening motion of the gating ring exerts a mechanical spring-like pulling action via the linker on the C-terminal ends of the 4 S6 helices to open the activation gate of the \( \text{K}^+ \) channel.\(^{38}\) In light of this model for channel opening, binding of DTX/BPTI to a site on the cytoplasmic portion of the \( \alpha \)-subunit could potentially affect the dynamics of the \( \text{Ca}^{2+} \)-dependent conformational change of the gating ring. Also, binding of DTX/BPTI could directly or indirectly affect the rate of conduction of \( \text{K}^+ \) ions through the \( \text{K}^+ \) pore domain. Either or both of these effects could underlie the appearance of the toxin-induced subconductance states illustrated in Figs. 2 and 3.

Aside from their similar 3-dimensional shape, a common feature of BPTI and DTX homologs that induce substates in the \( \text{BK}_{\text{Ca}} \) channel is their strongly cationic net charge ranging from +5 to +10 (Fig. 1). In contrast, a structural homolog and Kunitz proteinase inhibitor (KID) with a net negative charge (−5) that occurs as a modular domain of amyloid \( \beta \)-protein precursor is completely inactive in substate production in comparison to its basic homolog BPTI (net charge = +6).\(^{20}\) The association rate of a collection of BPTI mutants is correlated with net positive charge; and, the association rates of DTX-I and BPTI are also greatly increased as a function of decreasing ionic strength.\(^{18,20}\) Such behavior implies that binding of such toxins to the \( \text{BK}_{\text{Ca}} \) gating ring involves an electrostatic interaction.\(^{39}\) However, electrostatic attraction is not the sole factor that determines this protein-protein interaction since other small basic proteins with a dissimilar protein fold are inactive in substate production; e.g., calciseptine (net charge = +6) (see Fig. 2). To further pursue the problem, we examined the surface electrostatics of the \( \text{BK}_{\text{Ca}} \) gating ring and the substrate-inducing toxins.

The crystal structure of the \( \text{Ca}^{2+} \)-bound ‘open’ form of the tetrameric \( \text{BK}_{\text{Ca}} \) gating ring from zebrafish (PDB entry 3U6N)\(^{25}\) was computationally refined to predict optimal solution orientations of amino acid side chains without altering the position of the main chain atoms. The refined structure of the \( \text{BK}_{\text{Ca}} \) gating ring and crystal structures of the Kunitz inhibitor BPTI (PDB entry 5PTI) and \( \alpha \)-DTX (PDB entry 1DTX) were analyzed to calculate a map of electrostatic surface potential of each protein based on the Poisson-Boltzmann equation.\(^{40}\) Results of these calculations for the \( \text{BK}_{\text{Ca}} \) gating ring (Fig. 7) and the toxins (Fig. 8) show that the gating ring surface is strongly electronegative in comparison to the basic toxins. In Fig. 7, the gating ring surface is colored using a scale of red to blue to represent an electrostatic surface potential ranging from −20 to +5 kT/e (or −513 to +128 mV). The corresponding color scale for the toxins (Fig. 8) ranges from −1 to +5 kT/e (or −25.7 to +128 mV). The surface charge distribution of the \( \text{BK}_{\text{Ca}} \) gating ring thus exhibits large areas of electronegativity consistent with evidence for an attractive electrostatic interaction with the positive toxins, DTX and BPTI, as summarized above. However, the non-uniform distribution of surface charge implies that certain regions of the gating ring may interact more strongly with the toxins.

The overall shape of the gating ring may be described as a cylindrical disc, \( \sim 60 \) Å thick by \( \sim 140 \) Å in diameter, as estimated from the distance between residues at the extremity of the structure. The gating ring also has a prominent star-like hole through the center of the disc that could potentially

\[ \text{Figure 4. Examples of dwell time histograms of substate-blocked and unblocked events for dendrotoxin homologs, DpL1 (A) and DpE4 (B). Smooth curves indicate best fit to an exponential function.} \]
accommodate a spherical molecule with a maximum diameter of approximately 19 Å. As shown in Fig. 7, the opposite faces of the gating ring disc also have different electrostatic character. The surface facing the membrane is more uniformly electronegative than the opposite surface facing the cytoplasm. In contrast, the cytoplasm-facing surface of the gating ring has a distinct area (~300 Å²) of positive surface charge on each of the 4 ‘subunits’ of the homo-tetramer. This positively-charged area is primarily due to the surface location of side chains of the following residues: K688, R689, K779, R976, and R980 (numbered according to the zebrafish α-subunit). A side view of the gating ring also shows a prominent electronegative crevice corresponding to the intersubunit contact region where 7 acidic residues of the ‘Ca²⁺-bowl’ sequence D896-D904 are located at the surface (Figs. 8 and 9). In addition, the star-like hole in the center of the gating ring has a predominantly negative surface potential. The results of Figs. 7 and 8 show that the positively charged toxins, DTX and BPTI, would experience electrostatic attraction to negatively charged regions of the gating ring. Since the surface of the gating ring closest to the intracellular entrance of the conduction pore has a negative surface potential, it is also conceivable that electrostatic attraction of K⁺ ions by the gating ring may contribute to a ~30% enhancement of unitary K⁺ conductance observed for the whole BKCa channel (307 pS) vs. a truncated form of the channel lacking the gating ring (213 pS).

**Discussion**

Recent crystal structures of the BKCa cytoplasmic gating ring from 2 different vertebrate species disproved the hypothesis of a serine proteinase-like domain in the C-terminal region of BKCa α-subunit and instead revealed a 3D-fold comprised of 2 tandem RCK domains with mixed α/β secondary structure. An important structural feature of the gating ring is a conserved Ca²⁺-binding loop within the RCK2 domain known as the “Ca²⁺-bowl” (DQDDDDDPD) included within residues Q889-D900 of the human BKCa α-subunit. In the crystal structure of the Ca²⁺-bound gating ring, this loop makes direct contact with a Ca²⁺ ion via main chain oxygen atoms of Q889 and D892 and with side-chain carboxyl oxygens of residues D895 and D897. Here we use this structural information and current results with dendrotoxin homologs to consider alternative mechanisms for the substate phenomenon.

At the single-channel level, ‘subconductance’ behavior may arise from: 1. partial occlusion or electrostatic interference of the ion conduction pathway by an ion or small molecule or 2. modification of channel gating behavior that mimics the appearance...
of a stable lower conductance state. Likely examples of the former partial-occlusion subconductance mechanism include the following: 1. Certain mutations of δ-conotoxin GIIIA that partially block voltage-dependent Na⁺ channels (NaV) at an external binding site known to be near the pore entrance; 2. Binding of certain dendrotoxin homologs off center to the outer pore entrance of the Kir inward rectifier channel; 3. Binding of Zn²⁺ at the outer pore entrance of the mammalian cardiac NaV channels as observed in the presence of batrachotoxin, a stabilizer of the open-state.

In contrast to partial occlusion, the rectifying subconductance effect of DTX/BPTI acting from the intracellular side of the BKCa channel was previously ascribed to the second type of mechanism, an alteration of gating. When captured at higher time resolution accessible with patch-clamp recordings (e.g., using a filter cut-off frequency in the range of 1–10 kHz), substate events induced by BPTI and DTX exhibit prominent excess noise due to fast fluctuations that can be described as a rapid, open-closed gating process. This type of behavior would not ordinarily be expected for a blocker that physically occluded the ion conduction pathway to produce a lower unitary current of permeant ions through an open pore. Given current understanding of the BKCa gating ring as a dynamically active mechanical nanodevice that opens the aperture of the activation gate formed by apposition of 4 S6 helices, it seems possible that binding of small toxin proteins such as BPTI/DTX could alter the normal

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**Figure 7.** Electrostatic surface potential of the tetrameric gating ring structure of the zebrafish BKCa channel. The reported crystal structure (PDB entry 3O6N) determined in the presence of 10 mM CaCl₂ was used to plot the surface potential calculated from the Poisson-Boltzmann equation as a color map using the solvent-accessible surface. Color scale ranges from −20 kT/e (dark red) to +5 kT/e (dark blue). (A) view from the cytoplasmic surface of the membrane closest to BKCa pore domain, (B) view from the cytosol farthest from the BKCa pore domain, (C) side view oriented with the surface closest to the membrane at the top.

**Figure 8.** Orthogonal views of (A) BPTI (PDB entry 5PTI) and (B) α-DTX (PDB entry 1DTX) shown as a color map of electrostatic surface potential plotted on the solvent-accessible surface. Color scale ranges from −1 kT/e (dark red) to +5 kT/e (dark blue).
dynamics of BKCa gating ring and give rise to subconductance behavior. A possible scenario might involve binding of a toxin molecule at a site on or near the upper layer of the gating ring involved in the conformational change that opens that channel. For example, if a bound toxin partially hinders movement of one or more of the 4~16-residue linkers that connect the S6 helix to the N-terminus of RCK1, this might result in altered gating dynamics akin to fast, flickering behavior characteristic of BPTI- and DTX-induced substrates.\textsuperscript{42} It is also worth noting that mechanisms involving partial occlusion of the ion conduction pathway and alteration of channel gating are not necessarily mutually exclusive. For example, a mutant of \(\mu\)-conotoxin that blocks single-channel current of voltage-dependent Na\(^+\) channels to a discrete subconductance level binds close to the external pore entrance and also suppresses channel opening by electrostatic inhibition of outward movement of the S4 voltage sensors associated with activation gating.\textsuperscript{44}

Direct evidence for the ‘alteration-of-gating’ hypothesis would require precise identification of the actual sites of interaction of DTX/BPTI with the BKCa gating ring. Indirect evidence from previous work showed that the kinetics and/or affinity of 2 pore blockers, Ba\(^{2+}\) and tetraethylammonium (TEA\(^+\)), are not affected by occupancy of the channel with BPTI or DTX. Detailed analysis showed that blocking affinities of both Ba\(^{2+}\) and TEA\(^+\) applied from either side of the membrane are not altered by the peptide toxin.\textsuperscript{18,46} The latter results indicate that binding of BPTI or DTX to the intracellular gating ring does not obstruct entry or alter kinetic barriers for entry and exit of Ba\(^{2+}\) and TEA\(^+\) pore blockers to/from the ion conduction pathway. This is consistent with the idea that the substate effect is not due to physical occlusion of the primary ion conduction pathway defined as the pore that extends from the extracellular vestibule to the cytoplasmic end of the 4 S6 helices.

In contrast, discrete block of single BKCa channels by a 20-residue homolog of the N-terminal Shaker inactivation ball peptide (BP) is non-competitively inhibited by simultaneous binding of DTX. The binding affinities of BP and DTX as measured by their respective equilibrium dissociation constants (\(K_d\)) are reciprocally reduced by \~8-fold when either one of these ligands binds to the BKCa channel already occupied by the other ligand.\textsuperscript{46} These latter experiments revealed a negatively-coupled allosteric interaction between BP and DTX in which binding of one ligand interferes with, but does not exclusively prevent, binding of the other ligand. How might this occur?

Ball peptide from the N-terminus of the Shaker K\(^+\) channel of Drosophila is known to emulate the blocking behavior of similar loosely structured peptides at the N-terminus of certain \(\beta\)-subunits of BKCa channels (e.g., \(\beta_2\), \(\beta_3\)) that mediate a form of rapid “ball-and-chain” inactivation by entering and physically occluding the ion conduction pore from the internal side of the membrane.\textsuperscript{47-51} Trypsin-cleavage studies of the mechanism of \(\beta\)-subunit-mediated inactivation of BKCa reveal that as many as 16 N-terminal residues of the highly flexible inactivation peptide can enter any of 4 presumably identical antechambers at the base of the K\(^+\) pore domain.\textsuperscript{27,52} These antechambers apparently form a natural entryway to the K\(^+\) conduction pathway near inner gate, which is formed by the 4 S6 helices at the cytoplasmic surface of the membrane. Furthermore, kinetic analysis indicates that the inactivation peptide first associates with the BKCa channel to form a transient pre-inactivated open state before transitioning to a non-conducting inactivated state.\textsuperscript{28} Because the 3D structure of the whole intact vertebrate BKCa channel is not yet available, homology models of the separate pore domain docked with the gating ring (e.g., see Fig. 9) have been used as a surrogate composite model to consider functional implication of these antechambers.\textsuperscript{27}

Such a composite structural model (e.g., Fig. 9) as previously discussed by Zhang et al.\textsuperscript{27} suggests that the external opening of the 4 antechambers, termed a “side-portal,” is wide enough to accommodate entry of the \(\beta_2\) inactivation peptide. The side-portal window in the native channel is proposed to have an opening shaped like a truncated square pyramid with approximate dimensions of \~19 A \times \~52 A as measured for the narrowest width and longest side.\textsuperscript{27} Such a window was found to be large enough to accommodate entry of the N-terminal part of \(\beta_2\) inactivation peptide whose solution structure exhibits 2 short \(\alpha\)-helical regions as determined by NMR.\textsuperscript{53} However, this window is not

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**Figure 9.** Homology model of BK\(\alpha\) subunit with \(\alpha\)-DTX. This model is a ribbon diagram of the BKCa channel similar to that illustrated in Contreas et al.\textsuperscript{2} where the membrane pore domain is a homology model of the human channel (HSlo) based on the crystal structure of MthK (PDB: 1LNQ). The approximate location of the lipid bilayer is represented by the blue lines with the extracellular side at the top. The separate gating ring tetramer docked beneath the pore domain is the Ca\(^{2+}\)-bound form of zebrafish BKCa (PDB: 3U6N). The \~16 residue linkers that connect the 4 S6 helices to the gating ring domains are missing from this model. The small protein at the lower left is the crystal structure of \(\alpha\)-DTX (PDB: 1DTX). Secondary structure is colored as \(\alpha\)-helix (red) and \(\beta\)-strand (cyan). Residues of the Ca\(^{2+}\) ions are magenta spheres sized at 1.5x. Possible sites of DTX binding include the side portals at the interface between the membrane domain and the gating ring and the subunit interfaces of the gating ring near the Ca\(^{2+}\) bowl.
wide enough to allow entry of the larger trypsin molecule, a ~30 kDa soluble enzyme which cleaves the inactivation peptide at exposed basic residues to abolish inactivation.27

Relevant to the present results, a window of this size could potentially accommodate partial entry of DTx or BPTI. BPTI is roughly a pear-shaped molecule with a long dimension of ~30 Å and a shorter width dimension ranging from ~11 Å at the skinny end where the trypsin specificity loop is located to a maximum width of ~27 Å at the opposite fat end of the molecule.15 Based on these size considerations, it is conceivable that the narrow end of a BPTI/DTx molecule could partially enter one of the side portals and perturb channel gating dynamics without preventing entry of the ball peptide through one of the remaining 3 portals. However, a positively charged BPTI/DTx molecule that partially plugs the antechamber could affect the simultaneous binding of an inactivation peptide by slowing both its association rate to the toxin-occupied channel and also by slowing its dissociation rate from the toxin-bound channel, as previously documented.46 In this scenario, with DTx or BPTI bound part of the way into a side portal, apparent subconductance behavior might result from alteration of gating dynamics that destabilizes the fully open state of the S6 activation gate and/or a contact effect of the toxin partially plugged into a side portal. A model where BPTI/DTx acts like a partial plug bound in one of 4 side portals could also explain the observation that BPTI can bind with ~120-fold lower affinity to a DTx-occupied subconductance state,46 if 2 different side portals can be simultaneously occupied by one molecule each of DTx and BPTI.

Another possible explanation for the mechanism of substate production could involve entry of BPTI/DTx into the central star-like hole of the gating ring via the opening distal to the membrane and perturbation of channel gating similar to that described above. However, the relative dimensions suggest that the toxin molecule would not be able to traverse very far into the star-like hole. One further idea is that BPTI/DTx may simply bind at a superficial site on the surface of the gating ring, perhaps near one of the 4 Ca-bowl sites and exert its effects on channel gating and the inactivation peptide by long-range allosteric interactions. However, in this case, it would be difficult to explain why simultaneous binding of more than one toxin molecule to 4 identical superficial sites on the surface of the tetrameric gating ring is highly disfavored.46

Summary

We studied the behavior of a collection of natural DTx homologs and Ala substitution mutations of δ-DTx to further investigate the structure-activity basis of their subconductance effect on single BKCa channels. All of the tested toxins induced the appearance of discrete substate events in current records as previously documented for DTx-I and the homologous Kunitz inhibitor, BPTI. Modest variations in binding kinetics and mean current level of the apparent substate among the different DTx variants were observed. The accumulated evidence suggests that the specificity of the binding interaction with the cytoplasmic gating ring of BKCa is largely determined by complementarity of the Kunitz/DTx protein fold and electrostatics of interaction between the positively charged toxin molecule and the channel binding site. Calculation of an electrostatic surface potential map for the 3D structure of the BKCa tetrameric gating ring identified predominant areas of negative surface charge, especially on the surface proximal to the membrane and 4 identical crevices corresponding to the location of the Ca2+ bowl. Current structural models of the pore domain attached to the cytoplasmic gating ring predict the existence of 4 internal antechambers at the base of the pore domain that are accessible via side portal windows to the entry of ions, small molecules, and the N-terminus of the inactivation peptide of the β2 accessory subunit. Consideration of the latest structural and functional information suggests that electrostatic attraction between cationic BPTI/DTx molecules and negatively charged regions of the gating ring may facilitate binding of the toxin to sites on the surface or part way into the side portals that interfere with normal conformational dynamics of the gating ring. Suppression of the normal range of motion of the gating ring and/or the spring-like linker that controls opening of the activation gate of the pore domain may account for the apparent subconductance effect exhibited by these toxins.

Materials and Methods

Dendrotoxin sources and purity

DTx-I (Toxin I) and α-DTx were obtained from Alomone Labs (Jerusalem, Israel) and purity was assayed by the manufacturer. Recombinant δ-DTx (DaK) and selected Ala mutants (K6A, R10A, K16A, K17A, R44A, R53A) were produced and purified as described by Imredy et al.11,12 Purified mamba venom toxins DjR2, DTx-K (DpK), DpL1, DjT2, DaP1, and DpE4 as described in Schweitz and Moinier29 were kindly provided by Hugues Schweitz and Michel Lazdunski (Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, France). The identity of the dendrotoxins was confirmed by mass spectrometry and sequencing. The DaP1 sample was found to contain less than 10% of another toxin from *Dendroaspis angusticeps* venom. Toxin stock solutions were prepared in water at 200 μM and stored at ~80°C between experiments. The sequences of DTx homologs used in this study are summarized in Figure 1.

Planar bilayer recordings

Plasma membrane vesicles prepared from rat skeletal muscle46 were used as source of native BKCa channels for incorporation into planar lipid bilayers. Lipid bilayers were formed by painting a 25 mg/mL lipid solution composed of a 4:1 weight ratio of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine and 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (Avanti Polar Lipids, Alabaster AL) in n-decane with a fine glass rod on a hole with a diameter of 200 μm in a polystyrene or teflon chamber (Warner Instruments, Hamden CT). Membrane thinning and bilayer formation was monitored by capacitance measurement. The solution on both sides of the bilayer was 100 mM KCl and 10 mM MOPS-KOH, pH 7.4. During channel incorporation,
CaCl₂ concentration was 200 µM on the side of the bilayer (cis/intracellular) to which membrane vesicles were added. EDTA (0.1 mM) was added to the opposite (trans/extracellular) side to ensure the orientation of active BKCa channels as cis-intracellular. Only bilayers containing single BKCa channels as determined from the current amplitude were used for quantitative analysis. Ca²⁺ concentration on the intracellular side was increased as necessary up to 800 µM to maintain 90% open state probability for measurement of dwell times and to collect I-V data in the negative voltage range. Dendrotoxins were added from a 200 µM stock solution to the intracellular side of BKCa channels and the chamber was stirred for at least 1 min before commencing recordings.

Single-channel currents were recorded at room temperature (20–23°C) using a commercial patch-clamp amplifier (Axon Instruments, Foster City, CA). The patch-clamp headstage was connected to the bilayer chambers using Ag/AgCl electrodes and agar-KCl bridges. Single-channel records typically lasting 1–3 hour per bilayer were digitally stored using Clampex 8.1 software (Axon Instruments, Foster City, CA). Current records were filtered at a final corner frequency of 20-500 Hz using an 8-pole low-pass Bessel filter as required for analysis or display and sampled at 5 times greater than the filter frequency for kinetic analysis. Data samples of at least 100 substate events recorded at +30 mV were used for determination of toxin binding rate constants.

Data analysis

PCLAMP (Axon Instruments, Foster City, CA) Clampfit 9.0 software was used for analysis of single-channel data. The absolute zero-current level was defined by well-resolved closed state events in the single-channel records. The mean current level of the open state and DTX-induced substates was measured from appropriately filtered signals by mouse-driven cursors or from peaks of all-points amplitude histograms. The relative current of the major resolved subconductance level for each toxin was measured as the ratio of the unitary current of the subconductance state to open state at +30 mV.

Rate constants for toxin binding were measured from histograms of substate dwell times and inter-substate durations, as described previously. Histograms of such events were well-described by single-exponential distributions (e.g., first-order rate constant for DTX dissociation, koff, was calculated as the reciprocal of the time constant of the exponential distribution of inter-substate events. The bimolecular rate constant for DTX association, kon, was calculated from the reciprocal of the time constant of the exponential distribution of inter-substate events with an appropriate correction for missed events divided by the DTX concentration.

Molecular modeling and electrostatic calculations

The x-ray structure of the BK channel gating ring (PDB code 3U6N, biological assembly 2) was refined using the Monte Carlo energy minimization protocol in the ZMM program. AMBER force field was used for energy calculations. The refinement did not affect the protein folding since α carbons were constrained to crystallographic positions with the help of PINS (parabolic penalty functions with the force constant of 10 kcal mol⁻¹ Å⁻¹), but predicted energetically optimal orientations of side chains, which are not resolved in the X-ray structure, and also removed clashes between atoms. To visualize electrostatic potential at solvent-accessible and van der Waals surfaces of the gating ring and toxins, we performed Poison-Boltzmann calculations using the Adaptive Poisson-Boltzmann Solver, APBS plugin to the PYMOL program. Atomic charges and radii were generated by program PDB2PQR server that uses PROPKA program to predict ionization states of titratable residues at pH 7. Default values of the dielectric constant (water, ε = 80; protein, ε = 2) were used. Ionic strength was varied from 0 to 0.3 M. Results of Figs. 7 and 8 are shown for zero ionic strength.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We especially thank Carlos Gonzalez (University of Valparaiso, Chile) for the homology model of the pore domain of the human BKCa channel and Hugues Schweitz and Michel Lazdunski (Institut de Pharmacologie Moleculaire et Cellulaire, Valbonne, France) for the purified snake dendrotoxins.

Funding

Experimental work was funded by NIH Grant P01 NS42202. EGM was supported by an Early Career LDRD award from Sandia National Laboratories. Sandia National Laboratories is a multi-program laboratory managed and operated by Sandia Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, a wholly owned subsidiary of Lockheed Martin Corporation, for the US Department of Energy’s National Nuclear Security Administration under contract DE-AC04-94AL85000. Electrostatic computations were made possible by the facilities of the Shared Hierarchical Academic Research Computing Network (SHARCNET:www.sharcnet.ca). This part of the work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada to BSZ [Grant GRPIN/238773-2009].

References

1. Rothberg BS. The BK channel: a vital link between cellular calcium and electrical signaling. Protein Cell 2012; 3:883-92; PMID:22996175; http://dx.doi.org/10.1007/s13238-012-0207-8
2. Contreras GF, Castillo K, Enrique N, Carrazquel-Ursuláez W, Castillo JP, Milesi V, Neely A, Alvarez O, Ferreira G, Gonzalez C, et al. A BK (Slo1) channel journey from molecule to physiology. Channels 2013; 7:1-17; PMID:23247505; http://dx.doi.org/10.4161/chann.26242
3. Yan J, Aldrich RW. LRRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium. Nature 2010; 466:513-6; PMID:20613726; http://dx.doi.org/10.1038/nature09162
4. Yan J, Aldrich RW. BK potassium channel modulation by leucine-rich repeat-containing proteins. Proc Natl Acad Sci U S A 2012; 109:7917-22; PMID:22547800; http://dx.doi.org/10.1073/pnas.1205435109
5. Yang C, Zeng XH, Zhou Y, Xiao XM, Lingle CJ. LRRRC32 (leucine-rich-repeat-containing protein 52), a testis-specific auxiliary subunit of the alkalization-
activated Slo 3 channel. Proc Nat Acad Sci U S A 2011; 108:19419-24; PMID:22084117; http://dx.doi.org/10.1073/pnas.1111041108.

6. Tanaka Y, Kose K, Toro L. MaxiK channel roles in blood vessel relaxations induced by endothelium-derived relaxing factors and their molecular mechanisms. J Smooth Muscle Res 2004; 40:125-33; PMID:15653302; http://dx.doi.org/10.1540/jsmr.40.125.

7. Singh PN, Stefani E, Toro L. Intracellular BK(Ca) (IBK) channels. J Physiol 2012; 590 (Pt 23):5937-47; PMID:22992068; http://dx.doi.org/10.1111/j.1469-7793.2011.215533.x.

8. Miller C. The charybdotoxin family of K+ channel-blocking peptides. Neuropharmacology 2004; 46:165-74; PMID:15379882; http://dx.doi.org/10.1016/j.chroma.2003.06.022.

9. Imredy JP, Chen C, MacKinnon R. A snake toxin inhibitor of inward rectifier potassium channel ROMK1. Biochemistry 2001; 40:7367-74; PMID:11597882; http://dx.doi.org/10.1021/bi010925k.

10. Banerjee A, Lee A, Campbell E, MacKinnon R. Structure of a pore-blocking toxin in complex with a eukaryotic voltage-gated K+ channel. eLife 2013; e00594, 1-22; PMID:23795070; http://dx.doi.org/10.7554/eLife.00594.

11. Zhang Z, Zhou Y, Jiang Y. Structure of the gating ring from the human large-conductance Ca2+-activated K+ channel. Nature Struct Mol Biol 2011; 18:540-7; PMID:21504420; http://dx.doi.org/10.1038/nature09525.

12. Yuan P, Leonardi MD, Pico AR, Hsiung Y, MacKinnon R. Open structure of the Ca2+-gating ring in the high-conductance Ca2+-activated K+ channel. Nature 2012; 483:94-7; http://dx.doi.org/10.1038/nature10670.

13. Schreiber S, Salkoff L. A novel calcium-sensitive domain in the BK channel. Biophys J 1997; 73:1355-63; PMID:9284303; http://dx.doi.org/10.1016/S0006-3495(96)73274-6.

14. Lingle CJ. Stereospecific binding of a disordered peptide to the cytosolic domain of the BK channel. J Neurosci 2006; 26:11833-43; PMID:17180156; http://dx.doi.org/10.1523/JNEUROSCI.0186-06.2006.

15. Gonzalez-Perez V, Zeng X-H, Henzler-Wildman K, Marzillo D, Scheidereit C, Hruska KA, Cao J, Chan LS, Lengerfeld D, Calegro F, Caracciolo M, Rothberg JM, Flatley A, Hollenbeck G, Lazdunski M. Calcicludine, a venom peptide of the triggerfish, activates Ca2+ channels. Neuron 2004; 42:285-96; PMID:15182715; http://dx.doi.org/10.1016/j.neuron.2004.05.001.

16. Alabagiu R, Zhou H-X. Electrostatic enhancement and transient complex of protein-protein association. Proteins Struct Funct Bioinforma 2008; 71:320-35; http://dx.doi.org/10.1002/prot.21769.

17. Baker NA, Sepf D, Joseph S, McCammon JA. Electrostatics of native protein application to microtubules and the ribosome. Proc Nat Acad Sci U S A 2001; 98:10037-41; PMID:11517324; http://dx.doi.org/10.1073/pnas.181342397.

18. Bader G, Geng Y, Busetti G, Salkoff L. Properties of Slo1 K+ channels with and without the gating ring. Proc Nat Acad Sci U S A 2013; 110:16657-6662; PMID:24067659; http://dx.doi.org/10.1073/pnas.1313453110.

19. Moss GWJ, Moczydlowski E. Rectifying conductance substates in a large conductance Ca2+-activated K+ channel: evidence for a fluctuating barrier mechanism. J Gen Physiol 1996; 107:68-824; http://dx.doi.org/10.1083/jgp.107.1.47.

20. Fogelson GL, Isenberg HA, French RJ. Electrostatic and steric contributions to block of the skeletal muscle sodium channel by α-conotoxin. J Gen Physiol 2002; 119:45-54; PMID:11775237; http://dx.doi.org/10.1085/jgp.107.1.45.

21. Moss GWJ, Currie JE, Moczydlowski E. Voltage-activated K + channels: a model system for analysis of calcium-channel function. Neuron 1992; 9:1501-15; PMID:1370897; http://dx.doi.org/10.1016/S0896-6273(00)80838-8.
51. Li W, Aldrich RW. State-dependent block of BK channels by synthesized shaker ball peptides. J Gen Physiol 2006; 128:423-41; PMID:16966472; http://dx.doi.org/10.1085/jgp.200609521

52. Zhang Z, Zeng X-H, Xia X-M, Lingle CJ. N-terminal inactivation domains of β-subunits are protected from trypsin digestion by binding within the anechoamber of BK channels. J Gen Physiol 2009; 133:263-82; PMID:19237592; http://dx.doi.org/10.1085/jgp.200810079

53. Bentrop D, Beyermann M, Wissmann R, Faker B. NMR structure of the “ball-and-chain” domain of KCNMB2, the β2-subunit of large conductance Ca²⁺-, and voltage-activated potassium channels. J Biol Chem 2001; 276:42116-21; PMID:11517232; http://dx.doi.org/10.1074/jbc.M107118200

54. Garden DP, Zhorov BS. Docking flexible ligands in proteins with a solvent exposure- and distance-depen- dent dielectric function. J Computer-aided Mol Design 2010; 4:91-105; http://dx.doi.org/10.1085/jcm.201007118200

55. Weiner SJ, Kolman PA, Case DA, Singh UC, Ghio C, Alagona G, Profeta S, Weiner P. A new force field for molecular mechanical simulation of nucleic acids and proteins. J Am Chem Soc 1984; 106:765-84; http://dx.doi.org/10.1021/ja00315a051

56. Weiner SJ, Kolman PA, Nguyen DT, Case DA. An all atom force field for simulations of proteins and nucleic acids. J Comput Chem 1986; 7:230-52; http://dx.doi.org/10.1002/jcc.540070216

57. Dolinsky TJ, Nielsen JE, McCammon JA, Baker NA. PDB2PQR: an automated pipeline for the setup of Poisson-Boltzmann electrostatics calculations. Nucl Acids Res 2004; 32:W665-667; PMID:15215472; http://dx.doi.org/10.1093/nar/gkh381

58. Li H, Robertson AD, Jensen JH. Very fast empirical prediction and rationalization of protein pKa values. Proteins 2005; 61:704-21; PMID:16231289; http://dx.doi.org/10.1002/prot.20668