NMR Structure of the “Ball-and-chain” Domain of KCNMB2, the β2-Subunit of Large Conductance Ca\(^{2+}\)- and Voltage-activated Potassium Channels*§

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The auxiliary β-subunit KCNMB2 (β2) endows the non-inactivating large conductance Ca\(^{2+}\)- and voltage-dependent potassium (BK) channel with fast inactivation. This process is mediated by the N terminus of KCNMB2 and closely resembles the “ball-and-chain”-type inactivation observed in voltage-gated potassium channels. Here we investigated the solution structure and function of the KCNMB2 N terminus (amino acids 1–45, BKβ2N) using NMR spectroscopy and patch clamp recordings. BKβ2N completely inactivated BK channels when applied to the cytoplasmic side; its interaction with the BK α-subunit is characterized by a particularly slow dissociation rate and an affinity in the upper nanomolar range. The BKβ2N structure comprises two domains connected by a flexible linker: the pore-blocking “ball domain” (formed by residues 1–17) and the “chain domain” (between residues 20–45) linking it to the membrane segment of KCNMB2. The ball domain is made up of a flexible N terminus anchored at a well ordered loop-helix motif. The chain domain consists of a 4-turn helix with an unfolded linker at its C terminus. These structural properties explain the functional characteristics of BKβ2N-mediated inactivation.

Large conductance K\(^{+}\) channels (BK\(^{+}\) or MaxiK channels) are key modulators of excitability in many types of cell (1, 2).

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The atomic coordinates and chemical shifts of the final 24 structures (code 1JO6) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/) and the BioMagResBank (accession number 5092).

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1 The abbreviations used are: BK, large conductance Ca\(^{2+}\)- and voltage-dependent K\(^{+}\) channel; Kv, superfamily of voltage-dependent K\(^{+}\) channels; BKβ2N, synthetic peptide covering the N-terminal 45 amino acids of the β2-subunit of the human BK channel (human KCNMB2); NOE, nuclear Overhauser effect; ID, inactivation domain; MES, 4-morpholineethanesulfonic acid; r.m.s., root mean square.

They are formed from four identical α-subunits encoded by the Slo gene and are activated by membrane depolarization and/or increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)), (3–8). This dual activation is unique among the large family of K\(^{+}\) channels and provides a direct feedback mechanism to regulate Ca\(^{2+}\) influx.

In many tissues, the activation gating of BK channels is modulated by accessory β-subunits, a family of membrane proteins (KCNMB) closely associated with the α-subunit (7). Four KCNMB proteins have been identified (KCNMB1–4), and they all share a prototypic topology of two transmembrane domains with intracellular N and C termini (9–13). Functionally, each of these KCNMB proteins distinctly changes the rates of channel activation and deactivation as well as the apparent sensitivity of the channel for Ca\(^{2+}\) (9).

In addition, one of the β-subunits, KCNMB2 (β2), was found to confer rapid and complete inactivation to the BK channel complex (11, 12) in a manner similar to that observed in chromaffin cells of the adrenal gland or in hippocampal CA1 neurons (14, 15). Analysis of this KCNMB2-mediated inactivation gating showed that it closely resembled the famous ball-and-chain-type inactivation of voltage-gated K\(^{+}\) channels (Kv): (i) it is determined by the N terminus of KCNMB2; (ii) it occludes the open channel pore and competes with the pore-blocking agent tetraethylammonium (11, 12); and (iii) recovery from inactivation is speeded up by an increase of the extracellular K\(^{+}\) concentration (11).

Moreover, the N-terminal stretch of the KCNMB2 N terminus (19 amino acids) was shown to be a functional entity, i.e. its fusion to the N terminus of KCNMB1 (β1) conferred rapid inactivation to this non-inactivating β-subunit, and it occluded BK channels as a synthetic peptide very similar to the “pore plugging” observed for the synthetic inactivation domains (ID) derived from various α-subunits and one β-subunit of Kv-type K\(^{+}\) channels (11).

The three-dimensional structure of Kv-derived IDs was analyzed with NMR spectroscopy in solution and revealed a wide range of structural variability for these proteins. Thus, the ID of Kv3.4 was found to exhibit well defined and compact folding although the backbone lacks secondary structural elements. In contrast, the ID from Shaker B and the inactivating N terminus of Kvβ1.1 (amino acids 1–62) showed no uniquely folded structure but rather behaved like random-coil peptides (16–19).

To gain structural insight into KCNMB2-mediated inactivation of BK channels, we investigated the solution structure and function of the hydrophilic N terminus of KCNMB2 using NMR spectroscopy and giant patch clamp recording on a synthetic...
Fig. 1. Functional characteristics of BKβ\_2N. A, inactivation of homeric human BK channels (BK\_α) by 27 \(\mu\)M BKβ\_2N continuously present at the cytoplasmic side of a giant inside-out patch. Channels were activated by voltage steps from \(-100\) mV to \(-10\) or \(+40\) mV at a \([Ca^{2+}]_i\) of 10 \(\mu\)M. Time and current scaling were as indicated; intra- and extracellular K\textsuperscript+ concentrations were 120 and 5 mM, respectively. B, time constant of channel inactivation mediated by 27 \(\mu\)M BKβ\_2N as a function of the transmembrane voltage. Data are mean \(\pm\) S.D. from five experiments. The continuous line represents the fit of the equation \(A_0 + A_1\exp[-V/\alpha]\) to the data with a value for \(\alpha\) of 117.1 mV. Inset, current response to the transmembrane voltage stepped from \(-100\) mV to potentials between \(-80\) and 80 mV with 27 \(\mu\)M BKβ\_2N and 10 \(\mu\)M Ca\textsuperscript{2+} present on the cytoplasmic side of the patch. C, Piezo-driven fast application of 27 \(\mu\)M BKβ\_2N to open BK channels as indicated by the horizontal bar. Note the slow dissociation of BKβ\_2N from the channel. D, rates of the inactivation process mediated by BKβ\_2N. The rates determined for the well structured Kv\_3.4-ID and the random-coil Kv\_1.1 N terminus (both at non-inactivating Kv\_1 channels) are given for comparison (19). Values are mean \(\pm\) S.D. of eight experiments.

MATERIALS AND METHODS

Electrophysiology—BK channels were expressed heterologously in Xenopus oocytes as described elsewhere (20). Giant patch recordings were made at room temperature (\(-23\) \(^\circ\)C) 3–7 days after injection of capped cRNA encoding hBK\_α (GenBank\textsuperscript{TM} accession no. U23767). Pipettes were made from thick-walled borosilicate glass, had resistances of 0.3–0.6 MΩ (tip diameter of about 20 μm), and were filled with (in mM) 5 KOH, 115 NaOH, 10 HEPES, and 0.5 CaCl\textsubscript{2}, pH adjusted to 7.2 with MES. Currents were sampled at 10 kHz and corrected for capacitative transients with an EPC9 amplifier (HEKA electronics, Lamprecht, Germany) with the analog filter set to 3 kHz (\(-3\) db).

The fast application system used is described elsewhere (17) and allowed for a complete solution exchange in less than 2 ms. BKβ\_2N was dissolved in K\_ion solution and applied via one barrel of the application system. K\_ion was composed as follows (in mM): 119 KOH, 1 KCl, 10 HEPES, and 0.5 CaCl\textsubscript{2}, pH adjusted to 7.2 with MES. The amount of CaCl\textsubscript{2} required to yield a free Ca\textsuperscript{2+} concentration of 10 \(\mu\)M was calculated according to Fabiato (36) and added to the EGTA solution under pH control. Thereafter, pH was readjusted to 7.2 with KOH.

Rates of inactivation were determined as described previously (17). Briefly, \(k_{\text{off}}\) was determined from the time constant of the wash-off of \(\tau_{\text{off}}\) as \(k_{\text{off}} = 1/\tau_{\text{off}}\) and \(k_{\text{on}}\) was then calculated as \(k_{\text{on}} = (1/\tau_{\text{on}} - k_{\text{off}})/[\text{peptide}]\), with \(\tau_{\text{on}}\) the time constant for wash-in. Affinity for the peptide-receptor interaction was calculated as \(k_{\text{off}}/k_{\text{on}}\). All values throughout the paper are given as mean \(\pm\) S.D. of \(n\) experiments.

Peptide Synthesis and Sample Preparation—The BKβ\_2N protein was synthesized by standard solid-phase synthesis and purified by high pressure liquid chromatography. The mass was confirmed by mass spectrometry.

5.4 mg of BKβ\_2N were dissolved in 500 μl of 90% H\textsubscript{2}O/10% D\textsubscript{2}O (v/v), pH 3.0, resulting in a final peptide concentration of 2 mM. To verify structural properties under physiological conditions, NMR experiments were carried out on BKβ\_2N dissolved in physiological salt solution (90 mM KCl, 10 mM KH\textsubscript{2}PO\textsubscript{4}, 2 mM MgCl\textsubscript{2}) at pH 6.0. All NMR samples contained 2,2-dimethyl-2-silapentane-5-sulfonate as the internal standard for \(^1\)H chemical shift referencing.

NMR Spectroscopy—Homonuclear NMR spectra were acquired on a Bruker Avance 600 spectrometer at either 293 or 288 K with a spectral window of 11.5 ppm. Standard pulse sequences were used to record NOESY (21) (mixing times between 100 and 250 ms), CLEAN-TOCSY (22) (isotropic mixing time of 80 ms), and DQF-COSY (23) spectra with 4096 data points in F\textsubscript{2} and 512 increments in F\textsubscript{1}. All two-dimensional \(^1\)H NMR spectra employed the method of time-proportional phase incrementation for quadrature detection in the F\textsubscript{1} dimension (24). Water suppression was achieved either by presaturation or by the WATER-GATE technique (25).

NMR data were processed with the Bruker XWINNMR software using shifted squared sine window functions prior to Fourier transformation. The final matrix size was 4096 \(\times\) 1024, except for the DQF-COSY spectrum, which was transformed to 16384 \(\times\) 1024 (corresponding to a digital resolution of 0.42 Hz/point in the F\textsubscript{2} dimension) to extract \(J_{\text{HH}}\), coupling constants through a fit of the COSY cross-peaks to two antiphase Lorentzian lines. The programs AURELIA (26) and XEASY (27) were used for analysis of two-dimensional spectra.

Structure Determination—NOE distance constraints were derived from a 250-ms NOESY spectrum in H\textsubscript{2}O and a 200-ms NOESY spectrum in D\textsubscript{2}O solution, both recorded at 288 K, pH 3.0. Unambiguously assigned NOESY cross-peaks were integrated manually with XEASY, and the resulting volumes were converted into proton-proton upper distance limits with the program CALIBA (28) using five different classes of NOEs. Constraints for the backbone dihedral angle \(\Phi\) were obtained from the \(3J_{\text{HH}}\) coupling constants \(\pm 6\) Hz. In these cases, a \(\Phi\) angle between \(-85^\circ\) and \(-35^\circ\) was imposed. Structures of BKβ\_2N were calculated with the program DYANA (version 1.5) (29) employing a simulated annealing algorithm in the torsion angle space. Structures from preliminary DYANA calculations were used to recalculate the
Fig. 2. NMR data of BKβ2N in aqueous solution. A, NOE connectivities and J-coupling constants observed for BKβ2N in aqueous solution. Sequential and medium range NOEs are shown as a function of the amino acid sequence; the intensity of NOEs is reflected by the line thickness. Filled circles represent 3JHNH coupling constants between 5 and 6 Hz, and open circles represent coupling constants between 6 and 8 Hz. B, upper panel, deviation of Ha chemical shifts from random-coil values (31). ΔδHa was calculated as the difference between the experimentally determined δHa and the random-coil δHa. Lower panel, bar diagram of NOE constraints by residue. Intraresidual contacts are shown in gray, sequential contacts in white, and medium range contacts in black.

distance restraints and to obtain stereospecific assignments by the GLOMSA (28) routine within DYANA. The final family of structures was generated in a calculation with 300 random starting structures and 9000 annealing steps. 30 structures with target function values < 0.92 Å and no violations of dihedral angle constraints were selected for further analysis.

Visualization of structures and preparation of figures were done with the program MOLMOL (30).

RESULTS

BKβ2N Inactivated BK Channels in a Ball-like Manner—The functional characteristics of BKβ2N were tested in inside-out patches from Xenopus oocytes expressing non-inactivating homomeric BK channels. As shown in Fig. 1A, BKβ2N induced rapid inactivation of the BK α-subunit when present at the cytoplasmic side of the patch. Moreover, BKβ2N-mediated inactivation occurred only at open channels. Despite the long-lasting presence of BKβ2N, channels first opened upon depolarization before they were inactivated by the peptide (Fig. 1, A and B).

The time course of inactivation was strongly dependent on the BKβ2N concentration (not shown) and exhibited mild voltage dependence (Fig. 1B). Thus, the time constant as obtained from a monoexponential fitted to the current decay (τinact) changes e-fold with a change in membrane potential of 117 mV, which is equivalent to a valence (z) of 0.21.

These results suggested that very similar to IDs derived from Kvα or Kvβ1.1 subunits, BKβ2N blocks BK channels in a “ball-like manner” via interaction with a receptor site on the α-subunit that becomes accessible once the channel is in the open state.

Therefore, interaction between BKβ2N and the channel α-subunit was more closely investigated by the “fast application” technique. This technique allows for complete solution exchanges at inside-out patches in less than 2 ms and enables separate determination for on- and off-rates of channel-peptide interaction (17). Fig. 1C shows rapid application and wash-off of BKβ2N at a concentration of 27 μM. Channels were activated prior to peptide application by a voltage-step to 0 mV at a [Ca2+]i of 10 μM. Inactivation occurred with a time constant of ~17 ms (16.9 ± 1.5 ms, n = 8), identical to that induced by the continuously present BKβ2N (Fig. 1B). Wash-off of BKβ2N, which should reflect unbinding of the peptide from the receptor, exhibited a time constant of ~850 ms (851.5 ± 69.3 ms, n = 8) and could be well fitted with a monoexponential (Fig. 1C).

This was an indication that interaction between BKβ2N and its receptor on the α-subunit could be described as a bimolecular reaction as suggested (11) with on- and off-rates (kon, koff) of 2.0×10⁶ (Ms)⁻¹ and 1.2 s⁻¹ (Fig. 1D), respectively. The affinity (IC50) of BKβ2N for its receptor as calculated from these rates is 0.59 μM (Fig. 1D), which is very similar to the value obtained from a steady-state concentration-inhibition relationship (not shown).
NMR Structure and Function of the N Terminus of the BK β-Subunit, KCNMB2

Interestingly, a comparison among the inactivation rates of various IDs shows that \( k_{\text{off}} \) of BKⅡN closely resembles that of the compactly folded Kv3.4-ID, whereas \( k_{\text{on}} \) of BKⅡN is very similar to that of Kvβ1.1 or the Shaker B-ID, IDs that both lack ordered three-dimensional structure in solution (Fig. 1D and Ref. 19). Next we investigated the structural properties of BKⅡN in solution with NMR spectroscopy.

Assignment and NOE Connectivity of BKⅡN—NMR experiments were performed under various conditions in aqueous solution at pH 3.0 and in a physiological salt solution at pH 6.0 (see "Materials and Methods"). The \(^1\)H NMR resonances of BKⅡN were completely assigned by two-dimensional NMR methods in the low pH solution and verified under physiological conditions.

As illustrated in Fig. 2A, NOE contacts between nonadjacent amino acids \((i,j+x)\) indicative for structured domains were only observed on the sequence stretch roughly extending from Ser14 to Leu31. This "core domain" exhibited NOE patterns typically observed with α-helices. Thus, connectivities between the α-proton of one amino acid and the amide (\(\delta i\)N(i+3)) or β-proton (\(\delta j\)H(i,i+3)) of the third amino acid following were observed. Most residues throughout this stretch show sequential contacts between backbone amide protons (dNN NOEs). Moreover, \(\delta j\)HNL, coupling constants were determined in this region for 8 of 22 residues. Five of these J-couplings showed values between 5.3 and 6 Hz, indicative of helical conformations, and three were between 6 and 8 Hz.

The NOE-based indication of secondary structural elements in BKⅡN was corroborated by results of an H/D exchange experiment, where one-dimensional spectra were recorded 15, 30, 45, and 65 min after dissolving lyophilized BKⅡN in D₂O. Thus, a number of amide protons (HN) including those of Ile25, Gln23, Ile25, Asp29, and Leu31 were identified in the first and second one-dimensional spectrum (see supplemental material). Resonances of the HN of Ile25 and Leu31 were present even in the spectrum recorded 65 min after dissolution, indicating significant protection from exchange with the solvent as would be expected for hydrogen bonding in a helical conformation. This view is further supported by the deviations of the α-proton chemical shifts from random-coil values (31). As shown in Fig. 2B (upper panel), the α-protons of all residues in the core region are shifted up-field as typically seen in helical structures (32).

Together, the NOE pattern, the H/D exchange, the J-couplings, and the chemical shifts suggest that BKⅡN consists of an ordered mostly helical core domain flanked by flexible N and C termini.

Structure of BKⅡN in Solution—A total of 728 experimentally determined NOE constraints (average of 16.2/residue; Fig. 2B, lower panel) together with the restraints for dihedral angles and stereospecific assignment of protons were used to calculate the solution structure of BKⅡN (Table I). After structure calculations using the simulated annealing protocol of DYANA (29) in the torsion angle space 24 structures with the lowest values of the target function and without NOE violations of >0.4 Å were selected as the final family of BKⅡN structures (for structural statistics see Table I).

Fig. 3 shows 18 representatives of this family of best structures, superimposed either between residues 10 and 17 (Fig. 3A) or residues 18 and 31 (Fig. 3B). Both superpositions display reasonable convergence with similar r.m.s. deviation values to the mean structure (0.65 ± 0.22 and 0.48 ± 0.27 Å for the backbone atoms in Figs. 3, A and B, respectively), whereas superposition on the entire range was not meaningful because of the divergent orientation of residues Glu17—Arg29 that connect both stretches. The Glu17—Arg29 linker thus divides BKⅡN into an N- and a C-terminal domain. The N-terminal domain consists of a disordered part made up of residues 1–10 and a loop-helix motif formed by amino acids Ser11—Asp16 (Fig. 3A). Superposition of residues 10–16 revealed r.m.s. deviations from the mean structure of 0.60 ± 0.17 Å for backbone atoms and of 1.14 ± 0.17 Å for all atoms. The C-terminal domain is made up of an extended helical structure formed by residues 20–31 and a flexible C terminus (residues 32–45; Fig. 3B). Within the helical structure, residues 22–30 form a regular

| TABLE I Structural statistics of BKⅡN |
|----------------------------------------|
| Total number of experimental distance restraints | 728 |
| Intraresidual | 486 |
| Sequential | 191 |
| Medium range (\(i-j\) ≤ 5) | 51 |
| Long range (\(i-j\) > 5) | 5 |
| \(\phi\) dihedral angle restraints from \(\delta j\)NNNOE ≤ 6 Hz | 11 |
| Stereospecific assignments | 0 |
| Distance restraint violations > 0.4 Å (per structure) | 0 |
| Dihedral angle restraint violations (per structure) | 0 |
| Average number of distance restraint violations > 0.2 Å (per structure) | 1.1 |
| Average sum of distance restraint violations (per structure) | 5.1 ± 0.6 Å |
| Average maximum distance restraint violation | 0.25 ± 0.08 Å |
| Average target function | 0.66 ± 0.13 Å² |
| r.m.s. deviations to the respective mean structure: |
| Superimposed residues | Backbone N, Cα, and Cα atoms | All heavy atoms |
| 1–10 | 2.01 ± 0.49 Å | 3.30 ± 0.67 Å |
| 10–17 | 0.65 ± 0.22 Å | 1.26 ± 0.32 Å |
| 18–31 | 0.48 ± 0.27 Å | 1.04 ± 0.28 Å |
| 20–30 | 0.29 ± 0.17 Å | 0.57 ± 0.23 Å |
| 30–40 | 2.00 ± 0.46 Å | 3.62 ± 0.50 Å |

Ramachandran plot statistics for residues 17–32 (9–32)

- Residues in most favored regions, % | 84 (69) |
- Residues in additional allowed regions, % | 16 (30) |
- Residues in generously allowed regions, % | 0 (1) |
- Residues in disallowed regions, % | 0 (0) |

* Structural statistics for the final family of 24 structures. All 45 residues of BKⅡN(1–45) were included in the analysis unless otherwise noted. None of the 24 best structures has a target function > 0.92 Å², distance restraint violations > 0.4 Å, or violations of J-coupling restraints.
α-helix that is preceded by one turn of a $3_1^\alpha$-helix (Fig. 3B). Superposition of structures over the range of the helix domain results in r.m.s. deviations of 0.43 ± 0.26 Å for backbone atoms and of 0.90 ± 0.25 Å for all atoms.

When correlated with functional properties, it is only the N-terminal domain that is required for occlusion of the channel pore, as seen in experiments with this domain fused to the KCNMB1 subunit or applied to BK channels as a synthetic peptide (11). Accordingly, this domain was termed the ball domain (Fig. 4). In contrast, the C-terminal domain, which links the ball to the transmembrane segment of KCNMB2, may be regarded as the chain domain (Fig. 4).

**Discussion**

BKβ2-N inactivates BK channels with characteristics known from the "pore plug-in" described for inactivation domains of Kv-type IK channels. Accordingly, BKβ2-N-mediated pore occlusion exhibits shallow voltage dependence and is competed by the pore-blocking agent TEA (11, 13). As determined from NMR experiments, BKβ2-N presents with a unique solution structure; it consists of two domains connected by a flexible linker, the ball domain, made up of a disordered N terminus anchored at a loop-helix motif, and the chain domain, a 4-turn helix with an unfolded region at its C terminus.

As shown in Fig. 1B, the structure of BKβ2-N is accompanied by functional properties that are unique with respect to those of Kv-derived IDs. Thus, the association rate of BKβ2-N with its receptor on the channel is very similar to that observed with the unstructured IDs of Kvβ1.1 or Shaker B, but considerably slower than that determined for the compactly folded Kv3.4-IDs (17, 19, 33). The dissociation rate of BKβ2-N, on the other hand, is more than 10-fold lower than that of Kvβ1.1-IDs or the ID of Shaker B and even about 2-fold lower than that of Kv3.4-IDs.

These correlations between structural and functional properties are consistent with our earlier observations that well ordered IDs exhibit a faster $k_{on}$ and a much slower $k_{off}$ than unfolded domains (17, 19). The latter seems to be caused either by the higher number of molecular contacts (hydrogen bonds, etc.) formed between the folded domain and the receptor or by the higher flexibility of the unfolded IDs, which destabilizes the ID-receptor interaction.

Together with the observation that the actual pore block is realized by the N-terminal 19 or 26 residues (11), BKβ2-N-mediated inactivation may be imagined to occur as follows. The ball domain (Fig. 4) will approach the open channel and, in a second step, bind to its receptor, which finally results in occlusion of the channel pore. Channel approach and binding are reflected by $k_{on}$ and are determined by the flexible part of the ball domain as suggested recently for Kvβ1.1-mediated inactivation (34). Unbinding of the ID from its receptor, as reflected by $k_{off}$, should be controlled by the structured part of the ball domain. As $k_{off}$ of BKβ2-N is the lowest of all ID peptides investigated to date, the ball-receptor interaction must be particularly strong. Interestingly, the structured region of the ball domain contains a cluster of charged residues suggesting that hydrophilic interactions may be an important determinant for the BKβ2-N-BKα interaction. This premise would be in line with work by Toro et al. (35) who investigated the interaction between the ID from Shaker B with BK channels and concluded that the ID receptor of BK channels in the inner vestibule of the channel may contain hydrophilic residues and a "pocket" that favors binding of helical structures.

The molecular identity of the BK ID receptor, however, must remain open at this point as well as the question of how far BKβ2-N enters the channel pore and whether interactions between charges on the ball domain and the channel wall are involved in ID receptor interaction.
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