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NMR Structure and Functional Characteristics of the Hydrophilic N Terminus of the Potassium Channel β-Subunit Kvβ1.1*

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Rapid N-type inactivation of voltage-dependent potassium (Kv) channels controls membrane excitability and signal propagation in central neurons and is mediated by protein domains (inactivation gates) occluding the open channel pore from the cytoplasmic side. Inactivation domains (ID) are donated either by the pore-forming α-subunit or certain auxiliary β-subunits. Upon coexpression, Kvβ1.1 was found to endow non-inactivating members of the Kv1α family with fast inactivation via its unique N terminus. Here we investigated structure and functional properties of the Kvβ1.1 N terminus (amino acids 1–62, βN-(1–62)) using NMR spectroscopy and patch clamp recordings. βN-(1–62) showed all hallmarks of N-type inactivation: it inactivated non-inactivating Kv1.1 channels when applied to the cytoplasmic side as a synthetic peptide, and its interaction with the α-subunit was competed with tetraethylammonium and displayed an affinity in the lower micromolar range. In aequous and physiological salt solution, βN-(1–62) showed no well defined three-dimensional structure, it rather existed in a fast equilibrium of multiple weakly structured states. These structural and functional properties of βN-(1–62) closely resemble those of the "unstructured" ID from *Shaker* B, but differ markedly from those of the compactly folded ID of the Kv3.4 α-subunit.

Fast N-type inactivation of voltage-gated potassium (Kv)[1] channels shapes the action potential, governs the firing rate (spiking), and controls signal propagation in central neurons (1). Biophysically, N-type inactivation has long served as the model for gating transitions in ion channels and is realized by a "ball plug-in" mechanism. In this mechanism a protein domain termed "inactivation gate" or "inactivation ball" binds to its receptor at the inner vestibule of the open channel and thereby occludes the ion pathway (2–5). Such inactivation gates have been localized in the N terminus of various Kvα subunits and were shown to be functional entities, *i.e.* they conferred rapid inactivation to "ball-less" Kvα subunits when applied to the cytoplasmic side of the channels as synthetic peptides (5–8). Identical to protein-harbored inactivation domains, the synthetic gates interacted with channels in the open state, blocked the pore with low voltage dependence, and were competed with the channel blocker tetraethylammonium (TEA) (9–11). Recently, the structures of the inactivation domains (ID) from Kv1.4 and Kv3.4 were determined with NMR spectroscopy. Both IDs were found to exhibit well defined and compact folding in aequous solution (12). In contrast, the ID from *Shaker* B showed no unique, folded structure (13, 14).

Besides with Kvα subunits owning an N-terminal ID, fast inactivation was observed for a subset of non-inactivating Kvα1 channels when coexpressed with certain β-subunits (15–17). These auxiliary subunits constitute a family of cytoplasmic proteins (subdivided into subfamilies Kvβ1, -2, and -3) that are made up of two distinct regions: a highly conserved core region that shows homology to the superfamily of aldo-keto reductases and a variable N terminus (18, 19). Fig. 1*A*, depicts the mechanism for inactivation of a non-inactivating Kvα1 subunit by the coexpressed β-subunit, Kvβ1.1. Kvβ constitutively (and specifically) binds to the Kvα1-subunit via the conserved core region (20, 21) and mediates fast inactivation via its unique N terminus (15, 16, 18). The latter should contain a "ball-like" domain that occludes the channel pore from the cytoplasmic side similar as known for IDs derived from α-subunits. This view is mainly supported by two findings. First, β-induced inactivation was absent when the N terminus of Kvβ1.1 was deleted, and second, inactivation was sensitive to oxidation, similar to that reported for inactivation in fast inactivating Kv channels (16). Although this suggested an N-type mechanism of inactivation, the "β-ball" was never tested for competition with TEA. This competition is considered indicative for N-type inactivation (9, 10). Moreover, there is puzzling controversy on the functionality of a peptide that comprises the N-terminal 24 amino acids of Kvβ1.1 and is supposed to constitute the β-ball. This peptide induced inactivation in some experiments (16) but failed in others (22), although the coexpressed Kvβ1.1 subunit inactivated the Kvα1 subunit in either case.

To get insight into the molecular mechanism of fast β-mediated inactivation, we investigated structure and function of the hydrophilic Kvβ1.1 N terminus using NMR spectroscopy and giant patch clamp recording on a synthetic peptide (βN-(1–62)) that covers the entire hydrophilic domain N-terminal to the well conserved core region of Kvβ1.1.

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[1] The abbreviations used are: Kv, superfamily of voltage-dependent potassium channels; βN-(1–62), synthetic peptide covering the N-terminal 62 amino acids of Kvβ1.1; TEA, tetraethylammonium; NOE, nuclear Overhauser enhancement; NOESY, nuclear Overhauser enhancement spectroscopy; ID, inactivation domain; DQF, double quantum filtered.

MATERIALS AND METHODS

Electrophysiology—Kv1.1 channels were heterologously expressed in *Xenopus* oocytes as described elsewhere (23). Giant patch recordings were made at room temperature (approximately 23° C) 3–7 days after injection of capped Kv1.1-specific cRNA. Pipettes used were made from thick walled borosilicate glass, had resistances of 0.3–0.6 megaohm (tip diameter of about 20 μm), and were filled with 5 mM KCl, 115 mM NaCl, 10 mM HEPES, and 1.8 mM $CaCl_2$ (pH 7.2). Currents were sampled at 10 kHz and corrected for capacitative transients with an EPC9 amplifier (HEKA Electronics, Lamprecht, Germany) with an analog filter set to 3 kHz (−3 decibel). The fast application system used is described elsewhere (8, 24) and allowed for a complete solution exchange in less than 2 ms (24). βN-(1–62) or TEA were dissolved in K_{int} solution and applied via one barrel of the application system; K_{int} was composed as follows: 120 mM KCl, 10 mM HEPES, 10 mM EGTA (pH 7.2).

Rates of inactivation were determined as described previously (8, 24). All values are given as mean ± S.D. of 3–5 experiments.

Peptide Synthesis and Sample Preparation—The βN-(1–62) peptide was made by conventional solid-phase synthesis and high pressure liquid chromatography-purified (25); the mass was confirmed by mass spectrometry. 5.4 mg of βN-(1–62) was dissolved in 99.9% D_2O as well as in a solution of 90% H_2O, 10% D_2O. The final peptide concentration was 1.6 mM, pH 3.3. To verify structural properties under physiological conditions, NMR experiments were also performed on βN-(1–62) dissolved in a physiological salt solution (90 mM KCl, 10 mM KH_2PO_4, 2 mM Mg_2Cl) at pH 6.6.

Structural Analysis—NMR spectra were recorded at 283 K with a Bruker DRX 600 and Bruker DMX 800 spectrometer. Spin system identification and sequential assignments were accomplished by two-dimensional homonuclear NOESY (26), total correlation spectroscopy (27), and DQF-COSY (28) experiments. NOESY spectra were acquired with mixing times of 250 ms; total correlation spectroscopy experiments were acquired with isotropic mixing times of 80 ms. $^3J_{H^NH}\alpha$ coupling constants were measured by high-resolution DQF-COSY spectra. The digital resolution of the time domain data used in the t_2 direction was 0.77 Hz/point. The time proportional phase increments method was used for signal detection in the ω_1-direction; for water suppression a binomial pulse sequence with gradients as well as presaturation technique was employed. ^1H chemical shifts were referred to the internal standard of 2,2-dimethyl-2-silapentane-5-sulfonate.

NMR data were processed with XWINNMR (Bruker). Spectral assignment was performed by AURELIA (29) according to standard procedures using total correlation spectroscopy and DQF-COSY spectra for identification of spin systems and NOESY spectra for sequence-specific assignment. Amide-H_α coupling constants $^3J_{H^NH}\alpha$ were obtained by fitting the corresponding DQF-COSY peaks to two antiphase Lorentzian functions. Proton-proton distances were calculated from the volumes of the NOESY spectra using δ-proline protons with a known separation of 0.18 nm as reference. NOESY cross peaks were integrated by the automated segmentation procedure, which is part of the AURELIA software package.

Prior to first order H/D exchange rate determination the peptide was lyophilized of a 90% H_2O, 10% D_2O solution. To evaluate the exchange rates, the peptide was dissolved in 99.9% D_2O, and a one-dimensional NMR spectrum was recorded within less than 3 min.

RESULTS

βN-(1–62) Interacts with the α-Subunit of Kv 1.1 Channels in a Ball-like Manner—The functional characteristics of βN-(1–62) were tested in inside-out patches from *Xenopus* oocytes expressing non-inactivating Kv1.1 channels. As shown in Fig. 1*B*, βN-(1–62) induced rapid inactivation of these channels when present at 50 μM on the cytoplasmic side of the patch; the time-course of inactivation depended on the peptide concentration (not shown). βN-(1–62) blocked Kv1.1 channels only in the open state (Fig. 1*B*), similar to IDs derived from Kvα subunits (6, 10). The latter are known to interact with a receptor that becomes accessible upon opening of the channel and that is competed by the "open channel blocker" TEA (9, 10). Therefore, βN-(1–62)-induced inactivation was investigated in the presence of 1 mM TEA, a concentration which blocked about two-thirds of open Kv1.1 channels. As shown in Fig. 1, *C–E*, the time-course of βN-(1–62)-mediated inactivation was slowed down by about a factor of 3 (time constants for inactivation in the absence and presence of TEA were 8.0 ± 1.7 and 22.2 ± 3.0, respectively) consistent with the peptide and TEA competing for an overlapping binding site in the pore (11). These results suggested that βN-(1–62) inactivated Kv1.1 channels in a "ball-like manner" via interaction with a receptor site on the α-subunit that is accessible only in the open state.

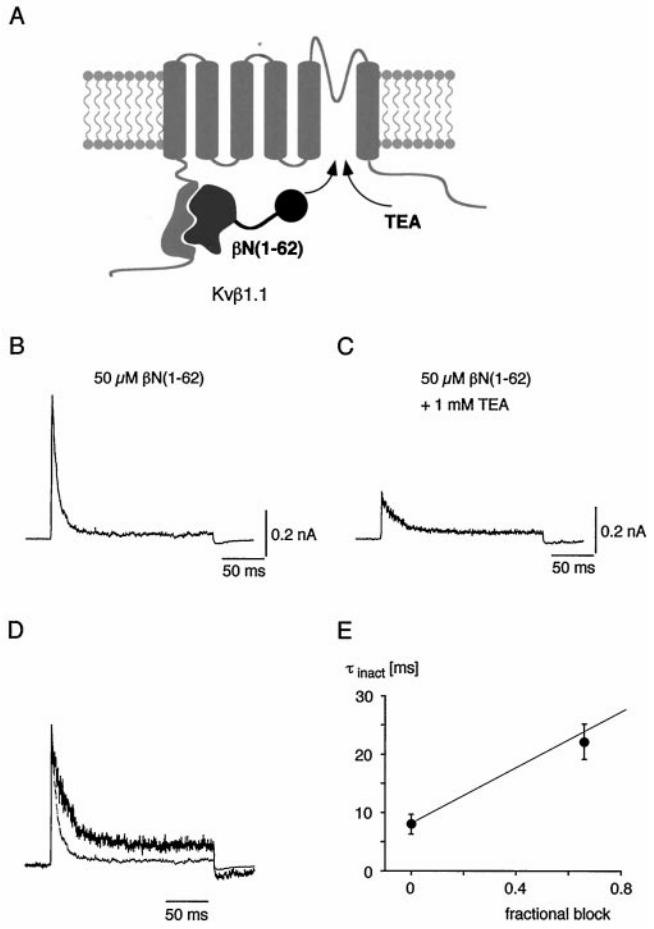

FIG. 1. **βN-(1–62) acts as an open channel blocker.** *A*, drawing representing constitutive and functional interaction between Kvβ1.1 and the Kvα subunit. *B*, block of non-inactivating Kv1.1 channels by 50 μM βN-(1–62) present on the cytoplasmic side. Currents, recorded in a giant inside-out patch, were activated by a voltage step to 120 mV (to rapidly activate channels) from a holding potential of −80 mV; $[K^+]$ on the cytoplasmic side was 120 mM and 5 mM on the extracellular side. *C* and *D*, block by βN-(1–62) is competed by 1 mM TEA. For better comparison, traces recorded in the absence and presence of TEA (scaled to the maximum in outward current) are overlaid. *E*, time constants (τ_{inact}) for βN-(1–62)-mediated inactivation (mean ± S.D. of four experiments) determined in the absence and presence of 1 mM TEA. *Line* represents theoretical dependence of τ_{inact} on fraction of channels blocked by TEA assuming competition between TEA and βN-(1–62).

The interaction between βN-(1–62) and the channel α-subunit was more closely investigated by the "fast application" technique. This technique (see "Materials and Methods") allows solution exchange at giant inside-out patches in less than 2 ms and enables separate determination for on and off rates of channel-peptide interaction (8, 24). Fig. 2, *A* and *B,* shows rapid application and wash-off of 50 μM βN-(1–62); these experiments were performed at a membrane potential of 0 mV under asymmetrical K^+ conditions ($[K^+]_{ex}$ 5 mM, $[K^+]_{in}$ 120 mM). Inactivation occurred with a time constant of ≈10 ms (10.3 ± 1.6 ms, $n = 3$) and was the same whether determined by fast application (*left panel*, first activation) or in the continuous presence of βN-(1–62) (*left*, second activation). Wash-off of βN-(1–62), which should reflect unbinding of the peptide from the

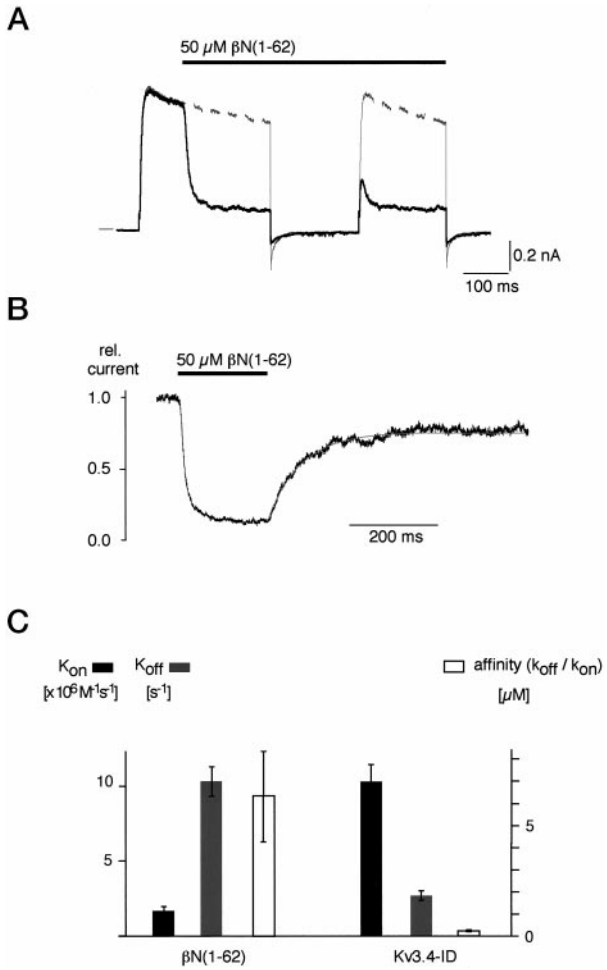

FIG. 2. **Kinetics of βN-(1-62)-mediated inactivation.** *A*, inactivation of non-inactivating Kv1.1 channels mediated by 50 μM βN-(1–62) applied to the cytoplasmic side of a giant inside-out patch by the fast application system. Current trace recorded with the peptide applied (application as indicated by the horizontal bar) is in a *continous line*; the control trace is a *dashed line*. The first part of the recording is inactivation by rapid application of the peptide, the second part is inactivation in the continuous presence of βN-(1–62). Outward currents were elicited by voltage steps from −120 to 0 mV; $[K^+]$ concentrations as in Fig. 1; *scale bars* are as indicated. *B*, application and wash-off of 50 μM βN-(1–62). Current was normalized to the control trace and a monoexponential (line) fitted to the wash-in and wash-off, respectively. Time constants obtained from the fits were 12.3 ms for wash-in and 83.1 ms for wash-off. *C*, binding affinity and rates of the inactivation process mediated by βN-(1–62) and the ID of Kv3.4 (Kv3.4-ID). Values are mean ± S.D. from four experiments.

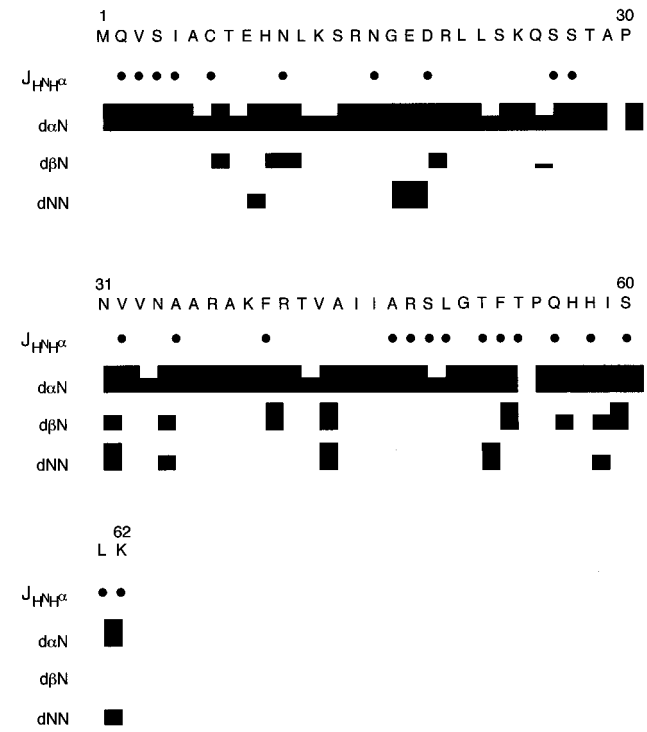

FIG. 3. **NOE contacts observed for βN-(1–62) in aequous solution.** Sequential NOE contacts and *J* coupling constants as a function of the amino acid sequence of βN-(1–62). The NOEs were classified as strong (1.8–2.8 Å), medium (2.8–3.8 Å), and weak (3.8–5 Å), and their intensities are reflected by the thickness of line. *dαN* denotes the NOEs between H^α of amino acid i and the H^N of amino acid i+1; *dβN* is NOE between Hβ of amino acid i and the H^N of amino acid i+1, *dNN* denotes the NOE between H^N of amino acid i and the H^N of amino acid i+1. NOEs between nonadjacent residues ($|i-j| \geq 2$) were not detected. *Filled circles* represent $^3J_{H^NH}\alpha$ coupling constants between 6 and 8 Hz.

receptor, exhibited a time constant of ≈100 ms (99.9 ± 16.8 ms, $n = 3$) and could be well fitted with a monoexponential (Fig. 2*B*, *right*). This indicated that interaction between βN-(1–62) and its receptor on the α-subunit is a first order reaction, with on and off rates (k_{on}, k_{off}) calculated as 1.7×10^6 mol^{-1} s^{-1} and 10.3 s^{-1} (Fig. 2*C*). These values were close to the rates determined for the "α-ball" from *Shaker* B channels (k_{on}, 4.4 10^6 mol^{-1} s^{-1}, k_{off} 13.8 s^{-1}, (5)), but differed from those found for the ID from Kv3.4 channels (Kv3.4-ID: k_{on}, 10.2×10^6 mol^{-1} s^{-1}; k_{off}, 2.6 s^{-1}, Fig. 2*C*). The binding affinity of βN-(1–62) was about 25-fold lower than that of Kv3.4-ID, but almost equals that of the *Shaker* B-inactivation domain (5, 8).

βN-(1–62) Does Not Exhibit a Defined Structure in Solution—The reason(s) for the largely different kinetics observed with the IDs from *Shaker* B and Kv3.4 channels are not well understood but might be related to their different tertiary structure. Kv3.4-ID shows a well defined and compactly folded three-dimensional structure, whereas the *Shaker* B ID does not have a unique structure in solution (13, 14, 30).

To get more insight into the structure-function basis of N-type inactivation in general and β1.1-mediated inactivation in particular, we investigated the structural properties of βN-(1–62) with NMR spectroscopy. Experiments were performed under various conditions, in aequous solution at low pH and in a physiological salt solution at neutral pH (see "Materials and Methods"). The spin systems were completely assigned by two-dimensional NMR methods in the low pH solution and verified under physiological salt and pH conditions. As illustrated in Fig. 3, only sequential NOE contacts were found, with the pattern of NOEs dominated by dαN, the NOE between the α-proton of an amino acid and the amide proton of the following ones. Stretches of consecutive amide-amide NOEs (dNN), indicative for formation of local structures, were absent as were intermediate or long range NOE contacts. All of the experimentally determined coupling constants between amide and α-protons showed values very close to those reported for random coil peptides (31) (Fig. 3).

This view was further supported by the deviations of amide and α-proton chemical shifts ($\Delta\delta H^N$ and $\Delta\delta H^\alpha$) from the random-coil values (32). As depicted in Fig. 4, some of the shifts slightly deviated from random-coil values. However, analysis of the data with the chemical shift index by Wishard *et al.* (33) did not lead to identification of a pattern typical for canonical secondary structures (Fig. 4, *A* and *C*). Nevertheless, upfield shifts (characteristic for helical structures) and downfield chemical shifts (characteristic for β-pleated structures) can be observed and are distributed nonuniformly over the sequence. This suggests transient formation of these structures in the

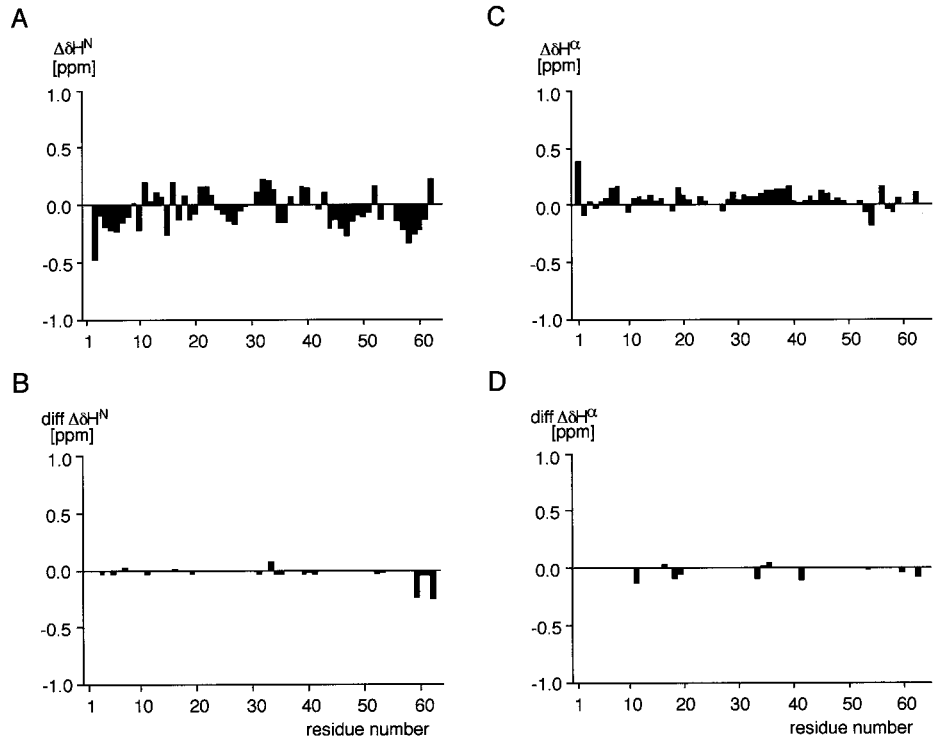

FIG. 4. **βN-(1–62) is a nonstructured peptide.** *A* and *C*, deviation of chemical shifts for amide (H^N) and α-protons (H^α) from random coil values (values given by Wüthrich). *B* and *D,* difference in chemical shift values (*diff*$\Delta\delta H^N$ and *diff*$\Delta\delta H^\alpha$) obtained for H^N and H^α protons in aequous solution at pH 3.2 and in physiological salt solution (see "Materials and Methods") at pH 6.6.

thermal equilibrium. In addition, there are three stretches of downfield shifted amide resonances (between amino acids 2–10, 44–52, and 56–61) indicating a helical propensity in these regions. These results were essentially independent of whether the chemical shifts were determined under low pH or physiological salt and pH conditions (Fig. 4, *B* and *D*). Furthermore, experiments performed to determine the rates of H/D exchange of amide protons showed that all these protons were exchanged in <3 min., indicating that none of these protons are protected by formation of hydrogen bonds within an "internal structure." Rather all amide protons are easily accessible from the solvent, as is typical for not compactly folded peptides. In summary, these results indicated that βN-(1–62) does not exhibit a well defined structure in solution, but rather behaves like a flexible peptide showing only transient formation of local structures.

DISCUSSION

The results presented here show that Kvβ1.1 mediates fast inactivation of Kv1α subunits via an N-type mechanism of inactivation. Accordingly, the unique N terminus of Kvβ1.1 comprises a ball-like domain that blocks the channel via interaction with a receptor site that becomes accessible upon channel opening. This interaction is competed with TEA, very similar to what is known from α-derived IDs. As determined from NMR experiments, the hydrophilic N terminus of Kvβ1.1 does not exhibit a well defined, unique three-dimensional structure. Rather it can be described by a fast conformational equilibrium of weakly structured substates.

With the structural and functional properties described above, the ID of Kvβ1.1 closely resembles the ID from *Shaker* B, but clearly differs from that of Kv3.4 (5, 8, 12–14, 30, 34). Thus, on rates of inactivation were significantly slower for the unstructured IDs compared with the structured Kv3.4-ID, whereas the respective off rates were considerably faster for the IDs from *Shaker* B and Kvβ1.1 than for Kv3.4-ID. Interestingly, on and off rates of inactivation mediated by the well ordered Kv3.4-ID changed considerably when its structure was disturbed by protein phosphorylation (8). Partial unfolding of the N-terminal hemisphere of the Kv3.4-ID resulted in a significant slowing of the on rate, whereas destabilization of the C-terminal hemisphere lead to an increase in the off rate of inactivation (8).

These correlations suggest that the structural properties of the IDs in solution affect the characteristics of their receptor binding. Thereby, a compactly folded ID exhibits faster access to its receptor than an unfolded domain, even if the latter is more positively charged (net charges are +2, +6, and +4 for IDs from *Shaker*, Kvβ1.1, and Kv3.4, respectively). Unbinding is much faster for the nonstructured domains compared with the folded. This may either be because of the higher number of molecular contacts (hydrogen bonds, etc.) formed between the folded domain and the receptor or because of the higher flexibility of the unfolded IDs that destabilizes the ID-receptor interaction. Taken together, the overall structural stability may represent a major determinant of the ID-channel interaction in addition to overall charge and the presence of hydrophobic domains, which were reported previously to govern on and off rates of this interaction (5, 34).

The results about inactivation of Kv1.1 channels with the "core-free" N terminus of Kvβ1.1 differs from the results obtained from coexpression of both subunits. βN-(1–62) induced almost complete inactivation, whereas inactivation mediated by the entire Kvβ1.1 protein was found to be considerably less effective (15, 16, 35, 36). This discrepancy might reflect some variation in binding of Kvβ1.1 to the N terminus of Kv1.1 or might be because of interaction of the α-β complex with other proteins affecting the mobility of the β-inactivation domain. Thus, it was recently suggested that the Kv1.1-Kvβ1.1 complex may interact with the Gβγ dimer, thereby increasing the degree of β-mediated inactivation (35).

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