Structure-Activity Relationships of the Kvβ1 Inactivation Domain and Its Putative Receptor Probed Using Peptide Analogs of Voltage-gated Potassium Channel α- and β-Subunits∗

(Received for publication, July 20, 1998, and in revised form, August 31, 1998)

Stephen J. Lombardi†, Amy Truong, Paul Spence, Kenneth J. Rhodes, and Philip G. Jones
From Wyeth-Ayerst Research, CNS-Disorders, Princeton, New Jersey 08543-8000

Certain β-subunits exert profound effects on the kinetics of voltage-gated (Kv) potassium channel inactivation through an interaction between the amino-terminal “inactivation domain” of the β-subunit and a “receptor” located at or near the cytoplasmic mouth of the channel pore. Here we used a bacterial random peptide library to examine the structural requirements for this interaction. To identify peptides that bind Kv1.1 we screened the library against a synthetic peptide corresponding to the predicted S4-S5 cytoplasmic loop of the Kv1.1 α-subunit (residues 313–328). Among the highest affinity interactors were peptides with significant homology to the amino terminus of Kvβ1. We performed a second screen using a peptide from the amino terminus of Kvβ1 (residues 2–31) as “bait” and identified peptide sequences with significant homology to the S4-S5 loop of Kv1.1. A series of synthetic peptides containing mutations of the wild-type Kvβ1 and Kv1.1 sequences were examined for their ability to inhibit Kvβ1/Kv1.1 binding. Amino acids Arg20 and Leu32 in Kvβ1 and residues Arg234 and Leu328 in Kv1.1 were found to be important for the interaction. Taken together, these data provide support for the contention that the S4-S5 loop of the Kv1.1 α-subunit is the likely acceptor for the Kvβ1 inactivation domain and provide information about residues that may underlie the protein-protein interactions responsible for β-subunit mediated Kv channel inactivation.

Voltage-gated (Kv) K+ channels are heteromorphic protein complexes composed of four integral membrane ion-conducting α-subunits and four cytoplasmic β-subunits. These channels are critical for action potential conduction and neurotransmitter release and are fundamental to the control of neuronal excitability (1–5). The diversity of function of Kv channels is reflected in the heterogeneity of channels that have been found in the nervous system, both in terms of electrophysiological properties and in the multitude of genes that encode them (6–11, 32). Electrophysiological studies have broadly classified Kv channels into two types: delayed-rectifier channels, which are characterized by their slow inactivation, and A-type Kv channels, which inactivate rapidly. Although the majority of Kv channels are composed of α-subunit cDNAs give rise to delayed-rectifier-type currents when expressed in heterologous cells, it has been demonstrated recently that certain β-subunits can dramatically alter the kinetics of channel inactivation when co-expressed with particular α-subunits (12–17). For example, co-expression of Kvβ1 with the delayed rectifier α-subunit Kv1.1 results in a channel that inactivates rapidly (12).

Analysis of the sequences of cloned β-subunits taken together with mutagenesis studies indicates that the amino-terminal 30 amino acids of Kvβ1 contains a “ball” domain that is necessary and sufficient to rapidly inactivate Kv1.1 (12). This NH2-terminal inactivation domain of Kvβ1 shares primary amino acid sequence homology with the inactivation ball of the A-type Kv channels Kv1.4 and the Drosophila Shaker channel. Point mutations in the S4-S5 cytoplasmic loop of the Kv1.4 α-subunit, which lies near the inner mouth of the channel pore, indicate that this may be the acceptor site for the NH2-terminal inactivation ball (18) and suggest that analysis of the interaction of NH2-terminal ball and S4-S5 “receptor” domains may provide clues to the structural requirements for N-type channel inactivation.

Recently, a method to study protein-protein interactions has been developed in which constrained random peptides are displayed on the surface of bacteria as functional fusions to the protein flagellin (19). This enables large libraries of random and diverse polypeptides to be screened and specific peptides selected and characterized following their binding to an immobilized target protein. Due to the immense diversity of these libraries and the relative ease at which sequences can be identified, it is possible to rapidly obtain information about the structural requirements of high-affinity protein-protein interactions (20–25). Random peptide libraries displayed on the surface of bacteria and phage have been employed successfully to identify protein phosphatase-1-binding motifs (26) and potent rhodopsin-binding peptide sequences related to a COOH-terminal Gαi peptide (27), as well as for selecting peptide ligands for the erythropoietin receptor (28).

In the present study, we used this bacterial peptide display library screening strategy to examine the interaction of Kv channel α- and β-subunit domains. In addition, we utilized an in vitro protein interaction assay to examine analogs of these α- and β-subunits and identified key amino acid residues that are important for the interaction. We provide support for the contention that the S4-S5 loop of the Kv1.1 α-subunit is the likely acceptor for the Kvβ1 inactivation domain and provide information about residues that may underlie the protein-protein interactions responsible for β-subunit-mediated Kv channel inactivation.

MATERIALS AND METHODS

Proteins and Peptides—A synthetic peptide (QILGQTLKASMR-ELGL) corresponding to amino acids (313–328) of the human Kv1.1 potassium channel α-subunit polypeptide was synthesized in both biotinylated and nonbiotinylated form (Genosys Biotechnologies, Woodlands, TX). A full-length Kvβ1 and a truncated version corresponding to...
amino acids (2–31) were expressed in *Escherichia coli* as GST fusions (Amersham Pharmacia Biotech). The GST-Kv\(\beta\)1 fusions were purified by affinity chromatography on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) and cleaved from the GST by the addition of thrombin. All mutant peptides were synthesized as constrained 12-mers by Genosys Biotechnologies (Woodlands, TX).

**Growth of the E. coli Peptide Library**—The FliTrx random peptide bacterial library was obtained from Invitrogen (San Diego, CA); growth of the bacterial cultures and general panning methods are based on the system described previously (19).

**Immobilization of Kv\(\beta\)1 and Kv1.1 on Culture Plates**—100 \(\mu\)g of Kv\(\beta\)1 or Kv1.1 was immobilized on 60-mm plastic Petri dishes. The plates were washed with 10 ml of sterile water and nonspecific sites blocked with 10 ml of blocking solution (1% non-fat dry milk, 150 mM NaCl, 1% methylmannoside, and 100 \(\mu\)g/ml ampicillin in IMC medium) at room temperature for 1 h.

**Selection for Kv1.1/Kv\(\beta\)1 Binding**—All incubations were performed at 25 °C, and all other manipulations were at room temperature. An aliquot of the FliTrx library was grown to saturation for 15 h. 10\(^10\) cells were added to IMC medium (Invitrogen) containing 100 \(\mu\)g/ml ampicillin and 100 \(\mu\)g/ml tryptophan to induce expression of the FliTrx plasmid and incubated for an additional 6 h. After library induction the culture was adjusted to a final concentration of 1% non-fat dry milk, 200 mM NaCl, and 1% methylmannoside. Subsequently, 10 ml of the induced cells were added to each culture plate for 1 h. Following binding each plate was washed three times with IMC/amp100 medium containing 1% methylmannoside. The bound cells were removed by vortexing in a residual amount of buffer and incubated in 10 ml of IMC at 25 °C for 14–16 h. Up to five rounds of panning were performed, after which the cultures were plated out and individual colonies selected.

**Plasmid Isolation and Sequence Determination**—Plasmid minipreps were performed on the Qiagen BioRobot 9600 (Qiagen Inc., Santa Clarita, CA) and the resulting plasmid DNA sequenced on an Applied Biosystems 373A automated DNA sequencer (Perkin-Elmer/Applied Biosystems, Foster City, CA) using the FliTrx forward and reverse sequencing primers (Invitrogen). The resulting dodecamer DNA sequences were translated using DNAstar (Madison, WI) and Clustal analysis used to align the sequences and generate a consensus sequence. The resulting consensus sequences generated from days 2, 3, and 5 were similarly aligned to the wild-type sequences. Gaps in the consensus sequences signify that no consensus residue was identified at this position.

**Plate Assay**—The full-length Kv\(\beta\)1 protein was immobilized onto Microtiter plates (Corning Costar, Cambridge, MA,) for 1 h at 37 °C at a final concentration of 10 ng/well. The wells were then washed twice with Hepes-buffered saline (10 mM Hepes, pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% v/v Surfactant P20) followed by blocking with 1% bovine serum albumin in Hepes-buffered saline. For binding, 100 ng of a biotinylated K1.1 (S4-S5 loop) were added to each well and incubated for 1 h at 37 °C, followed by detection with streptavidin, biotin-alkaline

### Table I

| Alignment of peptide sequences that bind to the K\(\beta\)1 S4–S5 loop peptide |
|---|
| **Peptide Sequence** |
| VOSIACTELNLKSNGGDLRLRQLS |
| RDDGRLERQK |
| 100 |

### Table II

| Alignment of peptide sequences that bind to the K\(\beta\)1 NH\(\_\)terminal peptide |
|---|
| **Peptide Sequence** |
| QILGSTTLKASHRMELGL |
| 1-11 |

---

*K\(\beta\) Channel Inactivation Domain Analogs* 30903
were calculated using the computer program Microsoft Excel 6.0 and p values calculated using the InStat 1.14 statistical program.

The results of days 2 (22% stop codons, 8/36) and 3 (17% stop codons, 6/36) were analyzed and the IC$_{50}$ for K$_{\beta}$1 wild-type peptide was calculated to be 9 ± 2 nm, the IC$_{50}$ for K$_{\beta}$1.1 wild-type peptide was 15 ± 2 nm. The single mutants K$_{\beta}$1 R20A and L21A showed greatly reduced affinity (200- and 30-fold, respectively) when compared with the wild-type control sequence. Interestingly, these amino acids were conserved following just 2 days of panning, substantiating our belief that key residues would be conserved at these earlier days. K$_{\beta}$1 residues Arg$_{15}$, Asn$_{16}$, and Ser$_{23}$ were also observed at day 3, although their mutation had only a small 2–3-fold effect on binding. Among the mutants tested for K$_{\beta}$1 was the triple mutant K$_{\beta}$1 G17S,E18G,D19N, since it had been reported in the literature to enhance the K$_{\beta}$1 mediated K$_{\beta}$1 inactivation. Surprisingly, in this study the triple mutant had no inhibitory effect (Fig. 3). Of the single mutants tested for K$_{\beta}$1, R324A completely abolished peptide inhibition of K$_{\beta}$1/K$_{\beta}$1 binding, S322A and L328A demonstrated marked decreases in affinity (60- to 100-fold, respectively) (Fig. 4). T318A (100 ± 2 nm) and E325A (100 ± 3 nm) showed smaller 6-fold decreases in binding affinity. Other residues investigated showed no effect.

**RESULTS**

The panning procedure was applied to identify peptides that bind the K$_{\beta}$1.1 channel S4-S5 loop and K$_{\beta}$1 NH$_2$ terminus, using immobilized K$_{\beta}$1.1 (313–328) and K$_{\beta}$1 (2–31) as “baits.” Following five successive rounds of panning against immobilized K$_{\beta}$1 were aligned to the wild-type sequence (amino acids 313–328) of K$_{\beta}$1.1, phosphatase, and developed with p-nitrophenyl phosphate. Peptides were tested in the assay system for inhibition of binding. IC$_{50}$ values were calculated using the computer program Microsoft Excel 6.0 and p values calculated using the ImStat 1.14 statistical program.

**DISCUSSION**

Voltage-activated potassium currents are vital for neuronal function and are carried by membrane channels composed of 4$\alpha$- and 4$\beta$-subunits arranged as a hetero-octamer (31). Some $\beta$-subunits, such as K$_{\beta}$1, can convert noninactivating “delayed rectifying” currents of certain $\alpha$-subunits (e.g. K$_{\alpha}$1.1) into rapidly inactivating currents (12). In this study we used a random peptide library to investigate the interacting domains of K$_{\beta}$1.1 and K$_{\beta}$1. The N terminus of K$_{\beta}$1 (amino acids 2–31), when used as bait, bound peptides homologous to the S4-S5 loop of K$_{\beta}$1.1, whereas sequences related to K$_{\beta}$1 NH$_2$ terminus were observed when the S4-S5 loop of K$_{\beta}$1 was used as bait. This correlates well with studies that have implicated these regions as the sites of interaction between the K$^+$ channel and NH$_2$-terminal inactivation domains (12, 18). Furthermore, we were able to restrict the interacting regions to small motifs, amino acids 12–23 in K$_{\beta}$1 and residues 317–325 of K$_{\beta}$1.1, which form core domains important for the interaction.

In the motif identified in K$_{\beta}$1, Arg$_{20}$$^a$ and Leu$_{21}$$^a$ were represented in 55 and 77% of the sequences analyzed at day 5. 69% of the clones had a basic residue (KR) at position 20, suggesting that a positive charge at this position may be important for structure/function of the K$_{\beta}$1. In contrast, a strong preference for a hydrophobic residue (AIIFWPM) was seen at position 21 (80% of the clones had a hydrophobic residue). In a protein interaction assay the K$_{\beta}$1 mutations R20A and L21A had the largest effect on binding, causing a 200- and 30-fold decrease in affinity, respectively. The other residues had little or no effect, and this is perhaps indicative of their having a more structural role in presenting the key residues in a conformation to maximize the interaction rather than participating directly in K$_{\beta}$1 binding. A sequence comparison of K$_{\beta}$1, K$_{\beta}$1.2, and K$_{\beta}$1.3 indicates that the NH$_2$ terminus of these proteins are quite divergent (16, 17). Interestingly, the key residues identified by our study are not found in K$_{\beta}$1.2, K$_{\beta}$1.3, K$_{\beta}$2, or K$_{\beta}$3, which could explain the lower effectiveness of the K$_{\beta}$1.2 in inactivating K$_{\alpha}$ channels (12, 30). Retig et al. (12) have shown that the triple mutation G17S,E18G,D19N increased the inactivating ability of K$_{\beta}$1. In our study, this triple mutant was unable to inhibit the K$_{\beta}$1.1- K$_{\beta}$1 interaction even though this triplet was seen at early days of panning, suggesting that these amino acids play a role in the K$_{\beta}$1/K$_{\beta}$1.1 binding. One might speculate that this triplet is important for the structural integrity of the K$_{\beta}$1 interacting domain, and the conformational changes due to the mutation have less of an impact on the...
of TLKASMRELGLL found in the Kv1.1 S4-S5 loop is important for channel inactivation domain analogs. Other substitutions, which decreased Shaker channel inactivation (T388S/A, S392C/A), also reduced the affinity of the inactivating ball peptide for the R324A mutation in our hands. This residue was conserved even at day 2 and was found in 70% of day 5 clones. Surprisingly, mutation of this residue to glutamine in Shaker was without effect. This may indicate the increased sensitivity of our assay to changes in conformation of a small peptide.

This study demonstrates that random peptide libraries are useful tools for the study of protein-protein interactions. Our results support and extend the literature indicating that the β1 NH₂ terminus is likely to mediate its action by binding the S4-S5 loop. Furthermore, we have identified key residues which are likely to participate in the protein-protein interactions required for β-subunit-mediated K⁺ channel inactivation.

### Table III

| Synthetic peptides | IC₅₀ (nM) |
|--------------------|----------|
| Kβ1 wild-type      | 9 ± 2    |
| Kβ1 K13A           | 9 ± 4    |
| Kβ1 S14A           | 10 ± 6   |
| Kβ1 R15A           | 25 ± 4   |
| Kβ1 N16A           | 35 ± 5   |
| Kβ1 G17S,E18G,D19N | N.I     |
| Kβ1 G17A           | 10 ± 3   |
| Kβ1 E18A           | 11 ± 3   |
| Kβ1 D19A           | 9 ± 4    |
| Kβ1 R20A           | 2000 ± 300 |
| Kβ1 L21A           | 300 ± 5  |
| Kβ1 L22A           | 10 ± 5   |
| Kβ1 S23A           | 35 ± 4   |
| K,1.1 wild-type    | 15 ± 2   |
| K,1.1 T318A        | 100 ± 2b |
| K,1.1 L319A        | 17 ± 3   |
| K,1.1 S322A        | 2000 ± 400 |
| K,1.1 M323A        | 45 ± 3b  |
| K,1.1 R232A        | N.I     |
| K,1.1 E325A        | 100 ± 3b |
| K,1.1 L326A        | 25 ± 3a  |
| K,1.1 G327A        | 18 ± 2   |
| K,1.1 S329A        | 900 ± 5b |

* p < 0.01.

** REFERENCES**

1. Rudy, B. (1988) *Neuroscience* **25**, 729–749
2. Hille, B. (1992) *Channels of Excitable Membranes*, 2nd Ed., Springfield, MA
3. Heinemann, S. H., Retzig, J., Graack, H.-R., and Pongs, O. (1996) *J. Physiol.* **493**, 625–633
4. Pongs, O. (1995) *Semin. Neurosci.* **7**, 137–146
5. Latorre, R., Coronado, R., and Vergara, C. (1984) *Annu. Rev. Physiol.* **46**, 485–495
6. Jan, L. Y., and Jan, Y. N. (1989) *Cell* **56**, 13–25
7. Jan, L. Y., and Jan, Y. N. (1990) *Trends Neurosci.* **13**, 415–418
8. Catterall, W. A. (1988) Science **242**, 50–61
9. Shi, G., Kleinlaus, A. K., Marrion, N. V., and Trimmer, J. S. (1994) *J. Biol. Chem.* **269**, 23204–23211
10. Nakamura, R. L., Anderson, J. A., and Gaber, R. F. (1997) *J. Biol. Chem.* **272**, 1911–1918
11. Sheng, M., Tsaur, M. L., Jan, Y. N., and Jan, L. Y. (1992) *Neuron* **9**, 271–284
12. Retzig, J., Heinemann, S. H., Wunder, F., Lorr, C., Parej, D. N., Dolly, J. O., and Pongs, O. (1994) *Nature* **369**, 269–294
13. England, S. K., Uebele, V. N., Kodali, J., Bennett, P. B., and Tamkun, M. M. (1995) *J. Biol. Chem.* **270**, 28531–28534
14. England, S. K., Uebele, V. N., Shear, H., Kodali, J., Bennett, P. B., and Yamkun, M. M. (1995b) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 6309–6313
15. McMormack, K., McMormack, T., Tanouye, M., Rudy, B., and Stuhmer, W. (1995) *FEBS Lett.* **370**, 32–36
16. Majumder, K., DeBiasi, M., Wang, Z., and Wible, B. A. (1995) *FEBS Lett.* **370**, 32–36
17. Shaganskiy, V. V., Andrianov, K. A., and Verkhovsky, A. B. (1995) *FEBS Lett.* **370**, 32–36
18. Isacoff, E. Y., Jan, Y. N., and Jan, L. Y. (1991) *Nature* **353**, 86–90
K⁺ Channel Inactivation Domain Analogs

19. Lu, Z., Murray, K. S., Van Cleave, V., LaValle, E. R., Stahl, M. L., and McCoy, J. M. (1995) Bio/Technology 13, 366–372
20. Scott, J. K., and Craig, L. (1994) Curr. Opin. Biotechnol. 5, 40–48
21. Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6378–6382
22. Devlin, J. J., Panganiban, L. C., and Devlin, P. E. (1990) Science 249, 404–406
23. Cull, M. G., Miller, J. F., and Schatz, P. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 1865–1869
24. Smith, G. P. (1985) Science 226, 1315–1317
25. Parmley, S. F., and Smith, G. P. (1988) Gene (Amst.) 73, 305–318
26. Zhao, S., and Lee, E. Y. C. (1997) J. Biol. Chem. 272, 28368–28372
27. Martin, E. L., Rens-Domiano, S., Schatz, P. J., and Hamm, H. E. (1996) J. Biol. Chem. 271, 361–366
28. Wrighton, N. C., Farrell, F. X., Chang, R., Kasahyap, A. K., Barbone, F. P., Mulecky, L. S., Johnson, D. L., Barrett, R. W., Jolliffe, L. K., and Dower, W. J. (1996) Science 273, 458–464
29. Rettig, J., Wunder, F., Stocker, M., Lichtinghagen, R., Masteiaux, F., Beckh, S., Kues, W., Pedarzani, P., Schroder, K., Ruppersberg, P., Veh, R., and Pongs, O. (1992) Eur. Mol. Biol. Org. J. 11, 2473–2486
30. Leicher, T., Roepel, J., Weber, K., Wang, X., and Pongs, O. (1996) Neuropharmacology 35, 787–795
31. Trimmer, J. S. (1998) Curr. Opin. Neurobiol. 8, 370–374
32. Rhodes, K. G., Strassle, B. W., Monaghan, M. M., Bekele-Arcuri, Z., Matos, M. F., and Trimmer, J. S. (1997) J. Neurosci. 17, 8246–8258