Mapping the Functional Anatomy of BgK on Kv1.1, Kv1.2, and Kv1.3

CLUES TO DESIGN ANALOGS WITH ENHANCED SELECTIVITY*

(Received for publication, June 28, 1999, and in revised form, September 7, 1999)

Nicole Alessandri-Haber‡§, Alain Lecoq§§, Sylvaine Gasparini†, Geraldine Grangier-Macmath‡, Guy Jacquet‡, Alan L. Harvey†, Cleane de Medeiros‡, Edward G. Rowan‡, Maurice Gola‡, André Ménez‡, and Marcel Crest‡**

From the ‡Laboratoire de Neurobiologie, CNRS UPR 9024, 31 chemin J. Aiguier, 13402 Marseille, France, the †Department of Physiology and Pharmacology, University of Strathclyde, Glasgow G1 1XW, United Kingdom, and the §Département d’Ingénierie et d’Études des Protéines, CEA, Saclay, 91191 Gif-sur-Yvette cedex, France

BgK is a peptide from the sea anemone Bunodosoma granulifera, which blocks Kv1.1, Kv1.2, and Kv1.3 potassium channels. Using 25 analogs substituted at a single position by an alanine residue, we performed the complete mapping of the BgK binding sites for the three Kv1 channels. These binding sites included three common residues (Ser-23, Lys-25, and Tyr-26) and a variable set of additional residues depending on the particular channel. Shortening the side chain of Lys-25 by taking out the four methylene groups dramatically decreased the BgK affinity to all Kv1 channels tested. However, the analog K25Orn displayed increased potency on Kv1.2, which makes this peptide a selective blocker for Kv1.2 (K_D 50- and 300-fold lower than for Kv1.1 and Kv1.3, respectively). BgK analogs with enhanced selectivity could also be made by substituting residues that are differentially involved in the binding to some of the three Kv1 channels. For example, the analog F6A was found to be 300-fold more potent for Kv1.1 than for Kv1.2 and Kv1.3. These results provide new information about the mechanisms by which a channel blocker distinguishes individual channels among closely related isoforms and give clues for designing analogs with enhanced selectivity.

Voltage-gated K^+ channels (Kv1 channels) take part in various cellular functions by modulating the cell membrane potential. Among all Kv channels, the Shaker-related Kv1 channels (Kv1.1–Kv1.7) (1, 2) have been particularly studied, using numerous peptidic blockers isolated from snakes, scorpions, cone snails, and sea anemones (3–13). These blockers have proved to be useful tools to understand the relationships between potassium currents and potassium channels. Furthermore, they have brought new insights into the structure of the pore-forming region of these channels (14–20). Finally, in brain, the different subtypes of Kv1 channels have been shown to co-assemble into heteromultimers, and the use of radiolabeled blockers has been essential for studying the molecular composition of these channels (21–23).

The peptidic blockers have a variable range of specificities, but none of them appears to be strictly selective toward the different Kv1.1–Kv1.7 channel subtypes. For instance, the scorpion toxin charybdotoxin blocks with dissociation constants in the nanomolar range both Kv1.2 and Kv1.3 but not Kv1.1, even at 1 µM concentration, whereas the snake venom toxin α-dendrotoxin (αDTX) blocks Kv1.1, Kv1.2, and Kv1.3 with dissociation constants equal to 20, 17, and 250 nM respectively (24). However, potent blockers endowed with high selectivity toward desired channels would be of great interest. For example, homeric Kv1.3 channels in T-lymphocytes are being considered as a therapeutic target for immunosuppression, and a selective blocker of Kv1.3 could become an interesting immunosuppressant (25–27). The use of more selective blockers would bring further insights in identification of the molecular composition of the native heteromultimers Kv1 channels. In this respect, it would be particularly interesting to study the Kv1.1 containing channels because they are involved in episodic ataxia (28), and Kv1.1-deleted mice display frequent spontaneous seizures (29).

One way to design a blocker that would be selective for one subtype of Kv1 channel may consist of modifying the pharmacological profile of a natural blocker and altering its selectivity for Kv1 subtypes, as suggested previously (27). Therefore, the first requirement in such a strategy is to elucidate, as precisely as possible, all the topographies by which a selected blocker binds to the different channel subtypes that are associated with its own pharmacological profile. Although various animal toxins have been previously submitted to extensive mutational studies, no such a complete mapping has been reported for a single toxin against a number of K^+ channels.

We approached this question with BgK, a 37-amino acid peptide, isolated from the sea anemone Bunodosoma granulifera (7), which blocks with equal potencies the homomeric Kv1.1, Kv1.2, and Kv1.3 channels (30). Using alanine scanning, the binding determinants of BgK to heteromeric αDTX targets in rat brain that mainly contain Kv1.2 (31) were identified (32). In the present study, we reported the complete mapping of the binding sites of BgK to homomeric Kv1.1, Kv1.2, and Kv1.3 channels. Our results show that these binding sites include (i) three common residues (Lys-25, Tyr-26, and Ser-23) that seem to form a conserved binding core for all Kv1 channel blockers from sea anemones (7–11), and (ii) a differential number of additional residues that ensure binding to each of the three channel subtypes. The deduced size of these binding sites increases in the order Kv1.1 < Kv1.2 < Kv1.3.

In addition, we showed that some introduced substitutions already confer the toxin with substantially higher selectivity

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* This work was supported by the CNRS, by Association Française contre les Myopathies Grant 5050, and by the Association de Recherche contre la Sclérose en Plaques. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

** To whom correspondence should be addressed. Tel.: 33-491-16-43-18; Fax: 33-491-16-46-15; E-mail: crest@lhn.cnrs-mrs.fr.

† The abbreviations used are: DTX, dendrotoxin; HEK, human embryonic kidney; hKv1.1, human Kv1.1; rKv1.1, rat Kv1.1; KTX, kalotoxin; MgTX, margatoxin; NTX, noxiustoxin.
for some channels, offering an experimental basis for designing, in the future, BgK analogs with predetermined selectivity toward the different Kv1.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis and Physicochemical Characterization**

All the analogs in which one residue was substituted by an alanine were synthesized as described previously by Dauplais et al. (32). The analogs in which Lys-25 was substituted by an ornithine (K25Orn), a norleucine (K25Nle), a diaminobutyrate (K25Dab), or a diaminopropionate (K25Dap), and the analog K25AY26A were synthesized according to the same procedure. Circular dichroic spectra were recorded using a Jobin-Yvon CD6 dichrograph, as described previously (32).

**Expression in Human Embryonic Kidney (HEK)-293 and B82 Cell Lines**

HEK-293 cells stably transfected with human Kv1.1 (hKv1.1) cDNA were kindly provided by O. Pongs (Zentrum für Molekulare Neurobiologie, Universität Hamburg, Hamburg, Germany). Cells were maintained in a Dulbecco’s modified Eagle’s medium/Ham’s F-12 (HEK-293 cells, BioMedia) or a Dulbecco’s modified Eagle’s medium (B82 cells, Life Technologies, Inc.) culture medium supplemented with 10% fetal calf serum, 50 μg/ml streptomycin, 50 units/ml penicillin (Biowittaker), 4 mm l-glutamine (Life Technologies, Inc.), and 1 mg/ml geneticin (G418, Life Technologies, Inc.) in a humidified 5% CO2 incubator at 37 °C. HEK-293 cells were plated onto Nun dishes and allowed to grow for 3 days before electrophysiological experiments. B82 cells were plated onto glass coverslips for 2 h at 37 °C before being used.

**Expression in Xenopus Oocytes**

Rat Kv1.1, Kv1.2, Kv1.3 cDNAs and subsequent cRNAs (stored in water at 1 μg/ml) were a generous gift from S. Alper (Beth Israel Hospital, Havard Medical School, Boston MA). cRNAs injections in oocytes were done at 4 ng/ml (50 nl injected), and the currents were recorded 1–6 days after injection.

**Electrophysiological Recordings**

**Whole-cell Experiments in HEK-293 and B82 Cells—Patch pipettes were filled with 130 mM KCl, 2 mM MgCl2, 10 mM EGTA, 7 mM Hepes-KOH, pH 7.35. Cells were bathed in 137 mM NaCl, 5.3 mM KCl, 0.8 mM MgSO4, 0.4 mM Na2HPO4, 1.8 mM CaCl2, 10 mM Hepes-NaOH, pH 7.4. BgK and analogs were diluted in this medium on the day of experiment. Stock solutions (10 μM) were stored in water at −20 °C. Drugs and toxins were applied with a pneumatic Pico-Pump system (WPI) or with a bath application by gravity from several reservoirs connected to a short common outlet. Liquids in excess in the bath were constantly sucked. Holding potential was set at −80 mV for HEK-293 cells and −60 mV for B82 cells.

Voltage-Clamp Experiments in Oocytes—Two-electrode voltage clamp recordings were performed 1–6 days after cRNAs injection, using a Gene Clamp 500 amplifier (Axon Instruments). Oocytes were bathed in a calcium-free saline containing: 88 mM NaCl, 1 mM KCl, 0.8 mM MgSO4, 2.4 mM Na2CO3, 10 mM Hepes-NaOH, pH 7.4. Intracellular electrodes were filled with 3 μM KCl. The electrode resistance was <1 MΩ. The holding potential was set at −80 mV. The perfusion system was built in order to ensure a fast change in toxin concentration as described previously (30).

**RESULTS**

**Kv1.1 Channel Blockade by BgK**

Kv1.1 currents were recorded from HEK-293 cells expressing hKv1.1 and from Xenopus oocytes injected with rKv1.1 cRNA. Upon depolarization, the current activated with a sigmoidal time course and was almost devoid of inactivation (Fig. 1, A and B). The voltage dependence was conventionally determined by calculating the peak conductance at various potentials and by fitting the resulting normalized curve to Boltzmann functions. The current reversal potential was evaluated from the tail current kinetic upon repolarization and set at −80 mV. Mean values of V1/2 (voltage corresponding to half-conductance activation) and p (Boltzmann coefficient) were V1/2 = −22 ± 1 mV and p = 11 ± 2 mV (n = 18) for hKv1.1 expressed in HEK-293. These values are in good agreement with those obtained for rKv1.1 in Xenopus oocytes, V1/2 = −32 ± 5 mV and p = 9 ± 3 mV. The 10 mV positive shift in V1/2 observed in HEK-293 as compared with oocytes may be accounted for the junction potential, which results from the low ionic strength of the saline used in patch electrodes (33).

The blocking potency of BgK on hKv1.1 (Fig. 1A) and rKv1.1 (Fig. 1B) were determined by step increasing the BgK concentration from 0.1 to 200 nM (Fig. 1C). At each concentration and in the −40 to 0 mV range, the blocking potency slightly increased with the pulse level (Fig. 1D). The voltage dependence was particularly obvious with rKv1.1. Therefore, the K+ cur-
current persisted in the presence of BgK was measured between +20 and +40 mV. Experimental points were fitted to hyperbolic curves. For hKv1.1 in HEK-293, we found an IC$_{50}$ = 30 ± 5 nM, and a Hill coefficient of $n = 0.97 ± 0.4$ (Fig. 1C). The corresponding values for rKv1.1 in Xenopus oocyte were IC$_{50}$ = 0.72 ± 0.06 nM and $n = 0.92 ± 0.09$ (Fig. 1C). The Hill coefficient was not significantly different from 1, which confirmed that the BgK action follows a bimolecular mechanism. Therefore, the above IC$_{50}$ values were considered to reflect the apparent dissociation constant $K_D$.

Identification of BgK Residues Involved in the Binding to Kv1 Channels

In order to define the residues of BgK that bind to Kv1 channels, we synthesized analogs corresponding to the alanine substitution of all the residues with the exception of the six half-cysteines, four alanines, and one glycine (32). Among the 26 analogs, D4A could not be properly oxidized. A similar observation has already been mentioned for ShK (D5A analog) (34), suggesting that this aspartic acid, conserved in all the Kv1 channels blockers from sea anemone, may be important for their folding pathway.

**Kv1.1**—In preliminary experiments, the relative affinity of the 25 alanine-monosubstituted analogs was determined as the percentage (mean ± S.E. of 3–7 experiments) of hKv1.1 current blocked by a 50 nM concentration of each peptide. In the case of BgK, this concentration blocks 62% of the current. The mutants were classified in two groups, including, respectively, peptides that induced a percentage of block higher (Fig. 2A, white column) or lower (black column) than 25%, a value corresponding to an estimated 5-fold increase in $K_D$ (Fig. 2A, right axis).

In order to precisely determine the $K_D$ values for the nine analogs of which the affinity was shifted by more than 5-fold, we performed a series of dose-response experiments. We also considered the substitution F6A because this residue was shown to participate in the binding of BgK to rat brain Kv channels (32). The experimental points (mean ± S.E., 4–6 experiments) were fitted to hyperbolic curves with a Hill coefficient, $n$, set to 1, because no change in the stoechiometry of the binding was observed for all the analogs. To illustrate this point, the Fig. 3A shows the results for N19A, K25A, and Y26A, for which $n = 0.95 ± 0.07, 1.02 ± 0.03, and 1.09 ± 0.1$, respectively.

The results from dose-response experiments are given in Fig. 2B. The three most important residues for BgK binding to hKv1.1 are Lys-25, the alanine substitution of which induced an affinity decrease of 60-fold, and Asn-19 and Tyr-26, the substitution of which induced an affinity decrease of 20-fold. In addition, alanine substitution of Ser-23, Gln-24, and Asn-29 induced a moderate, 5–20-fold affinity decrease.

Analogs F6A, K7A, S16A, N19A, T22A, S23A, Q24A, K25A, Y26A, R27A, and N29A were also tested for their ability to block the rKv1.1 currents expressed in Xenopus oocytes. The $K_D$ values were determined as described above from dose-response experiments. Fig. 3B shows that the relative changes in toxin affinity are similar to those observed in HEK-293 transfected cells. However, as observed with native BgK, the $K_D$ value of all the analogs subjected to dose-response experiments was systematically smaller for Kv1.1 expressed in oocytes than in HEK-293 cells. The $K_D$ ratio between the two expression systems was almost constant: $K_{D(hKv1.1)} / K_{D(rKv1.1)} = (45 ± 6) × K_{D(hKv1.1)}$ (see legend of Fig. 3B). Such a difference may result from differences in species of cDNA (human versus rat) or in expression system (mammalian cell line versus oocyte) used. The sequences of the rat and human Kv1.1 cDNAs are identical between the second and the sixth transmembrane segments, and the binding sites of various sea anemone and scorpion toxins have been identified between the fifth and the sixth transmembrane segment of Kv1 channels (14–17, 27). Variations in the affinity of charybdotoxin, α-DTX and 4-aminopyridine for Kv channels has been observed depending on the expression system used, either in mammalian cell lines or in Xenopus oocytes (1, 13, 16, 24, 35). Moreover, the expression of the rat Kv1.2 cDNA in the B82 cells instead of Xenopus oocytes increased by 28-fold the $K_D$ of the BgK (see the following section). These data suggest that depending on the expression system, endogenous molecules located around the channels
Tyr-26), the substitution of which increased the dissociation constant of BgK was 7.6 nM in B82 cells. Preliminary experiments highlighted a group of 13 residues, Phe-6, Lys-7, Asn-19, Ser-23, Gln-24, Lys-25, Tyr-26, Arg-27, Asn-29, and Leu-36. These residues are in the vicinity of the disulfide 11–30 and 20–34, might alter more precisely, the methyl group of Thr-33 and one of Leu-36 are totally buried in a hydrophobic core. More solvent accessibility indicated that the side chains of Thr-33 and Leu-36 are in the vicinity of the disulfide 11–30 and 20–34, might alter the three-dimensional structure of the protein. For the analogs K32A and E35A, the distortions observed in their CD spectra could not be readily interpreted. The dichroic spectra of the corresponding proteins (Fig. 6). The superimposed spectra of the four analogs K32A, T33A, Glu-35, and Leu-36 displayed minimum at 215 nm and maximum at 192 nm. This observation shows that they all possess a similar content in secondary structure, suggesting that their overall conformation is similar to that of the wild-type BgK.

By contrast, substitution of five residues (Asn-29, Lys-32, T33A, Glu-35, and Leu-36) induced some distortions in the dichroic spectra of the corresponding proteins (Fig. 6). The superimposed spectra of the four analogs K32A, T33A, E35A, and L36A displayed minimum at 215 nm and maximum at 192 nm, but with substantially different intensities as compared with the spectrum of BgK. Close inspection of the 15 best NMR structures of BgK (32) and calculation of side chain relative solvent accessibility indicated that the side chains of Thr-33 and Leu-36 are totally buried in a hydrophobic core. More precisely, the methyl group of Thr-33 and one of Leu-36 are close to the methylene groups of half-cysteines 11, 30, and 34. Therefore, alanine substitution of Thr-33 and Leu-36, which are in the vicinity of the disulfide 11–30 and 20–34, might alter the three-dimensional structure of the protein. For the analogs K32A and E35A, the distortions observed in their CD spectra could not be readily interpreted. The dichroic spectra of the analog N29A and BgK displayed the same overall shape, al-

FIG. 5. Blockade by BgK and alanine-substituted analogs of rKv1.3 expressed in Xenopus oocytes. A, percentage (± S.E.) of current block induced by 50 nM peptide. Thirteen analogs (black columns) were selected and used in dose-response experiments. B, Kp was determined from dose-response experiments performed with the 13 analogs selected in A. Black columns, Kdp/BgK > 20-fold; gray columns, Kdp/BgK between 5- and 20-fold.

Structural Properties of BgK Analogs

Before concluding that an affinity decrease observed upon substitution reflects the functional contribution of the substituted residue, we assessed by circular dichroism that the secondary structure of the protein mutants was not different from that of the wild-type BgK. In the 185–250 nm region, 20 of the monosubstituted analogs displayed circular dichroic spectra similar to that of BgK with a minimum at 215 nm and a maximum at 192 nm. This observation shows that they all possess a similar content in secondary structure, suggesting that their overall conformation is similar to that of the wild-type BgK.

In conclusion, irrespective of the expression system used, three residues (Asn-19, Lys-25, and Tyr-26) appear to be important for BgK binding to Kv1.1, and three others (Ser-23, Gln-24, and Asn-29) appear to contribute more moderately to this binding.

Kv1.2—BgK and the 25 analogs were tested on rKv1.2 channels expressed in Xenopus oocytes and B82 cells. The dissociation constant of BgK was 1.3 ± 0.4 nM in oocytes and 37 ± 5 nM in B82 cells. Preliminary experiments highlighted a group of six residues (Phe-6, Arg-12, His-13, Ser-23, Lys-25, and Tyr-26), the substitution of which increased the Kp by >5-fold (Fig. 4A). The corresponding analogs were subjected to dose-response experiments (Fig. 4B). Three residues (Phe-6, Lys-25, and Tyr-26) highly contribute to BgK binding to Kv1.2, because their alanine substitution induced an affinity decrease larger than 20-fold (above 500-fold in the case of K25A and F6A). The substitution of three other residues (Arg-12, His-13, and Ser-23) induced a moderate, 5–20-fold increase in Kp.

Kv1.3—On rKv1.3 channels expressed in Xenopus oocytes, the dissociation constant of BgK was 7.6 ± 0.9 nM. Preliminary experiments were performed using a 50 nM concentration of each peptide, and the scan distinguished a group of 13 residues, the substitution of which increased the Kp by >5-fold (Fig. 5A). These are Phe-6, Lys-7, His-13, Leu-17, Asn-19, Thr-22, Ser-23, Gln-24, Lys-25, Tyr-26, Arg-27, Asn-29, and Leu-36.

The respective Kp values of analogs were determined by performing dose-response experiments. Fig. 5B summarizes the results and shows that seven residues (Phe-6, Lys-7, Asn-19, Ser-
though the minimum and maximum signals were shifted to 208 and 189 nm respectively (Fig. 6). This finding suggests that substitution N29A might have reinforced the helicity within the helical region 24–30. Because of the observed modification in the CD spectra of analogs N29A, K32A, T33A, E35A, and L36A, the functional effects associated with these substitutions should be interpreted with caution.

**Binding of BgK-Lys-25 Analogs to rKv1.1, rKv1.2, and rKv1.3**

Lys-25 of BgK has been shown to be the most important or one of the most important residues for BgK binding to Kv1.1, Kv1.2, and Kv1.3 (this study), as well as to αDTX targets in rat brain (32). These observations raised the question of whether or not this residue plays the same binding role toward all these channel subtypes. To approach this question, we synthesized analogs of BgK in which Lys-25 has been substituted by non-natural residues in order to (i) maintain the positive charge but progressively decrease the chain by one (K25Orn), two (K25Dab), and three (K25Dap) methylene groups, and (ii) maintain the side chain length of four methylene groups while substituting the ammonium group by an hydrogen atom (Nle). All these analogs but K25Dap displayed CD spectra similar to that of BgK. In the spectrum of the BgK-K25Dap (Fig. 6), we observed an increase of the intensities of positive and negative bands, suggesting that shortening the side chain 25 by three methylene groups induced conformational modifications. Very recent results have shown that the equivalent substitution in ShK (ShK-K22Dap) induced some structural differences as compared with the native protein (27).

All of these analogs were tested on rKv1.1, rKv1.2, and rKv1.3 expressed in *Xenopus* oocytes, and their *K*D values were determined from dose-response experiments as illustrated in Fig. 7A. The results are presented in Fig. 7B in terms of relative change in dissociation constant. Removing the charged group at position 25 (K25Nle) decreased the affinity of BgK for the three channels, suggesting that the presence of a positive charge at position 25 is necessary for BgK binding with high affinity to Kv1.1, Kv1.2, and Kv1.3.

Reducing the side chain length of position 25 decreased differentially the affinity of BgK for the three channels. For Kv1.1 and Kv1.3, the decrease in affinity approximately correlated to the side chain shortening, whereas for Kv1.2, shortening the side chain of Lys-25 by a single methylene group (K25Orn) increased 3-fold the toxin affinity for this channel (*K*D, BgK, 1.3 ± 0.2 nM; *K*D, BgK-K25Orn, 0.38 ± 0.06 nM). Because the *K*D values of the analog K25Orn were 20 ± 1 and 111 ± 20 nM for Kv1.1 and Kv1.3, respectively, BgK-K25Orn was 50- and 300-fold more selective for Kv1.2 than for Kv1.1 and Kv1.3.

**Contribution of the Diad Lys-25/Tyr-26 in the BgK Blocking Capacity**

In addition to Lys-25, Tyr-26 is also found to be one of the most important residues for BgK binding to Kv1.1, Kv1.2, and Kv1.3 (this study), as well as to αDTX targets in rat brain (32). Indeed, it was previously proposed that in BgK, Lys-25 and Tyr-26 constitute the two elements of a critical diad formed by a lysine and a spatially close hydrophobic residue (aromatic or aliphatic) found in all Kv1 channels blockers from sea anemone, snakes, and scorpions (32, 36). In order to evaluate the functional contribution of the diad, we synthesized the analog possessing both substitutions, K25A/Y26A. Having checked that its CD spectrum was identical to that of BgK, we examined its blocking capacity on rKv1.1, rKv1.2, and rKv1.3. The *K*D (± S.E.) values were determined from dose-response experiments. The analog K25A/Y26A had a *K*D of 157 ± 5 nM for rKv1.1, 30 ± 5 μM for rKv1.2, and 12 ± 1 μM for rKv1.3, which correspond to affinity decreases of 214 for rKv1.1, 23,000 for rKv1.2, and 1580 for rKv1.3.

**DISCUSSION**

Various peptides blocking Kv1 channels have been isolated from scorpions, snakes, and sea anemones. Although these peptides are organized on the basis of three different folds (32,
37–41), they are all likely to bind to the P-region of Kv1 channels (32, 42–46). Recent progress has characterized the sites by which these peptides bind to the vestibule of Kv1 channels (14, 16–17, 27, 32–33, 36, 47–50), and comparison of these sites revealed that all of these blockers possess at least a common diad composed of two functionally important residues: a lysine and an hydrophobic residue (32, 36). A model of the interaction of these blockers with Kv1 channels is now commonly accepted in which this critical lysine plugs the pore of the channel (14–17, 27).

However, regarding the more subtle interactions of these blockers with the different Kv1 subtypes, little is still known. In other words, we do not know how one blocker binds to different Kv1 subtypes because no extensive comparison of the binding surfaces of one blocker to different Kv1 subtypes has yet been made. In this study, we addressed this question using synthetic analogs of a sea anemone blocker, BgK (7, 30), and thus identified the residues by which it binds to Kv1.1, Kv1.2, and Kv1.3 channels. Due to structural distortions, described under “Results,” the functional consequences of substitutions at five positions (N29A, K32A, T33A, E35A, and L36A) have not been considered in the delineation of the interacting surfaces of BgK.

Our results allowed us to (i) extend the comparison of the Kv1 binding sites of structurally related or unrelated Kv1 blockers, and (ii) distinguish two ways for enhancing the selectivity of BgK.

The Binding Site of BgK to Kv1.1 and Comparison with Scorpion Kv1 Blockers—We identified five residues involved in the binding of BgK to homomeric Kv1.1 channels: Asn-19, Ser-23, Gln-24, Lys-25, and Tyr-26. These residues are located either in the Leu-17–Ser-23 loop or in the Gln-24–Ala-31 helix, forming a surface of approximately 400 Å². In Fig. 8A, BgK residues are shown in red for alanine substitutions causing affinity decreases above 20-fold (Asn-19, Lys-25, and Tyr-26) and shown in orange for affinity decreases between 5- and
20-fold (Ser-23 and Gln-24). For affinity decreases lower than 5-fold, residues are shown in green. If we observe the interaction surface formed by these residues, from a virtual axis formed by the side chain of Lys-25, the side chains of the four significant residues are oriented perpendicularly to this axis, and are all located on one side. If we consider that the side chain of Lys-25 plugs in the pore of the channel, the four other residues should interact with one side of the channel.

A recent mutagenesis analysis on noxiustoxin (NTX) (48) identified, as implicated in its binding to Kv1.1 channels expressed in Xenopus oocytes, the residues Lys-6, Thr-8, and Lys-28 and the C-terminal tripeptide Tyr-37/Asn-38/Asn-39. Lys-28, which corresponds to the functional lysine 29 of scorpion Kv1 blockers as defined by Miller (6), and Tyr-37 together constitute the functional diad of NTX. Fitting it with that of BgK (Lys-25/Tyr-26) revealed that the carbonyl of the side chains of Asn-19 (in BgK) and Asn-39 (in NTX) are separated by 6.3 Å, suggesting that they contact a common channel residue (Fig. 9). Therefore, this analysis suggests that BgK and NTX may bind to Kv1.1 channel by common (the diad and the two homologous asparagine) and different (the unrelated Ser-9, Arg-11, and Tyr-38 (NTX)) binding sites. The Binding Site of BgK to Kv1.3—Six residues were identified as being involved in the binding to Kv1.2 channel: Phe-6, Arg-12, His-13, Ser-23, Lys-25, and Tyr-26. They delineate a 540-Å² surface shown in Fig. 8B. To our knowledge, this is the first delineation of the functional site by which a peptide blocks the current through homomeric Kv1.2.

The Binding Site of BgK to Kv1.3: Comparison with Scorpion and Sea Anemone Kv1 Blockers—Eleven residues were found to interact with the homomeric Kv1.3 channel: these are Phe-6, Lys-7, His-13, Leu-17, Asn-19, Thr-22, Ser-23, Gln-24, Lys-25, Tyr-26, and Arg-27. They form a 918-Å² surface that includes both Kv1.1 and Kv1.2 binding sites. If we observe this surface in the same manner as for the Kv1.1 and Kv1.2 binding sites, Phe-6, Lys-7, Gln-24, and Arg-27 on the one hand and His-13, Leu-17, Asn-19, and Tyr-26 on the other hand form two sets of residues located on two virtual perpendicular axes, the intersection of which is materialized by Lys-25 (Fig. 8C). The three basic residues Lys-7, Arg-27, and His-13 are located at the extremities of these axes in an almost perpendicular fashion.

Elements of the Kv1.3 binding sites of ShK (50), kallotoxin (KTX) (16), and margatoxin (MgTX) (47) have been identified using mutagenesis on the blockers combined with either electrophysiological or binding experiments. The binding site of ShK comprises the three basic residues Lys-9, Arg-11, and Lys-22 and probably Tyr-23 (50), that of KTX comprises Lys-27 and probably Arg-24 and Phe-25, and that of MgTX comprises Lys-28, Met-30, Asn-31, His-39, and Tyr-37. Fitting the four diads Lys-22/Tyr-23 (ShK), Lys-25/Tyr-26 (BgK), Lys-28/Tyr-37 (MgTX), and Lys-27/Phe-25 (KTX) revealed a closed topological location of Arg-11 in ShK, His-13 in BgK and Arg-24 in KTX (Fig. 10). Similarly, and based on the 4-fold symmetry of the Kv1.3 channel, the His-39 in MgTX may also be fitted with the three previous residues if a π/2 rotation around the central Lys-27 of MgTX is done. The distance between the Cα of the lysine and the center of the imidazole or guanidinium group was estimated to be 13 ± 1 Å. Interestingly, in agreement with our comparison, it has been suggested, using quantitative methods, that Arg-24 in KTX interacts electrostatically with Asp-386 and His-404 of Kv1.3 (16) and that Arg-11 of ShK interacts with His-404 (27).

Searching for other similarities between the binding surfaces of BgK and MgTX interacting with Kv1.3, we observed that Asn-19 in BgK and Asn-31 in MgTX are separated by 9.0 and 9.4 Å from the Cα of Lys-25 and Lys-28, respectively, and located at 180° around this central lysine. Observations that residues of two blockers may be located in an homologous position after rotation of π and π/2 around the side chain of the pivotal lysine illustrate how a toxin can take advantage of the 4-fold symmetry of Kv1.3 channels.

In conclusion, the surfaces of BgK (918 Å²) and MgTX (700 Å²) that interact with Kv1.3 are much larger than the surface of BgK that binds to Kv1.1 and Kv1.2 channels. This surface seems to include in both scorpion and sea anemone blockers a central lysine plugging the pore (16, 27) assisted by an aromatic residue at 6 ± 1 Å, a polar binding site at 9 ± 1 Å, and a distal basic residue at 13 ± 1 Å. Comparison of the Different Kv1 Binding Sites of BgK—Comparison of the sites by which BgK binds to Kv1.1, Kv1.2, and Kv1.3 allowed us to classify the residues of BgK. First, three residues (Ser-23, Lys-25, and Tyr-26) contribute to the binding of BgK to the three channels. These three residues are conserved in the five identified Kv1 channels blockers from sea anemones (7–11). Interestingly, in ShK, they have also been identified as being involved in the binding to DTX-I targets in rat brain synaptosomes (34).

Besides these residues, we found some additional amino acids that are differentially implicated depending on the particular channel: Asn-19 and Gln-24 contribute to the binding of...
BgK to Kv1.1 and Kv1.3 but not to Kv1.2, whereas Phe-6 and His-13 contribute to the BgK binding to Kv1.2 and Kv1.3 but not to Kv1. Finally, the third category comprises residues implicated in the binding to only one channel: Arg-12 for binding to Kv1.2 and Lys-7, Leu-17, Thr-22, and Arg-27 for Kv1.3.

Implications for the Design of Blockers with Enhanced Selectivity toward Kv1 Subtypes—The binding sites of BgK to different Kv1 are organized around a minimal functional core formed by the diad lysine-hydrophobic residue (Lys-25/Tyr-26) also found in scorpion and snake blockers. It is assisted by a serine in BgK. The most likely role for these residues is to interact with residues common to all the different Kv1 subtypes to which these blockers bind. It has been shown, using mutant cycle-based analysis, that the lysine of the diad from the sea anemone blocker ShK (27) and of the scorpion blockers Agitoxin 2 (17) and KTX (16) interact with the conserved tyrosine of the selectivity filter of voltage-gated potassium channels (51). Furthermore, it has been shown that the hydrophobic residue of the diad of agitoxin2 (Phe-25) interacts with the conserved methionine residue from the same channel region (17). Thus, the three residues of the minimal functional core could be implicated in a conserved set of interactions that anchor the blocker in the external vestibule of the channel in a similar manner, whatever the considered channel.

However, despite this common role in the binding to different Kv1 channels, substituting these residues induced differential effects depending on the Kv1 channels subtypes considered. Thus, we have shown that substituting both Lys-25 and Tyr-26 by an alanine decreased the affinity of BgK for rKv1.1, rKv1.2, and rKv1.3 by 241, 23,000, and 1580, respectively. Furthermore, we showed that shortening the side chain of Lys-25 by a single methylene group (K25Orn) increased 3-fold the toxin affinity for rKv1.2 although decreasing its affinity for rKv1.1 and rKv1.2 by 30- and 15-fold, respectively.

Although these results are difficult to interpret, they indicate that appropriate substitutions of the residues from the conserved functional core can be used to create selective blockers of Kv1 subtypes. However, the effects of a substitution are different, depending on the blocker considered. Indeed, in BgK, substituting the critical lysine by an ornithine residue (K25Orn) affected the affinity for Kv1.1 and Kv1.3 but not for Kv1.2, thus increasing the selectivity of the protein for Kv1.2, whereas in ShK the equivalent substitution (K22Orn) had no effect on the affinity of the blocker for Kv1.3. In the same way, in BgK, substituting this lysine by a diaminopropionic residue (K25Dap) affected similarly the affinity of the protein for Kv1.1, Kv1.2, and Kv1.3, whereas in ShK, the equivalent substitution (K22Dap) significantly altered the affinity of the protein for Kv1.1 but had no effect on its affinity for Kv1.3 and Kv1.2 (27).

Another way to generate selective blockers is to substitute residues that are differentially involved in the binding to the different Kv1 subtypes. In this study, we showed that Phe-6 of BgK is important for the binding to Kv1.2 and Kv1.3 but not to Kv1. Finally, the analog BgK-F6A is at least 500-fold more potent on Kv1.1 (K_{D_{\text{F6A}}} 0.7 \text{ nM}) than on Kv1.3 (K_{D_{\text{F6A}}} 400 \text{ nM}) or on Kv1.2 (K_{D_{\text{F6A}}} 800 \text{ nM}).

In conclusion, we not only mapped the sites by which BgK binds to Kv1.1, Kv1.2, and Kv1.3 but also produced two analogs with enhanced selectivity as compared with the native protein: BgK-K25Orn, which is 50- and 300-fold more selective for Kv1.2 than for Kv1.1 and Kv1.3, respectively, and BgK-F6A, which is at least 500-fold more potent on Kv1.1 than on Kv1.2 and Kv1.3.

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