Characterization of a Novel Radiolabeled Peptide Selective for a Subpopulation of Voltage-gated Potassium Channels in Mammalian Brain*

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A number of peptides isolated from scorpion, snake, and sea anemone venoms have been characterized and shown to block Kv1 channels by binding to residues located in the external vestibule of the channel (12). Despite their limited selectivity for a given Kv1 subtype, these peptides represent unique pharmacological tools for studying the structure-function relationship of Kv1 channels and have proved to be important for: 1) defining the physiological role that channels play in native tissue, 2) purifying channels from native tissues and determining their subunit composition, and 3) developing the pharmacology of potassium channels (4, 12–20). Design of peptides with new pharmacological profiles should help to develop our understanding of both structure and function of Kv channels.

In a previous study (21), it was shown that the pharmacological profile of BgK, a 37-amino acid peptide from the sea anemone Bunodosoma granulifera (22–24), can be modified by substituting certain residues. In the present study, we report the design and characterization of two novel radioligands derived from BgK, 125I-BgK(W5Y/Y26F) and 125I-BgK(W5Y/F6A/Y26F), and its monoiodotyrosine derivative, 125I-BgK(W5Y/F6A/Y26F). The design of these two BgK analogs that have been radiolabeled with 125INa. Whereas BgK(W5Y/Y26F) and its radiolabeled derivative, 125I-BgK(W5Y/Y26F), bind to Kv1.1, Kv1.2, and Kv1.6 channels with potencies similar to those for the parent peptide, BgK, BgK(W5Y/F6A/Y26F) and its monoiodo-tyrosine derivative, 125I-BgK(W5Y/F6A/Y26F), display a distinctive and unique pharmacological profile; they bind with high affinity to homomultimeric Kv1.1 and Kv1.6 channels, but not to Kv1.2 channels. Interaction of BgK(W5Y/F6A/Y26F) with potassium channels depends on the nature of a residue in the mouth of the channel, at a position that determines channel sensitivity to external tetraethylammonium. In native brain tissue, 125I-BgK(W5Y/F6A/Y26F) binds to a population of Kv1 channels that appear to consist of at least two sensitive (Kv1.1 and/or Kv1.6) subunits, in adjacent position. Given its unique pharmacological properties, 125I-BgK(W5Y/F6A/Y26F) represents a new tool for studying subpopulations of Kv1 channels in native tissues.

Voltage-gated potassium (Kv)1 channels regulate numerous cellular processes by controlling plasma membrane potential and electrical excitability (1). The existence of a large number of pore-forming subunit genes contributes to the large diversity of potassium channels found in native tissues (2, 3). Because potassium channels are tetrameric structures (4) made up by association of four identical or closely related subunits (5–11), it is difficult to determine the subunit composition of a given channel in vivo based on the biophysical properties of the resultant proteins.

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tion site at the 3'-end. PCR products were generated by using Stratagene Pfu DNA polymerase and standard protocols for this enzyme. The PCR fragments were restriction-digestion, gel-purified, and ligated into the pCI-neo vector (Promega). The ligation reaction was transformed in XL1blue bacteria. From the resulting colonies, DNA was prepared and analyzed by restriction digest and sequencing. DNA sequences of hKv1.1, hKv1.3, and hKv1.3(H399Y) were cloned into the mammalian expression vector pcDNA3 (Invitrogen), and was kindly provided by Prof. Olaf Pongs (Zentrum für Molekulare Neurobiologie, Hamburg, Germany). 

Peptides—Native BgK, BgK analogs and dDTX were synthesized as described previously (24, 25). dDTX-K(26) was synthesized using the same procedure as for dDTX (25). Synthetic charybotoxin (CtxTX) was purchased from Latoxan (Valence, France).

Iodination of Peptides—0.5 nmol of BgK, BgK(W5Y/Y26F), or dDTX were incubated with 80 μl of 75 mM sodium phosphate, pH 7.4, containing 1 mCl of 125I (Amersham Biosciences, Inc.), in the presence of 1 unit (7.5 μg) of bovine milk lactoperoxidase (Sigma). Two additions of 10 μl of a 50,000-fold dilution of 30% hydrogen peroxide (w/w) were carried out, at 1-min intervals. At the end of the 2-min incubation period, the reaction was stopped by addition of 0.1% trifluoroacetic acid, and the reaction mixture was injected onto a C18 column (Vydac, 4.6 × 250 mm) equilibrated with 20 mM sodium acetate, 0.085% (w/v) trifluoroacetic acid in solvent A. The A (0.1% trifluoroacetic acid). A 40-min linear gradient of either 20% (B) or 25% (B) solvent B in solvent A, at a flow rate of 1 ml/min, led to the isolation of one nonradioactive and two radioactive species. Using nonradioactive iodine, these species were characterized by mass spectrometry. The first peak corresponds to native peptide, whereas the second and third peak represent the monoiodo- and diiodo-derivatives of the peptide, respectively. Monoiodinated peptides (2000 Ci/ml) were stored at 4 °C in the presence of 1 mg/ml bovine serum albumin. Higonotoxin(A19Y/Y37F) (HgTX1(A19Y/Y37F) was iodinated as described previously (18).

Heterologous Expression of Kv1 Channels in Mammalian Cells—HEK-293 or tsA-201 cells were maintained in minimal essential medium (Sigma) supplemented with 10% fetal calf serum (Sigma), 100 units/ml penicillin (Sigma), 2 mM l-glutamine (Invitrogen), 10 mg/ml streptomycin (Sigma), and 10% (w/v) fetal bovine serum in a humidified 5% CO2 incubator at 37 °C. HEK-293 or tsA-201 cells were maintained in minimal essential medium (Sigma) supplemented with 10% fetal calf serum (Sigma), 100 units/ml penicillin (Sigma), 2 mM l-glutamine (Invitrogen), 10 mg/ml streptomycin (Sigma), and 10% (w/v) fetal bovine serum in a humidified 5% CO2 incubator at 37 °C.

Data Analysis—Data from saturation experiments were analyzed according to Equation 1, where \( B_{max} \) is the amount of ligand bound at equilibrium, \( B_{max} \) the maximum receptor concentration, \( K_d \) the ligand dissociation constant, and \( L^* \) the free ligand concentration.

\[
B_{max} = \frac{B_{max} \cdot I^*}{1 + (I/C_{50})^{H}}
\]  

(1) Eq.

Competition experiments were analyzed according to the Hill equation in Equation 2 where, \( B_{max} \) is the amount of ligand bound at equilibrium, \( K_d \) the dissociation constant, and \( n_H \) the Hill coefficient.

\[
B_{max} = \frac{B_{max}}{1 + (I/C_{50})^{H}}
\]  

(2) Eq.

\[
K_d = \frac{IC_{50}(1 + (L/K_d))}{IC_{50}(1 + (L/K_d))} + (80\% \cdot B_{max})/(1 + (I/C_{50})^{H})
\]  

(3) Eq.

RESULTS

Synthesis of BgK Analogs—To develop new pharmacological tools for studying Kv1 channels, two analogs of BgK, a Kv1 channel blocker isolated from the sea anemone, B. granulifera (22, 23), have been radiolabeled and used to characterize Kv1 channels. Direct iodination of BgK leads to a peptide that lacks biological activity, as inferred from its failure to bind to rat brain synaptosomal membranes. This finding is consistent with the observation that the only Tyr residue of BgK, Tyr26, is critical for conferring high affinity interaction of the peptide to either homomultimeric or native Kv1 channels (21, 24, 30). Substitution of Phe at Tyr26 and Tyr at Trp 5 led to a peptide, BgK(W5Y/Y26F) as an inhibitor of 125I-HgTX1(A19Y/Y37F) (18), that can be radiolabeled without loss of biological activity. Moreover, a substitution that has been shown previously to modify the pharmacological profile of BgK for homomultimeric Kv1 channels (21, 30) was introduced to yield BgK(W5Y/F6A/Y26F). The two BgK analogs were radiolabeled with 125IIna and their monoiodo-derivatives used in radioligand binding studies using either heterologous expressed channels or channels present in native tissues.

Binding of BgK and Its Analogs to Homomultimeric Kv1 Channels—The ability of BgK peptides to interact with homomultimeric Kv1 channels was determined in radioligand binding studies using 125I-dDTX or 125I-HgTX1(A19Y/Y37F) (18) as radiolabeled tracers. 125I-dDTX binds to Kv1.1, Kv1.2, and Kv1.6 channels with dissociation constants (\( K_d \)) of 24 ± 9 ps (n = 4), 12 ± 2 ps (n = 3), and 36.5 ± 5 ps (n = 4), respectively. Under identical experimental conditions, 125I-HgTX1(A19Y/Y37F) binds to Kv1.3 channels with a \( K_d \) of 0.3 ± 0.04 ps (n = 3). Table I presents the results of competition experiments in which BgK analogs were evaluated for their ability to inhibit binding of these radioligands to homomultimeric Kv1.1, Kv1.2, Kv1.3, or Kv1.6 channels. BgK(W5Y/Y26F) inhibits binding of 125I-dDTX to Kv1.1, Kv1.2, and Kv1.6 channels with \( K_d \) values of 104 ± 6, 369 ± 17, and 14 ± 5 ps, respectively. These values are similar to those obtained with native BgK. Likewise, the potency of BgK(W5Y/Y26F) as an inhibitor of 125I-HgTX1(A19Y/Y37F) binding to Kv1.3 channels is not significantly different from that of native BgK. However, both BgK and BgK(W5Y/Y26F) display lower affinity for Kv1.3 channels than for the other Kv1 channels investigated (Table I).

Iodination of BgK(W5Y/Y26F) results in a peptide, 125I-BgK(W5Y/Y26F), that displays a binding capacity of 87–89% as determined in an analysis of specific binding versus protein concentration using rat brain synaptosomal mem-
membranes derived from HEK-293 or tsA-201 cells expressing either homomultimeric Kv1.1, Kv1.2, Kv1.6, or Kv1.3(H399Y) channels. ND, not determined.

**Experimental Procedures.**

A, inhibition of $^{125}$I-oDTX (*) or $^{125}$I-HgTx(A19Y/Y37F) (**) binding to membranes derived from HEK-293 or tsA-201 cells expressing either homomultimeric Kv1.1, Kv1.2, Kv1.6, or Kv1.3(H399Y) channels. B, binding of $^{125}$I-BgK(W5Y/Y26F) and $^{125}$I-BgK(W5Y/F6A/Y26F) to membranes derived from HEK-293 or tsA-201 cells expressing either homomultimeric Kv1.1, Kv1.2, Kv1.6, or Kv1.3(H399Y) channels. ND, not determined.

|       | $^{125}$I-BgK(W5Y/Y26F) | $^{125}$I-BgK(W5Y/F6A/Y26F) |
|-------|-------------------------|-----------------------------|
| $K_i$ | 35 ± 18                 | 88 ± 32                     |
|       | 77 ± 18                 | 77 ± 18                     |
|       | 4 ± 1                   | 70 ± 27                     |
|       | 40 ± 3                  | ND                          |

| $^{125}$I-BgK(W5Y/Y26F) | $^{125}$I-BgK(W5Y/F6A/Y26F) |
|-------------------------|-----------------------------|
| $K_d$                   |                             |
| 35 ± 18                 | 88 ± 32                     |
| 77 ± 18                 | 77 ± 18                     |
| 4 ± 1                   | 70 ± 27                     |
| 40 ± 3                  | ND                          |

**Table I**

 Binding of BgK, BgK analogs, ChTX, DTX-K, $^{125}$I-BgK(W5Y/Y26F), and $^{125}$I-BgK(W5Y/F6A/Y26F) to homomultimeric Kv1 channels

A, inhibition of $^{125}$I-oDTX (*) or $^{125}$I-HgTx(A19Y/Y37F) (**) binding to membranes derived from HEK-293 or tsA-201 cells expressing either homomultimeric Kv1.1, Kv1.2, Kv1.6, or Kv1.3(H399Y) channels. B, binding of $^{125}$I-BgK(W5Y/Y26F) and $^{125}$I-BgK(W5Y/F6A/Y26F) to membranes derived from HEK-293 or tsA-201 cells expressing either homomultimeric Kv1.1, Kv1.2, Kv1.6, or Kv1.3(H399Y) channels. ND, not determined.

|       | $^{125}$I-BgK(W5Y/Y26F) | $^{125}$I-BgK(W5Y/F6A/Y26F) |
|-------|-------------------------|-----------------------------|
| $K_i$ | 35 ± 18                 | 88 ± 32                     |
|       | 77 ± 18                 | 77 ± 18                     |
|       | 4 ± 1                   | 70 ± 27                     |
|       | 40 ± 3                  | ND                          |

**Fig. 1.** Binding of $^{125}$I-BgK(W5Y/F6A/Y26F) to homomultimeric hKv1.1 or hKv1.6 channels. Membranes prepared from cells expressing either homomultimeric Kv1.1 (A) or Kv1.6 (B) channels were incubated with increasing concentrations of $^{125}$I-BgK(W5Y/F6A/Y26F) in a total volume of 0.5 ml (hKv1.1) or 1 ml (hKv1.6), as indicated under “Experimental Procedures.” Non-specific binding (▽) was determined in the presence of 200 nM BgK(W5Y/F6A/Y26F) (Kv1.1) or 250 nM BgK(W5Y/Y26F) (Kv1.6). Specific binding (■) was assessed from the difference between total binding (●) and non-specific binding (▽). Kv1.1 ($K_i = 68\, \mu M, B_{max} = 6\, \mu M$), Kv1.6 ($K_i = 42\, \mu M, B_{max} = 3.1\, \mu M$).

branes (data not shown). $^{125}$I-BgK(W5Y/Y26F) binds with picomolar affinities to Kv1.1 (K_d = 35 ± 18 pM (n = 3)), Kv1.2 (K_d = 77 ± 18 pM (n = 3)), and Kv1.6 channels (K_d = 4 ± 1 pM (n = 3)) (Table I), but no specific binding was detected with homomultimeric Kv1.3 channels.

Consistent with previous results using BgK(F6A) (21), BgK(W5Y/F6A/Y26F) displays much lower affinity for Kv1.2 and Kv1.3 channels than BgK(W5Y/Y26F). The affinity of BgK(W5Y/F6A/Y26F) for Kv1.6 and Kv1.1 channels is, however, not much different from that of BgK(W5Y/Y26F). As a consequence, the potency of BgK(W5Y/F6A/Y26F) for either Kv1.1 or Kv1.6 channels (K_d values of 215 ± 11 and 162 ± 74 pM, respectively) is 3 orders of magnitude higher than for Kv1.2 and Kv1.3 channels (289,200 ± 11,200 and 134,400 ± 200 pM, respectively).

Iodination of BgK(W5Y/F6A/Y26F) yields a peptide, $^{125}$I-BgK(W5Y/F6A/Y26F), that displays a binding capacity of 87% as determined using the same procedure described above for $^{125}$I-BgK(W5Y/Y26F) (data not shown). $^{125}$I-BgK(W5Y/F6A/Y26F) binds to Kv1.1 and Kv1.6 channels with K_d values of 88 ± 32 pM (n = 3), or 70 ± 27 pM (n = 3), respectively (Fig. 1, Table I), but no specific binding of the ligand can be detected with either Kv1.2 or Kv1.3 channels.

Binding of BgK and Its Analogs to Kv1.3(H399Y)—It has recently been shown that substitution of Ala at position 6 alters the affinity of BgK for a Kv1.1 channel in which Tyr is replaced by the corresponding residue present in Kv1.3 (30). These data suggest that the nature of the residue at that position could be a major determinant in the interaction of BgK(W5Y/F6A/Y26F) with Kv1 channels. To examine this in further detail, we determined the ability of BgK and its analogs to inhibit $^{125}$I-BgK(W5Y/F6A/Y26F) binding to a Kv1.3 mutant, Kv1.3(H399Y), in which His was replaced by the corresponding residue, Tyr, present in Kv1.1. Results of these experiments are presented in Table I and Fig. 2 (A and B). Modification of this single residue in Kv1.3 is sufficient for enhancing the affinity of both BgK and BgK(W5Y/Y26F) by 30–40-fold (K_d values of 29.5 ± 1.9 and 44 ± 4 pM, respectively). These values are similar to those of Kv1.1 channels (Table I). The difference in affinity between BgK(W5Y/F6A/Y26F) and BgK(W5Y/Y26F) for Kv1.3(H399Y) is 3–4-fold (K_d values of 171 ± 5 and 44 ± 4 pM, respectively), whereas it is 75-fold for Kv1.3 channels (289,200 ± 11,200 and 134,400 ± 200 pM, respectively).
FIG. 2. Binding of BgK and BgK analogs to Kv1.3(H399Y). Membranes prepared from either HEK-293 cells stably transfected with hKv1.3 (A) or tsA-201 cells transiently transfected with Kv1.3(H399Y) (B) were incubated with 0.5 pM $^{125}$I-HgTX1(A19Y/Y37F) in the absence, or presence of increasing concentrations of BgK (■), BgK(W5Y/Y26F) (♦), or BgK(W5Y/F6A/Y26F) (◇), for 20 h at room temperature. Other experimental conditions are described under “Experimental Procedures.” Inhibition of binding was assessed relative to an untreated control.

C, double-mutant cycle analysis for the substitutions Phe6 to Ala in BgK, and His399 to Tyr in Kv1.3. $\Delta \Delta G$ was calculated from the equation $\Delta \Delta G = RT \ln K_D$, where $K_D = [K_D(BgK(W5Y/Y26F) - Kv1.3)]/[K_D(BgK(W5Y/F6A/Y26F) - Kv1.3)]/[K_D(BgK(W5Y/Y26F) - Kv1.3)], K_D(BgK(W5Y/Y26F) - Kv1.3)].

D, binding of $^{125}$I-BgK(W5Y/Y26F) to Kv1.3(H399Y). Membranes derived from tsA-201 cells transiently transfected with Kv1.3(H399Y) were incubated with increasing concentrations of $^{125}$I-BgK(W5Y/Y26F) in a total volume of 0.8 ml. Nonspecific binding (◇) was determined in the presence of 200 nM BgK(W5Y/Y26F). Specific binding (♦) was assessed from the difference between total binding (●) and nonspecific binding (◇). $K_D = 41$ pm, $B_{max} = 2$ pm.

FIG. 3. Binding of $^{125}$I-BgK(W5Y/Y26F) and $^{125}$I-BgK(W5Y/F6A/Y26F) to rat brain synaptosomal membranes. Rat brain membranes were incubated with increasing concentrations of either $^{125}$I-BgK(W5Y/Y26F), in a total volume of 3 ml (0.25 µg of protein/ml) (A) or $^{125}$I-BgK(W5Y/F6A/Y26F) in 1 ml (15.2 µg of protein/ml) (B). Nonspecific binding (◇) was determined in the presence of 100 nM BgK(W5Y/Y26F) (A) or 200 nM BgK(W5Y/F6A/Y26F) (B). Specific binding (●) was assessed from the difference between total binding (●) and nonspecific binding (◇). $^{125}$I-BgK(W5Y/Y26F); $K_D = 4.85$ pm, $B_{max} = 0.73$ pm (2.9 pmol/mg protein). $^{125}$I-BgK(W5Y/F6A/Y26F); $K_D = 74$ pm, $B_{max} = 6.9$ pm (0.46 pmol/mg protein).
are not independent; the effect of F6A substitution in BgK (W5Y/Y26F) depends on the nature of the residue at the position that determines sensitivity of the channel to external tetraethylammonium. These results are consistent with data that indicate that the affinity of BgK(W5Y/F6A/Y26F) for Kv1.6, which also possesses a Tyr residue at the equivalent position, is only 10-fold lower than that of BgK(W5Y/Y26F), but is much more reduced for Kv1.2, which possesses a valine residue at that position (Table I).

In contrast to results obtained with Kv1.3 channels, $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ binds to Kv1.3(H399Y) with a $K_i$ of 40 ± 3 pm (n = 4) (Fig. 2D, Table I). The affinity of $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ for Kv1.3(H399Y) could not be accurately measured because of high levels of nonspecific binding.

**Binding of BgK Analogs to Heteromeric Channels**—Given that BgK(W5Y/Y26F) and BgK(W5Y/F6A/Y26F) differ in their pharmacological profile toward homomultimeric Kv1 channel subtypes, we then evaluated the effects of these peptides on heteromeric channels present in rat brain synaptic membranes. $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ binds to a single class of sites with a $K_i$ of 4.5 ± 1 pm and a $B_m$ of 2.7 ± 0.3 pmol/mg of protein (n = 4) (Fig. 3A). Using the same membrane preparation, and under the same experimental conditions, $^{125}\text{I}-\text{dDTX}$ binds to a single class of sites (B$_{max}$ value of 2.6 ± 0.7 pmol/mg of protein) with a $K_i$ of 3.5 ± 1.7 pm (n = 5), a value virtually identical to that published previously (25). Binding of both $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ and $^{125}\text{I}-\text{dDTX}$ to brain membranes is sensitive to inhibition by dDTX, BgK, BgK(W5Y/Y26F), and BgK(W5Y/F6A/Y26F), and $K_i$ values for these peptides are similar regardless of the radioligand used (Table II). These data indicate that the concentration of binding sites labeled by both, $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ and $^{125}\text{I}-\text{dDTX}$, as well as the affinities of these sites for dDTX, BgK, BgK(W5Y/Y26F), and BgK(W5Y/F6A/Y26F) are similar and suggest that both radioligands bind to the same receptor population in rat brain membranes. DTX-K inhibits binding of $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ (Fig. 4A) and $^{125}\text{I}-\text{dDTX}$ (data not shown) with Hill coefficients lower than 1, indicating that this peptide distinguishes between several subpopulations of these receptors. These data are consistent with previous observations suggesting that $^{125}\text{I}-\text{dDTX}$ receptors do not form a homogeneous population (14).

The potency of BgK(W5Y/F6A/Y26F) as inhibitor of $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ binding to brain membranes (Fig. 4C) ($K_i = 2.256 ± 970$ pm) is lower than its affinity for BgK(W5Y/F6A/Y26F)-sensitive subunits, Kv1.1 and Kv1.6 (Table II). These data indicate that the concentration of binding sites labeled by BgK(W5Y/Y26F) in rat brain and to 44 ± 14% of the heteromeric Kv1.1–Kv1.2 channels recognized by $^{125}\text{I}-\text{dDTX}$ and $^{125}\text{I}-\text{HgTXI(A19Y/Y37F)}$ contain at least one Kv1.2 subunit (14, 18).

In rat brain membranes, $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ binds to a single class of sites with a $K_i$ of 71 ± 25 pm and a $B_m$ of 0.49 ± 0.12 pmol/mg of protein (n = 4) (Fig. 3B). Thus, whereas the number of receptors labeled by $^{125}\text{I}-\text{dDTX}$ and $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ appears to be the same (2.6 ± 0.7 pmol/mg of protein), the total number of $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ receptors is much lower. BgK(W5Y/F6A/Y26F) inhibits binding of $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ to brain membranes with a $K_i$ of 229 ± 42 pm. Thus, BgK(W5Y/F6A/Y26F) displays 10-fold higher affinity as an inhibitor of $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ than $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ binding, which suggests that the two radioligands do not bind to the same receptor population. However, because binding of both radioligands to brain membranes is inhibited by both BgK and BgK(W5Y/Y26F) with similar potencies (Table II), the data are more consistent with the idea that $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ receptors constitute a subpopulation of those labeled by $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$. These findings would have predicted biphasic curves for inhibition of $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ binding to rat brain synaptosomal membranes by BgK(W5Y/F6A/Y26F); one component representing ~20% of $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ binding would display a $K_i$ of ~229 pm, and the second component a much higher $K_i$. In Fig. 4D, data corresponding to BgK(W5Y/F6A/Y26F) inhibition of $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ binding to rat brain membranes were analyzed with either a one-site model or a two-site model, in which BgK(W5Y/F6A/Y26F) inhibits 20% of the sites with a $K_i$ of 229 pm. Fitting the data to a single-site model yields a $K_i$ of 1,405 ± 98 pm, whereas the two-site model predicts a $K_i$ of 2,110 ± 175 pm for the low affinity site. However, the two curves in Fig. 4C can almost be superimposed, indicating that it is not possible to determine from such competition experi-

### Table II

| A.                          | Rat brain Kv1 channels | Heteromeric Kv1.1–Kv1.2 channels |
|-----------------------------|------------------------|----------------------------------|
| $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ | 4.5 ± 1                | 8.6 ± 1.8                        |
| $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ | 71 ± 25$^a$           | 225 ± 110$^a$                    |

| B.                          | Rat brain Kv1 channels | Heteromeric Kv1.1–Kv1.2 channels |
|-----------------------------|------------------------|----------------------------------|
| $^{125}\text{I}-\text{BgK(W5Y/Y26F)}$ |                       |                                  |
| $^{125}\text{I}-\text{BgK(W5Y/F6A/Y26F)}$ |                       |                                  |

$^a$ Data were fitted using the Hill equation for one-site model.

$^b$ Data were fitted using a two-site model in which the high affinity represents 20% of the total sites and has a $K_i$ of 229 pm (see text and Fig. 4C).
ments whether or not BgK(W5Y/F6A/Y26F) distinguishes sub-populations of 125I-BgK(W5Y/Y26F) receptors. These findings illustrate the importance of using 125I-BgK(W5Y/F6A/Y26F), instead of its unlabeled analog, in binding studies. Although 125I-BgK(W5Y/Y26F) binds to a subpopulation of brain 125I-BgK(W5Y/Y26F) receptors, these sites do not appear to form an homogeneous population, as indicated by the fact that DTX-K inhibits binding of 125I-BgK(W5Y/F6A/Y26F) with Hill coefficient lower than 1 (Fig. 4B).

Given that the BgK(W5Y/F6A/Y26F)-insensitive subunit, Kv1.2, is the prevalent Kv1 subunit in brain and that Kv1.1 has been shown to be associated with Kv1.2 in this, binding of 125I-BgK(W5Y/Y26F) and 125I-BgK(W5Y/F6A/Y26F) to channels expressed in HEK-293 cells after transient transfection with a Kv1.1-Kv1.2 dimer channel tandem was also characterized. Several lines of evidence indicate that the expressed channels are likely to contain both types of subunits. For instance, 125I-HgTX1(A19Y/Y37F), which displays equivalent affinities for both Kv1.1 and Kv1.2 channels (18), binds with high affinity to these channels, and solubilized 125I-HgTX1(A19Y/Y37F) receptors can be 100% immunoprecipitated by either specific Kv1.1 or Kv1.2 antibodies (data not shown). In addition, 125I-HgTX1(A19Y/Y37F) binding to these channels is completely inhibited by increasing concentrations of peptides that bind Kv1.2 but not Kv1.1, such as charybdotoxin (ChTX) (32) (Table I), or by peptides that bind Kv1.1 but not Kv1.2 such as DTX-K or ShK (33, 34) (Table I) (data not shown). Finally, both Kv1.1 and Kv1.2 antibodies recognize a band of /H11011130,000 in Western blots that corresponds to the calculated mass of the Kv1.1-Kv1.2 dimer protein (data not shown).

125I-BgK(W5Y/F6A/Y26F), a New Radioligand for Kv1 Channels

**FIG. 4.** Binding of 125I-BgK(W5Y/Y26F) and 125I-BgK(W5Y/F6A/Y26F) to rat brain synaptosomal membranes: effects of DTX-K, BgK(W5Y/Y26F), and BgK(W5Y/F6A/Y26F). A and B, rat brain membranes were incubated with either 12 pM 125I-BgK(W5Y/Y26F) (A) or 9 pM 125I-BgK(W5Y/F6A/Y26F) (B) in the absence or presence of increasing concentrations of either BgK(W5Y/Y26F) or BgK(W5Y/F6A/Y26F) (●) or DTX-K (■). Data were analyzed using the Hill equation as described under "Experimental Procedures." Hill coefficients were as follows: A, BgK(W5Y/Y26F) = 0.90 and DTX-K = 0.44; B, BgK(W5Y/F6A/Y26F) = 0.97 and DTX-K = 0.37. C, rat brain membranes (8 ml) were incubated with 0.77 pM 125I-BgK(W5Y/Y26F) in the absence or presence of increasing concentrations of BgK(W5Y/Y26F) (●) or BgK(W5Y/F6A/Y26F) (■). Data were analyzed as described under "Experimental Procedures" using the Hill equation for a one-binding site model (solid lines). In addition, data with BgK(W5Y/F6A/Y26F) were analyzed using a two-binding site model (dotted line), in which the high affinity site represents 20% of the sites and has a $K_i$ of 229 pM (see "Experimental Procedures").
channels recognized by $^{125}$I-BgK(W5Y/F6A/Y26F) are likely to contain both BgK(W5Y/F6A/Y26F)-sensitive Kv1.1 and BgK(W5Y/F6A/Y26F)-insensitive Kv1.2 subunits because ChTX completely inhibits binding with an affinity, ($K_i$) of $94 \text{ pM}$, that is 3 orders of magnitude higher than that for homomultimeric Kv1.1 channels ($K_i$) of $101,150 \text{ pM}$, but close to that of Kv1.2 channels (Fig. 5D, Tables I and II).

$^{125}$I-BgK(W5Y/F6A/Y26F) is 1 order of magnitude more potent as inhibitor of $^{125}$I-BgK(W5Y/F6A/Y26F), ($K_i$) of $289 \text{ pM}$, than of $^{125}$I-BgK(W5Y/Y26F) binding, ($K_i$) of $289 \text{ pM}$, whereas BgK(W5Y/Y26F) inhibits with similar potencies binding of both radioligands ($K_i$) of $9$ and $32 \text{ pM}$, respectively (Fig. 5, C and D; Table II). These data resemble the situation found with native brain membranes and suggest that $^{125}$I-BgK(W5Y/F6A/Y26F) binds to a subpopulation of the Kv1.1-Kv1.2 channels recognized by $^{125}$I-BgK(W5Y/Y26F). The geometry of the resulting channels formed by association of two dimer molecules must be a determinant for high affinity $^{125}$I-BgK(W5Y/F6A/Y26F) binding (see “Discussion”).

**DISCUSSION**

This study concerns the development of new pharmacological tools for studying the composition of potassium channels expressed in native tissues. Two analogs, derived from BgK, a high affinity blocker of certain Kv1 channels (22–24), have been characterized in radioligand binding studies using homomultimeric Kv1 channels expressed in mammalian cells. Furthermore, the two BgK analogs were radiolabeled and used to study the molecular composition of native Kv1 channels present in brain tissue.

To obtain a radiolabeled derivative of BgK that retains biological activity, two substitutions, Trp5 to Tyr and Tyr26 to Phe, had to be introduced in the molecule to: 1) prevent conformational changes caused by the incorporation of iodine in the functionally important residue Tyr26 (21, 24, 30), and 2) provide a iodination site at a position, Trp5, that is not critical for the interaction of the peptide with its target channels (21, 24, 30). Both BgK(W5Y/Y26F) and its radiolabeled derivative bind to Kv1 channels with similar affinities as BgK.

**FIG. 5.** Binding of $^{125}$I-BgK(W5Y/Y26F) and $^{125}$I-BgK(W5Y/F6A/Y26F) to Kv1.1-Kv1.2 channels. Membranes prepared from HEK-293 cells transiently transfected with the Kv1.1-Kv1.2 dimer tandem were incubated with increasing concentrations of $^{125}$I-BgK(W5Y/Y26F) (A) or $^{125}$I-BgK(W5Y/F6A/Y26F) (B) in a total volume of 3 ml (A) or 0.4 ml (B). Nonspecific binding (■) was determined in the presence of 100 nM BgK(W5Y/Y26F) (A) or 500 nM BgK(W5Y/F6A/Y26F) (B). Specific binding (▲) was assessed from the difference between total binding (●) and nonspecific binding (■). A, $K_i$ of $9.2 \text{ pM}$, $B_{max}$ of $1.16 \text{ pm}$; B, $K_i$ of $242 \text{ pM}$, $B_{max}$ of $18 \text{ pm}$. C and D, membranes (0.5 or 0.25 ml) were incubated with 18 pm $^{125}$I-BgK(W5Y/Y26F) (C) or 50 pm $^{125}$I-BgK(W5Y/F6A/Y26F) (D) in the absence or presence of increasing concentrations of BgK(W5Y/Y26F) (●), BgK(W5Y/F6A/Y26F) (▲), or ChTX (■). Hill coefficients were as follows: C, BgK(W5Y/Y26F) = 1.08, BgK(W5Y/F6A/Y26F) = 0.90, and ChTX = 0.74; D, BgK(W5Y/Y26F) = 0.85, BgK(W5Y/F6A/Y26F) = 0.93, and ChTX = 0.78.
It has been shown previously that the selectivity of BgK for Kv1 channels can be altered by substitution of certain residues in the peptide (21). Among them, Phe to Ala substitution is of interest because it reduces the affinity of BgK for homomultimeric Kv1.2 and Kv1.3 channels, while having no effect on homomultimeric Kv1.1 channels (21). In this study, we show that this effect depends on the nature of a residue in the mouth of the channel, at a position that determines channel sensitivity to external tetraethylammonium. The substitution F6A does not affect the affinity of BgK for channels bearing a tyrosine at that position, such as Kv1.1, Kv1.6, or Kv1.3 (H399Y), but it significantly decreases affinity for channels containing a His residue such as Kv1.3 or a valine residue such as Kv1.2. Consistent with this, BgK(W5Y/F6A/Y26F) and BgK(W5Y/Y26F) display similar affinities for Kv1.1 and Kv1.6 channels, but the affinity of BgK(W5Y/F6A/Y26F) for Kv1.2 and Kv1.3 channels is much lower than that of BgK(W5Y/Y26F).

In rat brain membranes, 125I-BgK(W5Y/F6A/Y26F) binds to a subpopulation of 125I-BgK(W5Y/Y26F) receptors. Because these two peptides bind with different affinities to homomultimeric Kv1.2 channels (Table I), their receptors in brain membranes are likely to differ in the amount of Kv1.2 subunits. In mammalian brain, Kv1.2 is the most abundant Kv1 subunit (10), and it has been shown to be present in all the receptors labeled by two other Kv1 ligands, such as 125I-a-DTX and 125I-HgTX3(A19Y/Y37F) (14, 18). Moreover, association of two Kv1.1-Kv1.2 subunits is important for conferring high affinity to a subpopulation of 125I-BgK(W5Y/Y26F) receptors because BgK(W5Y/F6A/Y26F) could bind with low affinity to these receptors (see Table I). In this study, we show that the geometry of the tetrameric channels resulting from the association of two Kv1.1-Kv1.2 subunits is important for conferring high affinity 125I-BgK(W5Y/F6A/Y26F) binding. Recently, a model describing the interaction of BgK with homomultimeric Kv1 channels has been proposed (30), using distance constraints derived from double-mutant cycle analysis, and the structure of the bacterial potassium channel KcsA as a template (35). In this model, BgK lies on two adjacent subunits. Thus, BgK(W5Y/F6A/Y26F) could bind with low affinity to channels containing two sensitive Kv1.1 subunits in a diagonal orientation and with high affinity to those containing adjacent Kv1.1 subunits. Expression of constructs containing the four subunits in a tandem, so that the geometry of the tetramer is fixed, will be necessary to validate this hypothesis.

Modification of BgK has led to a new radioligand, 125I-BgK(W5Y/F6A/Y26F), that recognizes a restricted population of Kv1 channels in mammalian brain. All results, taken together, suggest that native 125I-BgK(W5Y/F6A/Y26F) receptors are formed by the association of at least two toxin-sensitive Kv1.1 and/or Kv1.6 subunits in adjacent position. Therefore, 125I-BgK(W5Y/F6A/Y26F) represents a novel tool for studying structure-function relationships of native potassium channels.
Characterization of a Novel Radiolabeled Peptide Selective for a Subpopulation of Voltage-gated Potassium Channels in Mammalian Brain
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