A $K_v$2.1 gating modifier binding assay suitable for high throughput screening

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Keywords: $K_v$2.1, gating modifier, tarantula toxin, voltage-gated, potassium channel, binding assay, high throughput screen, automated electrophysiology

Abbreviations: $K_v$ channel, voltage-gated potassium channel; GxTX-1E, guangitoxin-1E; ChTX, charybdotoxin; IbTX, iberiotoxin; ScTx1, stromatoxin; MS, mass spectroscopy; CHO, chinese hamster ovary; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PPC, population patch clamp

Gating modifier peptides alter gating of voltage-gated potassium ($K_v$) channels by binding to the voltage sensor paddle and changing the energetics of channel opening. Since the voltage sensor paddle is a modular motif with low sequence similarity across families, targeting of this region should yield highly specific channel modifiers. To test this idea, we developed a binding assay with the $K_v$2.1 gating modifier, GxTX-1E. Monoiodotyrosine-GxTX-1E ($^{125}\text{I}-\text{GxTX-1E}$) binds with high affinity ($IC_{50} = 4 \text{ nM}$) to CHO cells stably expressing h$K_v$2.1 channels, but not to CHO cells expressing Maxi-K channels. Binding of $^{125}\text{I}$-GxTX-1E to K2.1 channels is inhibited by another $K_v$2.1 gating modifier, stromatoxin ($IC_{50} = 30 \text{ nM}$), but is not affected by iberiotoxin or charybdotoxin, pore blocking peptides of other types of potassium channels, or by ProTx-II, a selective gating modifier peptide of the voltage-gated sodium channel Na1.7. Specific $^{125}\text{I}$-GxTX-1E binding is not detectable when CHO-K2.1 cells are placed in high external potassium, suggesting that depolarization favors dissociation of the peptide. The binding assay was adapted to a 384-well format, allowing high throughput screening of large compound libraries. Interestingly, we discovered that compounds related to PAC, a di-substituted cyclohexyl $K_v$ channel blocker, displayed inhibitory binding activity. These data establish the feasibility of screening large libraries of compounds in an assay that monitors the displacement of a gating modifier from the channel's voltage sensor. Future screens using this approach will ultimately test whether the voltage sensor of $K_v$ channels can be selectively targeted by small molecules to modify channel function.

Introduction

Voltage-gated potassium ($K_v$) channels regulate many fundamental physiological processes, including neurotransmitter release, muscle contractility and hormone secretion. Not surprisingly, molecular defects in $K_v$ channels can lead to inherited human diseases and pharmacological modulation of $K_v$ channel function has the potential of providing therapeutic benefit. Unwanted inhibitory activity on certain $K_v$ channels, such as the cardiac delayed rectifier channel hERG, can pose a significant risk to the safety profile of drugs. The development of therapeutic agents targeting $K_v$ channels has been slow, with a very limited number of compounds undergoing clinical evaluation. This has been due, in part, to the uncertainty in establishing the exact composition of the channel in the target tissue, and to the fact that identifying compounds that selectively target a particular $K_v$ channel is difficult.

The opening and closing of $K_v$ channels is steeply dependent on the voltage gradient across the membrane. The precision of this mechanism is exquisite, with the open probability varying several orders of magnitude over the physiological range of membrane potentials. Each subunit of a voltage-gated potassium ($K_v$) channel possesses six transmembrane segments (S1–S6). Neutralization of the positive charges in S4 provided the first indication that this region is important in voltage-sensing and contributes significantly to the charge movement during gating. Structural studies have shown that the S3b and S4 helices contain a conserved helix-turn-helix motif, termed the voltage-sensor paddle. Although the mechanism of movement of the gating charge is still very much in debate, there is wide acceptance that this region is involved in voltage sensing.

The venoms of poisonous invertebrates have been a rich source of peptidyl $K_v$ channel inhibitors. Two types of inhibitory peptides can be distinguished based on their molecular
mechanism of action. One such type, typified by the scorpion toxin charybdotoxin (ChTX), inhibits Kᵥ channels by binding to the outer vestibule and blocking ion conduction through physical occlusion of the pore.26,27 Tarantulas are a rich source of peptides that modify the gating of Kᵥ channels.28 These so-called gating modifier peptides shift Kᵥ channel opening to more positive potentials29 by stabilizing the resting conformation of the voltage sensor.30 Not surprisingly, the S3b helix in the voltage-sensor paddle has been implicated in the binding of these peptides.31-33 Interestingly, these peptides appear to partition into lipid membranes.34-37 We recently identified and characterized a potent gating modifier peptide of Kᵥ 2 channels from the venom of the tarantula Plesiophrictus guangxicus.38 This peptide, guan-gitoxin-1E (GxTX-1E), can be made synthetically, enabling further characterization of the channel-peptide interaction.

The voltage sensor paddle of Kᵥ channels is a highly mobile and modular motif attached to the pore region,39,40 which makes it an attractive pharmacological target for identifying distinct channel modulators. In voltage-gated sodium (Naᵥ) channels, each of the four paddle motifs has a unique sequence and can impart distinct functional and pharmacological properties.41 In contrast, in a homomeric Kᵥ channel, the four voltage sensors are equivalent and thus provide four identical binding sites. We reasoned that targeting the voltage sensor paddle of Kᵥ channels should yield novel probes for this class of channels. Toward this goal, we report here a cell-based binding assay for hKᵥ2.1 channels using monoiiodotyrosine-GxTX-1E (125I-GxTX-1E) and demonstrate the utility of the assay in a high-throughput screen of small molecule libraries.

Results

Iodination of GxTX-1E. GxTX-1E was reacted with 125I-Na, under the conditions described in the Materials & Methods and resulting products were separated by C₁₈ reverse phase chromatography (Fig. 1A). To confirm the identity of these peptides, GxTX-1E was iodinated under identical conditions with 127I-Na and the resulting peptides were characterized by electrospray mass spectroscopy (MS) (Fig. 1B). The injection of 10 μl of the HPLC peak corresponding to unmodified GxTX-1E (peak 1) reveals the presence of triply (1317.7±) and quadruply (988.5±) charged ions that reconstruct to yield a molecular weight of 3950.1 Da. Injection of the HPLC peak corresponding to the mono-iodinated shoulder (peak 2) reveals the presence of triply (1360.2±) and quadruply (1020.0±) charged ions that reconstruct to yield a molecular weight of 4076.8 Da. This change in mass (126.7 Da) is consistent with the incorporation of a single iodine atom in the peptide.

In addition to the above-mentioned peaks, the iodination reaction produced two additional unexpected peaks, 3 and 4, with much shorter retention times. Mass spectrometry of peak 3 revealed the presence of triply (1523.0±) and quadruply (992.6±) charged ions that reconstruct to yield a molecular weight of 3966.2 Da, while MS of peak 4 identified triply (1364.6±) and quadruply (1024.0±) charged ions that reconstruct to yield a molecular weight of 4091.4 Da. Notably, peaks 3 and 4 are related to peaks 1 and 2 by a change in mass of 16.1 and 14.6 Da, most likely due to peptide oxidation of a tryptophan or methionine residue.

The functional activity of 127I-GxTX-1E on hKᵥ2.1 channels was determined by recording currents using IonWorks® Quattro™ automated electrophysiology.42 A robust IonWorks assay for Kᵥ2.1 channels stably expressed in CHO cells has been described previously.43 127I-GxTX-1E inhibited hKᵥ2.1 channels with an IC₅₀ of 19 nM (Fig. 2), and appears to display weaker activity than the native GxTX-1E peptide (3 nM).38 This difference in affinity between native and iodinated GxTX-1E may reflect a modest loss in potency upon iodination or may result from errors in estimating the small quantity of 127I-GxTX-1E recovered. Nevertheless, these data suggest that the iodination of GxTX-1E does not result in a major loss in the functional activity of the peptide for Kᵥ2.1 channels.

125I-GxTX-1E binding to hKᵥ2.1.CHO cells. CHO cells stably expressing hKᵥ2.1 channels were used to determine if a cell-based 125I-GxTX-1E binding assay would be feasible. This cell line expresses a high density of Kᵥ2.1 channels and extremely low levels of other voltage-gated ion channels.38,43 In normal physiological media, specific binding of 125I-GxTX-1E to hKᵥ2.1.CHO occurs in a manner that is dependent on cell density (Fig. 3A). 125I-GxTX-1E binding was not detected in a CHO cell line stably expressing high levels of Maxi-K channels (Fig. 3A), suggesting specificity in 125I-GxTX-1E-Kᵥ2.1 interaction. To further test the specificity of 125I-GxTX-1E binding to hKᵥ2.1.CHO cells, competition experiments were performed using various peptidyl ion channel inhibitors. Native GxTX-1E inhibited 125I-GxTX-1E binding to hKᵥ2.1.CHO cells with an IC₅₀ of 4.1 nM (Fig. 3B), similar to the potency of GxTX-1E at inhibiting hKᵥ2.1 channels when using modest membrane depolarization protocols.38 On average, GxTX-1E inhibited 125I-GxTX-1E binding to hKᵥ2.1.CHO cells with an IC₅₀ of 3.8 ± 0.3 nM (n = 7). The Maxi-K channel pore-blocking peptides stromatoxin (ScTx1), another Kᵥ gating modifier peptide, inhibited 125I-GxTX-1E binding to hKᵥ2.1.CHO cells when tested at concentrations up to 300 nM (Fig. 3B). Stromatoxin (ScTx1), another Kᵥ gating modifier peptide, inhibited 125I-GxTX-1E binding with an IC₅₀ of 30 nM (Fig. 3B). Taken together, these data suggest that binding of 125I-GxTX-1E to hKᵥ2.1.CHO cells is due to specific high affinity interaction between the peptide and the channel.

We next studied the kinetics of 125I-GxTX-1E binding and unbinding to hKᵥ2.1.CHO cells. Incubation of hKᵥ2.1.CHO cells with 147 pM 125I-GxTX-1E resulted in a time-dependent association of ligand that reached equilibrium in approximately 2 h (Fig. 3C). A semilogarithmic plot of these data was well described by a linear fit (Fig. 3C, inset), the slope of which yields a kₐ of 0.024 min⁻¹. At ligand concentrations about 150 pM, kₐ was estimated at 0.002 ± 0.002 min⁻¹ (n = 5). Although time-dependent association determinations were accurate and reproducible at the low ligand concentrations of ~150 pM, the calculation of kₐ from kₐ values requires knowledge of the maximum receptor density (Bₘₐₓ). However, at the concentrations of 125I-GxTX-1E required to achieve >80% occupancy, the specific to nonspecific binding ratio is greatly diminished.
**Figure 1.** Iodination of GxTX-1E. (A) Upper: Amino acid sequence of GxTX-1E. Lower: HPLC separation of reaction products. GxTX-1E was subjected to iodination using the Enzymobead method as described in Materials and Methods. The reaction mixture was loaded onto a HPLC C\(_18\) reverse-phase column equilibrated with 10 mM TFA, and elution was achieved with a linear gradient of 80% acetonitrile in 5 mM TFA (0–30% at 2 min and 30–60% at 60 min) at a flow rate of 0.5 ml/min. Material eluting from the column was monitored by measuring the absorbance at 210 nm. (B) Electrospray ionization mass spectra of eluted HPLC peaks 1–4 in positive ion mode. Peaks 1 and 2 correspond to unmodified GxTX-1E and mono-iodotyrosine-GxTX-1E respectively while peaks 3 and 4 likely correspond to the oxidative products of native GxTX-1E and mono-iodotyrosine-GxTX-1E respectively.

which precludes an accurate estimation of \(K_d\) and \(B_{max}\) values. Dissociation of \(^{125}\)I-GxTX-1E bound to hKv2.1.CHO cells was measured following rinsing of the wells with media to remove unbound ligand. Bound \(^{125}\)I-GxTX-1E followed a monoexponential decay with a time constant of 30.7 min (Fig. 3D), corresponding to a \(k_1\) of 0.033 min\(^{-1}\). On average, \(k_1\) was 0.054 ± 0.014 min\(^{-1}\) (n = 5).
When dissociation of $^{125}$I-GxTX-1E was initiated by addition of an excess of unlabeled GxTX-1E (200 nM), $^{125}$I-GxTX-1E dissociated with a $\tau = 5.0$ min (Fig. 4A), yielding a $k_1$ of 0.20 min$^{-1}$. On average, the $k_1$ estimated by excess of GxTX-1E was 0.25 ± 0.04 min$^{-1}$ (n = 7), approximately 5-fold faster (p < 0.01) than when dilution was used. One major difference between the two methods relates to the occupancy of the voltage sensor with GxTX-1E during the dissociation time course. After addition of excess unlabeled GxTX-1E, occupancy of the channels is near maximal, while with the dilution approach occupancy is small to start and steadily decreasing with time. If $K_{V_{2.1}}$ channels contribute to setting the resting membrane potential of cells used to start and steadily decreasing with time. If $K_{V_{2.1}}$ channels contribute to setting the resting membrane potential of cells used to start and steadily decreasing with time.

High throughput screening using $^{125}$I-GxTX-1E binding to $K_{V_{2.1}}$ channels. To enable a high throughput screen of $^{125}$I-GxTX-1E binding to $K_{V_{2.1}}$ channels that would identify novel channel modulators, the assay described above was converted to a 384-well format. Miniaturization required optimization of cell density, compound addition, washing steps and scintillation counting (see Materials and Methods for details). The final volume of the assay was 30 μl and compounds were added from DMSO stocks (1–10 mM) using 250 nl PocketTips®, yielding a final DMSO concentration of 0.8%. We first screened 96,233 compounds at a final concentration of 16.7 μM. The results of a typical screening plate are shown in Figure 5A. In this plate, test compounds were in compound positions 33–350 from hKV2.1.CHO cells (data not shown). Membrane vesicles from hKV2.1.CHO cells (data not shown). Membrane vesicles typically have a membrane potential near 0 mV. Thus, these data suggest that specific binding of $^{125}$I-GxTX-1E to hK$_{V_{2.1}}$.CHO cells is strongly influenced by the membrane potential of the cell which may position the voltage sensor in a favorable conformation for $^{125}$I-GxTX-1E binding to occur.

Compounds that were confirmed as active inhibitors of $^{125}$I-GxTX-1E binding were profiled by IonWorks® automated electrophysiology for functional effects on hK$_{V_{2.1}}$ channels using a voltage protocol optimized to detect use-dependent block of channels (see Methods). A total of 90,000 compounds in each well) were screened. In total, 459,000 compounds (995 plates) were screened with this assay. Although the screening window tended to decrease with cell passage number due to reduced hK$_{V_{2.1}}$ channel expression over time in culture, plate failures were very rare. For 108 plates, no additions took place. On this plate, test compounds were in compound positions 33–350. The histogram of this set is shown in Figure 5C. The number of compounds that inhibited binding greater than 50% was 303 (47%). In addition to the 96,000 compounds library, two convoluted libraries (multiple compounds in each well) were screened. In total, 459,000 compounds (959 plates) were screened with this assay. Although the screening window tended to decrease with cell passage number due to reduced hK$_{V_{2.1}}$ channel expression over time in culture, plate failures were very rare. For 108 plates, the calculated Z-factor was 0.73 ± 0.01.

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**Figure 3.** Binding of $^{125}$I-GxTX-1E to cells stably expressing hK$_{v}$.2.1. (A) Cell density dependency. CHO cells stably transfected with hK$_{v}$.2.1 (hK$_{v}$.2.1.CHO) were seeded in a 96-well plate at increasing densities and incubated with 227 pM $^{125}$I-GxTX-1E for 3 h at 37°C. Separation of bound from free ligand was carried out as indicated in Materials and Methods. Specific binding of $^{125}$I-GxTX-1E to hK$_{v}$.2.1.CHO occurs in a manner that is dependent on cell density. $^{125}$I-GxTX-1E binding was not detected in a CHO cell line stably expressing high levels of Maxi-K channels. (B) Pharmacology. Cells were incubated with approximately 100 pM $^{125}$I-GxTX-1E in the presence or absence of increasing concentrations of GxTX-1E (●), ScTX1 (○), IbTX (◇), ChTX (△), or ProTx-II (▽), for 3 h at 37°C. Inhibition of binding was assessed relative to an untreated control. Specific binding data were fit to a single-site inhibition model, yielding IC$_{50}$ values of 4.1 nM (●) and 30 nM (○). Inhibition of $^{125}$I-GxTX-1E binding by IbTX (◇), ChTX (△), and ProTx-II (▽) was not detected. (C) Association kinetics. Cells were incubated with 147 pM of $^{125}$I-GxTX-1E for indicated periods of time at 37°C. Nonspecific binding, determined in the presence of 200 nM GxTX-1E, has been subtracted from the experimental points. Inset: a semi-logarithmic representation of the pseudo-first order association reaction, where $B_0$ and $B_t$ represent ligand bound at time 0 and time t, respectively, yielded $k_2 = 2.4 \times 10^{-2}$ min$^{-1}$. A value for $k_1$ could not be calculated. (D) Dilution dissociation kinetics. After incubation with $^{125}$I-GxTX-1E, cells were rinsed twice with growth medium then incubated in growth medium for different periods of time at 37°C. $^{125}$I-GxTX-1E dissociation followed mono-exponential kinetics, indicative of a first-order reaction with a time constant of 30.7 min and $k_1 = 3.3 \times 10^{-2}$ min$^{-1}$. Inset: a semi-logarithmic representation of the pseudo-first order reaction, where $B_0$ and $B_t$ represent ligand bound at time 0 and time t, respectively, yielded $k_1 = 3.3 \times 10^{-2}$ min$^{-1}$.

$^{125}$I-GxTX-1E binding screen actives. Investigation of the compounds active in the binding assay revealed several compounds structurally related to the di-substituted cyclohexyl compound, PAC. PAC blocks K$_{v}$.3.3 channels by accelerating channel closing during sustained depolarizations but also inhibits K$_{v}$.2.1 channels, albeit with weaker potency compared to K$_{v}$.1.3. PAC itself was not in any of the libraries screened and hence was not identified. Nine compounds related to PAC that were active in the $^{125}$I-GxTX-1E binding assay, as well as PAC itself, were chosen for further evaluation. This set of ten compounds was subjected to evaluation in 10-point titrations in the $^{125}$I-GxTX-1E binding assay and by automated electrophysiology on K$_{v}$.2.1 and K$_{v}$.1.2 channels. The data are presented in Table 1. Interestingly, there does not appear to be a strict correlation between functional K$_{v}$.2.1 blocking activity and the ability to displace $^{125}$I-GxTX-1E binding to K$_{v}$.2.1 channels. This lack of correlation is quite clear in the representative titrations shown in Figure 6. Compound A and Compound B are quite active in the $^{125}$I-GxTX-1E binding assay whereas PAC is not (Fig. 6A). In contrast, Compound A and PAC are much more active as functional K$_{v}$.2.1 blockers compared to Compound B (Fig. 6B).

Membrane potential measurements. Given that high external potassium and excess GxTX-1E increase K$_{v}$.2.1 channel activity, we reasoned that if compounds depolarized hK$_{v}$.2.1.CHO cells, either by blocking K$_{v}$.2.1 channels or by other mechanisms, such compounds might appear as active in the $^{125}$I-GxTX-1E binding assay. To test this idea, we measured the membrane potential of hK$_{v}$.2.1.CHO cells by conventional electrophysiology. Cells that expressed little K$_{v}$.2.1 current had resting potentials near 0 mV (data not shown). Cells with robust K$_{v}$.2.1 expression had a mean resting membrane potential of
The present data supports the view that the specific binding observed in our studies is due to direct interaction between the peptide and the KV2.1 channel. First, 125I-GxTX-1E binding is observed with KV2.1 expressing cells, but not with cells expressing high levels of Maxi-K channels. Second, binding is inhibited by the addition of unlabeled GxTX-1E and by another KV2 gating modifier, ScTx1, with IC50s similar to those observed for channel modification by electrophysiology. Third, other peptide ion channel inhibitors that do not target KV2 channels, including the sodium channel gating modifier ProTx-II, do not affect binding of 125I-GxTX-1E to hKV2.1.CHO cells.

Gating modifier peptides are known to partition into lipid membranes. For VSTX1, a gating modifier that inhibits the bacterial Kc channel, KvAP, lipid partitioning is thought to account for the difference between the Kc and KV2.1 channel. First, 125I-GxTX-1E binding is observed with KV2.1 expressing cells, but not with cells expressing high levels of Maxi-K channels. Second, binding is inhibited by the addition of unlabeled GxTX-1E and by another KV2 gating modifier, ScTx1, with IC50s similar to those observed for channel modification by electrophysiology. Third, other peptide ion channel inhibitors that do not target KV2 channels, including the sodium channel gating modifier ProTx-II, do not affect binding of 125I-GxTX-1E to hKV2.1.CHO cells.

**Discussion**

In this study we describe a cell-based binding assay for 125I-GxTX-1E and use the assay to execute the first high-throughput screen with a KV2 channel gating modifier peptide as a probe. The present data supports the view that the specific binding observed in our studies is due to direct interaction between the peptide and the KV2.1 channel. First, 125I-GxTX-1E binding is observed with KV2.1 expressing cells, but not with cells expressing high levels of Maxi-K channels. Second, binding is inhibited by the addition of unlabeled GxTX-1E and by another KV2 gating modifier, ScTx1, with IC50s similar to those observed for channel modification by electrophysiology. Third, other peptide ion channel inhibitors that do not target KV2 channels, including the sodium channel gating modifier ProTx-II, do not affect binding of 125I-GxTX-1E to hKV2.1.CHO cells.

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inhibitory action of the peptide on these channels.45 A systematic study with hanatoxin and SGTx1, two KV2 channel gating modifiers, revealed that these peptides do partition into membrane under physiological conditions but the partitioning is not sufficient to explain modification of channel gating, implying that a direct interaction with the channel is required.35,37 For GxTX-1E, the relative importance of membrane partitioning in contributing to the low nanomolar IC50 for KV2.1 modification is unknown. Nevertheless, the present data with 125I-GxTX-1E suggests that the observed binding to cells is dependent on the presence of KV2.1 channels, suggesting a direct gating modifier-channel interaction.

As illustrated in Figure 1A, the amount of 125I-GxTX-1E material that can be recovered from the iodination conditions is reproducibly small. This result could be due to lack of accessibility of the Tyr residue to solvent. In addition, a large amount of GxTX-1E is oxidized during the iodination reaction (peak 3, Fig. 1A). Whether Trp or Met residues are responsible for this modification remains to be determined. In order to improve the yield of biologically active 125I-GxTX-1E, it will be necessary to

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**Figure 5.** High throughput screening using 125I-GxTX-1E to hK,2.1 channels. (A) Typical screening plate. Cells were incubated with approximately 230 pM 125I-GxTX-1E in the presence or absence of 16.7 μM test compounds for 3 h at 37°C. Nonspecific binding was determined in the presence of 400 nM GxTX-1E. Data are shown as a scatter plot and expressed relative to an untreated control. (B) Screening results. A histogram representing screening results from a 96,233 compound library. A compound was considered active if it displayed 80% or greater inhibition at a test concentration of 16.7 μM. (C) Screening active follow-up. A histogram representing the confirmation rate for 642 compounds chosen as active from the initial screen. 303 compounds (47%) were considered to re-confirm by displaying 50% or greater inhibition at a test concentration of 27 μM. (D) Automated electrophysiology analysis of binding actives. A total of 690 confirmed actives identified from all screening sets were tested at 3 μM for functional inhibition of hK,2.1 channels using IonWorks® automated electrophysiology as described in the Materials and Methods. A histogram of the number of compounds versus percent inhibition of hK,2.1 current is shown.

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**Table 1.** Activity of PAC series compounds in 125I-GxTX-1E binding and in functional block of hK,2.1 and hK,1.2 channels

| Compound | 125I-GxTX-1E binding | IC50 (μM) |
|----------|----------------------|-----------|
|          |                      | KV2.1     | KV1.2      |
| PAC      | 27.6                 | 0.60      | 0.20       |
| Compound A | 0.41                | 0.21      | 0.04       |
| Compound B | 0.22                | 4.59      | 0.31       |
| Compound C | 5.09                | >20       | >20        |
| Compound D | 8.51                | 0.22      | 0.10       |
| Compound E | 2.85                | 0.69      | 0.23       |
| Compound F | 2.89                | 2.31      | 0.97       |
| Compound G | 1.20                | 0.16      | 0.06       |
| Compound H | 11.8                | 0.22      | 0.07       |
| Compound I | 11.8                | >20       | >20        |

Compounds were evaluated in the 125I-GxTX-1E binding assay and on hK,2.1.CHO and hK,1.2.CHO currents. Electrophysiology was performed using an automated instrument (IonWorks®). The reported electrophysiology IC50s were measured at the end of a 40 pulse train (see Materials & Methods for experimental details).
substitute the oxidation prone residue with a more stable amino acid, and to identify a more solvent accessible position for Tyr substitution.

The limited supply of $^{125}$I-GxTX-1E made a standard saturation (Scatchard) analysis with hKv2.1.CHO cells unfeasible. At concentrations of ligand near 150 μM, development of specific binding was slow, with a time constant of about 36 min. Dissociation measured by dilution was also slow, with a time constant of about 25 min. This time course is similar to the recovery from channel modification as measured by electrophysiology. Interestingly, dissociation measured by addition of excess cold GxTX-1E or high potassium was much faster, with time constants of 3 min and 4 min, respectively. Since both GxTX-1E and potassium depolarize hKv2.1.CHO cells, one potential explanation for the faster dissociation is that the reaction is accelerated by membrane depolarization. Consistent with this idea, specific binding was not detected in isolated membranes from hKv2.1.CHO cells or with cells incubated in high potassium. Dissociation of hanatoxin from the activated conformation of the voltage sensor of Kv2.1 (F274A) channels is clearly near 150 pM, development of specific binding was slow, with a time constant of about 36 min. Dissociation measured by dilution was also slow, with a time constant of about 25 min. This time course is similar to the recovery from channel modification as measured by electrophysiology.

As a test of the utility of the $^{125}$I-GxTX-1E binding assay, several small molecule libraries were screened. The hit rate was exceptionally low (~0.2%) and the assay in 384-well format was quite robust. Plate failures were usually the result of a diminished specific binding window resulting from decreased expression of channels with increasing passage number. The assay was relatively tolerant of DMSO up to at least 0.8%, allowing screening of compounds at concentrations up to 83 μM (from 10 mM DMSO stocks). Much to our surprise, the screen did not identify any compounds that showed a high degree of specificity for Kv2.1 over Kv1 channels. Further, none of the active compounds noticeably altered the kinetics and/or voltage-dependence of activation would be better suited for confirmation of binding assay actives.

Several representatives of the di-substituted cyclohexyl class of small molecules were identified from the $^{125}$I-GxTX-1E binding screen. The prototypical member of this family is PAC. PAC blocks Kv1.3 and, with weaker potency, Kv2.1 channels. This class of inhibitors has two identical binding sites per channel that display positive allosteric cooperativity. The binding site of 3H-trans-NPCO-DSC, a radiolabeled analogue of PAC, on Kv1.3 channels has not been mapped extensively, but may reside in the water-filled cavity, our data would suggest that GxTX-1E also binds less tightly to the activated conformation of Kv2.1.

As a test of the utility of the $^{125}$I-GxTX-1E binding assay, several small molecule libraries were screened. The hit rate was exceptionally low (~0.2%) and the assay in 384-well format was quite robust. Plate failures were usually the result of a diminished specific binding window resulting from decreased expression of channels with increasing passage number. The assay was relatively tolerant of DMSO up to at least 0.8%, allowing screening of compounds at concentrations up to 83 μM (from 10 mM DMSO stocks). Much to our surprise, the screen did not identify any compounds that showed a high degree of specificity for Kv2.1 over Kv1 channels. Further, none of the active compounds noticeably altered the kinetics and/or voltage-dependence of activation would be better suited for confirmation of binding assay actives.

Several representatives of the di-substituted cyclohexyl class of small molecules were identified from the $^{125}$I-GxTX-1E binding screen. The prototypical member of this family is PAC. PAC blocks Kv1.3 and, with weaker potency, Kv2.1 channels. This class of inhibitors has two identical binding sites per channel that display positive allosteric cooperativity. The binding site of 3H-trans-NPCO-DSC, a radiolabeled analogue of PAC, on Kv1.3 channels has not been mapped extensively, but may reside in the water-filled cavity. The correolide binding site on Kv1.3 has been mapped to residues in S5-S6, and residues in the S6 region of Kv1.5 have been identified as being important for block by PAC and correolide. The binding site on PAC for Kv2.1 has not been determined. If indeed the binding site is in the water-filled cavity, our data would suggest that a small molecule within the cavity destabilizes GxTX-1E binding to the voltage sensor.

Given the dependence of $^{125}$I-GxTX-1E binding to Kv2.1.CHO cells on membrane potential, it is possible that some of the compounds identified from the screen were active simply because they depolarize cells. If the Kv2.1 channel contributes to setting the membrane potential of these cells, blocking the channels would be expected to cause depolarization. Thus, we studied the effects...
of PAC compounds on the membrane potential of hK_\text{V}2.1.CHO cells. These cells have a rather modest resting potential (~-25 mV), which is close to the foot of the activation curve for these channels. Inhibiting K_\text{V}2.1 channels with GxTX-1E depolarizes the cells, suggesting that K_\text{V}2.1 channels do contribute to setting the resting potential. Consistent with this idea, PAC series compounds that were potent functional blockers of K_\text{V}2.1 also depolarized the cells (PAC and Compound A), whereas a weak K_\text{V}2.1 blocker (Compound B) did not. Since both Compounds A and B were potent in the $^{125}$I-GxTX-1E binding assay whereas PAC was not, there is not a strict correlation with the ability of a compound to depolarize the cells and its activity in the binding assay. Other classes of compounds that block K_\text{V}2.1 were not active in the $^{125}$I-GxTX-1E binding assay (Herrington J, unpublished observations). Taken together, these data suggest that functional block of K_\text{V}2.1 does not equate with inhibition of $^{125}$I-GxTX-1E binding and suggests that small molecule binding to K_\text{V}2.1 can influence GxTX-1E binding by a mechanism discrete from blocking potassium flux through the channel.

The identification of selective, small molecule K_\text{V} channel inhibitors has been slow to date. Given the divergent sequences between K_\text{V} channel classes in the voltage sensor, gating modifier-binding assays might provide a route to find novel, selective probes for these channels. Similarly, a recently described $^{125}$I-ProTx-II binding assay for Na_\text{V}1.7 channels may provide a useful tool for identifying inhibitors of this channel. Future studies exploring these assays will ultimately test the notion of whether the voltage sensor paddle is a unique pharmacophore that can be exploited to identify small molecules that display proper channel selectivity.

**Materials and Methods**

**Materials.** The CHO cell line stably expressing human K_\text{V}2.1 was obtained from Dr. O. Pongs (Institut fuer Neurale Signalverarbeitung, Hamburg, Germany). CHO cells stably expressing the Maxi-K (alpha and beta1 subunits) or K_\text{V}1.2 channels were prepared following procedures as described. Guangxitoxin-1E (GxTX-1E), iberiotoxin and charybdotoxin were purchased from Peptides International (Louisville, KY). ScTx1 was purchased from Alamone Labs (Jerusalem, Israel) and purified further by HPLC. All compounds were prepared by the Department of Medicinal Chemistry, Merck Research Laboratories, Rahway, NJ. All tissue culture media and additives were purchased from Invitrogen/Gibco (Carlsbad, CA) unless otherwise noted. Chemicals were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

**Iodination of GxTX-1E.** Iodination of GxTX-1E was accomplished using lactoperoxidase and glucose oxidase coupled to polyacrylamide beads as described. Briefly, one aliquot of beads was thawed on ice, centrifuged and washed once with 200 μl of ice-cold PAC compounds on the membrane potential of hK_\text{V}2.1.CHO cells. Membrane potential was measured in individual CHO cells stably expressing hK_\text{V}2.1 channels by whole-cell current clamp as described in the Materials and Methods. Membrane potential in control solution was monitored for approximately 200 sec to confirm stability prior to adding 100 nM GxTX-1E (A), 10 μM PAC (B), 10 μM Compound A (C) or 10 μM Compound B (D) to the bath at the times indicated by the solid bar.
NaPO₄, pH 8.0. To the washed beads, 35 μl of GxTX-1E solution (0.71 mg/ml in 100 mM NaPO₄, pH 8.0) and 14 μl of a solution containing either 5 mM of ¹²⁵I-Na (2,200 Ci/mmol) or 20 μg/ml ¹²³I-Na in 10 μM NaOH was added followed by 5 μl of 5% β-D-glucose. After incubating at room temperature for 20 min, the reaction was subjected to centrifugation and the supernatant was loaded onto a 300-Å pore size C₁₈ reverse phase HPLC column (Vydac, 0.46 x 25 cm) that had been equilibrated with 10 mM TFA. Elution was achieved with a linear gradient of acetonitrile in 5 mM TFA (0–30% 2 min, 30–60% 60 min) at a flow rate of 0.5 ml/min. Material eluting from the column was monitored by measuring absorbance at 210 nm. Individual peaks were lyophilized dry, and reconstituted in 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 0.1% bovine serum albumin. Aliquots were frozen in liquid N₂ and stored at -70°C. Following the iodination of peptide with ¹²⁷I-Na, chromatographically homogeneous peaks were collected and analyzed by electrospray mass spectrometry.

**Cell culture.** hK₂.1 CHO cells were maintained in MEM Alpha media with nucleosides supplemented with 10% certified FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.29 mg/ml L-glutamine and 2 μg/ml basicatin S HCl. høβ23.CHO cells expressing the alpha and beta1 subunits of the Maxi-K channel were maintained in Iscove's Modified Eagle Medium supplemented with 10% certified FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.29 mg/ml L-glutamine, 1X HT supplement, 0.5 mg/ml G418 and 12 μg/ml puromycin. hK₂.1.CHO cells were maintained in Iscove's Modified Eagle Medium supplemented with 10% certified FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 0.29 mg/ml L-glutamine, 1X HT supplement and 0.5 mg/ml G418. hK₂.1.CHO and høβ23.CHO cells were grown at 10% CO₂; hK V2.1.CHO cells were grown at 5% CO₂. All cells were grown at 37°C.

**¹²³I-GxTX-1E binding assay.** CHO cells stably expressing hK₂.1 channels were seeded at a density of 30,000 cells per well in 96 well tissue-culture treated clear-bottom white wall plates, and cells were allowed to attach for approximately 18 h at 37°C. ¹²³I-GxTX-1E was added to wells and incubation took place under normal growth conditions. Quadruplicate samples were averaged for each experimental point. At the end of the incubation, cells were washed twice with 200 μl Dulbecco’s phosphate buffered saline to separate bound from free ligand. Cells were lysed by addition of 200 μl of 0.2% SDS, and radioactivity associated with the cell lysate was determined using a gamma counter. Non-specific binding was defined as binding in the presence of 200–400 nM GxTX-1E. Competition binding experiments were carried out in the absence or presence of increasing concentration of a test compound. IC₅₀ values for inhibition of ¹²³I-GxTX-1E binding by the test compound were determined using the equation:

\[
B_{eq} = \frac{(B_{max} - B_{min})}{[1 + (D/IC_{50})^{βH}]} + B_{min},
\]

where \(B_{max}\) represents bound ¹²³I-GxTX-1E in the absence of test compound (D), \(B_{min}\) is the amount of ¹²³I-GxTX-1E bound at a saturating concentration of test compound, D is the concentration of test compound, \(nH\) is the Hill coefficient, and IC₅₀ is the compound concentration resulting in half maximal inhibition. To determine kinetics of ligand association, cells were incubated with ¹²³I-GxTX-1E for different periods of time. Dissociation kinetics were initiated either by addition of 200 nM GxTX-1E or by washing the wells twice with media, and incubating for different periods of time. The dissociation rate constant \(k_{1}\) was calculated by fitting the data to a single mono-exponential decay.

**High throughput screening.** High throughput screening followed the same protocol as the 96 well assay with the following modifications. Cells were seeded at 30,000 cells per well in 384 well tissue culture treated clear bottom white wall plates. Compound additions were performed using PocketTips® (FX384P30-250, Thermo Fisher Scientific, Hudson, NH), and all compound data points were single determinations. At the end of the incubation, cells were washed twice with 50 μl Dulbecco’s phosphate buffered saline to separate bound from free ligand. 50 μl Optiphase Supermix (Perkin Elmer, Shelton, CT) was added to each well and radiation associated with cells was determined using a Perkin Elmer MicroBeta. Data from test compounds was expressed as percent inhibition relative to an untreated control. The Z-factor for screening plates was calculated from the equation:

\[
Z-factor = 1 - 3 x (σ + μ)/|μ₁ - μ₂|,
\]

where \(σ\) is the standard deviation and \(μ\) is the mean of the high (H) or low (L) controls. The high control was defined by 32 wells with no compound addition and the low control was defined by 16 wells to which 400 nM unlabeled GxTX-1E was added.

**Automated 384-well electrophysiology.** K₂.1 currents were recorded using the IonWorks® Quattro™ system (MDS Analytical Technologies, Sunnyvale, CA) in Population Patch Clamp™ (PPC) mode as described previously. The standard voltage pulse protocol was a series of forty 100 msec pulses at a frequency of 5 Hz. The pre-pulse holding potential was -80 mV and the steps were to +50 mV. Currents were sampled at a rate of 1.25 kHz. To test the biological activity of ¹²³I-GxTX-1E, the voltage protocol was a single 100 msec step to +20 mV from a holding potential of -80 mV. Ten point titration series were created by serial diluting the 2 mM DMSO stock 1:3 in DMSO. The upper final concentration applied to cells was 20 μM. For the titrations in Table 1, 0.1% BSA was added to the external DPBS buffer to match the solution used for titrations in the ¹²³I-GxTX-1E binding assay.

**Conventional patch clamp electrophysiology.** Membrane potential was recorded at room temperature (23–25°C) using standard dialyzed, whole-cell current clamp techniques. The internal solution was (in mM): 100 K-Aspartate, 40 KCl, 10 EGTA, 10 HEPES, 4 mM MgATP, pH 7.2 with KOH. The external solution was (in mM): 150 NaCl, 4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 10 HEPES, 3 glucose, pH 7.4 with NaOH. BSA (0.1% w:v) was added to GxTX-1E solutions. Compounds were diluted in external solution from 10–20 mM stocks in DMSO. The final concentration of DMSO did not exceed 0.1%. Cells with small (<1 nA) hK₂.1 currents had membrane potentials between 0
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Acknowledgements

The authors would like to thank L. Ge for assistance with cell culture, A. Howard and Y.P. Zhou for scientific discussions, and B. Priest and J. Sack for careful reading of the manuscript.

Note

The authors William A. Schmalholzer, Kevin S. Ratliff, Adam B. Weinglass and James Herrington declare they are employees of Merck & Co., Inc., and potentially own stock and/or hold stock options in the company.