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# *Stichodactyla helianthus* Peptide, a Pharmacological Tool for Studying Kv3.2 Channels

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## ABSTRACT

Voltage-gated potassium (Kv) channels regulate many physiological functions and represent important therapeutic targets in the treatment of several clinical disorders. Although some of these channels have been well-characterized, the study of others, such as Kv3 channels, has been hindered because of limited pharmacological tools. The current study was initiated to identify potent blockers of the Kv3.2 channel. Chinese hamster ovary (CHO)-K1 cells stably expressing human Kv3.2b (CHO-K1.hKv3.2b) were established and characterized. *Stichodactyla helianthus* peptide (ShK), isolated from *S. helianthus* venom and a known high-affinity blocker of Kv1.1 and Kv1.3 channels, was found to potently inhibit $^{86}Rb^+$ efflux from CHO-K1.hKv3.2b ($IC_{50} \sim 0.6$ nM). In electrophysiological recordings of Kv3.2b channels expressed in *Xenopus laevis* oocytes or in planar patch-clamp studies, ShK inhibited hKv3.2b channels with $IC_{50}$ values of ~0.3 and 6 nM, respectively. Despite the presence of Kv3.2 protein in human pancreatic $\beta$ cells, ShK has no effect on the Kv current of these cells, suggesting that it is unlikely that homotetrameric Kv3.2 channels contribute significantly to the delayed rectifier current of insulin-secreting cells. In mouse cortical GABAergic fast-spiking interneurons, however, application of ShK produced effects consistent with the blockade of Kv3 channels (i.e., an increase in action potential half-width, a decrease in the amplitude of the action potential after hyperpolarization, and a decrease in maximal firing frequency in response to depolarizing current injections). Taken together, these results indicate that ShK is a potent inhibitor of Kv3.2 channels and may serve as a useful pharmacological probe for studying these channels in native preparations.

Voltage-gated potassium channels (Kv) represent a large family of proteins that regulate numerous physiological functions. Kv channels are tetrameric structures formed by the association of identical or closely related subunits. The study of potassium channels has been greatly facilitated by the discovery of high-affinity and selective channel inhibitors in the venoms of different organisms (Garcia et al., 1998; Corzo and Escoubas, 2003; Lewis and Garcia, 2003). Such peptidyl inhibitors have provided invaluable pharmacological tools for the isolation, purification, tissue localization, and the study of the structure and gating mechanism of ion channels (Garcia-Calvo et al., 1994).

To date, many subtype-selective Kv channel inhibitors have been identified. Dendrotoxins (DTXs) from the venom of *Dendroaspis angusticeps* and BgK from the sea anemone *Bunodosoma granuliferaare* are potent blockers of Kv1.1, 1.2, and 1.6 channels (Harvey, 2001). Charybdotoxin (ChTX), a minor component of *Leiurus quinquestriatus* venom, is a potent blocker of Kv1.3 and Kv1.2 channels (Castaneda et al., 1995; Garcia et al., 1999), whereas ShK, a peptide isolated from the sea anemone *Stichodactyla helianthus,* inhibits Kv1.1 and 1.3 channels with picomolar affinities (Middleton et al., 2003). Blood-depressing substances I and II from the venom of sea anemone *Anemonia sulcata* block Kv3.4 channels specifically (Diochot et al., 1998). Finally, phrixotoxins I and II from venom of the tarantula *Phriotrichus auratus* and hanatoxin from the venom of the spider *Grammostola spatulata* represent a class of gating-modifying peptides with ac-

**ABBREVIATIONS:** Kv, voltage-gated potassium channel; CHO, Chinese hamster ovary; ShK, *Stichodactyla helianthus* peptide; Dap, diaminopropionic acid; ShK-Dap22, *Stichodactyla helianthus* lysine[22]-diaminopropionic acid; DTX, dendrotoxin; FS cells, neocortical GABAergic fast-spiking interneurons; ChTX, charybdotoxin; TEA, tetraethylammonium; low-K, low external potassium; high-K, high external potassium; AHP, afterhyperpolarization; h, human; AP, action potential.

tivity against Kv4.2 and 4.3 (Diochot et al., 1999) and Kv2.1 channels (Swartz and MacKinnon, 1995), respectively.

Kv3.2 is a member of the Kv3 channel subfamily. These channels possess rather unique fast activation at voltages positive to $-20$ mV and very fast deactivation rates (Coetzee et al., 1999). Such biophysical properties could enable cells to fire high-frequency trains of action potentials, and therefore Kv3.2 could play a role in neuronal excitability. Consistent with this proposal, Kv3.2 is expressed in neurons that are known to fire at a high frequency, such as the inhibitory cortical GABAergic interneurons (Chow et al., 1999; Hernandez-Pineda et al., 1999; Tansey et al., 2002). In addition, Kv3.2 is expressed in the insulin-secreting, pancreatic $\beta$ cells (Yan et al., 2004) and could contribute to the delayed-rectifier current in these cells. The $\beta$ cell delayed-rectifier current is believed to regulate glucose-dependent firing, making it an attractive candidate for the development of novel glucose-dependent insulin secretagogues that could have usefulness in the treatment of type-2 diabetes. Although Kv3.2 channels are sensitive to low concentrations of tetraethylammonium ion (TEA) (Hernandez-Pineda et al., 1999), potent and selective Kv3.2 inhibitors with which to study the role of these channels in native tissues are not currently available.

To search for Kv3.2 inhibitors, we generated CHO-K1 cells stably expressing the human Kv3.2b channel (CHO-K1.hKv3.2b cells) and established a functional $^{86}Rb^+$ efflux assay with which to monitor the activity of the channel. Using this assay, ShK was identified as a potent inhibitor ($IC_{50} = 0.59$ nM) of heterologously expressed hKv3.2b channels and was used to evaluate the role of the channel in pancreatic $\beta$ cells and in cortical GABAergic fast-spiking interneurons. ShK may serve as a useful pharmacological tool for studying Kv3.2 channels in native tissues.

## Materials and Methods

**Reagents.** CHO-K1 cells stably expressing human Kv2.1 channels (CHO.K1.hKv2.1 cells) were obtained from Dr. O. Pongs (Institut fuer Neurale Signalverarbeitung, Hamburg, Germany). Rabbit polyclonal antibodies directed against the peptide (TPDLIGGDPG-DDEDLAAKR) corresponding to residues 171 to 189 of human Kv3.2b were obtained as described previously (Chow et al., 1999). $^{86}RbCl$ was obtained from PerkinElmer Life and Analytical Sciences (Boston, MA). ShK, ShK-lysine$^{22}$-diaminopropionic acid (ShK-Dap22), apamin, and iberiotoxin were purchased from Peptides International Inc. (Louisville, KY). Kaliotoxin, agitoxin-I, agitoxin-II, and margatoxin were prepared as described previously (Suarez-Kurtz et al., 1999). TEA was purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were obtained from commercial sources and were of the highest purity available.

**Preparation of CHO-K1 Cells Stably Expressing hKv3.2b (CHO-K1.hKv3.2b Cells).** CHO-K1 cells were maintained at 37°C in Ham's F-12 medium (Cellgro, Mediatech, VA) supplemented with 10% fetal bovine serum and appropriate antibiotics. cDNA coding for hKv3.2b (GenBank accession number AF268896), a splicing variant of hKv3.2 (Haas et al., 1993) was subcloned into pCI-neo mammalian expression vector (Promega, Madison, WI), and CHO-K1 cells were transfected using PolyFect Transfection Kit (QIAGEN, Valencia, CA), according to the manufacturer's instructions. Transfected cells were subjected to selection in the presence of 1 mg/ml G418. G418-resistant clones stably expressing functional hKv3.2b (CHO-K1.hKV3.2b cells) were identified by Western blot immunostaining and patch-clamp electrophysiological recordings.

**Western Blot Analysis.** CHO-K1 or CHO-K1.hKv3.2b cells were rinsed with ice-cold phosphate-buffered saline and lysed in ice-cold lysis buffer containing 20 mM HEPES, pH 7.4, 1% Triton X-100, 150 mM NaCl, and $1\times$ concentration of a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). The cell lysates were cleared by centrifugation. Protein concentrations were determined using Bradford reagent (Bio-Rad, Hercules, CA). Cell lysates were resuspended in SDS loading buffer (Invitrogen, Carlsbad, CA) and proteins were separated using precast 4 to 20% gradient NuPAGE SDS- polyacrylamide gel electrophoresis gels (Invitrogen) and transferred to polyvinylidene difluoride membrane (Immobilon; Millipore Corporation, Billerica, MA). The membrane blot was first incubated with blocking buffer (5% nonfat dried milk, 2.5% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS in $1\times$ Tris-buffered saline) overnight and then with blocking buffer containing rabbit hKv3.2 antiserum (1:500 dilution) for 2 h at room temperature. The blot was washed three times with blocking buffer, for 15 min each, and then incubated for 1 h in the same buffer with alkaline phosphatase-conjugated anti-rabbit secondary antibody. An enhanced chemifluorescence Western blotting kit (Amersham Biosciences, Piscataway, NJ) was used for detection. The blot was scanned using a Storm gel and blot imaging system (Amersham Biosciences).

**Conventional Patch-Clamp Electrophysiology.** Membrane currents were recorded using standard whole-cell voltage-clamp techniques (Hamill et al., 1981) with a Dagan 3900A amplifier (Dagan, Minneapolis, MN). Microelectrodes fabricated from borosilicate glass were coated with Sylgard (Dow Corning, Midland, MI) and fire-polished. Electrode resistances were generally 2 to 4 M$\Omega$ when filled with standard internal saline. The reference electrode was a silver-silver chloride wire within an agar bridge (4% agar in 170 mM KCl). Voltages given in the figures have not been corrected for the liquid junction potential between the internal and external solutions. All experiments were performed at room temperature (22–25°C). Generation of analog voltage commands and digitization of membrane currents were controlled with PULSE software (HEKA Elektronik, Lambrecht, Germany) and an ITC-16 computer interface (InstruTECH Corporation, Port Washington, NY). Currents were digitized at 5 kHz and digitally filtered at 2 kHz. Digital subtraction of leakage and capacitive currents was performed by the P/n procedure, where $n = 5$. Voltage steps were applied every 5 to 30 s from a holding potential of $-80$ mV.

The standard pipette solution contained 130 mM potassium aspartate, 5 mM NaCl, 10 mM EGTA, 2 mM $MgCl_2$, and 10 mM HEPES, with pH adjusted to 7.2 with 22 mM KOH. The standard external solution consisted of 160 mM NaCl, 1 mM KCl, 2.7 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5 mM HEPES, pH 7.5, with $N$-methyl-D-glucamine. For recordings of human islet cells, the pipette solution was 120 mM KCl, 20 mM KF, 10 mM EGTA, 10 mM HEPES, and 2 mM MgATP, with pH adjusted to 7.2 with KOH, and external solution was 160 mM NaCl, 4.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 3 mM glucose, pH 7.2, with NaOH. Solutions were applied to cells by bath perfusion via gravity. The experimental chamber had a volume of 0.2 ml, and the perfusion rate was 1 to 2 ml/min. Flow of solution through the chamber was maintained at all times. TEA was added directly to the standard extracellular solution from a 1 M stock in water. Measurement of current amplitudes was performed with PULSEFIT software (version 8.53) (HEKA Elektronik). Fitting of current waveforms or extracted data was performed with PULSEFIT or Igor Pro 4.0 (WaveMetrics, Lake Oswego, OR). Results are reported as mean $\pm$ S.E.M.

**$^{86}Rb^+$ Efflux Assay.** CHO-K1 hKv3.2b cells were plated into 96-well cell culture plates at a density of $\sim 2 \times 10^5$ cells/well and incubated with 6 $\mu$Ci/ml $^{86}RbCl$ in 100 $\mu$l of culture medium overnight at 37°C. To each well, 100 $\mu$l of low-potassium buffer (135 mM NaCl, 4.6 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.2% bovine serum albumin, and 10 mM HEPES, pH 7.4, with NaOH) with or without test samples was added, and incubation continued for 30 min at 37°C. At the end of the incubation, the medium was replaced with 200 $\mu$l of high-potassium buffer (140 mM KCl, 1 mM $CaCl_2$, 2 mM $MgCl_2$, 0.2% bovine serum albumin, and 10 mM HEPES, pH 7.4,

with KOH), with or without test samples, and $^{86}Rb^+$ efflux was monitored for determined periods of time. To quantify the amount of $^{86}Rb^+$ efflux, medium was removed from the wells, and 100 $\mu l$ was mixed with 100 $\mu l$ of MicroScint-20 (PerkinElmer Life and Analytical Sciences). $^{86}Rb^+$ content of the cells was determined by the addition of 100 $\mu l$ of MicroScint-20. Efflux was defined as the percentage of $^{86}Rb^+$ in each well in the low- or high-potassium buffer, normalized to the total radioactivity of the efflux solution and cells. $IC_{50}$ values represent the mean $\pm$ S.E.M.

**Expression and Two-Electrode Voltage-Clamp Studies of hKv3.2 in *X. laevis* oocytes.** *X. laevis* oocytes were prepared and injected using standard methods described previously (Arena et al., 1991). Adult female *X. laevis* oocytes were anesthetized with 0.17% tricaine methanesulfonate, and the ovaries were surgically removed and placed in a solution consisting of 82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 100,000 U/l penicillin G, and 1000 mg/l streptomycin sulfate, pH 7.5 (modified OR-2). Ovarian lobes were broken open, rinsed several times in modified OR-2, and incubated in 0.2% collagenase (type 1; Sigma-Aldrich) in modified OR-2 with gentle shaking at room temperature. After 1 h, the collagenase solution was renewed, and the oocytes were incubated for an additional 30 to 90 min until approximately 50% of the oocytes were released from the ovaries. Stage V and VI oocytes were selected and placed in media containing 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 2.5 mM sodium pyruvate, 0.5 mM theophylline, and 50 mg/ml gentamicin, pH 7.5 (ND-96) for 16 to 24 h before injection. Oocytes were injected with 50 nl of RNA encoding hKv3.2b at a concentration of 0.2 mg/ml. Oocytes were incubated at 18°C for 1 to 6 days in ND-96 before recording.

Recordings were made at room temperature in modified ND-96 consisting of 96 mM NaCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 3.5 mM $BaCl_2$, and 5 mM HEPES, pH 7.5. Oocytes were voltage-clamped using a Dagan CA1 two-microelectrode amplifier (Dagan Corporation) interfaced to a Macintosh 7100/80 computer (Apple Computer, Cupertino, CA). The current-passing electrode was filled with 0.7 M KCl and 1.7 M potassium citrate, and the voltage-recording electrode was filled with 1 M KCl. Throughout the experiment, oocytes were superfused with modified ND-96 (control solution) or with ND-96 containing test peptides at a rate of approximately 3 ml/min. Data were acquired at 5 kHz and filtered at 1.7 kHz using Pulse software (version 8.53) from HEKA Elektronik. All recordings were performed from a holding potential of −80 mV. $IC_{50}$ values represent the mean $\pm$ S.D.

**Planar Patch-Clamp Electrophysiology.** Parallel patch-clamp electrophysiology was performed essentially as described previously (Kiss et al., 2003). In brief, CHO-K1.hKv3.2b cells or CHO-K1.hKv2.1 cells were removed from culture plates using Versene (Invitrogen), subjected to centrifugation, and resuspended in 4 ml of Dulbecco's phosphate-buffered saline (Invitrogen) containing 138 mM NaCl, 8.1 mM $Na_3PO_4$, 2.67 mM KCl, 1.47 mM $K_3PO_4$, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$, pH 7.4. The internal solution consisted of 100 mM potassium gluconate, 40 mM KCl, 3.2 mM $MgCl_2$, 3 mM EGTA, and 5 mM HEPES, pH 7.4, with KOH, supplemented with 120 $\mu g$/ml amphotericin B. Voltage protocols and the recording of membrane currents were performed using the IonWorks HT software/hardware system (Molecular Devices, Sunnyvale, CA). Currents were sampled at 2.5 kHz, and leakage subtraction was performed using a 10-mV step from the holding potential (−80 mV) and assuming a linear leak conductance. No correction for liquid junction potentials was used. Wells with seal resistances less than 70 MΩ or less than 0.1 nA of Kv current at the test potential (+40 mV) were excluded from analysis. The mean seal resistance of the remaining wells was 169 MΩ. Current amplitudes were calculated with the IonWorks software, fraction block was calculated in Microsoft Excel (Microsoft, Redmond, CA), and fitting was performed with Sigma Plot (SPSS Inc., Chicago, IL).

**Human Islet Cells.** Human pancreatic islets were obtained from Dr. Jonathan Lakey (University of Alberta, Surgical-Medical Research Institute, Edmonton, AB, Canada) and the JDRF Human Islet Distribution Program at the University of Alberta (Edmonton, AB, Canada). Islets were isolated as described previously (Shapiro et al., 2000) using procedures approved by the University of Alberta Ethics Committee. Upon receipt of the tissue, islets were hand-picked, dissociated into single cells using trypsin, resuspended in culture medium, and plated on poly-L-lysine–coated glass chips. Cells were used for electrophysiology within 72 h of plating.

**Slice Preparation and Electrophysiology.** Mice (14 days old) were anesthetized via intraperitoneal injection of pentobarbital and decapitated. All animal procedures complied with National Institutes of Health guidelines for the ethical use of animals. The brain was rapidly removed to oxygenated, ice-cold artificial cerebrospinal fluid, and sagittal slices (300 $\mu M$ thick) were cut and incubated in a holding chamber at room temperature for at least 1 h before electrophysiological recording, at which point they were transferred to a submersion-type recording chamber. The artificial cerebrospinal fluid used for cutting, incubation, and recording contained 125 mM NaCl, 2.5 mM KCl, 1.25 mM $NaH_2PO_4$, 26 mM $NaHCO_3$, 20 mM glucose, 2 mM $CaCl_2$, 2 mM $MgSO_4$, and 0.5 mM ascorbic acid and was continuously bubbled with 95% $O_2$ and 5% $CO_2$ to maintain a pH of 7.40. Whole-cell current clamp recordings were made using an AxoClamp 2B amplifier (Axon Instruments Inc., Union City, CA). Patch electrodes (3–5 MΩ) were filled with a solution containing 130 mM potassium gluconate, 10 mM HEPES, 0.5 mM EGTA, 4.0 mM magnesium ATP, and 0.3 sodium GTP, with pH to 7.4 with KOH. Voltage output was filtered at 10 kHz, and data were acquired with a sampling frequency of 20 kHz. Neocortical GABAergic fast-spiking interneurons (FS cells), identified via multipolar morphology, lack of a prominent apical dendrite, and characteristic response to depolarizing current injection (Connors and Gutnick, 1990), were recorded in layer V primary somatosensory ("barrel") cortex. Values represent the mean $\pm$ S.D.

## Results

**Stable Expression of hKv3.2b in CHO-K1 Cells.** To establish a robust, functional assay for identifying hKv3.2 inhibitors, we stably expressed the splice variant, hKv3.2b, in CHO-K1 cells. CHO-K1 cells were chosen because it is known that these cells express low levels of endogenous potassium channels, and they are routinely used for functional heterologous expression of Kv channels. Figure 1A shows a Western blot analysis of the CHO-K1.hKv3.2b cell clone that was selected for all subsequent studies. Two bands with estimated molecular masses of ~57.5 and 74 kDa are specifically recognized by the Kv3.2 antibody in the lysate of these cells (lane 2) but not in the lysate of the parental, untransfected CHO-K1 cells (lane 1). The upper band agrees well with the predicted molecular mass of the full-length protein, whereas the lower band probably represents a proteolytic product of the higher molecular mass product.

**Electrophysiological Properties of CHO-K1.hKv3.2b Cells.** The biophysical properties of heterologously expressed hKv3.2b channels were evaluated in patch-clamp recordings. Characteristics of the Kv3 channel subfamily (including Kv3.2) include activation at potentials more positive than approximately −20 mV and fast deactivation upon repolarization (Rudy and McBain, 2001). Whereas the parental untransfected CHO-K1 cells display little if any Kv current (data not shown), CHO-K1.hKv3.2b cells displayed large outward currents in response to step depolarizations (Fig. 1B). The currents activated rapidly ($\tau = 3.0 \pm 0.9$ ms at +40 mV,

$n = 5$) and deactivated rapidly ($\tau = 5.6 \pm 0.2$ ms at $-40$ mV, $n = 5$). The voltage dependence of activation was studied by measuring the amplitude of tail currents after step depolarizations to various potentials. For the cell shown in Fig. 1B, the voltage-dependence of activation was well-described by a Boltzmann distribution with a $V_{50}$ of $+22.6$ mV and a slope of 13.5 mV (Fig. 1C). On average, the $V_{50}$ was $+20.2 \pm 0.9$ mV and the slope was $14.2 \pm 0.5$ mV ($n = 5$). To study current inactivation, long depolarizations (7 s) to various potentials were given (Fig. 1D). Currents decayed slowly at all potentials tested. At $+40$ mV, the current decayed $28 \pm 7\%$ ($n = 3$) over the 7-s step. Kv3 currents are known to be sensitive to low concentrations of TEA (Hernandez-Pineda et al., 1999), and this was also true for the channels present in CHO-K1.hKv3.2b cells (Fig. 1E). In these experiments, TEA reversibly inhibited hKv3.2b currents in a dose-dependent fashion. The combined data from experiments carried out in four cells illustrate that TEA blocked hKv3.2b with an $IC_{50}$ of 0.3 mM (Fig. 1F). Taken together, the data indicate that CHO-K1.hKv3.2b cells display a TEA-sensitive current with characteristics consistent with Kv3.2.

**TEA-Sensitive $^{86}Rb^+$ Efflux from CHO-K1.hKv3.2b Cells.** A functional, high-capacity assay for identifying hKv3.2 inhibitors was developed on the basis of the ability of $^{86}Rb^+$ to permeate through potassium channels. Cells incubated with $^{86}RbCl$ accumulate $^{86}Rb^+$ through operation of the Na/K-ATPase pump. When CHO-K1.hKv3.2b cells were then exposed to physiological, low external potassium (low-K) conditions, a time-dependent efflux of $^{86}Rb^+$ from the cells occurred. In the presence of high external potassium, however, $^{86}Rb^+$ efflux occurred much more rapidly, reflecting the activity of Kv channels. The Kv channel-mediated component of $^{86}Rb^+$ efflux, defined as the difference between efflux under high-K and low-K conditions, reached a plateau in approximately 10 min (Fig. 2A). $^{86}Rb^+$ efflux from CHO-K1.hKv3.2b cells was inhibited by TEA in a dose-dependent manner with an $IC_{50}$ of $0.25 \pm 0.01$ mM ($n = 3$) (Fig. 2B). High-K dependent $^{86}Rb^+$ efflux was not observed in the parental, untransfected CHO-K1 cells, although the basal levels of efflux in low-K were similar to those of CHO-K1.hKv3.2b cells (data not shown). These data suggest that in CHO-K1.hKv3.2b cells, high-K–induced $^{86}Rb^+$ efflux reflects the activity of hKv3.2 channels. The high-capacity, $^{86}Rb^+$ functional assay provides a means for identifying Kv3.2 inhibitors.

**ShK Is a Potent Inhibitor of hKv3.2b Channels.** Using the $^{86}Rb^+$ efflux assay described above, we evaluated a number of scorpion venoms and several known potassium channel inhibitors for their ability to block hKv3.2b channels. All venoms and the majority of potassium channel inhibitors
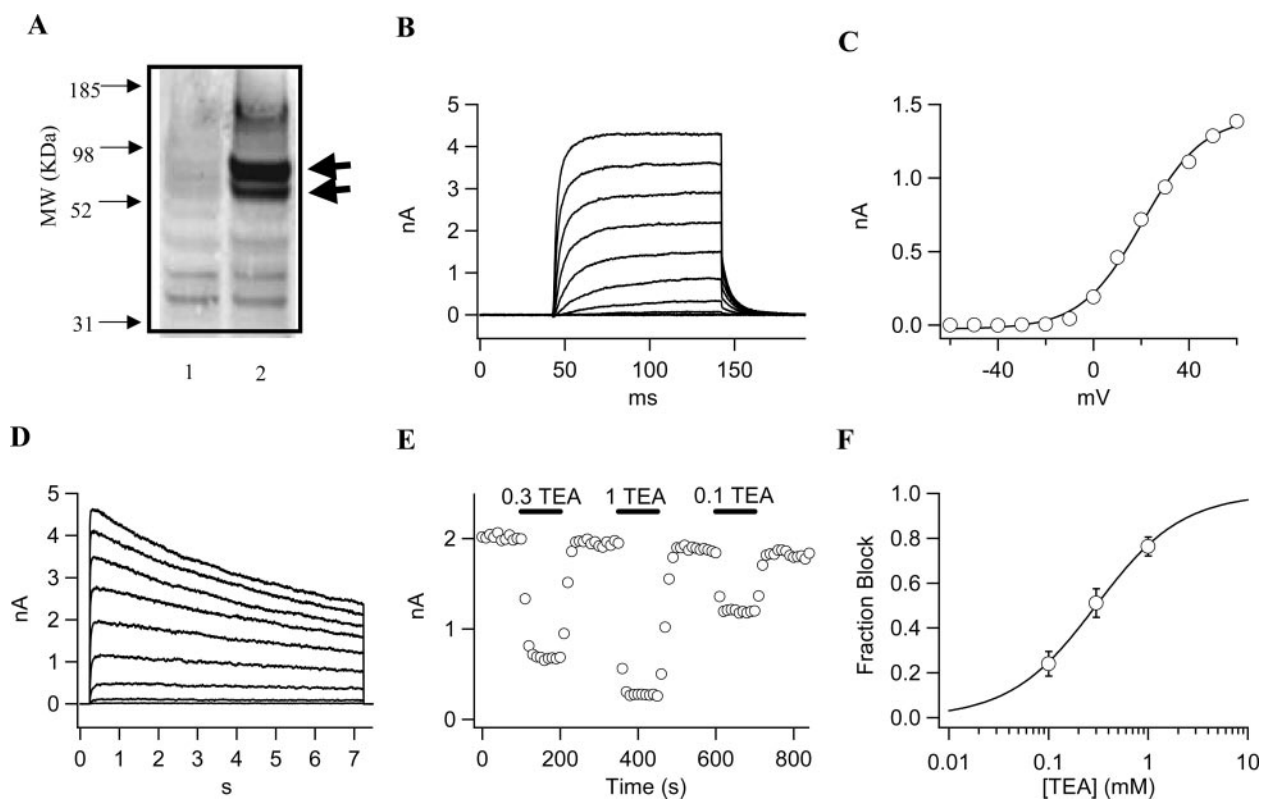


**Fig. 1.** Stable expression of hKv3.2b protein in CHO-K1 cells. A, Western blot analysis of protein extracts from the parental, untransfected CHO-K1 cells (lane 1) or from CHO-K1.hKv3.2b cells (lane 2). Equal amounts of total protein ($\sim$50 $\mu$g) were loaded onto each lane, and blots were probed with a rabbit polyclonal antibody raised against a sequence of Kv3.2b. The position of the molecular mass markers is indicated. B to F, whole-cell patch-clamp recordings from CHO-K1.hKv3.2b cells. B, outward currents in response to 100-ms step depolarizations ranging from $-60$ to $+60$ mV (in 10-mV increments) from a holding potential of $-80$ mV. C, voltage dependence of activation for the same cell shown in B determined from the amplitude of tail currents. The solid line is a fit of the Boltzmann equation to the data. Parameters of the fit are given in the text. D, inactivation of hKv3.2b currents by long depolarizations (7 s) ranging from $-20$ to $+60$ mV (in 10-mV increments). E, plot of the peak current amplitude in response to 100-ms step depolarizations to $+20$ mV from a holding potential of $-80$ mV versus time. The periods of application of 0.3, 1, or 0.1 mM TEA are denoted by the solid bars. F, dose-response relationship for TEA on hKv3.2b current. Average fraction block ($\pm$ S.E.M.) is plotted versus TEA concentration ($n = 4$ per concentration). The solid line is a fit of the Hill equation to the data with an apparent $IC_{50}$ value of 0.3 mM and a slope of 1.0.

such as apamin, kaliotoxin, iberiotoxin, agitoxin-I, agitoxin-II, margatoxin, and ChTX, when tested at concentrations up to 300 nM, did not significantly inhibit $^{86}$Rb$^+$ efflux from the CHO-K1.hKv3.2b cells (data not shown). In marked contrast, 100 nM ShK caused complete inhibition of $^{86}$Rb$^+$ efflux, and ShK-Dap22, in which diaminopropionic acid (Dap) was substituted at lysine 22 of ShK (Kalman et al., 1998), also inhibited $^{86}$Rb$^+$ efflux to a lesser extent. Dose-response curves for inhibition of $^{86}$Rb$^+$ efflux from CHO-K1.hKv3.2b cells indicate that ShK and ShK-Dap-22 block hKv3.2b-mediated $^{86}$Rb$^+$ efflux with IC$_{50}$ values of $0.62 \pm 0.10$ ($n = 3$) and $83.2 \pm 22.1$ ($n = 3$) nM, respectively, and Hill coefficients of 1 (Fig. 2C). These results provide the first evidence that ShK is a potent inhibitor of hKv3.2b channels.

**ShK Inhibits hKv3.2b but Not hKv2.1 Channels.** Because both Kv2.1 and Kv3.2 channels are expressed in human pancreatic $\beta$ cells and either one or both channels could contribute to the delayed-rectifier potassium current that regulates the firing frequency of the cells in the presence of high glucose, we compared the ability of ShK to inhibit both channels. *X. laevis* oocytes expressing hKv3.2b exhibited rapidly activating currents in response to step depolarizations to $+20$ and $+80$ mV (Fig. 3A) that were not seen in uninjected or water-injected oocytes. Similar to hKv3.2b currents expressed in mammalian cell lines, the hKv3.2b current in oocytes was blocked by TEA with an IC$_{50}$ of $0.10 \pm 0.01$ mM ($n = 3$) (data not shown). Bath application of 0.3 nM ShK reduced the current activated by depolarizations to $+20$ and $+80$ mV (Fig. 3A, gray line). This inhibition of current amplitude was concentration-dependent, shown in Fig. 3B for the current at $+80$ mV, and displayed an IC$_{50}$ value of $0.31 \pm 0.06$ nM ($n = 6$).

In an automated patch-clamp system (IonWorks HT) that performs synchronous perforated patch recordings in a 384-wells planar array (Schroeder et al., 2003), the blocking potency of ShK against hKv3.2b or Kv2.1 channels was evaluated after a 10-min incubation period in either peptide or vehicle. ShK inhibited hKv3.2b current in a dose-dependent manner with an IC$_{50}$ value of 6 nM but had no effect on hKv2.1 current at concentrations up to 1 $\mu$M (Fig. 3D). These

results suggest that ShK can be used as a pharmacological tool to evaluate the contribution of Kv3.2 channels to the whole-cell currents of cells that express both Kv3.2 and Kv2.1 channels. One such cell type is the human pancreatic $\beta$ cell.

**ShK Does Not Inhibit the Delayed Rectifier Current of Human $\beta$ Cells.** Whole-cell voltage-clamp recordings from human $\beta$ cells, identified by the presence of insulin mRNA through single-cell reverse-transcriptase polymerase chain reaction, show a prominent delayed-rectifier current with a voltage-dependence consistent with Kv2 and Kv3 channels (J. Herrington, M. Sanchez, D. Wunderler, L. Yan, R. M. Bugianesi, I. E. Dick, S. A. Clark, R. M. Brochu, B. T. Priest, M. G. Kohler, and O. B. McManus, submitted manuscript). Application of 100 nM ShK did not significantly inhibit ($7 \pm 4\%$, $n = 12$) this current (Fig. 4, A and B). TEA is known to inhibit the delayed-rectifier current of rodent $\beta$ cells and enhance glucose-dependent insulin secretion (MacDonald and Wheeler, 2003). Likewise, the delayed-rectifier current of human $\beta$ cells was blocked by TEA (Fig. 4, C and D). Application of 1 and 10 mM TEA inhibited the delayed rectifier current by $51 \pm 2\%$ ($n = 20$) and $89 \pm 2\%$ % ($n = 19$), respectively. Thus, the delayed rectifier of human $\beta$ cells is TEA-sensitive and ShK-insensitive.

**The Effects of ShK on Fast-Spiking Cortical Interneurons Are Consistent with Block of Kv3.2 Channels.** The presence of Kv3 channels in cortical GABAergic FS cells is required for the rapid repolarization of action potentials as well as for the initiation and maintenance of high-frequency firing. Application of low concentrations of TEA (a nonselective blocker of Kv3 channels) or genetic elimination of Kv3 channels produces broadening of the FS cell action potential, ablation of the afterhyperpolarization (AHP), and an attenuation of maximal achievable firing frequencies by these cells (Erisir et al., 1999; Lau et al., 2000; Rudy and McBain, 2001). Bath application of 100 nM ShK had no effect on resting membrane potential ($V_m$; $-73.0 \pm 5.5$ and $-75.0 \pm 5.2$ mV in control and after addition of ShK, respectively; $n = 4$) but produced a small increase in membrane resistance ($R_m$) from $112 \pm 52$ M$\Omega$ under control conditions to $139 \pm 61$ M$\Omega$ after ShK application (an increase of $26 \pm 20\%$ over control; $n = 4$).
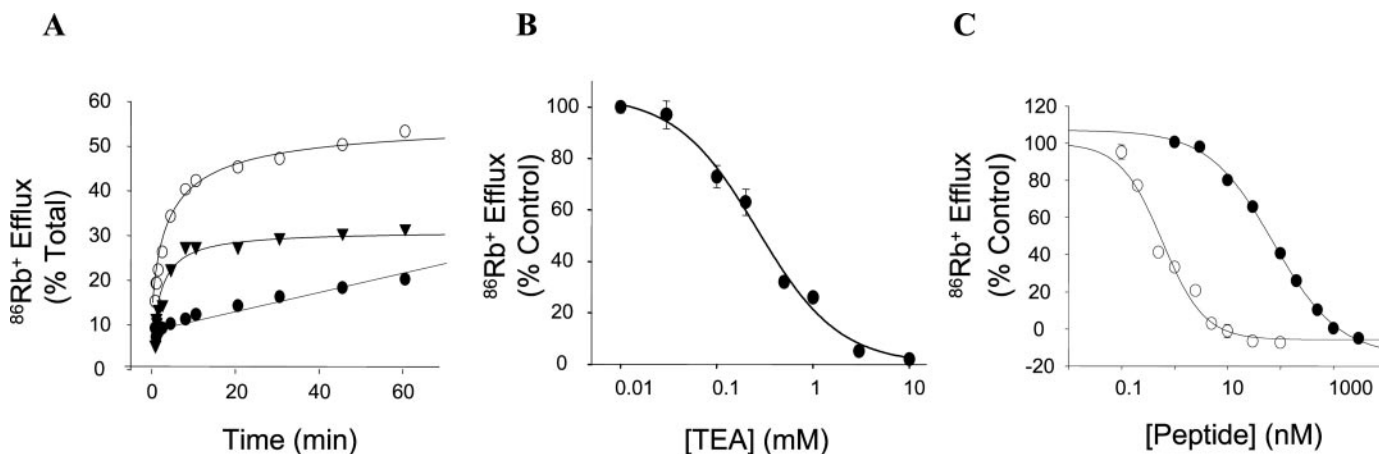
**Fig. 2.** $^{86}$Rb$^+$ efflux from CHO-K1.hKv3.2b cells. A, time course of $^{86}$Rb$^+$ efflux from CHO-K1.hKv3.2b cells in low-K ($\bullet$) and high-K ($\circ$) conditions. The Kv3.2 component of $^{86}$Rb$^+$ efflux, defined as the difference between high- and low-K, is indicated ($\blacktriangledown$). B, inhibition of $^{86}$Rb$^+$ efflux from CHO-K1.hKv3.2b cells by TEA. The Kv3.2 component of $^{86}$Rb$^+$ efflux at 10 min is plotted relative to the concentration of TEA. Each point represents the average of four replicates. The solid line is a fit of the Hill equation with an apparent IC$_{50}$ value of 0.24 nM. C, concentration-dependent inhibition of $^{86}$Rb$^+$ efflux from CHO-K1.hKv3.2b cells by ShK ($\circ$) and ShK-Dap22 ($\bullet$). The Kv3.2 component of $^{86}$Rb$^+$ efflux at 10 min is plotted against the concentration of peptide. Each data point is the average of three replicates. The solid lines are fits of the Hill equation with apparent IC$_{50}$ values of 0.59 ($\circ$) and 57 ($\bullet$) nM, respectively.

However, consistent with blockade of Kv3 channels, bath application of 100 nM ShK produced an increase in action potential half-width (i.e., the width of the action potential at half-amplitude, measured from action potential threshold to action potential peak) from $0.41 \pm 0.05$ to $0.58 \pm 0.16$ ms ($n = 4$), a decrease in the amplitude of the AHP as measured from action potential threshold to the maximal negativity of the afterhyperpolarization from $19.9 \pm 8.1$ to $14.1 \pm 8.0$ mV ($n = 4$), and a decrease in maximum firing frequency in response to depolarizing current injections from $280 \pm 53$ to $181 \pm 93$ Hz ($n = 4$) (Fig. 5). Statistical significance at the $p < 0.05$ level was achieved via unpaired, two-tailed $t$ test for AHP and maximal firing frequency but not for the other parameters shown in Fig. 5F. In contrast, 100 nM dendrotoxin-I, a selective inhibitor of Kv channels containing Kv1.1, Kv1.2, and/or Kv1.6 subunits, has no effect on the FS cell action potential and, in fact, produces a slight increase in firing frequency (Erisir et al., 1999). However, ShK did produce a marked increase in the frequency of spontaneous postsynaptic potentials recorded from FS cells (data not shown). This effect is qualitatively similar to that produced by 100 nM dendrotoxin-I and is consistent with blockade of Kv1 channels, probably present in the presynaptic terminals of intracortical excitatory synapses (Coetzee et al., 1999; Bekkers and Delaney, 2001; Harvey, 2001).

## Discussion

The goal of the present study was to identify potent and selective inhibitors of Kv3.2 channels with which to investigate the physiological role of the channel in native preparations. To this end, we constructed a CHO-K1.hKv3.2b cell line that stably expresses hKv3.2b channels and established a functional $^{86}Rb^+$ efflux assay that was used to screen a variety of venoms and known potassium-channel inhibitors. ShK, a peptide isolated from *S. helianthus* venom, was found to inhibit hKv3.2b-mediated $^{86}Rb^+$ efflux from CHO-K1.hKv3.2b cells in a dose-dependent manner and with high potency ($IC_{50} = 0.62$ nM). In electrophysiological recordings from *X. laevis* oocytes expressing hKv3.2b channels, ShK



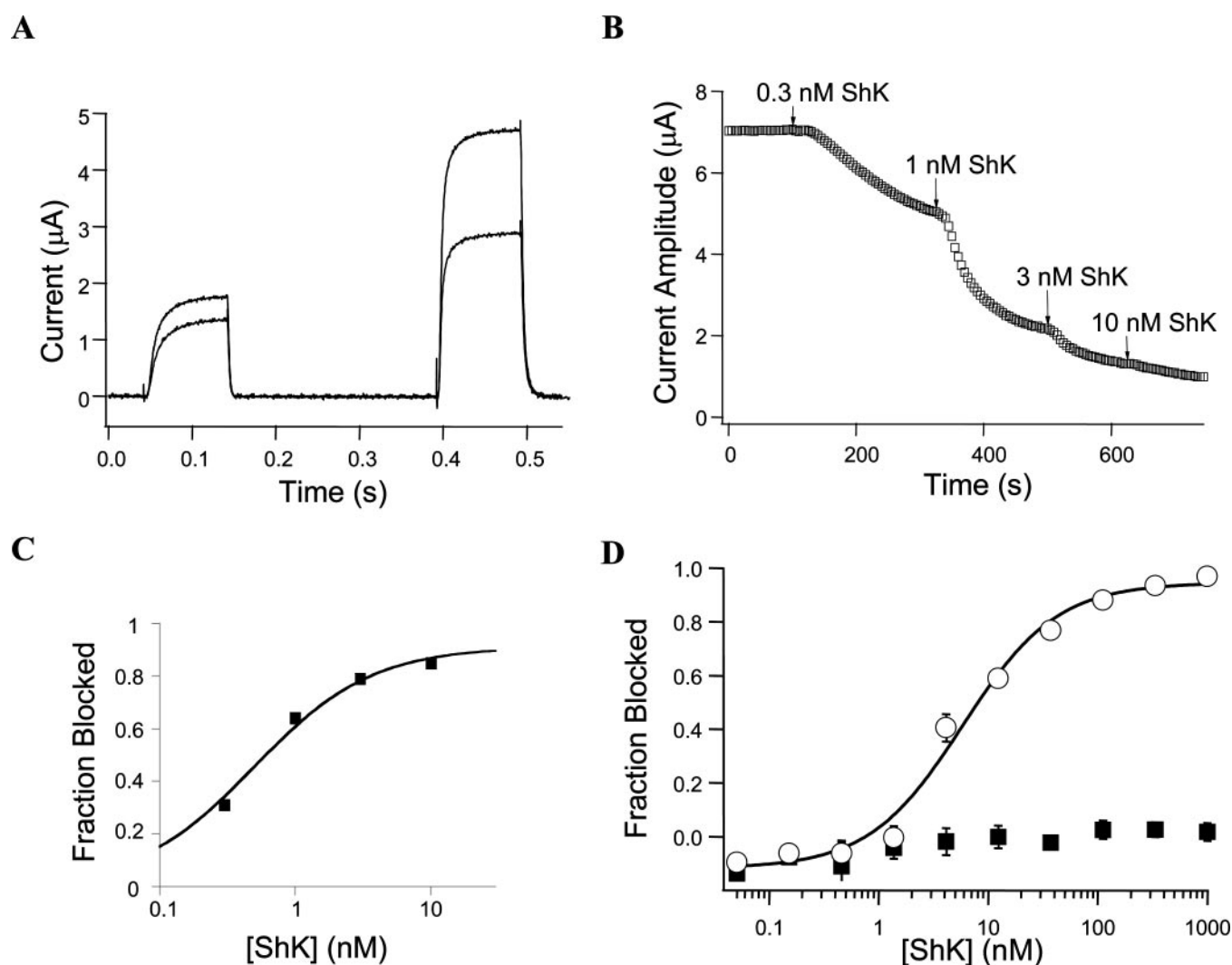**Fig. 3.** ShK inhibits hKv3.2b but not hKv2.1 channels. A, hKv3.2b currents expressed in *X. laevis* oocytes in response to depolarizations to +20 and +80 mV in control (black trace) and in 0.3 nM ShK (gray trace). B, time course of block of hKv3.2b currents, activated at +80 mV, by ShK. C, concentration-dependent inhibition of hKv3.2b currents by ShK for the cell shown in B. The solid line is a fit of the Hill equation with an apparent $IC_{50}$ value of 0.5 nM and a slope of 1. D, dose-response relationship for the inhibition of hKv3.2b (○) and Kv2.1 (■) by ShK studied with a planar patch-clamp device (IonWorks HT). The solid line is a fit of the Hill equation to the hKv3.2b data with an apparent $IC_{50}$ value of 6 nM and a slope of 1. In contrast, hKv2.1 was not blocked by 1 $\mu$M ShK.

displayed an $IC_{50}$ of 0.31 nM for channel inhibition, whereas in CHO-K1.hKv3.2b cells, the $IC_{50}$ was ~6 nM measured by planar patch-clamp electrophysiology. Taken together, these results suggest that ShK is a potent inhibitor of heterologously expressed hKv3.2b channels.

ShK is a potent inhibitor of certain members of the Kv1-channel family. In particular, ShK blocks Kv1.1 and Kv1.3 channels with $IC_{50}$ values of ~1 pM, a 1000-fold lower concentration than that required to inhibit Kv3.2 channels. Evidence also suggests that native Kv currents in the central nervous system, which are predominantly carried by Kv1.2 channels, are highly sensitive to the peptide (Middleton et al., 2003). Thus, the usefulness of ShK in discerning the physiological role of Kv3.2 channels must be considered with caution in preparations in which other ShK-sensitive channels may be present. This is particularly relevant to the central nervous system, given the large diversity of Kv channels that exist in different neurons and the wide distribution of Kv1 subunits. Nonetheless, in cells in which Kv1 channels are present, other Kv1 specific peptides, such as DTX, could be used to distinguish between Kv3- and Kv1-mediated effects of ShK. In other systems, in which channel distribution is more restricted and better defined, ShK provides a pharmacological tool for studying Kv3.2 channels. For instance, polymerase chain reaction, in situ hybridization, and immunostaining with specific Kv antibodies have provided strong evidence for the presence of Kv2.1- and Kv3.2-channel subunits in pancreatic β cells (MacDonald et al., 2002; Yan et al.,

2004). Because the association of Kv2.1 and Kv3.2 subunits has not been demonstrated either in vitro or in vivo, the delayed-rectifier Kv current in β cells is likely to be carried by channels that contain either Kv2.1 or Kv3.2 subunits. Defining the subunit composition of the delayed-rectifier current in β cells is important because this current regulates firing frequencies in the presence of glucose, and inhibitors of this current would be expected to enhance glucose-dependent insulin secretion and therefore have usefulness in the treatment of type-2 diabetes. ShK does not inhibit hKv2.1 currents, and because Kv1.1 subunits are not found in human pancreatic islets and functional Kv1.3 channels have not been reported in human β cells (MacDonald and Wheeler, 2003; Yan et al., 2004), the putative contribution of Kv3.2 subunits to the β-cell Kv current can be evaluated. Even when high concentrations of ShK were used, no inhibition of the human delayed-rectifier current in β cells was observed. Although these experiments strongly suggest that a homomultimeric Kv3.2 channel is not a component of the β cell delayed-rectifier current, it raises the question of why the channel subunit is expressed in these cells. It is possible that coexpression of Kv3.2 with other ShK-insensitive subunits could lead to a tetrameric complex insensitive to the peptide, and additional experiments are needed to evaluate this possibility.

Cortical GABAergic FS cells are named for their ability to fire sustained trains of brief action potentials at high frequency and, in fact, display the highest firing rates of any
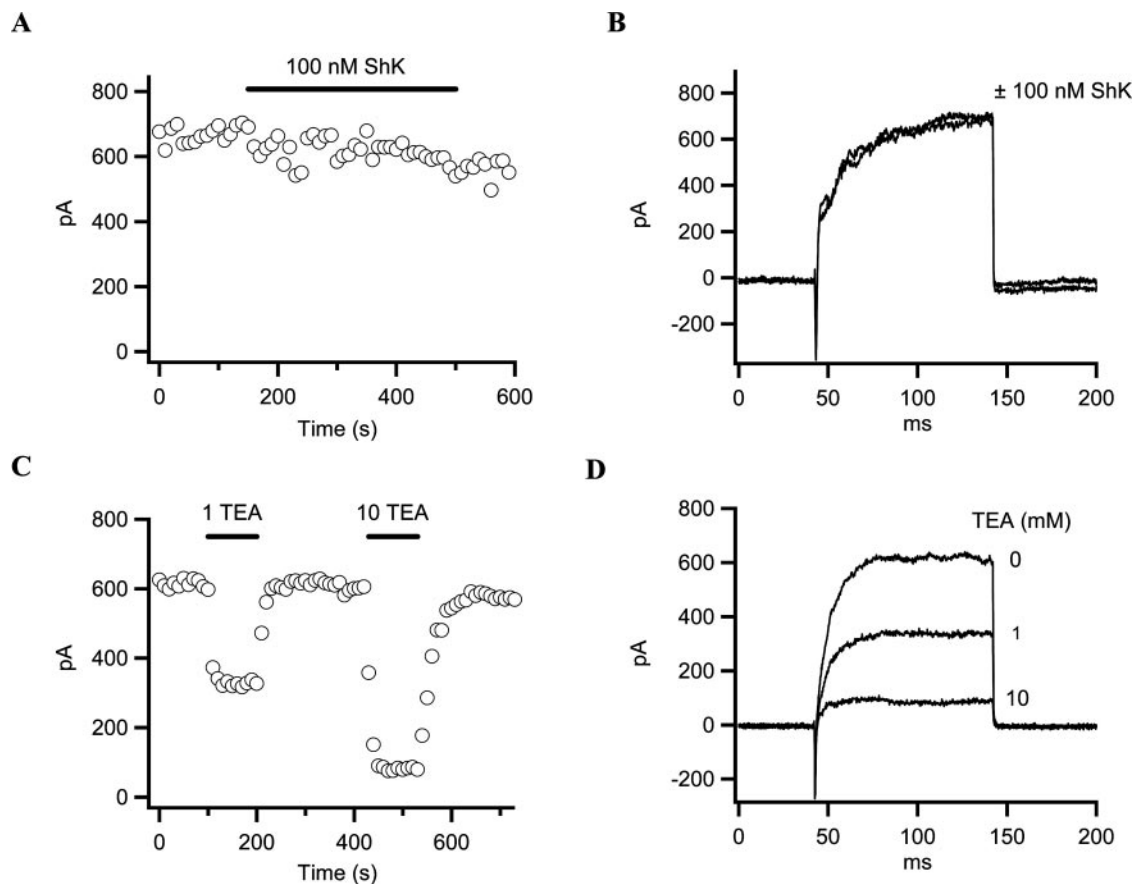


**Fig. 4.** ShK does not inhibit the Kv current of human pancreatic beta cells. A, plot of the peak current amplitude in response to 100-ms step depolarization to +20 mV from a holding potential of −80 mV versus time. The period of application of 100 nM ShK is denoted by the solid bar. B, representative current traces before and during application of ShK. C and D, effect of 1 and 10 mM TEA on peak Kv current for a different cell.

cortical cell (McCormick et al., 1985; Connors and Gutnick, 1990). Cortical FS cells express Kv3.1 and Kv3.2 subunits. A large body of evidence now exists to support the conclusion that the fast-spiking phenotype is, in fact, determined by the somatic expression of these Kv3 channels (Rudy and McBain, 2001; Lien and Jonas, 2003), which activate at depolarized potentials (relative to other Kv channels) and deactivate rapidly. Even at high firing frequencies, the effect of activation of Kv3.1/Kv3.2 channels is largely restricted to the re-

polarization phase of the action potential (Du et al., 1996), with little direct effect on the rate of rise or on spike threshold, and the interspike interval is established to a large degree by the accumulation of voltage-gated sodium-channel inactivation. However, by increasing the rate of spike repolarization (and keeping action potentials brief), Kv3.1/Kv3.2 channels limit sodium-channel inactivation. Moreover, the large, fast afterhyperpolarization elicited by Kv3.1/Kv3.2 currents accelerates recovery from inactivation of sodium
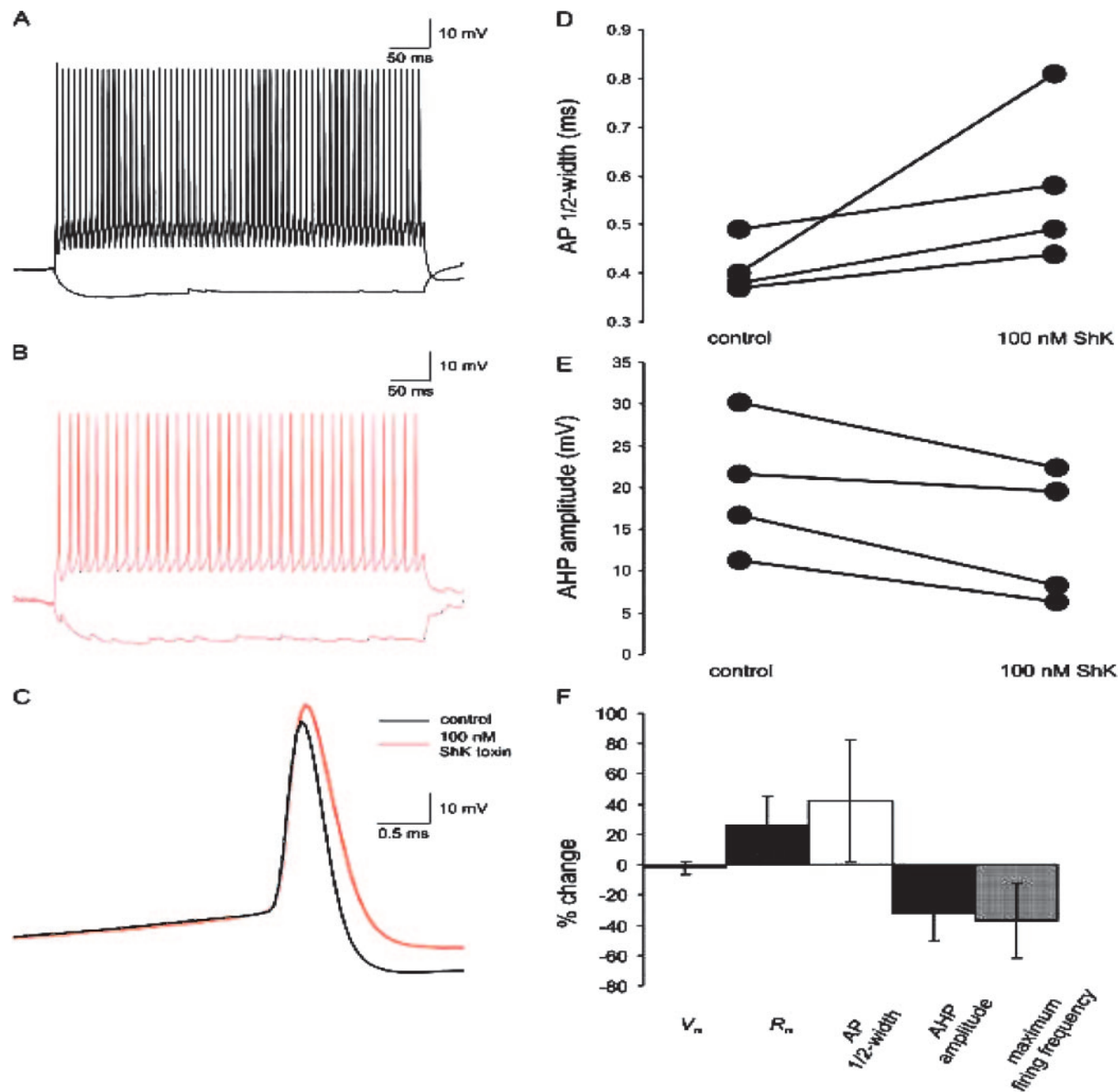


**Fig. 5.** The effects of ShK on neocortical FS cells are consistent with blockade of Kv3 channels. A, firing pattern of an FS cell in response to 300-ms hyper- and depolarizing current injections under control conditions. Note that the cell discharges with a sustained, nonadapting train of high-frequency action potentials. B, same cell after bath application of 100 nM ShK. C, the first action potential of the first suprathreshold sweep for control (black) and after bath application of 100 nM ShK (red). Note that ShK produces spike-broadening and attenuates the fast, deep afterhyperpolarization characteristic of the FS cell action potential. D, for all cells tested, bath application of 100 nM ShK produced an increase in the AP half-width (AP ½-width). E, same as D except for amplitude of the AHP (as measured from action potential threshold). F, summary data. Consistent with blockade of Kv3 channels, bath application of 100 nM ShK produced an increase in AP ½-width (from $0.41 \pm 0.05$ to $0.58 \pm 0.16$ ms; $p = 0.066$, $n = 4$), a decrease in the amplitude of the AHP (from $19.9 \pm 8.1$ to $14.1 \pm 8.0$ mV; $p = 0.028$, $n = 4$) and a decrease in maximum firing frequency (from $280 \pm 53$ to $181 \pm 93$ Hz; $p = 0.040$, $n = 4$).

channels (Rudy and McBain, 2001). Thus, Kv3.1/Kv3.2 channels ensure that sufficient sodium channels are available for a subsequent action potential, with little counteractive Kv current. The effects of pharmacological blockade or genetic elimination of Kv3.1/Kv3.2 channels has provided strong evidence that these channels are critical for high-frequency repetitive firing in FS cells (Martina et al., 1998; Erisir et al., 1999; Lau et al., 2000), as has a recent study using dynamic clamp technology (Lien and Jonas, 2003). In this context, the results of this study concerning the effect of ShK in FS cells are consistent with the proposed role for Kv3.2 channels and support the use of the peptide for studying these channels in other native preparations. Contribution of Kv1.1 and/or Kv1.3 channels to the observed effects of ShK in FS cells does not seem likely because DTX-I and ChTX, potent inhibitors of Kv1.1 and Kv1.3 channels, respectively, do not reproduce the effects of ShK on the shape of the FS cell action potential and on repetitive firing. In addition, Kv1.3 channels with their very slow recovery from inactivation would not be able to contribute to the fast firing frequency of FS cells.

A significant difference in the inhibitory potency of ShK on Kv3.2b channels was observed between *X. laevis* oocyte electrophysiology ($IC_{50} = 0.31$ nM) and planar patch-clamp electrophysiology on CHO-K1.hKv3.2b cells ($IC_{50} = 6$ nM) (Fig. 3, C and D). Indeed, $IC_{50}$ values for inhibition of $^{86}Rb^+$ flux from CHO-K1.hKv3.2b cells are closer to those obtained from electrophysiological experiments on oocytes. Although the reasons for the block affinity of ShK in the planar patch-clamp system are not fully understood, several possibilities, such as an incomplete equilibration and adsorption to the planar array caused by the high surface-to-volume ratio, could account for the observed differences.

In summary, we have provided evidence that ShK is a potent inhibitor of the hKv3.2b channel. Although much remains to be learned about the structural basis of the interaction between ShK and the Kv3.2 channel, it is likely that ShK will serve as an important pharmacological tool for the study of Kv3 family channels in native systems, particularly in those situations in which Kv1 channels are not present or can be selectively modulated.

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