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Synthesis of a Biotin Derivative of Iberitoxin: Binding Interactions with Streptavidin and the BK Ca^{2+} -activated K^{+} Channel Expressed in a Human Cell Line

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Abstract

Iberitoxin (IbTx) is a scorpion venom peptide that inhibits BK Ca^{2+} -activated K^{+} channels with high affinity and specificity. Automated solid phase synthesis was used to prepare a biotin-labeled derivative (IbTx-LC-biotin) of IbTx by substitution of Asp19 of the native 37-residue peptide with N-ε-(d-biotin-6-amidocaproate)-L-lysine. Both IbTx-LC-biotin and its complex with streptavidin (StrAv) block single BK channels from rat skeletal muscle with nanomolar affinity, indicating that the biotin-labeled residue, either alone or in complex with StrAv, does not obstruct the toxin binding interaction with the BK channel. IbTx-LC-biotin exhibits high affinity ($K_D = 26 \text{ nM}$) and a slow dissociation rate ($k_{\text{off}} = 5.4 \times 10^{-4} \text{ s}^{-1}$) in a macroscopic blocking assay of whole-cell current of the cloned human BK channel. Titration of IbTx-LC-biotin with StrAv monitored by high performance size exclusion chromatography is consistent with a stoichiometry of two binding sites for IbTx-LC-biotin per StrAv tetramer, indicating that steric interference hinders simultaneous binding of two toxin molecules on each of the two biotin-binding faces of StrAv. In combination with fluorescent conjugates of StrAv or anti-biotin antibody, IbTx-LC-biotin was used to image the surface distribution of BK channels on a transfected cell line. Fluorescence microscopy revealed a patch-like surface distribution of BK channel protein. The results support the feasibility of using IbTx-LC-biotin and similar biotin-tagged K^{+} channel toxins for diverse applications in cellular neurobiology.

Introduction

The human genome contains at least 75 different genes for K^{+} channel proteins as cataloged by the International Union of Pharmacology (Gutman et al., 2003). These membrane proteins are classified into 7 distinct subfamilies (K_V1-6 , K_V8 , K_V9 ; K_V7 ; K_V10-12 ; K_{Ca1} , K_{Ca4} , K_{Ca5} ; K_{Ca2} , K_{Ca3} ; K_2P ; K_{ir}) based on phylogenetic analysis of sequence similarity that reflects common structural relationships. From a parallel evolutionary perspective, at least 60 different peptide toxins that target K^{+} channels have thus far been discovered in scorpion venoms (Zhu et al., 2004). Sequence analysis of these toxin peptides has identified at least 12 subfamilies of scorpion K^{+} toxins that block various members of the K^{+} channel family in a complex pattern

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of selective and overlapping specificity (Tytgat et al., 1999; Rodriguez de la Vega et al. 2003). Evidently, over the course of evolution, scorpion species have waged a biological arms race to produce an ever more diverse array of venom peptide toxins that recognize a similarly diverse variety of K^+ channel proteins in the interplay of natural selection.

This natural peptide library constitutes an important resource for biomolecular engineering of derivatives of K^+ channel toxins with numerous basic and clinical applications. For example, fluorescent and biotin-labeled derivatives of such peptides have already been used for cellular localization, flow cytometry analysis, and single-molecule microscopy of various K^+ channels (Roibtaille et al., 1993; Schütz et al., 2000; Pragl et al., 2002; Jimenez-Gonzalez et al., 2003; Beeton et al., 2004; Hafadi et al., 2005). The growing database of X-ray crystal structures of K^+ channel proteins (MacKinnon, 2003; Gulbis and Doyle, 2004) and NMR structures of K^+ toxins (Bontems et al., 1992; Johnson and Sugg, 1992; Renisio et al., 1999; Rodriguez de la Vega et al., 2003) provides a unique vantage point for molecular modeling and design of toxin derivatives engineered for specific applications.

Converting a collection of natural peptide toxins into a practical toolbox of molecular probes requires concerted efforts to develop optimal methods for synthesis and pharmacological assay of activity, affinity, and stability of the designed molecules. Toward this goal we have synthesized and characterized a biotin-derivative of iberitoxin (IbTx¹), a toxin from the Indian red scorpion, *Mesobuthus tamulus* (formerly known as *Buthus tamulus*) (Galvez et al., 1990; Strong et al., 2001), for use as a probe in molecular and cellular studies of the large conductance Ca^{2+} -activated K^+ channel ($K_{Ca1.1}$). $K_{Ca1.1}$ is also called the BK channel, the maxi K_{Ca} channel, or HSlo, the latter term designating the human α -subunit protein that forms the channel tetramer (Wallner et al., 1995). The BK channel is synergistically activated by membrane depolarization and intracellular Ca^{2+} , and is known to function in neurotransmitter release at nerve terminals, regulation of smooth muscle contractility, and auditory functions of cochlear hair cells (Toro et al., 1998; Orio et al., 2002; Hafadi et al., 2005).

IbTx (α KTx1.3) is a 37-residue peptide with three disulfide bonds that belongs to the same K^+ toxin subfamily as charybdotoxin (ChTx or α KTx1.1), an extensively studied model of K^+ toxin-channel interaction (Galvez et al., 1990; Miller, 1995; Tenenholz et al., 2000). Whereas ChTx blocks various K^+ channels of both K_V and K_{Ca} gene families, IbTx appears to be a specific, high-affinity inhibitor of HSlo and other mammalian BK channels (Galvez et al., 1990; Rodriguez de la Vega et al., 2003). Extensive studies of the structure-activity relationships of ChTx, IbTx, K_V channels, BK channels, and the prokaryotic K^+ channel, KcsA, have led to a detailed model of the complex of IbTx docked to the external vestibule of the BK channel (Lucchesi et al., 1989; Giangiacomo et al., 1993; Goldstein et al., 1994; Stampe et al., 1994; Doyle, D. A. et al., 1998; MacKinnon et al., 1998; Mullmann et al., 1999; Cui et al., 2001; Gao and Garcia, 2003; Yu et al., 2005). Guided by this trove of information, we describe a direct method for synthesizing IbTx-LC-biotin and characterize its biochemical interactions with respect to block of BK channels, binding to streptavidin (StrAv), and cellular imaging of BK channels by fluorescence microscopy.

Experimental Procedures

Materials

Streptavidin, Alexa488-streptavidin, Alexa488-anti-biotin antibody, and biotin-4-fluorescein were obtained from Molecular Probes (Eugene, OR). The Fmoc derivatives of N- ϵ -(d-biotin-6-

¹Abbreviations: BK, large conductance Ca^{2+} -activated K^+ channel; ChTx, charybdotoxin; DIC, differential interference contrast microscopy; IbTx, iberitoxin; HPLC, high pressure liquid chromatography; HPSEC, high performance size exclusion chromatography; HSlo, human BK channel; IbTx-LC-biotin, iberitoxin-D19K-LC-biotin; TFA, trifluoroacetic acid, AcM; acetamidomethyl

amidocaproate)-L-lysine and S-acetamidomethyl-L-cysteine were purchased from Anaspec (AnaSpec, San Jose CA).

Synthesis and Purification of IbTx-LC-biotin

Automated peptide synthesis based on the linear sequence of IbTx was performed via solid phase Fmoc coupling chemistry by the Keck Peptide Facility at Yale Medical School (New Haven, CT). In place of the native Asp residue at position 19, N-ε-(d-biotin-6-amidocaproate)-L-lysine was substituted to produce the toxin derivative illustrated in Figure 1. Cleavage from the support resin and removal of protecting groups was accomplished using trifluoroacetic acid (TFA). Molecular mass determined by MALDI MS for the reduced product after cleavage, ether extraction and lyophilization was MH^+ 4588.0 Da vs. the theoretical monoisotopic mass of 4587.1 Da. A portion of the crude product (20 mg) was sonicated in 40 ml of buffer (2 M urea, 0.1 M NaCl, 0.1 M glycine, pH 7.8) and allowed to oxidize in air by stirring at 22° C for 18 hours. Final purification was achieved by C18 reverse-phase HPLC first on a semi-preparative column followed by a narrow-bore Vydac C18 column both using a linear separation gradient of 0.01% TFA/Aq. to 60% CH₃CN in 0.08% TFA/Aq over 60 min. UV absorption was monitored at 223 nm. Electrospray mass spectroscopy of pure oxidized peptides was performed on a Thermo Finnigan spectrometer. Peptide concentration of IbTx-LC-biotin was determined from integrated area of the HPLC peak (absorbance monitored at 280 nm) of the sample relative to that of a reference standard of synthetic IbTx previously quantified by amino acid analysis.

IbTx-D19C-Acm was synthesized in a similar manner by automated Fmoc solid phase chemistry. In place of the native Asp residue at position 19, S-acetamidomethyl-L-cysteine was substituted to produce the toxin derivative. IbTx-D19C-Acm was oxidized, purified and quantified, as described above.

Planar Bilayer Assay

Single-channel recording of BK channels from rat skeletal muscle in planar lipid bilayers was performed as previously described (Favre and Moczydlowski, 1999). The recording solution was 200 mM KCl, 10 mM Mops-KOH, pH 7.4 on both sides of the bilayer, with 0.2 mM CaCl₂ on the intracellular side and 0.1 mM EDTA plus 0.1 mg/ml bovine serum albumin on the extracellular side as defined by native asymmetry of the channel.

Whole-Cell Electrophysiology

HEK293 transformed human embryonic kidney cells were stably transfected using G418 selection with a pcDNA3 vector encoding a His₆-Flag-epitope-tagged version of the human BK channel (HSlo) originally cloned by Wallner et al. (1995). The tagged version of HSlo (kindly supplied by Dr. Andrew Tinker, University College London) contained the N-terminal tag sequence, MVHHHHHHHDYKDDDDK, linked directly to the second amino acid (D2) of the native HSlo sequence. HEK293/HSlo cells were grown in DMEM medium (Gibco) supplemented with 10% fetal bovine serum, 10,000 U/ml penicillin/streptomycin (Gibco), and 0.6 mg/ml Geneticin (G418, from Gibco) at 37° C in a humidified atmosphere of 5% CO₂ in air. Cells were seeded on small chips of glass cover slips coated with polylysine 2-4 days prior to patch-clamp recording.

Whole-cell patch recording was performed using a low volume cell recording chamber (RC24E, Warner Instruments) mounted on a Nikon TE200 inverted microscope. The recording chamber was continuously perfused at 0.5 ml/min with control and various bath solutions containing toxins and inhibitors. Patch pipettes with a resistance of 1-2 MΩ were fabricated from glass capillaries (Kimax-51, Fisher Scientific) with a PP-83 puller (Narishige). Whole-cell current was measured at room temperature with an Axon 200B amplifier controlled by

PC-based Clampex software (Axon Instruments) for voltage pulse delivery and digital current acquisition. Currents were low pass filtered at 5 kHz and corrected for capacitance transients and leak current using a negative P/5 pulse protocol. The composition of standard pipette (intracellular) solution with 500 nM free Ca^{2+} (Strobaek et al., 1996) was 150 mM KCl, 1 mM MgCl_2 , 1 mM CaCl_2 , 1.3 mM EGTA, 10 mM Hepes-KOH, pH 7.2. The bath solution contained 145 mM NaCl, 5 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM Hepes-KOH, pH 7.4 and various peptide toxins or 1 μM paxilline (Sigma) as described.

Streptavidin Binding Interaction

Streptavidin (StrAv) concentration was standardized by the method of Kada et al. (1999) based on fluorescence quenching of biotin-4-fluorescein (e.g., Fig. 6A), a ligand known to bind stoichiometrically to four sites of StrAv and avidin with high affinity. Sequential additions (5 nM) of biotin-4-fluorescein were made to a stirred cuvette containing ~11 nM StrAv in 100 mM NaCl, 50 mM Hepes-NaOH, pH 7.3. Fluorescence emission spectra excited at 484 nm were recorded five minutes after each addition of biotin-4-fluorescein using a QuantaMaster-4 spectrofluorimeter (Photon Technology International, Lawrenceville, NJ). The concentration of biotin-4-fluorescein was based on the reported extinction coefficient of $68,000 \text{ M}^{-1}\text{cm}^{-1}$ at 494 nm (Haugland, 2002).

Complex formation between standardized IbTx-LC-biotin and StrAv was analyzed by high performance size-exclusion chromatography using a Phenomenex BioSep-SEC-S 2000 column ($300 \times 7.5 \text{ mm}$) with buffer delivery and detection controlled by a Waters Alliance 2695 HPLC system equipped with a 995 photodiode array detector (wavelength scan at 200-400 nm). The column was equilibrated with buffer (100 mM NaCl, 50 mM Hepes-NaOH, pH 7.3) and run isocratically at a flow rate of 0.5 ml/min. Dextran blue (~2,000 kDa) and uridine (244.2 Da) were used to determine the void volume and column volume, respectively. Fixed samples of IbTx-LC-biotin (250 pmol) were incubated for 24 hours in separate vials containing increasing amounts of StrAv ranging from 0 to 375 pmol in 18 μl of running buffer. Each sample was separately injected on the column and monitored by absorbance at 280 nm. Binding of IbTx-LC-biotin (5 kDa molecular mass) to StrAv (52.8 kDa molecular mass) was followed by the decrease in area of the IbTx-LC-biotin peak and appearance of peaks corresponding to bound and free StrAv eluting at a higher mass range as observed upon increasing addition of StrAv (e.g., Fig. 5).

Fluorescence Microscopy

Native HEK293 cells and stably transfected HEK293/HSlo cells were grown either in tissue culture flasks for labeling of cells in suspension or on cover slips coated with poly-D-lysine in a 12-well culture plate for attached-cell labeling. Cells grown on cover slips were washed once with 125 mM NaCl, 25 mM NaP_i , pH 7.4 (PBS) followed by an optional endogenous biotin-blocking protocol following kit instructions (E-21390) from Molecular Probes. Cover slips were next incubated in PBS containing 1 mg/ml bovine serum albumin (BSA) and 50 to 100 nM IbTx-LC-biotin for 30 minutes at room temperature and then washed three times in PBS. This step was followed by incubation in the dark for 20 minutes at room temperature in the same buffer containing either 3 $\mu\text{g/ml}$ Alexa488-StrAv or 4 $\mu\text{g/ml}$ Alexa488-anti-biotin antibody. A final wash step, three times in PBS, was performed before observing cells using a Nikon Eclipse TE2000-E inverted epifluorescence microscope. Incubation and wash conditions were similar for labeling dissociated cells removed from tissue culture flasks by gentle pipetting except that the washing steps were performed by low-speed microcentrifugation. Labeled cells were either observed directly or after fixation with 2% formaldehyde in PBS for 20 minutes followed by washing in PBS and mounting on glass slides using VectaShield mounting media (Vector Laboratories). Digital Images were captured using with a Hamamatsu Orca CCD camera and IPLab acquisition software (Scanalytics).

Results

Design and Synthesis of a Biotin-derivative of IbTX

Similar to other α KTx1 toxins such as ChTx and Lq2 (Bontems et al., 1992; Renisio et al., 1999), the three-dimensional solution structure of IbTx (sequence given in Fig. 1A), is characterized by a short α -helix at residues 13-21 and a two-stranded antiparallel β -sheet comprised of residues 25-36 (Johnson and Sugg, 1992). Three disulfide bonds between Cys residues 7-28, 13-33, and 17-35 tightly constrain the structure of α KTx1 toxins and render them quite resistant to denaturation by heating and other treatments in the folded, oxidized state (Smith et al., 1986). Structure-activity studies including mutagenesis, x-ray crystallography, NMR, Brownian dynamics simulation, and molecular modeling approaches have yielded a rather detailed picture of the probable molecular basis of interaction of α KTx1 toxins with various K^+ channels (Goldstein et al., 1994; Stampe et al., 1994; Doyle et al., 1998; Tenenholz et al., 2000; Cui et al., 2001; Schroeder et al., 2002; Gao and Garcia, 2003; Yu et al., 2005). Binding of α KTx1 toxins is primarily determined by molecular interactions between critical residues that lie on the β -sheet face of the toxin and particular residues within the outer vestibule of K^+ channels. K^+ channel residues critical for toxin binding lie either in the “turret” formed by an extracellular linker between the outer membrane helix (M1 or S5) and the so-called pore helix, or in a second extracellular linker between the selectivity filter and the inner membrane helix (M2 or S6) (MacKinnon et al., 1998; Gao and Garcia, 2003). Eight residues predicted to lie on the interaction surface of IbTX (S10, W14, R25, K27, M29, G30, R34, Y36) are marked with asterisks in Fig. 1A. Modification of these latter residues typically lowers the affinity of the α KTx1 toxin-channel interaction, requiring the identification of other non-critical residues for toxin derivatization.

Based on the observation that certain residue substitutions on the toxin surface opposite to the channel interaction surface do not interfere with blocking activity, Shimony et al. (1994) identified residue R19 near the C-terminal end of the α -helical region of charybdotoxin as a favorable site for derivatization. Using a “spinster cysteine” strategy, *N*-[3 H]ethylmaleimide derivatives of the R19C mutant of charybdotoxin and a D19C mutant of IbTx have previously been developed as radioligands used in binding assays for Shaker and BK K^+ channels (Shimony et al., 1994; Sun et al., 1994; Knaus et al., 1996).

We followed a similar approach in this work by substituting N- ϵ -(d-biotin-6-amidocaproate)-L-lysine [Lys(ϵ -d-LC-biotin)] in place of native residue D19 of IbTx in automated solid phase synthesis of IbTx-LC-biotin. Figure 1B illustrates the chemical structure of the unnatural amino acid side chain substituted at residue 19 of IbTx-LC-biotin and also denotes the ~ 15 Å chemical linker between the α -carbon atom of the peptide backbone and the carboxy tail of the biotin moiety that is virtually buried upon binding to streptavidin (Henrickson et al., 1989). Lys(ϵ -d-LC-biotin) was selected as the replacement residue in order to evaluate the use of biotin-avidin labeling technology for biochemical and cellular studies of BK channels. As illustrated in Fig. 1C, a plausible extended conformation of a molecular model of IbTx-LC-biotin shows that the biotin-tagged side chain is physically well separated from the channel-interaction surface of the toxin. We expected that the relatively long linear linker of this particular biotin-amino acid derivative (17 atoms from the peptide backbone to the biotin ring) may facilitate unhindered binding of streptavidin or avidin to the toxin-channel complex.

After automated synthesis and cleavage from the resin, the crude peptide product was solubilized in folding buffer at pH 7.8, allowed to oxidize in air for 18 h, and analyzed by reverse phase HPLC as described in Experimental Procedures. The HPLC chromatogram exhibited a broad peak of heterogeneous product superimposed upon a major sharp peak at 32 minutes that comprised about $\sim 5\%$ of the total material (Fig. 2, top trace). Purification of this latter peak yielded about ~ 1 mg of pure IbTx-LC-biotin per 100 mg of the crude synthetic

product (Fig. 2A, lower trace). Electrospray mass spectroscopy of the latter peak confirmed the identity of oxidized IbTx-LC-biotin, with an observed deconvoluted mass of 4584.1 Da (Fig. 2B) compared to a theoretical average mass of 4584.5 Da predicted for the sequence in Fig. 1A with three disulfide bonds.

Electrophysiological Assays of BK Channel Block by IbTX-biotin

We first investigated the biological activity of synthetic IbTx-LC-biotin by the planar bilayer assay method (Miller et al., 1985; Smith et al., 1986; Lucchesi et al., 1989). Addition of IbTx-LC-biotin to the external side of bilayers containing single BK channels resulted in the appearance of discrete long-lived blocked states characteristic of α KTx1 toxins such as ChTx, Lq2, and IbTx (Miller et al., 1985; Lucchesi et al., 1989; Candia et al., 1992; Giangiacomo et al., 1992). As illustrated in Fig. 3A, under control conditions (200 mM symmetrical KCl, 0.2 mM internal Ca^{2+} , holding voltage = +30 mV) in the absence of toxin, typical current records of a single BK channel from rat skeletal muscle exhibit a high probability of opening with the majority of brief closures lasting less than 1 s. Long silent periods ranging from seconds to minutes observed after addition of 4 nM IbTx-LC-biotin to the external side of a single BK channel (Fig. 3B) correspond to blocking events or residence times of single toxin molecules similar to those produced by native IbTx (Candia et al., 1992; Giangiacomo et al., 1992). To test whether IbTx-LC-biotin retains blocking activity after complex formation with StrAv, we preincubated IbTx-LC-biotin with a 4-fold excess of StrAv tetramer and tested the mixture on single BK channels. As illustrated in Fig. 3C, single channel records in the presence of 4 nM complex of IbTx-LC-biotin /StrAv exhibit long-lived blocked events similar to those produced by IbTx alone, demonstrating that the toxin-StrAv complex is fully capable of binding to the toxin receptor site on BK channels.

Single-channel analysis of IbTX-blocking kinetics is technically rather difficult due to the long duration of IbTx-blocked state events; however, we estimated kinetic parameters with limited sets of blocking events pooled from several single-channel bilayers using a standard analysis for a 1-site blocking reaction (Moczydlowski, 1992; Favre and Moczydlowski, 1999). For IbTx-LC-biotin, this analysis yields the following kinetic constants: $k_{\text{off}} = 4.7 \times 10^{-3} \text{ s}^{-1}$ (dissociation rate constant), $k_{\text{on}} = 1.9 \times 10^6 \text{ s}^{-1}\text{M}^{-1}$ (association rate constant), and $K_D = 2.5 \text{ nM}$ (equilibrium dissociation constant). The corresponding values estimated for the complex of IbTx-LC-biotin and StrAv are: $k_{\text{off}} = 8.5 \times 10^{-3} \text{ s}^{-1}$, $k_{\text{on}} = 3.4 \times 10^6 \text{ s}^{-1}\text{M}^{-1}$, and $K_D = 2.5 \text{ nM}$. Although these kinetic parameters have large uncertainties in the range of 12-60%, they imply that the complex of IbTx-LC-biotin and StrAv has somewhat faster dissociation and association rates in comparison to IbTx-LC-biotin, but retains an equilibrium binding affinity in the low nanomolar range, similar to that of native IbTx (Candia et al., 1992; Giangiacomo et al., 1992).

We next investigated the effect of IbTx-LC-biotin in a macroscopic assay of whole-cell currents of the cloned human BK channel (HSlo) stably expressed in the HEK293 cell line. Using an intracellular solution containing 500 nM free Ca^{2+} , BK currents are activated at voltages more positive than +40 mV under these conditions (Fig. 4A, B). Greater than 95% of outward K^+ current recorded in this cell line is sensitive to inhibition by 1 μM paxilline (Fig. 4A, B), a potent inhibitor of mammalian BK channels (Knaus et al., 1994; Strobaek et al., 1996). Addition of 200 nM IbTx-LC-biotin to the bath solution substantially inhibits BK current (Fig. 4A, B) and such inhibition is slowly reversed by perfusion with toxin-free solution (Fig. 4C). The titration curve of steady-state inhibition of peak outward BK current by IbTx-LC-biotin is well described by one-site behavior with a K_D of 26 nM (Fig. 4D). By fitting the time course of development of IbTx-LC-biotin and washout with a single exponential and assuming one-site kinetics (Fig. 4C), we obtained values of apparent rate constants, $k_{\text{on}} = 9.9 \times 10^4 \text{ s}^{-1}\text{M}^{-1}$ and $k_{\text{off}} = 5.1 \times 10^{-4} \text{ s}^{-1}$, which are respectively, 19-fold and 9-fold slower than the

corresponding rate constants measured for the bilayer experiments of Fig. 3. Inhibition of whole-cell BK current by native ChTx in this assay system exhibited a K_D value of 13 nM (Fig. 4D), and apparent rate constants of $k_{on} = 1.3 \times 10^5 \text{ s}^{-1}\text{M}^{-1}$ and $k_{off} = 3.2 \times 10^{-3} \text{ s}^{-1}$ (Fig. 4C). These results demonstrate that synthetic IbTx-LC-biotin is a potent inhibitor of the cloned HSlo BK channel with a slower dissociation rate than charybdotoxin, as originally observed for blocking interactions of the native toxins (Candia et al., 1992; Giangiacomo et al., 1992; Stampe et al., 1994).

Binding Interaction of IbTx-LC-biotin with Streptavidin

To characterize complex formation between IbTx-LC-biotin and StrAv, a fixed amount of IbTx-LC-biotin (250 pmol) was preincubated for 24 hours with increasing amounts of tetrameric StrAv ranging from 0 to 375 pmol, and the resulting samples were analyzed by size exclusion chromatography. Superimposed absorbance profiles of the separate chromatography runs from this experiment are shown in Figure 5. In the absence of StrAv (mass $\approx 52,800 \text{ kDa}$), IbTx-LC-biotin (mass = 4.6 kDa) runs as a single peak (22.2 minutes) that elutes near the total column volume (12.4 ml; 24.8 minutes; 0.5 ml/min flow rate). Preincubation of the synthetic toxin with a low amount of StrAv results in a decrease in the absorbance of peak corresponding to the free toxin and the appearance of a higher mass peak (at 17.2 minutes) that corresponds to complex(es) of IbTx-LC-biotin and StrAv. As shown in Figure 5, at a molar ratio greater than 0.5 mole StrAv tetramer per mole IbTx-LC-biotin, a new peak is observed corresponding to the elution time (18.1 minutes) of free, uncomplexed StrAv. This observation indicates that the StrAv tetramer binds a maximum of two molecules of IbTx-LC-biotin under the conditions of this experiment in comparison to the theoretical capacity of four molecules corresponding to the four known biotin sites on the tetrameric protein (Hendrickson et al., 1989; Green, 1990). This surprising result was verified by measuring the concentration of total biotin binding sites in the commercial StrAv preparation using a method based on fluorescence quenching of biotin-4-fluorescein (Kada et al., 1999). As shown in Fig. 6A, this method reliably gives the total number of biotin binding sites in a StrAv sample from the break point of the fluorescence titration with biotin-4-fluorescein. Based on the standardized concentration of StrAv tetramer, titration experiments of IbTx-LC-biotin and StrAv (Fig. 5) clearly demonstrate an equivalence point of two binding sites for IbTx-LC-biotin per molecule of StrAv tetramer (Fig. 6B).

This result implies that binding of one molecule of IbTx-LC-biotin sterically interferes with binding of another IbTx-LC-biotin molecule on the StrAv tetramer. In fact, such “anti-cooperative” binding has been previously observed for titration of StrAv with certain long chain derivatives of biotin-fluorescein (Gruber et al., 1998). This unusual behavior is attributed to the particular three-dimensional relationship of biotin binding sites in StrAv. The quaternary structure of StrAv tetramer consists of a dimer of dimers, with two adjacent biotin sites on neighboring StrAv monomers located within $\sim 19 \text{ \AA}$ of each other on each of two separate faces of the tetramer (Hendrickson et al., 1989; Green, 1990; Freitag et al., 1997). Biotin derivative molecules with long chain linkers have a propensity to interfere with pairwise binding of each other across the two separate binding faces of the tetramer. In the present situation, the potential likelihood of this behavior can be appreciated by comparing the space filling models of StrAv and IbTx-LC-biotin (Fig. 6D). The molecular models show that the dimensions of IbTx-LC-biotin are comparable to the distance of separation of two biotin binding sites across the binding face of the tetramer. Steric interference or physical occlusion of an unoccupied site by a toxin molecule bound to an adjacent site may explain the 2:1 binding stoichiometry observed for titration of IbTx-LC-biotin with StrAv. Figure 6A also shows that preincubation of 11 pmol of StrAv with 3 equivalents (33 pmol) of IbTx-LC-biotin is sufficient to completely block binding of biotin-4-fluorescein to StrAv as shown by the lack of a detectable linear region of quenched fluorescence. This latter result implies that binding of IbTx-LC-biotin also interferes with binding of the smaller biotin-4-fluorescein molecule to an adjacent biotin site on StrAv.

Cellular Imaging of BK channels with IbTx-LC-biotin

The interactions with StrAv and BK channels described above suggest that IbTx-LC-biotin may be useful for studies of the cellular localization of BK channels by fluorescence microscopy. To evaluate IbTX-LC-biotin as an imaging probe, HEK293 cells stably expressing a version of the human BK channel, HSlo, were sequentially incubated with 100 nM IbTx-LC-biotin and 10 μ g/ml StrAv conjugated with Alexa488, a fluorophore with spectral properties similar to fluorescein (Panchuk-Voloshina et al., 1999). The labeled cells were washed, fixed, and examined with a 100 $\times$ oil immersion objective in an inverted fluorescence microscope. As shown by comparison of fluorescence and DIC (differential interference contrast) imaging of cells on processed coverslips, virtually all of the HEK293/HSlo cells exhibited intense fluorescence on the perimeter of the plasma membrane (Fig. 7A,C) consistent with the G418 antibiotic selection procedure for growth of cells stably transfected with the pcDNA3 vector containing the HSlo gene. In contrast, the plasma membrane of control untransfected HEK293 cells processed for imaging in an identical manner (Fig. 7B, D) showed only faint and diffuse background fluorescence, consistent with weak non-specific adsorption of the labeling reagents and the fact that this fibroblast-like cell line has very low levels of endogenous BK current (Yu et al., 1998).

Analysis of 0.5 μ m Z-sections of single cell images by fluorescence deconvolution enhancement showed that virtually all the fluorescence signal in HEK293/HSlo cells is localized to the cell surface, consistent with membrane impermeability and lack of cellular uptake of the imaging reagents under the labeling conditions. Supplementary Figure 1 is a movie file that shows a series of consecutive two-dimensional 0.5 μ m Z-sections through a single HEK293/HSlo cell labeled with IbTX-LC-biotin/Alexa488-StrAv, imaged by fluorescence microscopy, and processed by deconvolution enhancement. This visualization method reveals a non-random distribution of fluorescence on the cell plasma membrane. Supplementary Figures 2A-C are movie files of HEK293/HSlo cells imaged in a similar fashion, reconstructed in three-dimensions by software, and displayed by rotation of the reconstructed fluorescence image of the cell or cell pairs. These data also show a punctate-like distribution of fluorescence on the cell surface. A similar patch-like surface distribution of BK channels was also observed by processing fixed HEK293/HSlo cells for fluorescence microscopy with an anti-Flag antibody that recognizes the extracellular Flag epitope on the tagged HSlo channel (not shown). Thus the patch-like surface distribution of BK channels observed by IbTX-LC-biotin labeling is not a peculiar feature of the toxin-labeling method. The patch-like distribution of BK channels revealed in these experiments indicates that cellular mechanisms operating in this transfected cell line result in preferential clustering of BK channels on the membrane surface. Clustering and co-localization of BK channels with various signal-transduction proteins has previously been observed described in diverse cell types (Alioua et al., 2002; Eghbali et al., 2003; Liu et al., 2004) and is thought to be an important mechanism for coupling of BK channel activity to specific cellular functions.

In certain histochemical applications, StrAv-based labeling techniques are prone to background signals due to non-specific adsorption and endogenous biotin (Haugland, 2002). To test for this possibility we used a commercial biotin-blocking procedure according to the manufacturer's instructions (Molecular Probes; Eugene, OR), but found that this treatment made little difference in the background fluorescence (not shown). To further examine the specificity and versatility of IbTx-LC-biotin imaging, a number of additional control experiments were performed. As shown in Fig. 8A and 8B, fluorescence labeling of HEK293/HSlo cells by 50 nM IbTx-LC-biotin and 3 μ g/ml Alexa488-StrAv is effectively blocked by 5 μ M IbTX-D19C-Acm, a non-biotinylated derivative of IbTx. This result shows that excess untagged IbTx effectively competes with BK channel labeling by IbTx-LC-biotin, demonstrating that the fluorescence signal is specific for IbTx-binding sites. The latter

experiment was performed with unfixed cells demonstrating potential utility in live cell applications where fixation treatments are to be avoided. Figures 8C and 8D show results of a similar control experiment where IbTx-LC-biotin was simply omitted in the labeling procedure, again demonstrating specificity for the BK channel toxin. As an alternative to the use of StrAv, the experiment of Figs. 8E and 8F shows that a commercially available monoclonal antibody, Alexa488-antibiotin antibody, is also effective as a secondary fluorescent reagent in visualizing IbTx-LC-biotin bound to BK channels expressed on the cell surface.

Discussion

The growing inventory of natural K⁺ channel peptide toxins discovered in venoms of insects, scorpions, spiders, sea anemones, cone snails, and snakes has motivated efforts to develop radioactive and fluorescent derivatives of these molecules for applications in biochemistry and neuroscience. K⁺ channel toxins from scorpions are attractive candidates for probe development due to their small size (< 40 residues) and selective affinity for particular members of the K⁺ channel family. Peptide toxins and mutant toxin derivatives can be readily produced by synthetic gene technology and recombinant expression in *E. coli* (Park et al., 1991; Koschak et al., 1997); however, this approach involves numerous steps required to express, cleave, and purify the recombinant toxins. Alternatively, numerous disulfide-linked toxins such as ChTx and IbTx have also been produced by automated peptide synthesis (e.g., Sugg et al., 1990; Toth et al., 1995). The latter methodology permits direct incorporation of non-native amino acids during linear synthesis of the peptide as illustrated here by substitution of Lys-ε-d-LC-biotin at position 19 of the IbTx sequence. Biotin tags or fluorescent labels may also be coupled to peptide toxins by appropriate chemical modification reactions [(e.g., alkylation of the thiol group of a spinster cysteine (Shimony et al., 1994)] after synthesis and oxidation of paired disulfides. However, a practical coupling strategy requires high-yield labeling reactions and efficient purification of the bio-active derivative. Improvements in biomolecular engineering; i.e., design, synthesis, and labeling of K⁺ channel toxins, are needed to make these promising molecular tools more widely available to researchers in the field of ion channel biology.

Interesting findings previously reported with ChTx labeled with a biotin moiety at the N-terminus (Robitaille et al., 1993), IbTx randomly labeled with rhodamine red succinimidyl ester at native Lys residues (Jimenez-Gonzalez et al., 2003), or IbTx-D19C labeled with Alexa488 (Hafidi et al., 2005) warrant further development of ChTx and IbTx derivatives for fluorescence imaging applications in native cells and tissues. In this respect, the goal of the present work was to synthesize a derivative of IbTx with a biotin tag at a “backside” residue, and to characterize its relevant activities under defined model conditions. The results indicate that synthetic IbTx-LC-biotin avidly binds to mammalian BK channels as demonstrated by its blocking activity against rat muscle BK channels in planar bilayers (Fig. 3) and human BK channels expressed in a cultured cell line (Fig. 4).

Previous studies have shown that measured equilibrium constants for block and binding of charybdotoxin and IbTx are strongly dependent upon the ionic strength of the extracellular assay medium (Anderson et al., 1988; Garcia-Calvo et al., 1991; Vasquez et al., 1989; Koschak et al., 1997) and the association of particular isoforms of four known accessory beta subunits with the BK channel alpha subunit in native tissues (Hanner et al., 1997; Wallner et al., 1999; Giangiacomo et al., 2000; Meera et al., 2000). For example when the BK channel is coexpressed with the β1 subunit, the dissociation rate of ChTx is slowed by ~10-fold (Hanner et al., 1997; Giangiacomo et al., 2000). Thus, the performance of IbTx-LC-biotin in particular imaging applications may be expected to be strongly dependent upon the cellular preparation and the specific conditions of labeling. In transfected HEK293 cells, we find that incubation of cells with 50 nM IbTx-LC-biotin is sufficient to obtain high quality fluorescence imaging

(Fig. 8), but use of lower toxin concentrations may be possible. It may also be possible to further improve the specific fluorescence signal by exposing cells to a low concentration of IbTx-LC-biotin in combination with paxilline, an alkaloid that acts to enhance binding of scorpion toxins to BK channels by an allosteric mechanism (Knaus et al., 1994; Hanner et al., 1997; Koschak et al., 1997). The absolute affinity of a probe molecule for specific receptor sites is an important factor in fluorescence imaging, since the greater the difference between specific and non-specific binding to cell membranes, the greater the signal-to-noise ratio in visualization of receptor localization.

Our results generally support the use of biotin-StrAv methodology as a feasible approach to toxin-based imaging and localization of K^+ channels. Placement of a biotin-tag with a long chain linker on backside residue 19 of IbTx preserves both specific binding to the BK channel and efficient access to two of the four biotin sites of StrAv. A potential advantage of this method is the ability to combine IbTx-LC-biotin with a wide array of detection methods accessible through commercially available conjugates of StrAv and avidin including numerous fluorophores, enzyme conjugates, nanogold particles, and quantum dots (Woldin et al., 1999; Haugland, 2002; Watson et al., 2003). Since fluorescent conjugates of StrAv typically contain multiple fluorophores per StrAv molecule (Panchuk-Voloshina et al. 1999), this detection method may provide greater sensitivity than IbTx probes directly coupled to a single fluorophore. The apparent restriction of IbTx-LC-biotin binding to one site on each of the two binding faces of the StrAv tetramer would be expected to limit or prevent two-dimensional cross-linking of BK channels via multiple interactions with a single tetravalent StrAv molecule. The latter issue, also a problem with bivalent antibodies, may be of concern in situations where observation of natural clustering of BK channels is of special interest. In cellular preparations where StrAv-based secondary detection results in excessive background fluorescence, visualization of bound IbTx-LC-biotin with fluorescent anti-biotin antibody (Fig. 8E) may provide a satisfactory alternative.

In summary, we have described synthesis of a novel biotin-derivative of IbTx that has high binding affinity for both mammalian BK channels and StrAv. The combination of IbTx-LC-biotin and Alexa488-StrAv can be used to image the surface distribution of BK channels on stably transfected HEK293 cells. Such images reveal a non-random, patch-like distribution of BK channels indicative of an endogenous mechanism for BK channel clustering. Further improvements in design, synthesis, and imaging protocols for scorpion toxin probes may be expected to enhance research on numerous aspects of the cell biology of K^+ channels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Design Strategy and Molecular Model of IbTx-LC-biotin. (A) Sequence of synthetic IbTx-LC-biotin showing substitution of native residue D19 with Lys(ϵ -d-LC-biotin). Asterisks (*) mark eight residues important for toxin binding to BK channels. (B) Chemical structure of the side chain of the biotin derivative showing the ~ 15 Å linker between the Lys α -carbon atom and the biotin moiety buried in the complex with streptavidin. (C) Molecular model of IbTx-LC-biotin. Left panel is a side view of a model of the IbTx derivative with the BK channel binding surface at the bottom and the biotin-derivative side chain at the top. A 90° rotation of the latter structure back into the plane of the paper was used to generate the image for right panel, which shows green colored residues (marked * in A) at the toxin-channel interface.

Figure 2.

Purification and Mass Analysis of IbTx-LC-biotin. (A) Reverse phase HPLC chromatogram of crude oxidized (top trace) and purified (bottom trace) synthetic IbTx- LC-biotin. Samples were run on a Vydac C18 column and eluted with a linear gradient of Buffer A to buffer B (A = 0.1% TFA, B = 60% CH₃CN in 0.08% TFA, over 60 min.). (B) Electrospray MS analysis of pure oxidized IbTx-LC-biotin demonstrating a deconvoluted average mass of 4584.1 Da.

Figure 3.

Bilayer Assay of BK_{Ca} Channel Block by IbTx-LC-biotin in the Absence and Presence of StrAv. (A) Current record of a single BK_{Ca} channel from rat skeletal muscle under control conditions in the absence of toxin. (B) Record of a BK_{Ca} channel with 4 nM IbTx-LC-biotin on the external side. (C) Record of a BK_{Ca} channel in the presence of 4 nM IbTx-LC-biotin plus 16 nM StrAv on the external side. A small triangle in the third line marks where data is spliced from another bilayer recorded under identical conditions. Control conditions: Holding voltage = +30 mV. The solution on both sides of the bilayer was 10 mM Mops-KOH, pH 7.4, 200 mM KCl. The internal solution also contained 200 μ M CaCl₂ and the external solution contained 0.2 mM EDTA plus 0.1 mg/ml bovine serum albumin. Arrows to the right indicate

zero current level or the closed state of the channel. Low conductance noise superimposed on IbTx-blocked events in Figs. 3B and 3C is due to activity of extraneous, non-BK, channels occasionally observed in planar bilayer recordings.

Figure 4.

Electrophysiological Assay of IbTx-LC-biotin and ChTx on Whole-cell Current of HSlo Expressed in HEK293 Cells. (A) Effect of IbTx-LC-biotin and paxilline on whole-cell currents. Top panel shows a family of current traces elicited by stepping membrane voltage from a holding potential of -80 mV up to +100 mV in consecutive steps of +10 mV for a duration of 100 ms. Middle and bottom panels were recorded after addition of 100 nM IbTx-LC-biotin and 1 μ M paxilline, respectively, to the extracellular bathing solution. (B) Plot of whole-cell current from panel A at the end of the 100 ms depolarization vs. the step voltage. Solid and dotted lines for Control and 100 nM IbTx-LC-biotin correspond to current values before and after subtraction of background current measured in the presence of 1 μ M paxilline, respectively. (C) Time course of inhibition by toxins and recovery after perfusion. Peak current was monitored by 50 ms voltage steps from -80 mV to +50 mV once every 10 s. IbTx-LC-biotin (200 nM) or ChTx (100 nM) was added to the extracellular perfusion solution at the time of the first arrow and replaced by toxin-free solution at the time of the second arrow. Data points are normalized to the first few points before addition of toxin. Solid lines overlays are fits to an exponential time course. (D) Titration curves of toxin inhibition. HSlo current at steady-state in the presence of various concentrations of IbTx-LC-biotin or ChTx is normalized to values measured before toxin addition. Data is fit to a single-site inhibition isotherm ($I_{\text{Norm}} = K_D / (K_D + [\text{toxin}])$) with K_D values of 26 nM for IbTx-LC-biotin and 13 nM for ChTx.

Figure 5.

Analysis of Complex Formation between IbTx-LC-biotin and StrAv by Size Exclusion Chromatography. A fixed amount of IbTx-LC-biotin (250 pmol) was incubated with increasing amounts of StrAv in the range of 0 to 375 pmol and samples were subjected to HPSEC as described in Methods. Overlaid consecutive traces of the absorbance profile are alternated using solid and dashed lines for sake of clarity. The inset shows seven traces underlying the peak corresponding to unbound IbTx-LC-biotin that have been enlarged and vertically displaced. Triangles mark column calibrations: Vo, void volume; Ve, column volume.

Figure 6.

Stoichiometry of IbTx-LC-biotin Binding to StrAv. (A) Fluorescence titration of 11 pmol StrAv with biotin-4-fluorescein. Open circles, StrAv alone; open squares, StrAv preincubated with 33 pmol IbTx-LC-biotin. The arrow indicates the equivalence point at ~44 pmol biotin-4-fluorescein. (B) Titration of IbTx-LC-biotin with increasing StrAv. Peak areas corresponding to free IbTx-LC-biotin for various samples in Fig. 4 were normalized to the control taken in the absence of StrAv and plotted versus the amount of added StrAv. Titration data are consistent with an equivalence point near 125 pmol StrAv or a molar ratio of 1 : 0.5 for IbTx-LC-biotin / StrAv. Dashed line shows theoretical expectation for four IbTx-LC-biotin binding sites per StrAv. (C) Space-filling molecular models of StrAv complex with biotin on the left (structure 1SWE from Protein Data Bank) and IbTx-LC-biotin on the right at the same scale. Arrows mark the location of two adjacent biotin sites on the front face of the StrAv tetramer.

Figure 7.

Fluorescence Microscopy of Transfected Cells Expressing HSlo Using IbTx-LC-biotin. (A, C) Stably transfected HEK293/HSlo cells. (B, D) Untransfected HEK293 cells. (A, B) Fluorescence images acquired under identical conditions. (C, D) DIC image of the same fields in A and B, respectively. Cells grown on coverslips were processed for microscopy by incubation with 100 nM IbTx-LC-biotin, then washed and incubated with 10 μ g/ml Alexa488-StrAv, washed again, fixed in 2% formaldehyde, and viewed using a 100 $\times$ oil immersion objective as described in Methods. Scale bar = 10 μ m.

Figure 8.

Specificity of Fluorescence Labeling by IbTx-LC-biotin Assessed by Various Controls. (A, B) Live (unfixed) HEK293/HSlo cells after processing with 50 nM IbTx-LC-biotin and 3 $\mu\text{g/ml}$ Alexa488-StrAv. Control coverslip in B was treated identically to A except that 5 μM IbTx-D19C-Acm was added before exposure to IbTx-LC-biotin. (C, D) HEK293/HSlo cells after processing with 50 nM IbTx-LC-biotin, 3 $\mu\text{g/ml}$ Alexa488-StrAv, and fixation with 2% formaldehyde. Control coverslip in D was treated identically to C except that IbTx-LC-biotin was omitted. (E, F) HEK293/HSlo cells after processing with 50 nM IbTx-LC-biotin, 4 $\mu\text{g/ml}$ Alexa488-antibiotin antibody, and fixation with 2% formaldehyde. Control coverslip in F was treated identically to E except that IbTX-LC-biotin was omitted. Cells were processed for microscopy and viewed with a 40 $\times$ oil immersion objective as described in Methods. Scale bar = 30 μm .