Published in final edited form as:

Bioorg Med Chem Lett. 2011 September 1; 21(17): 5021–5024. doi:10.1016/j.bmcl.2011.04.099.

Chemical control of metabolically-engineered voltage-gated K⁺ channels

Zhengmao Hua, Anatoli Lvov, Trevor J. Morin, and William R. Kobertz*

Department of Biochemistry and Molecular Pharmacology, Programs in Neuroscience and Chemical Biology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324

Abstract

Metabolic oligosaccharide engineering is a powerful approach for installing unnatural glycans with unique functional groups into the glycocalyx of living cells and animals. Using this approach, we showed that K⁺ channel complexes decorated with thiol-containing sialic acids were irreversibly inhibited with scorpion toxins bearing a pendant maleimide group. Irreversible inhibition required a glycosylated K⁺ channel subunit and was completely reversible with mild reductant when the tether connecting the toxin to the maleimide contained a disulfide bond. Cleavage of the disulfide bond not only restored function, but delivered a biotin moiety to the modified K⁺ channel subunit, providing a novel approach for preferentially labeling wild type K⁺ channel complexes functioning in cells.

Keywords

KCNQ1; Shaker; glycosylation; charybdotoxin; tethered blockers

Voltage-gated K⁺ channels (Kv-type) are found in all types of cells and tissues where they permit the rapid and selective flux of potassium across the plasma membrane. Kv channels are comprised of four ion-conducting subunits that tetramerize to form a potassium-selective aqueous pore along the four-fold axis of symmetry.¹ During protein biogenesis and insertion into the ER membrane, many Kv subunits are either directly N-glycosylated² or co-assemble with N-glycosylated membrane-embedded regulatory subunits³. These carbohydrate handles aid in the folding and oligomerization of K⁺ channel complexes^{3,4}, which are subsequently trafficked to the cell surface where they open and close in response to changes in the plasma membrane potential. This voltage-dependent control enables the superfamily of Kv channels to maintain cardiac rhythmicity⁵, govern neuronal excitability⁶, regulate arterial tone⁷ and epithelial electrolyte homeostasis⁸. Consequently, Kv channels are targets for the treatment of arrhythmias⁹, epilepsy¹⁰ and high blood pressure.¹¹

© 2011 Elsevier Ltd. All rights reserved.

Address correspondence to: Dr. William R. Kobertz, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324, 508.856.8861 (phone), 508.856.8867 (fax), william.kobertz@umassmed.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Supplementary data

Supplementary data includes basic experimental procedures, metabolic-labeling protocols, and characterization of biotin bismaleimide 6.

Manipulating the function of Kv channels with chemical tools and methods—low and high affinity inhibitors, tethered blockers, unnatural amino acid incorporation, and high throughput screening—has been essential for investigating the structure and function of voltage-gated K⁺ channels.¹² In particular, cysteine-specific modifying reagents in combination with electrical recordings have defined the permeation pathway^{13,14}, determined the various voltage-dependent motions^{15,16}, and counted the number of regulatory subunits in K⁺ channel complexes.¹⁷ Since most K⁺ channels lack extracellular cysteines, this robust approach has relied on site-directed mutagenesis to install a target cysteine residue for chemical reactivity. Here, we utilize metabolic labeling with unnatural N-glycans¹⁸ to install thiol-containing neuraminic acids (sialic acids) on either the ion-conducting or regulatory subunits of K⁺ channel complexes. Using chemically-derivatized scorpion toxins^{17,19}, we show that K⁺ channel function can be controlled via toxin-directed reactions with the metabolically-incorporated sugars directly attached to K⁺ channel subunits.

Metabolic-labeling of glycosylated proteins with unnatural sugars is a robust method for installing a wide-variety of chemoselective and bio-orthogonal functional groups on integral membrane proteins at the cell surface.²⁰ Although the entire cell surface is coated with the engineered functional group, we reasoned that we could manipulate K⁺ channel function by selectively modifying the derivatized sialic acids on K⁺ channel subunits with a chemically-derivatized scorpion toxin (Figure 1A). These thiol-specific reagents behave as tethered blockers²¹, binding first to the ion-conducting pore before reacting rapidly with nearby cysteines and other thiols. At low nanomolar concentrations, we have shown that the non-specific bimolecular reaction between the reagent and cell surface thiols is essentially non-existent for the duration of most electrophysiology experiments (10 – 60 minutes).^{19,21} Thus, the chemoselectivity of the reaction comes from the toxin–K⁺ channel interaction and the length of the tether connecting the thiol modifying group to the toxin (Figure 1B). For the toxin, we chose charybdotoxin (CTX) for three reasons: (1) CTX is a highly affinity inhibitor of Kv1 and BK K⁺ channels²²; (2) CTX can be derivatized with bismaleimides to afford thiol reactive toxins that bind to the ion conduction pore with affinities similar to wild type CTX^{17,19}; (3) the CTX-binding site is transplantable, enabling the structural and functional investigation of Kv type channels that lack selective or high affinity inhibitors.^{23–25} For the unnatural sugar, we used Ac₅ManNTGc (1,3,4,6-tetra-*O*-acetyl-2-acetylthioacetamido-2-deoxy- α -D-mannopyranose; thiol sugar; **1**), which readily crosses the cell membrane where it is deacetylated in the cytosol and incorporated on newly synthesized proteins in the secretory pathway.^{26–28}

We first determined whether a chemically-derivatized scorpion toxin would irreversibly inhibit function by preferentially reacting with metabolically-labeled N-linked glycans on K⁺ channel complexes in the workhorse expression system for ion channels—*Xenopus* oocytes. Under this experimental paradigm, we used the Q1/E1 K⁺ channel complex, where the functional complex is composed of four Kv7.1 ion-conducting subunits co-assembled with two regulatory KCNE1 (E1) type I transmembrane glycopeptides (Figure 1A).^{17,24,29} Oocytes were injected with mRNAs encoding the CTX-sensitive Q1 and E1 subunits (15:5 ng respectively) and incubated in standard oocyte storage buffer³⁰ containing either vehicle (0.5% ethanol) or 50 μ M thiol sugar **1**²⁶, which was changed daily. One concern with oocytes is that they express low levels of Q1 subunits³¹; however, the endogenous currents are not inhibited by CTX and are readily subtracted out^{17,19}, enabling the visualization of the exogenously expressed Q1/E1 currents. Figure 2A shows representative CTX-sensitive traces and normalized currents elicited by a 20-mV pulse from an oocyte expressing Q1/E1 complexes that was incubated with thiol sugar. Treatment with 10 nM CTX-Mal¹⁹ resulted in rapid and irreversible inhibition of the Q1/E1 complex. At nanomolar CTX-Mal concentrations, irreversible inhibition required incubation with the thiol sugar since

incubation with vehicle rendered CTX-Mal inhibition of Q1/E1 currents completely reversible (Figure 2B). Besides the observed selective reactivity with CTX-Mal, the biophysical properties of the Q1/E1 complexes—including the hallmark slow opening kinetics³¹—were unperturbed by the inclusion of thiol-containing sialic acids on the E1 regulatory subunit. To show that the irreversible inhibition by CTX-Mal was due to specifically modifying the thiol-containing sialic acids on the E1 subunit, we metabolically-labeled oocytes expressing only Q1 channels, which are not glycosylated. The lack of E1 subunits was immediately noticeable in the current traces, as the currents from Q1 channels rapidly increase upon depolarization and then partially inactivate after ~ 100 msec (Figure 2C). In contrast to metabolically-labeled Q1/E1 complexes, CTX-Mal inhibition with Q1 channels was reversible. CTX-Mal reversibility was not due to a lower effective concentration since the slower washout in Figure 2C indicated that the average lifetime of CTX-Mal bound to the Q1 pore was longer in the absence of E1 subunits. Thus, these electrical recordings demonstrated that CTX-Mal can irreversibly inhibit Q1/E1 function by specifically modifying thiol-containing sialic acids on the E1 regulatory subunit.

To have full chemical control of K⁺ channel function, the reaction between the derivatized CTX and the K⁺ channel subunit needed to be chemically reversible. Although disulfide bond formation between CTX and thiol-containing sialic acids on the cell surface is an obvious chemoselective and cell friendly reaction, we chose to label CTX with a bismaleimide that had an internal disulfide bond because maleimides are inherently more stable in water than MTS reagents. Moreover, this subtle difference would allow for delivery of a small molecule probe to the modified K⁺ channel subunit after cleavage with reductant, which would be useful in subsequent biochemical, biophysical or imaging experiments. To simplify the synthesis of the bismaleimide, derivatization of CTX, and ensure delivery of a molecular probe to a K⁺ channel subunit, we set out to synthesize a symmetrical bismaleimide (Scheme 1) from cystamine dihydrochloride **2** that would allow for the facile incorporation of a molecular probe in the final step of the synthesis. The amino groups of cystamine **2** were capped with two equivalents of a doubly amino-protected, activated ester of L-lysine **3**. Selective deprotection of the Fmoc protecting groups gave the symmetric diamine **4**. Addition of two equivalents of the NHS-ester of 3-(maleimido)propionic acid and *N*-Boc deprotection afforded bismaleimide **5**, which was subsequently biotinylated with two equivalents of NHS-Biotin to give biotin bismaleimide **6**. CTX was then derivatized by labeling a cysteine mutant of CTX (R19C)³² with 100-fold molar excess of biotin bismaleimide **6** to yield CTX-Biotin, which was purified by reverse phase HPLC as we have previously described.^{17,19}

With CTX-Biotin in hand, we decided to change both the channel (Shaker-IR)³³ and the expression system (CHO-K1 cells) to demonstrate that our approach was versatile and could be used to label glycosylated ion-conducting subunits. Inactivation-removed Shaker is similar to Q1 in that it is an archetypical voltage-gated K⁺ channel with a pore-forming domain flanked by four voltage-sensing domains.¹ However, each Shaker voltage sensor is doubly N-glycosylated on the extracellular loop connecting the first two transmembrane segments (S1–S2 loop)². Previous studies have shown that the N-glycans on Shaker contain sialic acid residues³⁴. Therefore, each tetrameric Shaker channel will have eight N-glycans and potentially up to 24 – 32 sialic acids if every antenna on each glycoconjugate is capped. Figure 3 shows the effects of 10 nM CTX-Biotin on CHO cells expressing Shaker K⁺ channels incubated with either 50 μ M thiol sugar **1** or vehicle in perforated-patch whole cell recordings.³⁵ CTX-Biotin inhibition of Shaker in vehicle treated cells (circles) was completely reversible after 3 min. In striking contrast to control and Q1 channels (Figure 2C), metabolically-labeled Shaker channels (squares) were irreversibly inhibited after identical treatment with CTX-Biotin. Irreversible inhibition of the Shaker K⁺ currents was reversed with tris(2-carboxyethyl)phosphine (TCEP) (1 mM for 2 min), which reduced the

disulfide bond, freeing the toxin moiety and leaving a biotin moiety on a Shaker K⁺ channel subunit. Rewardingly, both metabolic-labeling and biotin deposition onto the Shaker voltage sensor had no effect on the voltage-gating properties of the channel, as the current amplitude, gating kinetics and voltage-dependence of the biotinylated Shaker K⁺ channel were the same as unlabeled (Figures 3B and 3C).

In conclusion, we have combined glycan engineering and chemically-derivatized scorpion toxins to switch K⁺ channel function off and on in living cells. Inhibition of K⁺ channel function required a glycosylation site on either the ion-conducting or regulatory subunits, demonstrating that the toxin-mediated reaction was specifically targeting the glycans directly attached to the K⁺ channel subunits. In addition, we have shown that unnatural glycans were incorporated into the glycocalyx of *Xenopus* oocytes, adding this well-known ion channel expression system to the many *in vitro* and *in vivo* studies where glycan engineering has found wide spread utility. Although the results of this study have focused on the functional consequences of the cell surface reactions, the unperturbed function of the biotinylated K⁺ channels demonstrated that this strategy has the potential to deliver molecular probes to both exogenously-expressed and endogenous K⁺ channel complexes. Given that most ion channel complexes require N-glycans to traffic to the cell surface, our combined approach can be readily applied to target a wide variety of ion channel subunits functioning at the plasma membrane.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by American Recovery and Reinvestment Act (ARRA) funding from the National Institutes of Health (R01-DC007669) and (RC1-HL099759).

References and Notes

1. Long SB, Campbell EB, Mackinnon R. Science. 2005; 309:897–903. [PubMed: 16002581]
2. Santacruz-Toloz L, Huang Y, John SA, Papazian DM. Biochemistry. 1994; 33:5607–5613. [PubMed: 8180185]
3. Chandrasekhar KD, Bas T, Kobertz WR. J. Biol. Chem. 2006; 281:40015–40023. [PubMed: 17065152]
4. Khanna R, Myers MP, Laine M, Papazian DM. J Biol Chem. 2001; 276:34028–34034. [PubMed: 11427541]
5. Marban E. Nature. 2002; 415:213–218. [PubMed: 11805845]
6. Cooper EC, Jan LY. Arch Neurol. 2003; 60:496–500. [PubMed: 12707061]
7. Ng FL, Davis AJ, Jepps TA, Harhun MI, Yeung SY, Wan A, Reddy M, Melville D, Nardi A, Khong TK, Greenwood IA. Br J Pharmacol. 2011; 162:42–53. [PubMed: 20840535]
8. Wangemann P. Hear Res. 2002; 165:1–9. [PubMed: 12031509]
9. Grunnet M, Hansen RS, Olesen SP. Prog Biophys Mol Biol. 2008; 98:347–362. [PubMed: 19351523]
10. Surti TS, Jan LY. Curr Opin Investig Drugs. 2005; 6:704–711.
11. Mackie AR, Byron KL. Mol Pharmacol. 2008; 74:1171–1179. [PubMed: 18684841]
12. Ahern CA, Kobertz WR. Biochemistry. 2009; 48:517–526. [PubMed: 19113860]
13. Liu Y, Jurman ME, Yellen G. Neuron. 1996; 16:859–867. [PubMed: 8608004]
14. Liu Y, Holmgren M, Jurman ME, Yellen G. Neuron. 1997; 19:175–184. [PubMed: 9247273]
15. Larsson HP, Baker OS, Dhillon DS, Isacoff EY. Neuron. 1996; 16:387–397. [PubMed: 8789953]
16. Rocheleau JM, Kobertz WR. J. Gen. Physiol. 2008; 131:59–68. [PubMed: 18079560]

17. Morin TJ, Kobertz WR. *Proc. Natl. Acad. Sci. USA*. 2008; 105:1478–1482. [PubMed: 18223154]
18. Mahal LK, Bertozzi CR. *Chem Biol*. 1997; 4:415–422. [PubMed: 9224572]
19. Morin TJ, Kobertz WR. *ACS Chem. Biol*. 2007; 2:469–473. [PubMed: 17602620]
20. Mahal LK, Yarema KJ, Bertozzi CR. *Science*. 1997; 276:1125–1128. [PubMed: 9173543]
21. Morin TJ, Kobertz WR. *J. Biol. Chem*. 2008; 283:25105–25109. [PubMed: 18541528]
22. Gutman GA, Chandy KG, Grissmer S, Lazdunski M, McKinnon D, Pardo LA, Robertson GA, Rudy B, Sanguinetti MC, Stuhmer W, Wang X. *Pharmacol Rev*. 2005; 57:473–508. [PubMed: 16382104]
23. Gross A, Abramson T, MacKinnon R. *Neuron*. 1994; 13:961–966. [PubMed: 7946339]
24. Chen H, Kim LA, Rajan S, Xu S, Goldstein SA. *Neuron*. 2003; 40:15–23. [PubMed: 14527430]
25. Soh H, Goldstein SA. *J Biol Chem*. 2008; 283:15072–15077. [PubMed: 18364354]
26. Sampathkumar SG, Li AV, Yarema KJ. *Nat Protoc*. 2006; 1:2377–2385. [PubMed: 17406481]
27. Sampathkumar SG, Li AV, Jones MB, Sun Z, Yarema KJ. *Nat Chem Biol*. 2006; 2:149–152. [PubMed: 16474386]
28. Sampathkumar SG, Jones MB, Yarema KJ. *Nat Protoc*. 2006; 1:1840–1851. [PubMed: 17487167]
29. Wang KW, Goldstein SA. *Neuron*. 1995; 14:1303–1309. [PubMed: 7605639]
30. Shih TM, Smith RD, Toro L, Goldin AL. *Methods Enzymol*. 1998; 293:529–556. [PubMed: 9711627]
31. Sanguinetti MC, Curran ME, Zou A, Shen J, Spector PS, Atkinson DL, Keating MT. *Nature*. 1996; 384:80–83. [PubMed: 8900283]
32. Shimony E, Sun T, Kolmakova-Partensky L, Miller C. *Protein Eng*. 1994; 7:503–507. [PubMed: 7518082]
33. Zagotta WN, Hoshi T, Aldrich RW. *Science*. 1990; 250:568–571. [PubMed: 2122520]
34. Johnson D, Bennett ES. *Pflugers Arch*. 2008; 456:393–405. [PubMed: 18043943]
35. Lvov A, Gage SD, Berrios V, Kobertz WR. *J. Gen. Physiol*. 2010; 135:607. [PubMed: 20479109]

Figure 1. Toxin-mediated modification of K^+ channel complexes in cells metabolically-labeled with thiol-containing sugars

(A) Cartoon of the reaction between a derivatized scorpion toxin (CTX) and a K^+ channel complex decorated with thiol-containing sialic acids. At low concentration ($\sim \text{nM}$), these thiol-reactive toxins bind first to the K^+ channel pore via the CTX moiety before the maleimide group reacts with nearby thiols. (B) Structures of the thiol-reactive portions of the chemically derivatized toxins.

Figure 2. Q1/E1 K^+ channel complexes metabolically-labeled with thiol sugar are irreversibly inhibited by CTX-Mal

(A) Q1/E1 complexes expressed in *Xenopus* oocytes incubated with (50 μ M) thiol sugar **1** in ND96³⁰ are irreversibly blocked by CTX-Mal. (B) Incubation with vehicle (0.5% ethanol) rendered CTX-Mal inhibition of Q1/E1 complexes completely reversible by washout with ND96. (C) CTX-Mal inhibition of Q1 channels was also reversible since these K^+ channel subunits are not glycosylated. (Left panel) CTX-sensitive currents shown were elicited by a 20-mV depolarization (5-s) before and after 10 nM CTX-Mal treatment; (Right panel) Reaction profiles upon addition and washout of 10 nM CTX-Mal. The current at 20 mV was

measured at the end of each 5-s pulse and normalized to the initial current before CTX-Mal addition. Black and gray data points correspond to the traces on the left.

Figure 3. Chemical control of metabolically-labeled Shaker channels with CTX-Biotin
 (A) CTX-Biotin (10 nM) inhibits Shaker-IR K^+ currents in CHO-K1 cells treated with either 50 μ M thiol sugar **1** ($\square$) or 0.5% ethanol as a vehicle ($\circ$). Only metabolically-labeled (+ Thiol Sugar) channels were irreversibly blocked by CTX-Biotin after a simple washout; inhibition was completely reversed by an application of 1 mM TCEP. Reaction profiles were monitored with a 200-ms, 40 mV pulse every 15 s. (B) Raw current traces before (black) and after (light gray) CTX-Biotin treatment. Traces of the recovered current after TCEP treatment are shown in the dark gray. (C) I-V relationship obtained from the same cell before inhibition with CTX-Biotin and after the current recovery by either washout ($-$ Thiol Sugar) or reduction with TCEP ($+$ Thiol Sugar).

Scheme 1.

(a) MeOH, Et₃N, rt; (b) piperidine, CH₂Cl₂, rt; (c) 3-(Maleimido)propionic acid N-hydroxysuccinimide ester, DMF, rt, 22% for 3 steps(a–c); (d) TFA, CHCl₃, H₂O, rt; (e) NHS-biotin, Et₃N, rt, 32% for 2 steps (d–e).