JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Communication

pubs.acs.org/JACS

Maleimide Conjugates of Saxitoxin as Covalent Inhibitors of Voltage-Gated Sodium Channels

William H. Parsons† and J. Du Bois*

Department of Chemistry, Stanford University, 333 Campus Drive, Stanford, California 94305-5080, United States

Supporting Information

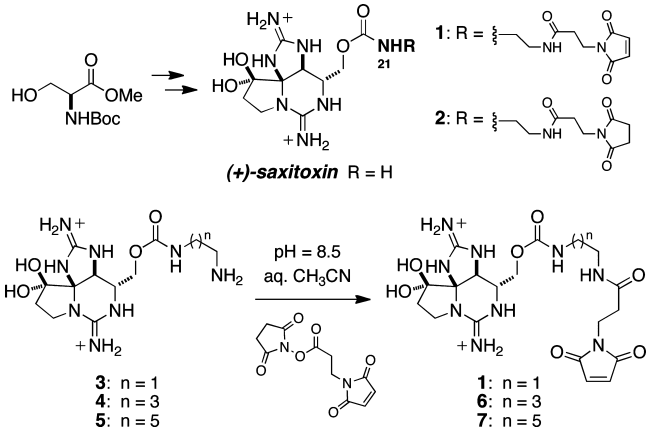
ABSTRACT: (+)-Saxitoxin, a naturally occurring guanidinium poison, functions as a potent, selective, and reversible inhibitor of voltage-gated sodium ion channels (Na_Vs). Modified forms of this toxin bearing cysteine-reactive maleimide groups are available through total synthesis and are found to irreversibly inhibit sodium ion conductance in recombinantly expressed wild-type sodium channels and in hippocampal nerve cells. Our findings support a mechanism for covalent protein modification in which toxin binding to the channel pore precedes maleimide alkylation of a nucleophilic amino acid. Second-generation maleimide-toxin conjugates, which include bioorthogonal reactive groups, are also found to block channel function irreversibly; such compounds have potential as reagents for selective labeling of Na_Vs for live cell imaging and/or proteomics experiments.

In electrically excitable cells, the coordinated action of voltage-gated sodium channels (Na_Vs) is responsible for the rising phase of the action potential.[1] These integral membrane protein complexes are comprised of a large, pore-forming α-subunit, coexpressed with two β-auxiliary glycoproteins. Genes encoding 10 sodium channel α-subtypes ($Na_V1.1-1.9$, Na_X) have been identified in mammalian cells.[2] Protein isoform expression levels are tightly regulated and varied within the cell and across different tissues. Chemical agents that act as specific modulators of Na_V function and/or as probes for live-cell imaging or affinity profiling are sought as tools for studies aimed at understanding the role of Na_V isoforms in shaping electrical signals in neuronal cells.[3,4] The potential utility of such compounds has motivated the studies described herein and has led to the design of a unique collection of small molecule, covalent inhibitors of Na_Vs.

Guanidinium toxins, which bind reversibly and with low nanomolar affinity to the outer pore of the sodium channel, have featured prominently in Na_V research.[5] Structure–activity experiments using tetrodotoxin (TTX), saxitoxin (STX), and a small number of congeners together with protein mutagenesis have informed modeling studies of the channel pore and toxin binding site.[6−9] Access to modified STXs through de novo chemical synthesis[10−15] enables further examination of the pore architecture and generation of novel reagents for Na_V labeling.

Use of a maleimide-conjugated STX (e.g., **1**) to covalently modify a cysteine mutant channel was envisioned as a strategy for examining the fidelity of our toxin pore model and for targeting selectively a single Na_V subtype.[16] Prior studies from our lab have demonstrated that functionalization of the carbamate moiety in STX can be accommodated with limited influence on the binding affinity between ligand and receptor.[17] As shown in Scheme 1, maleimide conjugates of STX (**1**, **6**, and **7**) can be obtained through selective *N*-hydroxysuccinimide (NHS) ester coupling to aminoalkyl-substituted STXs, starting materials made available through multistep synthesis. The same type of reaction has been employed to prepare succinimide (**2**), a structural analogue of **1** needed for control experiments.

Scheme 1. De Novo Synthesis of Maleimide and Succinimide Conjugates of (+)-Saxitoxin (STX)



Inhibition of sodium current by STX-maleimide conjugates was evaluated through whole-cell electrophysiology measurements. Experiments were performed as voltage-clamp recordings on the α-subunit of the rat skeletal muscle channel ($rNa_V1.4$) heterologously expressed in Chinese hamster ovary (CHO) cells. Initial recordings using saturating concentrations (5 μM) of **1** against *wild-type* $rNa_V1.4$ (CHO cells) gave unexpected results, as channel conduction was irreversibly disabled. Following 1 min of incubating cells with **1**, continuous perfusion with toxin-free external solution for 8 min restores only 51 ± 6% of initial peak current, I_0. As shown in Figures 1A,B and S1a,b (Supporting Information), longer incubation periods of 3 and 6 min lead to demonstrable decreases in % current recovery after equivalent washout periods (36 ± 5% and 18 ± 3% of I_0, respectively).[18] The inability to restore initial current levels following perfusion of the cell with toxin-free solution is in marked contrast to the fully reversible

Received: February 23, 2013

   dx.doi.org/10.1021/ja4019644 | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

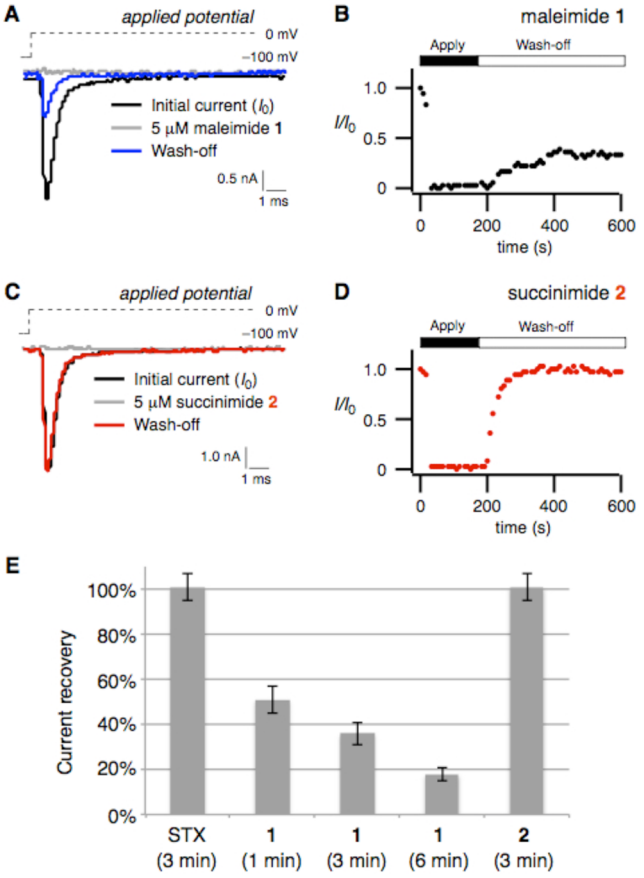


Figure 1. (A) Current recordings, elicited by a 10 ms voltage step from −100 to 0 mV, upon 3 min application and wash-off of 5 μM maleimide **1** to CHO cells expressing rNa$_V$1.4. (B) Representative wash-off time course for **1**. (C) Current recordings upon 3 min application and wash-off of 5 μM succinimide **2**. (D) Representative wash-off time course for **2**. Average current recovery after washout was 36 ± 5% of I_0 for **1**, compared to 101 ± 6% for **2**. Data represent the average of 3−5 cells ± SEM. (E) Current recovery following 8 min washout (incubation time for each compound shown in parentheses). Data represent the average of 3−7 cells ± SEM.

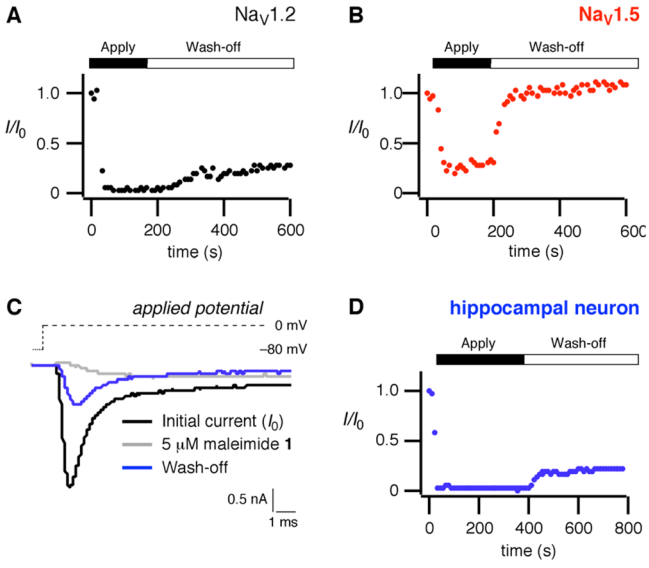


Figure 2. Representative time courses of wash-off of 5 μM **1** from CHO cells expressing rNa$_V$1.2 (A) and hNa$_V$1.5 (B). Average current recovery after washout was 25 ± 1% of I_0 on rNa$_V$1.2, compared to 100 ± 3% on hNa$_V$1.5. (C) Current recordings, elicited by a 10 ms voltage step from −80 to 0 mV, upon 6 min application and wash-off of 5 μM maleimide **1** to embryonic rat hippocampal neurons. (D) Representative wash-off time course of 5 μM **1** from embryonic rat hippocampal neurons. Average current recovery after washout was 25 ± 2% of I_0. Data represent the average of 3−5 cells ± SEM.

binding behavior of STX (Figure S1c, Supporting Information). The unique activity of **1** appears to be use-independent, as 3 min application and washout of this compound without active stimulation of the channel does not alter performance (current recovery = 35 ± 3% of I_0) (Figure S1d, Supporting Information). Other maleimide conjugates with extended linker groups (**6** and **7**) exhibit similar irreversible binding behavior (Figure S2, Supporting Information). Collectively, these results are in accord with reaction of **1** and a nucleophilic residue positioned near the toxin binding site in the wild-type rNa$_V$1.4 channel.

Support for the covalent modification of wt-rNa$_V$1.4 by **1** has been gained using a nonreactive, succinimide analogue **2**. Concentration−response measurements (Figure S4, Supporting Information) give an IC$_{50}$ value of 54 ± 7 nM for **2**, evidence that modification of the carbamate unit does not significantly perturb toxin affinity for the channel. Application of this compound at 5 μM concentration to rNa$_V$1.4-expressing CHO cells affords complete channel block. Perfusion of toxin-free buffer, however, results in rapid recovery of peak current (101 ± 6% of I_0), behavior that parallels STX binding (Figure 1D). Thus, irreversible block by **1** appears to be dependent on the presence of the electrophilic unsaturated dicarbonyl moiety, which can react with a nucleophilic amino acid group.

To explore further the activity of **1** as an irreversible Na$_V$ antagonist, whole-cell electrophysiology recordings were performed against two additional channel isoforms, rNa$_V$1.2 and hNa$_V$1.5. The measured inhibitory constant for **2** against rNa$_V$1.2 (IC$_{50}$ = 38 ± 5 nM) is comparable to that of rNa$_V$1.4 but is substantially higher for the TTX-resistant channel, hNa$_V$1.5 (IC$_{50}$ = 4.5 ± 0.8 μM) (Figure S4, Supporting Information). As shown in Figure 2, application of 5 μM **1** to CHO cells expressing rNa$_V$1.2 completely blocks sodium conduction; following a 3 min incubation period, subsequent washout of the toxin conjugate results in only partial restoration of channel current (25 ± 1% of I_0). Comparable results are obtained when **1** is perfused onto CHO cells coexpressing Na$_V\beta$1 and the α-subunit of Na$_V$1.2 (19 ± 4% of I_0, Figure S5b, Supporting Information); these findings are consistent with previous reports demonstrating that accessory subunit proteins do not affect STX binding.[19] By contrast, 5 μM solutions of **1** administered to CHO cells expressing hNa$_V$1.5 give only partial current block, and complete restoration of peak current is achieved following washout with toxin-free external solution (100 ± 3% of I_0). These data establish that high affinity binding of the toxin to the channel is a prerequisite for achieving efficient, covalent protein cross-linking.

To validate the utility of **1** for covalent modification of endogeneous Na$_V$s in primary cells, we have performed electrophysiology experiments with embryonic rat hippocampal neurons (Figure 2C,D).[20] Following a six minute application of 5 μM **1** to these cells, only a fraction of the initial current (25 ± 2% of I_0) is recovered after extended washout. Accordingly, the irreversible blocking behavior of **1** is not unique to one cell type

dx.doi.org/10.1021/ja4019644 | J. Am. Chem. Soc. XXXX, XXX, XXX−XXX

and does not appear to be influenced by auxiliary proteins known to associate with the Na_V α-subunit.[21]

Experiments with **8**, a maleimide conjugate prepared from β-saxitoxinol, offer additional support for our hypothesis that toxin association with the channel pore occurs prior to nucleophilic attack on the maleimide (Figure 3A,B).[22] β-

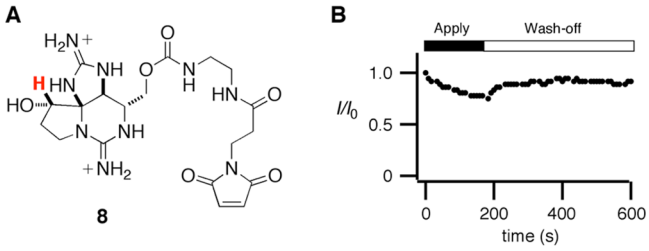


Figure 3. (A) Structure of maleimide-conjugated β-saxitoxinol **8**. (B) Representative time course of wash-off of 5 μM **8** from CHO cells expressing $Na_V1.4$. Current recovery after washout was 94 ± 7% of I_0, which represents the average of 5 cells ± SEM.

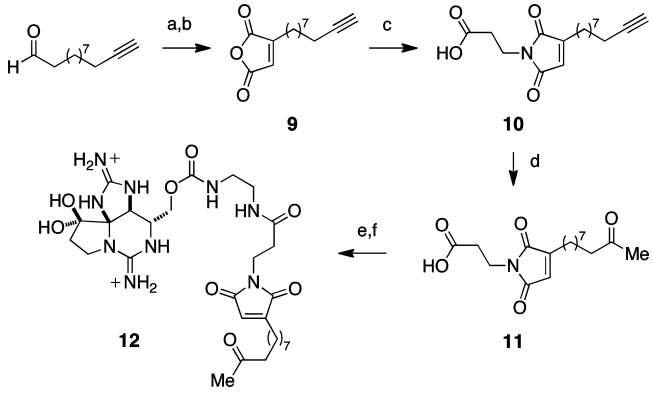
Saxitoxinol itself is a reduced form of the natural product that is 3 orders of magnitude less potent against $rNa_V1.4$.[23,24] Incubation of CHO cells expressing the 1.4 subtype for 3 min with 5 μM **8** affords only partial current block, and nearly full recovery of I_0 is observed following perfusion with external solution (94 ± 7% of I_0). Similarly, application of a 5 μM solution of a maleimide tethered to a single guanidinium group to $rNa_V1.4$ gives neither transient nor prolonged block of channel current (Figure S7b, Supporting Information).

Examination of the primary sequences of $rNa_V1.2$ and $rNa_V1.4$ as well as our homology model of the channel pore shows three native cysteines, C763, C1546, and C1561 ($Na_V1.4$ numbering), within proximity of the proposed toxin binding site. On the basis of our homology model of the p-loop region,[9] C763 appears to be the most likely candidate nucleophile (Figure S8, Supporting Information). To test this prediction, we have constructed two amino acid mutants, C763S and C763A, of the $rNa_V1.4$ isoform. Expression of these mutant proteins in both CHO and tsa201 cells failed to produce macroscopic current levels comparable to wild-type (0–0.3 nA for all n = 32 cells tested). Such low current densities were deemed insufficient for definitive characterization of the blocking behavior of **1**. Our present efforts are dedicated toward identifying the amino acid residue(s) modified by **1** through alternative means, which include protein isolation and mass spectrometric sequencing.

The surprising behavior of **1** against wt-Na_V isoforms can be exploited to mark endogenous channels in primary cells (see Figure 2C,D). To this end, we have prepared unique, C2-substituted maleimide groups bearing an attendant alkyne **10** or ketone moiety **11** (Scheme 2).[25] Selective coupling of activated ester forms of these reagents to STX-amines such as **3** proceeds without event. Modification at C2 on the maleimide core has a limited influence on electrophilic reactivity with cysteine nucleophiles (see Figure S9, Supporting Information, for a comparison of reaction rates).

Both maleimide **12** and succinimide **13** conjugates of STX have been prepared (Scheme 2, Figure 4) and evaluated against $rNa_V1.2$. As with toxin derivatives **1** and **2**, incubation of cells with 5 μM concentrations of **12** and **13** and subsequent washout reveals partial current recovery with the former (22 ± 5% of I_0) but rapid washout and complete restoration of I_0 with

Scheme 2. Synthesis of C2-Substituted Maleimide Derivatives[a]



[a]Conditions: (a) glyoxylic acid·H_2O, morpholinium hydrochloride, dioxane/H_2O, 100 °C, 65%; (b) Dess–Martin periodinane, CH_2Cl_2, 71%; (c) β-alanine, AcOH, 23 → 105 °C, 77%; (d) $HgSO_4$, H_2SO_4, MeOH, 59%; (e) *N*,*N*′-disuccinimidyl carbonate, *i*-Pr_2NEt, CH_2Cl_2, 62%; (f) **3**, aq. CH_3CN, pH 8.5, 58%.

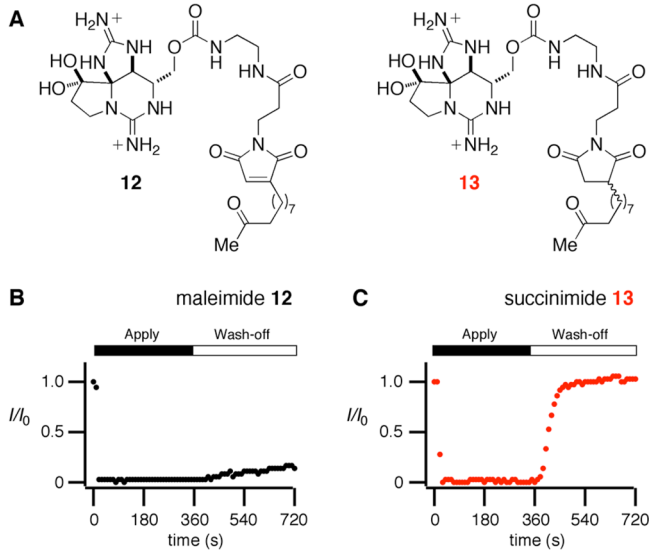


Figure 4. (A) Structures of C2-substituted maleimide (**12**) and succinimide (**13**) derivatives of STX. (B) Representative time course of wash-off of 5 μM **12** from CHO cells expressing $rNa_V1.2$. (C) Representative time course of wash-off of 5 μM **13** from CHO cells expressing $rNa_V1.2$. Average current recovery after washout was 22 ± 5% of I_0 for **12**, compared to 106 ± 6% for **13**. Data represent the average of 3–5 cells ± SEM.

the succinimide analogue **13** (Figure 4). Alkylation of wt-Na_Vs with **12** presents a ketone functional group on the extracellular protein surface that is suitable for bioorthogonal modification with a fluorescent dye or biotin cofactor.[26] Such probes could enable tracking of channel endocytosis or facilitate isolation and identification of Na_Vs expressed at the cell surface.[27]

In summary, we have prepared a unique collection of maleimide-bearing STX derivatives that display irreversible block of wild-type Na_Vs expressed in heterologous cells and in hippocampal neurons. Our experiments support a mechanism in which toxin binding precedes maleimide cross-linking with a proximal amino acid, the most likely candidate of which is C763 on the basis of our homology model of the channel pore and toxin binding site. These unique reactive probes should

dx.doi.org/10.1021/ja4019644 | J. Am. Chem. Soc. XXXX, XXX, XXX–XXX

have general applicability for live-cell investigations of Na_V function, studies of which are ongoing and will be reported in due course.

■ ASSOCIATED CONTENT

Supporting Information

Experimental synthetic procedures and characterization data for all novel compounds, details of cell culture and electrophysiology procedures, additional supplementary electrophysiological data, and full discussion of NMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

jdubois@stanford.edu

Present Address

[†]Department of Chemical Physiology, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037, United States.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. T. R. Cummins (Indiana University) for providing the expression plasmid for $Na_V1.5$, Dr. L. L Isom (University of Michigan) for providing the expression plasmid for $Na_V\beta1$, and Dr. W. A. Catterall (University of Washington) for the gift of a $Na_V1.2$ stably expressed cell line. We are grateful to Dr. Bianxiao Cui (Stanford University) and Wenjun Xie for providing embryonic rat hippocampal neurons for our experiments. We are indebted to Dr. M. Maduke for her helpful discussions and for generous use of her lab space. W.H.P. is supported by a Stanford Interdisciplinary Graduate Fellowship (SIGF). This work was financially supported by National Institutes of Health R01 NS045684, R21 NS070064.

■ REFERENCES

(1) Aidley, D. J. *The Physiology of Excitable Cells*; Cambridge University Press: Cambridge, UK, 1998; Chapter 5.

(2) Catterall, W. A.; Goldin, A. L.; Waxman, S. G. *Pharmacol. Rev.* **2005**, *57*, 397−409.

(3) Nardi, A.; Damann, N.; Hertrampf, T.; Kless, A. *ChemMedChem* **2012**, *7*, 1712−1740.

(4) Leterrier, C.; Brachet, A.; Fache, M.-P.; Dargent, B. *Neurosci. Lett.* **2010**, *486*, 92−100.

(5) Fozzard, H. A.; Lipkind, G. M. *Mar. Drugs* **2010**, *8*, 219−234.

(6) Lipkind, G. M.; Fozzard, H. A. *Biophys. J.* **1994**, *66*, 1−13.

(7) Tikhonov, D. B.; Zhorov, B. S. *Biophys. J.* **2005**, *88*, 184−197.

(8) Scheib, H.; McLay, I.; Guex, N.; Clare, J. J.; Blaney, F. E.; Dale, T. J.; Tate, S. N.; Robertson, G. M. *J. Mol. Model.* **2006**, *12*, 813−822.

(9) Walker, J. R.; Novick, P. A.; Parsons, W. H.; McGregor, M.; Zablocki, J.; Pande, V. S.; Du Bois, J. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 18102−18107.

(10) Tanino, H.; Nakata, T.; Kaneko, T.; Kishi, Y. *J. Am. Chem. Soc.* **1977**, *99*, 2818−2819.

(11) Jacobi, P. A.; Martinelli, M. J.; Polanc, S. *J. Am. Chem. Soc.* **1984**, *106*, 5594−5598.

(12) Fleming, J. J.; McReynolds, M. D.; Du Bois, J. *J. Am. Chem. Soc.* **2007**, *129*, 9964−9975.

(13) Iwamoto, O.; Shinohara, R.; Nagasawa, K. *Chem.—Asian J.* **2009**, *4*, 277−285.

(14) Bhonde, V. R.; Looper, R. E. *J. Am. Chem. Soc.* **2011**, *133*, 20172−20174.

(15) Sawayama, Y.; Nishikawa, T. *Angew. Chem., Int. Ed.* **2011**, *50*, 7176−7178.

(16) For related studies with a voltage-gated potassium channel: Morin, T. J.; Kobertz, W. R. *ACS Chem. Biol.* **2007**, *2*, 469−473.

(17) Andresen, B. M.; Du Bois, J. *J. Am. Chem. Soc.* **2009**, *131*, 12524−12525.

(18) As shown in Figure S3 (Supporting Information), maximal irreversible inhibition is ∼85% upon either extended incubation times or iterative application of **1**; see Supporting Information for additional details. A similar inability to completely saturate current levels using a high affinity, covalent toxin against nAChR has been noted previously; see: Ambrus, J. I.; Halliday, J. I.; Kanizaj, N.; Absalom, N.; Harpsøe, K.; Balle, T.; Chebib, M.; McLeod, M. D. *Chem. Commun.* **2012**, *48*, 6699−6701.

(19) Isom, L. L.; Scheuer, T.; Brownstein, A. B.; Ragsdale, D. S.; Murphy, B. J.; Catterall, W. A. *J. Biol. Chem.* **1995**, *270*, 3306−3312.

(20) Prior studies indicate that the TTX-sensitive isoforms $Na_V1.2$, 1.3, 1.6, and 1.7 are expressed in embryonic rat hippocampal neurons, see: Mechaly, I.; Scamps, F.; Chabbert, C.; Sans, A.; Valmier, J. *Neuroscience* **2005**, *130*, 389−396. We have determined an IC_{50} of 2.9 ± 0.4 nM for STX against these cells (Figure S6, Supporting Information).

(21) For a general review, see: Vacher, H.; Mohapatra, D. P.; Trimmer, J. S. *Physiol. Rev.* **2008**, *88*, 1407−1447.

(22) Experiments in which either **8** or a monoguanidine-maleimide construct **S1** (Figure S7c, Supporting Information) are applied in combination with 5 μM STX show no irreversible block of current. These data argue against the possibility that toxin binding simply promotes nonspecific alkylation. For reasons that are not evident, however, simultaneous application of a 20-fold excess of either STX or TTX with **1** does not significantly attenuate irreversible block of channel current.

(23) Koehn, F. E.; Ghazarossian, V. E.; Schantz, E. J.; Schnoes, H. K.; Strong, F. M. *Bioorg. Chem.* **1981**, *10*, 412−428.

(24) Andresen, B. M. Ph.D. Dissertation, Stanford University, 2010.

(25) Preparation of C2-substituted maleimides adapted from Miles, W. H.; Yan, M. *Tetrahedron Lett.* **2010**, *51*, 1710−1712.

(26) For preparation of a biotinylated saxitoxin derivative through semisynthesis, see: Robillot, C.; Kineavy, D.; Burnell, J.; Llewellyn, L. E. *Toxicon* **2009**, *53*, 460−465.

(27) Cravatt, B. F.; Wright, A. T.; Kozarich, J. W. *Annu. Rev. Biochem.* **2008**, *77*, 383−414.