A “Minimal” Sodium Channel Construct Consisting of Ligated S5-P-S6 Segments
Forms a Toxin-Activatable Ionophore

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Running Title: The Pore-only Na Channel

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Summary

The large size (six membrane spanning repeats in each of four domains) and asymmetric architecture of the voltage-dependent Na channel has hindered determination of its structure. With the goal of determining the minimum structure of the Na channel permeation pathway, we created two stable cell lines expressing the voltage-dependent rat skeletal muscle Na channel (µ1) with a polyhistidine tag on the C-terminus (µHis) and pore-only µ1 (µPore) channels with S1-S4 in all domains removed. Both constructs were recognized by a Na channel specific antibody on a Western blot. µHis channels exhibited the same functional properties as wild-type µ1. In contrast, µPore channels did not conduct Na currents, nor did they bind $^3$H-STX. Veratridine caused 40% and 54% cell death in µHis and µPore-expressing cells, respectively. However, veratridine-induced cell death could only be blocked by TTX in cells expressing µHis, but not µPore.

Furthermore, using a fluorescent Na indicator, we measured changes in intracellular Na$^+$ induced by veratridine and a brevetoxin analogue PbTx-3. When calibrated to the maximum signal after addition of gramicidin, the maximal percentage increases in fluorescence ($\Delta F$) were 35% and 31% in cells expressing µHis and µPore, respectively. Moreover, in the presence of 1µM TTX, $\Delta F$ decreased significantly to 10% in µHis but not in µPore expressing cells (43%). In conclusion, S5-P-S6 segments of µ1 channels form a toxin-activable ionophore, but do not reconstitute the Na channel permeation pathway with full fidelity.
Introduction

Sodium channels conduct the electrical impulse in excitable tissues and serve as the receptors for many drugs and toxins[1]. Their essential functional features include highly-selective permeation of Na\(^+\) and voltage-dependent gating[2-5]. The principal functional unit of the Na channel is the \(\alpha\) subunit, which consists of four internally homologous domains (labeled \(DI-DIV\)), each containing six transmembrane segments (S1-S6) and resembling a single \(\alpha\) subunit of a voltage-dependent K channel[6](Figure 1A). These four homologous domains are pseudosymmetrically arranged around a central pore whose structural constituents determine the selectivity and conductance properties of the channel. While the fourth transmembrane segment S4, studded with positively charged residues, confers voltage-sensitive gating[7], the S5 and S6 segments and the S5-S6 linker or the pore-lining (“P”) segment line the permeation pathway. Such a structure, while complex and asymmetrical, hints that essential features of the pore may be separable from those which confer gating.

MacKinnon and coworkers have solved the crystal structure of an inwardly rectifying bacterial K channel (KcsA) channel[8]. This important advance provides a framework for testing hypotheses concerning the pore structure of related channels. With only two transmembrane segments and a P segment in each subunit, KcsA channels function perfectly well as K\(^+\)-selective ionophores. Compared with KcsA and other Kir-like channels, voltage-dependent channels, sharing the same transmembrane topology of the core segments, have evolved 4 additional TM segments (S1-S4) in each domain, with S4 responsible for voltage gating and S1-S3 presumably insulating hydrophilic S4 from the lipid bilayer. In this view, the functional pore might be fully encoded by the S5-P-S6...
fragments, while the rest of the protein is required only for effecting voltage-dependent conformational changes (Figure 1B). Therefore, it is logical to ask whether a Na channel comprised only of S5-P-S6 from each domain will contain the minimal determinants of a functional pore.

In this study, we stably expressed polyHis-tagged wildtype and pore-only μ1 channel constructs in mammalian cell lines, and characterized them biophysically, biochemically, and functionally. The main goal was to test if the pore-only μ1 channel is functional as an ionophore and as a high-affinity toxin-binding scaffold. Such a reduced construct, if functional, would simplify structural determination of the pore region.

**Experimental Procedures**

**Materials and chemicals:** Materials are from following sources: STX dihydrochloride was kindly provided by Dr. Sherwood Hall (FDA); ³H-STX, Amersham Pharmacia Biotech, Piscataway, NJ; NMG, Veratridine, and TTX, Sigma Chemical Co., St. Louis, MO; PbTx-3 (pumiliotoxin), Calbiochem, San Diego, CA; HEK293 cells, American Type Culture Collections, Manassas, VA; CoroNa Red Sodium Indicator and gramicidin A, Molecular Probes, Eugene, OR; Anti-Na channel antibody, Upstate Biotechnology, Lake Placid, NY; Geneticin (G418 Sulfate), Life Technology, Grand Island, NY.

**Molecular biology:** A DNA fragment containing a c-myc epitope and a polyhistidine tag from pCDNA3.1 (Invitrogen) was inserted into XbaI site at the 3’ end of the skeletal muscle Na channel (μ1) to generate μ1 His tagged DNA (μHis). μHis DNA, coupled with a GFP reporter gene, was then cloned in a polycistronic IRES vector, allowing independent translation of two separate proteins from a single mRNA. The pore-only μ1
DNA (μPore) was generated by deleting S1-S4 of each domain (Figure. 1A & C).
Specifically, L128 to K228 in domain I, L567 to L680 in domain II, I1020 to E1137 in
domain III, F1342 to L1465 in domain IV, were deleted by PCR using primers with bases
covering regions adjacent to the sections of the channel that were deleted (Stratagene).
All constructs were verified by sequencing. For mRNA generation, these constructs were
cloned into pSP64T vector and mRNA was transcribed from the SP6 promoter (Ambion,
Inc.)

Cell biology: All cells were grown in Dulbecco’s Modified Eagle’s Medium (DMEM)
with 10% (v/v) fetal calf serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin at 37°C
with 5% CO₂. Linearized DNA of μHis and μPore were transfected into HEK293 cells,
respectively, using LipofectAMINE Plus Reagent (Life Technologies, Inc.). Two days
after transfection, cells were serially diluted with the addition of 0.5 mg/ml geneticin to
select for transfected cells. Colonies were screened initially by epifluorescence, and
further characterized by whole-cell current measurements and Western blot analysis.
Positive clones were again sorted by GFP signals using a FACStar Plus cell sorter. For
functional assays, 0.5 million cells were seeded and grown overnight in a 35 mm dish.
Veratridine was first dissolved in ethanol at a concentration of 150 mM and then in
culture medium. For blocking experiments, cells were incubated with TTX for 1 hour
before adding veratridine. Cell death was determined counting percentage of floating
cells and trypsinized attached cells that failed to exclude Trypan blue.

Electrophysiology. Patch clamp and two electrode voltage-clamp were performed as
previously described[9]. All recordings were carried out at room temperature (~21°C).
Membrane vesicle preparation: Cells were washed with cold PBS, pelleted, resuspended in 1 mM EDTA, 50 mM Tris-Cl (pH= 8.0) with protease inhibitors (Roche), and homogenized. The homogenate was brought up in a final concentration of 300 mM sucrose, 0.5 mM EDTA, 10 mM Hepes-Tris (pH=7.5) and homogenized again before spinning at 4,000 x g for 15 min at 4°C. The supernatant was retained and homogenization and centrifugation were repeated on the pellet. The supernatants were then mixed to a final concentration of 0.6 M KCl. The mixture was stirred gently for 30 min at 4 °C before spinning down at 100,000 x g for 1 hour. The pellet was resuspended in 300 mM sucrose, 10 mM MOPS-TRIS (pH=7.0) and homogenized using a glass-Teflon homogenizer. Protein concentration was determined by a Lowry assay[10]. For rat brain membrane vesicle preparation, rat brains were immersed in cold 20 mM MOPS-Tris (pH7.4) and homogenized using a glass-teflon homogenizer, then subjected to the previously described cell membrane vesicle isolation protocol. Normally, one rat brain yields about 80 mg total membrane protein.

STX binding assay. 3H-STX binding assay was performed as described previously by Vélez et al[11]. 250 µg of membrane vesicles were incubated on ice for one hour with buffer containing 2.5nM 3H-STX, 30 mM choline chloride, 10 mM, Hepes-Tris, pH=7.4, and a range of cold STX from concentration of 0 to maximum 4µM in a 250 µl reaction volume. Free 3H-STX was removed by passing each reaction mixture through a glass filter. The filter was washed twice and counted using a scintillation counter.

Fluorescent indicator loading and Microfluorimetry. For optimal dye loading, cells were incubated with 1 µM CoroNa Red Na indicator (from a freshly dissolved 1 mM stock
DMSO solution, Molecular Probes) in 140mM NaCl, 5mM KCl, 1mM MgCl$_2$, 1mM CaCl$_2$, 10 mM glucose, pH 7.4 at 37°C for 2 hours.

Cells were plated on 15 mm glass coverslips and mounted on a perfusion chamber (Warner Instruments, PH4) that was placed on the stage of a Nikon Diaphot inverted fluorescence microscope. A 20x Nikon oil-immersion lens was used, and all experiments were done at room temperature. The fluorescence cube in the microscope consisted of a 540±15 nm excitation filter, a 565LP dichroic mirror and a 620±30 nm emission filter. A 12-bit monochrome cooled CCD camera (5MHz readout rate, MicroMax, Princeton Instruments) was used to collect 650 x 514 pixel 16-bit grayscale images once every minute with either a 100 or 200 ms exposure time.

Experiments were performed during continuous perfusion of buffers and drugs at 0.6 to 1.0 mL/min. A run was initiated after a stable fluorescence signal was obtained during perfusion of control solution containing 140mM NaCl, 5mM KCl, 1mM MgCl$_2$, 1mM CaCl$_2$, 10 mM glucose, pH 7.4. At least 10 images were obtained in each condition. Addition of pumiliotoxin (PbTx-3), an analogue of brevetoxin, and veratridine elicited a slow increase of fluorescence that usually reached steady state within 10-15 minute. Normally only one addition of toxin could be done per run. The chamber was then perfused with control solution to dilute the added toxin, and the calibration sequence was initiated. In this case, perfusion was interrupted and 1 mL of 2 μM gramicidin dissolved in control solution was directly added to the chamber. After development of maximal fluorescence, typically 6 to10 min, perfusion was restarted and the calibration sequence was completed by the consecutive addition of buffers containing different sodium concentrations.
Data analysis. At least 5 regions of interest (ROI) covering all the cells were measured in each experiment. Average fluorescence intensity of each ROI was analyzed by ImageJ (http://rsb.info.nih.gov/ij/). The percentage changes for fluorescence signal, corresponding to the intracellular Na\(^+\) changes, were calibrated with gramicidin and calculated using the following formula:

\[
\frac{(F-F_{\text{min}})}{(F_{\text{max}}-F_{\text{min}})} \times 100\%
\]

where F is the fluorescence signal, F\(_{\text{min}}\) is the control signal in normal 140mM Na buffer; F\(_{\text{max}}\) is the maximum signal in the presence of gramicidin. At least 5 points in the plateau were averaged for each treatment. Data were further analyzed and plotted using Origin (Microcal Software, Inc.)

Results

Channel protein expression: We created two HEK293 stable cell lines expressing either wild-type \(\mu 1\) channels with a c-myc epitope and a polyhistidine (\(\mu \text{His}\)) tag fused to the C-terminus, or pore-only \(\mu 1\) (\(\mu \text{Pore}\)) channels with S1-S4 in all domains removed (Figure 1C). The stably-transfected cells all had a normal morphology under basal conditions. Membrane vesicles prepared from both cell lines were recognized by a Na channel specific antibody directed to the retained III-IV interdomain linker (SP19). \(\mu \text{His}\) channels, with a calculated molecular weight of 212KDa, ran at 240 KDa, while \(\mu \text{Pore}\), with a calculated molecular weight of 159KDa, ran at 210 KDa on a Western blot (Figure 2). Antibodies raised to the c-myc and polyHis-tags both recognized \(\mu \text{Pore}\) and \(\mu \text{His}\) channels (data not shown), indicating that the entire constructs were faithfully expressed. Using a polycistronic IRES vector, we found that the reporter GFP and the channel
construct were independently expressed and co-localized. In cells expressing either µHis or µPore channels, confocal microscopy indicated that the GFP signals were detected at the cell surface membrane (data not shown). These results indicated that µPore channels were expressed at the surface membrane.

**Channel activity:** Channel activity of the µHis and µPore channels was functionally characterized using the patch clamp technique. Whole-cell currents from cells stably expressing µHis channels exhibit the same electrophysiological properties as wild-type µ1 channels (Figure 3A), indicating they are fully functional. In contrast, µPore failed to conduct measurable time-dependent, Na⁺-selective currents despite expression of the protein (Figure 3B). In addition, no currents were observed in either mRNA-injected *Xenopus* oocytes or transiently-transfected HEK293 cells (data not shown). One explanation is that µPore channels may not be able to fold properly to form an ionophore; alternatively, µPore channels may generate a pore that favors a closed conformation. Since the voltage-sensing S4 segments are not present in µPore channels, voltage changes may not be able to generate conformational changes necessary to open µPore channels. Nevertheless, we explored the possibility that such channels might retain toxin sensitivity.

**STX binding assay:** Tetrodotoxin (TTX) and saxitoxin (STX) block the Na channels at neurotoxin receptor site 1, which is localized near the extracellular opening of the pore. High-affinity STX binding requires a highly stereotypical 3-dimensional structure with crucial contributions from numerous pore residues present in the extracellular linkers, including those from the P segments[12-16]. Thus, preservation of STX binding could be considered as evidence of proper processing and folding of µPore channels. We
performed STX binding assays on membrane vesicles of rat brain, stable cell lines expressing µ1 and µPore channels, and non-transfected HEK293 cells. Control HEK293 cells did not bind STX. In contrast, rat brain membrane vesicles exhibited strong $^3$H-STX binding with the total concentration of the binding sites ($B_{\text{max}}$) of 0.86 pmol $^3$H-STX binding per mg of total membrane protein, in agreement with previous reports[17, 18]. Membrane vesicles containing µ1 channels exhibited specific $^3$H-STX binding with a $B_{\text{max}}$ of 0.46 pmol/mg protein (Figure 4A). However, $^3$H-STX binding signal of membrane vesicles containing µPore channels was indistinguishable from background.

In addition, in $^3$H-STX displacement assays using non-radioactive STX, rat brain membrane vesicles and membrane vesicles containing µ1 channels exhibited specific $^3$H-STX binding (Figure 4B), but again µPore channels did not exhibit any $^3$H-STX displacement. These data suggest that µPore channels did not preserve a Na channel structure that specifically binds to STX. Nevertheless, we tested whether µPore channels remained sensitive to other Na channel toxins that bound to sites distinct from the STX binding site.

**Veratridine-induced cell death.** Veratridine causes persistent opening of Na channels and elevates intracellular Na$^+$ concentration, which perturbs ion homeostasis sufficiently in neurons and astroglia to trigger cell death[19, 20]. If our cells contain any toxin-activatable Na channels, they may likewise die during exposure to veratridine. Thus, we determined whether veratridine affects cell viability in our stable cell lines.

Control (non-transfected) HEK293 cells and cells expressing µPore and µHis channels were incubated with various concentrations of veratridine for 20 hours at 37°C, 5% CO$_2$. Cell death was assayed by the inability to exclude trypan blue (Figure 5A).
the absence of veratridine, none of the three cell lines exhibited measurable cell death. However, at veratridine concentrations of 200µM or higher, cells expressing µHis and µPore channels showed significantly more cell death than did control HEK293 cells. At 225µM veratridine, in control HEK293 cells, only 16% of cells were killed, but 40% and 54% of cells expressing µHis and µPore channels died, respectively (Figure 5B). This effect was specific, as the pro-apoptotic agents staurosporine and etoposide did not cause more cell death in µPore cells than in µHis cells (data not shown).

When STX/TTX binding is intact, TTX potently blocks the action of site 2 toxins. Therefore, TTX should block the veratridine-induced cell death in µHis cells[19], but not in µPore cells if µPore channels do not bind TTX. Under the same conditions, veratridine-induced cell death was significantly decreased in cells expressing µHis in 50 µM and 200 µM TTX (Figure 5B), confirming that TTX indeed blocked Na influx through µHis channels induced by veratridine. In addition, no significant changes were observed in control HEK293 cells (15% and 12% of cell death in 50 µM and 200 µM TTX, respectively). However, TTX did not reduce veratridine-induced cell death in cells expressing µPore channels, with cell death rates of 50% and 49% in 50 µM and 200 µM TTX, respectively (Figure 5B). Therefore, while blocking Na channels and antagonizing veratridine-induced cell death in µHis cells, TTX failed to block veratridine induced-cell death in µPore cells. Taken together, the findings were consistent with the idea that µPore channels retain veratridine sensitivity, but lose the ability to bind TTX and STX. In order to obtain additional independent evidence for a toxin-recruitable ionophore, we measured intracellular Na⁺ in cells expressing µHis and µPore channels.
Toxin-induced Na influx. If a µPore channel forms a veratridine-recruitable ionophore, one would expect an acute increase of Na\(^+\) influx when µPore is activated by veratridine. When veratridine alone was applied to cells expressing µPore channels, there was no consistent activation of Na\(^+\)-selective currents in patch clamp records (data not shown), perhaps due to the small density and time-independent nature of the currents. The lack of TTX sensitivity also made it impossible to use toxin sensitivity as a tool to specifically to isolate the current. Therefore, we developed an alternative strategy to assay intracellular Na\(^+\) changes in response to the opening of channels induced by toxin treatment. CoroNa Red, a Na-specific fluorescent dye, exhibits sensitive responses to Na\(^+\) concentration in the thin layer of fluid at the surface of large airways[21]. Here, we use CoroNa Red Na indicator to measure intracellular Na\(^+\) changes generated by Na\(^+\) influx through Na channels elicited by activating toxins.

The intracellular Na\(^+\) concentration changes were detected by changes in the CoroNa Red fluorescence, using a CCD camera. These signals were normalized to the percentage increase in fluorescence (ΔF) relative to the maximum signal after the addition of gramicidin, when intracellular and extracellular Na\(^+\) had presumably reached equilibrium. In all experiments, gramicidin elicited an average 2.6-fold increase over the background signal. Veratridine alone did not induce a significant increase in ΔF in either µHis or µPore cells, at least in the short term (over ~30 min; data not shown). Therefore, we used a combination of PbTx-3 and veratridine to activate these channels, exploiting the fact that PbTx-3 may allosterically stimulate veratridine-induced Na\(^+\) flux. Figure 6A shows representative data for µPore and µHis channels with summary plots shown in figure 6B. In control HEK293 cells, PbTX-3 and veratridine only generate an average of
2.5% increase in \( \Delta F \) (Figure 6B). In cells expressing either \( \mu \)His or \( \mu \)Pore channels, addition of the mixture of PbTx-3 and veratridine elicited a \( \Delta F \) increase of 35% and 31%, respectively, suggesting that there was a significant increase of intracellular Na\(^+\) due to Na influx through toxin-activated \( \mu \)His or \( \mu \)Pore channels. Furthermore, preincubation of 1\( \mu \)M TTX significantly decreased \( \Delta F \) in \( \mu \)His cells, but not in \( \mu \)Pore cells. Thus, PbTx-3 and veratridine activated both \( \mu \)Pore and \( \mu \)His channels and resulted in Na\(^+\) influx through the activated channel pore. The Na\(^+\) influx in activated \( \mu \)Pore channels is resistant to block by TTX, further confirming the insensitivity of \( \mu \)Pore channels to TTX.

**Discussion**

In a quest for a “minimal” Na channel structure, we trimmed the channels of all but those core segments that would be absolutely required for permeation. Based on the structure of KcsA and other Kir family K channels, we reasoned that the S5-P-S6 segments of a voltage-dependent Na channel might retain some aspects of a functional pore. Through biochemical, and physiological characterizations, we found that S5-P-S6 segments of \( \mu 1 \) channels do form functional ionophores, albeit ones which lack full fidelity when compared to the parent channels. Key structural motifs of Na channels are missing in \( \mu \)Pore channels, in that they fail to bind TTX or STX functionally and biochemically. Nevertheless, \( \mu \)Pore ionophores can be activated by Na channel toxins veratridine and PbTX-3, as gauged by Na\(^+\) fluorescence measurements and cell viability assays.

The following considerations support the notion that the fluorescence increases induced by PbTx-3 reflect the stimulation of Na influx through Na channels. First, *in situ* calibration curves (not shown) indicated that CoroNa Red can track sodium very
specifically, regardless of whether the remaining salt in the solution is NMG chloride or KCl. Second, we also have excluded potassium as a measurable interfering cation from examination of the in situ calibration curves that show that CoroNa Red fluorescence decreases in the presence of high K concentrations (NaCl/KCl buffers) as compared to the values obtained in the absence of K at the same Na concentration (i.e. in NaCl/NMG buffers). Due to the dye structure, the coordination of negative ions should be excluded from the crown moiety that coordinates sodium. The same would be the case for divalent cations, although there could be some competition as this dye indeed is derived from EDTA-like moieties. Third, the fluxes were only seen when PbTx-3 was added to cells expressing Na channels. The signals were not observed after high-K depolarization of HEK293 cells expressing Na channels or in control HEK cells with any of the treatments. The fluxes were also detected when cells expressing Na channels were exposed simultaneously to PbTx-3 and veratridine dissolved in a 45 K buffer, but they were similar to those in PbTx-3 and veratridine in a normal low-K buffer for both µpore and µHis cells. This treatment did not induce a fluorescence increase in control HEK293 cells, indicating that, during toxin activation, the fluxes are not affected in a measurable way by changes in the electrochemical gradient for potassium. If K efflux were to co-exist in µPore, but not in µHis cells, a measurable difference would have been expected in the fluorescence changes. Finally, during toxin activation in normal 140 Na buffer, potassium would be leaking out, and therefore the proportion of K-dye complexes should, if anything, decrease, producing a F decrease if K were indeed a serious contaminant. For these reasons, we believe that the toxin-activated fluorescence signals reflect Na flux through Na channels.
Gating of the µPore channel

The pore of Na channels exhibits a high degree of conformational flexibility and is involved in channel gating. The domain III-IV linker of Na channels is a prime determinant of rapid inactivation[7, 22, 23], while the external pore has been linked to slow inactivation[24-26]. We hypothesized that the inactivation mechanisms should be preserved in µPore channels as these structural elements are still intact. Indeed, like wild-type Na channels, µPore channels predominately are in closed or inactivated conformations at rest; otherwise, persistent Na⁺ influx at rest might be expected to kill cells expressing µPore. Since the voltage sensor is not present, µPore channels cannot be activated by depolarization. However, non-voltage-dependent activation appears to exist in µPore channels. Our results indicate that µPore channels can be activated by Na channel-opening toxins.

Veratridine and PbTx-3 mechanisms.

Veratridine and PbTx-3 are Na channel openers that bind to site 2 and site 5, respectively on the α subunit of the Na channel[27]. Site 2 neurotoxins, including the alkaloids batrachotoxin (BTX), veratridine, aconitine, and grayanotoxin, cause persistent activation of Na channels[28, 29]. Studies have shown that veratridine and BTX share a common binding site in D1-S6 and D4-S6[30, 31]. In wild-type channels, veratridine binds to open Na channels[32], blocking inactivation, shifting the voltage dependence of activation to more negative membrane potentials, and reducing selectivity. TTX and STX potently counteract the action of veratridine. Site 5 toxin brevetoxin and
pumiliotoxin (PbTx-3), lipid-soluble polyethers, bind to a receptor site located near the transmembrane interface between D1 and D IV near the extracellular side of D IV S5 segment and extracellular end of D I S6 segment of the α subunit[33, 34]. This interaction causes a shift in the voltage dependence of channel activation to more negative potentials and inhibits channel inactivation. In addition, the oxygen-rich nature of brevetoxins backbone interacts with the channel in a manner that stabilizes the open configuration[35]. Moreover, site 2 and site 5 lipid-soluble toxins are allosteric modulators of channel function. PbTx-3 allosterically stimulates Na influx generated by site 2 toxins including veratridine and BTX[36, 37]. As segments S5 and S6 are preserved in the µPore construct, the binding sites of veratridine and PbTx-3 might still be intact on µPore channels. Our functional data showed that the interactions of veratridine and PbTx-3 on µPore channel resulted in activation of the ionophore in a non-voltage-dependent fashion.

**Conclusions.** In an effort to define the minimal structure required to create a functional Na channel pore, we have found that the S5-P-S6 segments of the µ1 Na channel suffice to form a toxin-activatable ionophore. Unfortunately, the reduced construct does not provide a compelling platform for further protein purification and structure prediction, as it does not retain key biochemical markers (viz., STX binding) that would enable tracking of the protein during enrichment and purification. Nevertheless, our observations are conceptually valuable, as they identify the minimal determinants for two key features of pore function (Na⁺ flux and toxin activation).
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ABBREVIATIONS: TTX: Tetrodotoxin; STX: saxitoxin; PbTx-3: pumilioxotoxin; NMG: N-methyl-D-glucamine.

Figure legends

Figure 1. Schematic depictions of the Na channel α subunit. A, Transmembrane topology of the Na channel. The boxes enclose the S1-S4 segments that were deleted to generate the μPore channel. The amino acids of the start of S1 and the end of S4 in each domain are indicated. B, Schematic of the folding of the Na channel around the ion selective pore. The ring highlights the putative channel pore-lining segments. C, Transmembrane topology of the μPore Na channel, where S1-S4 segments were deleted in all domains.

Figure 2. Immunoblot analysis. A Na channel specific antibody that recognizes an epitope in the interdomain III-IV linker binds to both μPore and μHis channels. Membrane vesicles were prepared as described in Methods and Materials section. 10 μg of total membrane proteins for μPore, μHis and Hek293 cells, were subject to SDS-
PAGE (4-15% gradient) and immunoblotting with an anti-Na channel polyclonal antibody.

Figure 3. **Whole-cell Na currents recordings.** Currents elicited by a family of depolarizing voltage steps from a holding potential of –100 mV to test voltages of –60 to +50 mV in increments of 10 mV. A, HEK293 cells expressing µHis channels exhibit normal currents while B, cell expressing µPore channels did not have any time- or voltage-dependent currents.

Figure 4. **³H STX binding in membrane vesicles of rat brain, native Hek293 cells, and cells expressing µ1His and µPore channels.** A, Equilibrium binding of 2.5 nM ³H STX binding to membrane vesicles as described in Methods (n=3). B, ³H STX displacement by increasing concentrations of cold STX. in contrast to rat brain and µ1His-containing vesicles, µPore (n=3) did not exhibit any ³H STX displacement.

Figure 5. **Veratridine induced cell death.** A. In normal DMEM, veratridine reduced cell survival in µ1His and µPore expressing HEK293 cells as assessed by failure to exclude Trypan blue. B. Veratridine-induced cell death was partial blocked by TTX in cells expressing µ1His channels but not µPore channels (n=4, * p<0.01, one-way ANOVA).

Figure 6. **Toxins induced Na⁺ influx.** A, Representative experiments of the percent change in fluorescence in HEK293 cells loaded with CoroNa Red expressing µ1His and
µPore channels when exposed to different toxins. B, Summary data plotting the change in fluorescence when cells were exposed to 112nM PbTx-3 and 150µM veratridine with or without preincubation of 1 µM TTX. (n=4, ROI>25, * p<0.001).

References


Fig. 2

[Image of a gel electrophoresis with molecular weight markers and bands labeled for different samples: μPore, μHis, and HeLa293.]
Fig. 3

A  μHis

B  μPore

5 msec

5 mS

-100 mV

50 mV

-50 mV
Fig. 5

A

B

% Cell Death

Veratridine (μM) 0 100 200 300 0 100 200 300 0 100 200 300

HEK293 μ1His μ1Pore

% Cell Death

225μM Veratridine

TTX (μM) 0 50 200 0 50 200 0 50 200

HEK293 μ1His μ1Pore
Fig. 6

A  μPore   μHis

B

% Increase

TTX - μPore + μHis + HEK293

% Increase

-  -  -
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