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Minimal sodium channel pore consisting of S5-P-S6 segments preserves intracellular pharmacology

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Abstract

We studied the properties of a sodium channel comprised only of S5-P-S6 region of the rat sodium channel α-subunit Nav1.4 (μ1pore). Results obtained in HEK cell lines permanently transfected with the sodium channel α-subunit or with the μ1pore were compared with data of the native HEK cells. Sodium channel blockers, tetrodotoxin and tetracaine, protect cells transfected with the complete sodium channel against death produced by incubation with veratridine. Veratridine-induced cell death in cell lines expressing the μ1pore construct is antagonised by tetracaine, but not by tetrodotoxin. Whole-cell conductance also increases in the presence of veratridine in μ1pore transfected cells and tetracaine inhibits these currents. Our pharmacological and electrophysiological data suggest that μ1pore keeps binding sites for veratridine and tetracaine, but not for TTX, and reconstitutes the permeation pathway for Na^+ ions.

Keywords: Sodium channels; Toxins; Local anaesthetics; Pore; Pharmacology

Sodium channels conduct the electrical impulse in excitable tissues and serve as receptors for many drugs and toxins [1]. Their functional features include a highly selective permeation of sodium and voltage-dependent gating [2,3]. The principal functional unit of the sodium channel is the α-subunit, which consists of four homologous domains, each containing six transmembrane segments (S1–S6) resembling a single α-subunit of a voltage-dependent K^+ channel. These four domains are arranged around a central pore, which is responsible for the ion selectivity and for the conductance of the sodium channel. In each domain, the charged segment S4 confers voltage-sensitive gating, transmembrane segments S1–S3 probably insulate the S4 from the lipid bilayer, while segment S5–S6 and S5–S6 linkers (P) form the permeation pathway.

Chen et al. [4] observed that a polypeptide comprised only of S5-P-S6 from each domain of the sodium channel (μ1pore) forms a toxin-activatable ionophore. The μ1pore is apparently activated by veratridine, a plant alkaloid that binds to sodium channels and stabilises them in the open state, probably in a site in the S6 segment of first domain [5]. However, the ionic flux across μ1pore cannot be inhibited by tetrodotoxin (TTX), which selectively and reversibly blocks the sodium currents acting from the extracellular side. We have further explored the pharmacology of the μ1pore, by analysing the effect of tetracaine. This drug is a local anaesthetic that inhibits the sodium channels, acting from the intracellular side, binding a region in the S6 segment in domain IV [6]. We have found that, as occurs for the hydrophobic alkaloid veratridine, the integrity of the local anaesthetic binding site is conserved in the μ1pore, suggesting that the structure of the intracellular vestibule of the sodium pore would be preserved in this minimal pore construct.

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Materials and methods

Human embryonic kidney cell lines (HEK-293) were grown in standard conditions, in DMEM–F12 (1:1) medium supplemented with 10% foetal calf serum, 2 mM glutamine, and 0.05 mg/100 ml gentamicin at 37 °C with 5% CO_2. Cells were permanently transfected with sodium channel α-subunit (HEK-α) [7,8] or with the μ1pore (HEK-μ1pore) [4].

For veratridine-induced cell death assays, 5×10^5 cells were seeded and grown overnight in a 96-multiwell. Variable concentrations of veratridine were then added to the culture medium. Channel blockers, TTX or tetracaine, were added to the veratridine-containing medium. After 24 h of exposure to the drugs, the vital stain fluorescein diacetate (4.5 μl/ml) was added and incubated at 37 °C for 30 min. Cell death was determined by counting the percentage of cells that did not retain any fluorescein diacetate but that were stained by ethidium bromide. Cell counting was done in quadruplicate for each experiment.

Sodium currents were measured using the patch-clamp technique in the whole-cell configuration [9] using a standard amplifier EPC7 (List Medical Electronics, Darmstadt, Germany). Borosilicate glass micropipettes (Hilgemberg, Mansfield, Germany) were fire polished to a tip diameter yielding a resistance of 1.0–2.0 MΩ in the working solutions. Pipettes were filled with (in mM): NaCl 25, CsCl 110, EGTA 10, TEA-Cl 2, Hepes–NaOH 10, pH 7.3. The standard external solution had the following composition (in mM): NaCl 100, KCl 5, $CaCl_2$ 2, TEA-Cl 20, Hepes–NaOH 10, pH 7.4. Osmolarity was adjusted to 300 mOsm/kg with mannitol. Toxins (veratridine, PbTx-9) and tetracaine were added to the extracellular working solution at the desired concentration immediately before each experiment. All chemicals, including toxins, were purchased from Sigma–Aldrich (St. Louis, MO, USA).

The output of the patch-clamp amplifier was filtered by a low-pass 4-pole Bessel filter with a cut-off frequency of 5 kHz and sampled at 20 kHz. The cell was kept at a holding potential of −100 mV. Capacitive responses were partially compensated analogically. No analogical or digital leakage subtraction was used. Measurements were done at a room temperature (20 ± 1 °C). Data were expressed as mean values ± SEM (number of experiments).

Results

Veratridine-induced cell death

Control non-transfected cells (HEK-0), and expressing HEK-μ1pore and HEK-α cells were incubated with various concentrations of veratridine for 24 h at 37 °C. In the absence of veratridine, none of the three cell lines exhibited measurable cell death (⩽1%). When control HEK cells were incubated in a medium containing 200 μM veratridine, toxicity of the drug induced a 3% of death in HEK-0 cells. Differently, activation of the sodium channel and the μ1pore by veratridine caused 17% and 34% of cell death in HEK-α and HEK-μ1pore cells, respectively. As expected, sodium channel blockers significantly decreased the veratridine-induced cell death in HEK-α cells to 5% and 4% in 1 μM TTX and 300 nM tetracaine, respectively. However, application of 1 μM TTX did not reduce veratridine-induced death in HEK-μ1pore cells, yielding values of 26%. Differently, a significant decrease of death, to 3%, was observed on HEK-μ1pore cells incubated with 300 nM tetracaine.

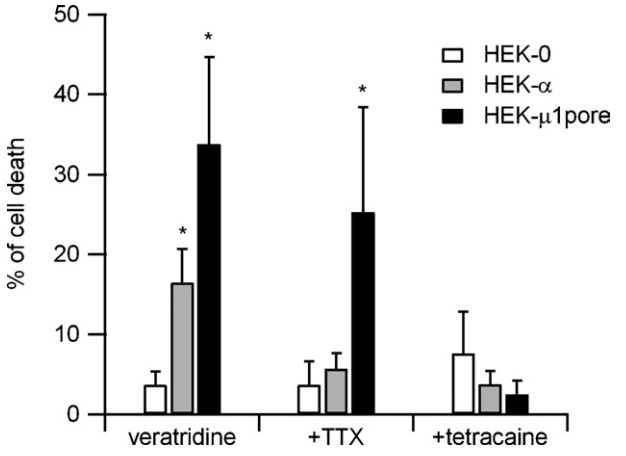

Fig. 1. Percentage of cell death of control non-transfected cells (HEK-0), and cells transfected with the sodium channel α-subunit (HEK-α) and the minimal sodium channel pore (HEK-μ1pore). Percentage of death was measured after 24 h incubation with 200 μM veratridine and with the addition of 1 μM TTX or 300 nM tetracaine. Asterisks indicate that is significantly different from HEK-0 control cells. Data were obtained from at least eight independent experiments for each cell line.

No significant changes were observed in control HEK-0 cells. A summary of these data is shown in Fig. 1.

Minimal sodium channel pore electrophysiology

We recorded whole-cell currents elicited by 50 ms depolarising pulses from a holding potential of −100 mV to a variable voltage from −60 to +70 mV. Currents recorded from HEK-μ1pore cells did not show any voltage- or time-dependent behaviour, and were not different from those recorded on non-transfected HEK-0 cells (Figs. 2A and D). To activate the μ1pore, we applied 200 μM veratridine and 110 nM PbTx-9 [10]. Application of 200 μM veratridine on HEK-α cells induces non-inactivating currents with a considerable delay in the deactivation, indicating that the drug stabilises the sodium channels on the open state (data not shown). For electrophysiological experiments on HEK-μ1pore, we used the brevetoxin PbTx-9, a lipid-soluble polyether marine toxin, to further potentiate the effect of veratridine [4,11,12]. Application of the sodium channel activators resulted in a slight increase in current in control HEK-0 cells (Figs. 2B and 3A), probably by the destabilisation of the membrane due to the lipid-soluble nature of veratridine. However, when the toxins were applied to HEK-μ1pore cells, a significant bigger increase in the current was detected (Figs. 2E and 3B). The equilibrium potential of the current elicited by veratridine is 24.9 ± 2.9 mV, that is near to the equilibrium potential of sodium.

We tested whether the μ1pore could be blocked by a typical intracellular ligand, the local anaesthetic

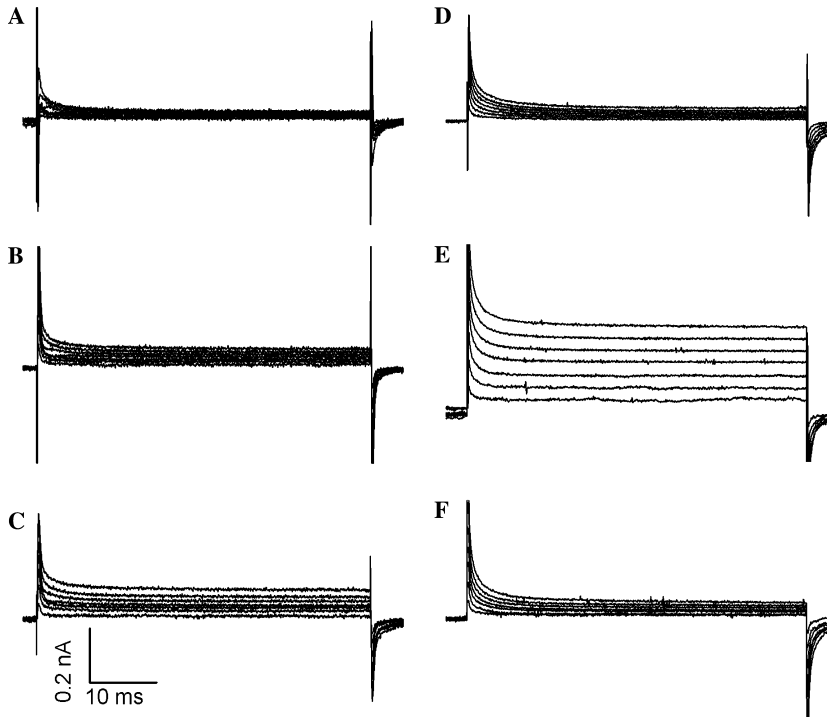

Fig. 2. Whole-cell currents recorded from non-transfected cells (A–C), HEK-0, and cells transfected with the minimal sodium channel pore (D–F), HEK-μ1pore. Cells were kept at −100 mV and current was measured from a 50 ms pulse from −50 to 70 mV, in 20 mV steps. Traces represent the raw data without any leakage subtraction. Control traces, recorded in normal extracellular solution, do not show any significant current in HEK-0 (A) or in HEK-μ1pore cells (D). Application of 200 μM veratridine and 110 nM PbTx-9 induces a significant increase of current for μ1pore transfected cells (E), compared to the non-transfected controls (B). This increase in current in HEK-μ1pore is inhibited by the addition of 300 nM tetracaine (F), while no significant effects occur in HEK-0 control cells.

tetracaine. Application of 300 nM tetracaine (in the presence of 200 μM veratridine) reduced significantly the membrane conductance increase induced by veratridine in HEK-μ1pore cells (Figs. 2F and 3B), while it did not produce any effect on control HEK-0 cells (Figs. 2C and 3A). Differently, extracellular application of 1 μM TTX did not change significantly the current elicited by veratridine (data not shown). Conductance for each experimental condition was measured from the slope of the current to voltage curves as those shown in Fig. 3. Data were normalised by the cell capacity, as measured from the capacity compensation in the patch-clamp amplifier. Increase in the cell conductance by veratridine and the antagonism of tetracaine is shown in Table 1. We concluded that expression of the minimal sodium channel pore induces a veratridine-activated current, which can be inhibited by tetracaine.

Discussion

The minimal sodium channel construct was designed with the rationale that the S5-P-S6 segments of the voltage-dependent sodium channels have structural similarities with Kcsa and Kir potassium channels, and therefore they may form a functional pore [4]. Expression of the l1pore on HEK cells results in a phenotype sensitive to the sodium channel activator veratridine. Veratridine binds to sodium channels and stabilises them in the open state. Probably its binding site is in the S6 segment of first domain [5]. This persistent opening of sodium channel causes the elevation of the intracellular Na^+ concentration, which perturbs ion homeostasis sufficiently to trigger cell death [13]. Hence, if cells contain any toxin-activatable sodium channel, they may likewise die during exposure to veratridine. When HEK-l1pore cells are exposed to this alkaloid, there is a reduced viability, compared to the HEK-0 non-transfected cells. Veratridine reduces the viability also on cells expressing the complete sodium channel, HEK-a (Fig. 1). The slightly reduced effect observed on HEK-l1pore cells could be due to the complete channel that has the voltage sensors and the inactivation mechanisms that may partially antagonise the effect of veratridine. Conversely, the minimal sodium channel pore expressed in HEK-l1pore cells has only the intracellular gating mechanism, being more sensitive to veratridine. The properties of the structure formed by the sodium channel S5-P-S6 segment construct differs in some properties of the complete sodium channel, as it fails to bind guanidinium toxins [4]. This characteristics

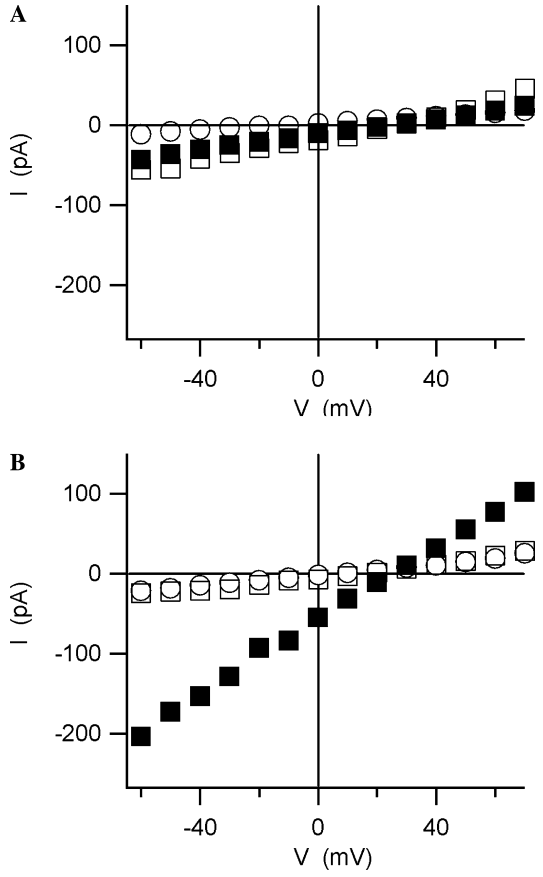

Fig. 3. Current–voltage relationships measured from non-transfected cells (A) and cells transfected with the minimal sodium channel pore (B). Data were obtained from the same experiments shown in Fig. 2. Points represent the mean current evaluated in the last 30 ms interval of the pulse obtained in control conditions (circles), after perfusion with 200 μM veratridine and 110 nM PbTx-9 (open squares), and after the addition of 300 nM tetracaine.

is confirmed in our laboratory by the lack of protection to veratridine-induced death by TTX (see Fig. 1).

In a previous report, Chen et al. [4] were unable to record veratridine-evoked currents from the same construct. Perhaps the small density and the time- and voltage-independent nature of these currents concealed their identification. Moreover, the lack of TTX sensitivity made it impossible to use toxin sensitivity as a tool to specifically isolate the current. We used a combination of the brevetoxin PbTx-6 and veratridine to activate these channels, exploiting the fact that brevetoxins may allosterically stimulate veratridine-induced sodium channel activation [4,11,12]. On the other hand, the local anaesthetic tetracaine could be used to block the veratridine-evoked current, overcoming the lack of integrity of the guanidinium receptor in the μ1pore construct. Hence, we succeeded in recording an increase in current upon application of veratridine, potentiated by the addition of PbTx-9, on cells expressing the μ1pore. This veratridine-evoked current completely lacks any voltage-dependence (see Fig. 2), indicating that the voltage-sensitive mechanisms are absent on the μ1pore. An interesting finding is that the channels expressed in HEK-μ1pore cells are preferentially in the closed state, and the activation by veratridine is necessary to record measurable currents.

By analogy to the inward rectifier potassium channels, the gating mechanism of the voltage-dependent sodium channel may reside on the C-terminus of the S6 segments, and this mechanism would be preserved in the μ1pore. This hypothesis is supported by the veratridine-evoked currents and the veratridine-induced cell death, as this alkaloid would bind S6 segment of first domain. Inhibition of the veratridine-evoked currents (Fig. 2) and the veratridine-induced cell death (Fig. 1) by tetracaine further advocates in favour of this hypothesis. Tetracaine is a local anaesthetic that inhibits the voltage-gated sodium channels binding to the S6 segment in domain IV of the sodium channel [6]. This fact will be very useful for further investigations on this construct, as consists of a functional probe to confirm the activity of the μ1pore.

In conclusion, the minimal sodium channel pore, μ1pore, expressed a channel that lacks the guanidinium toxin receptor in the extracellular side, but seems to preserve the intracellular vestibule of the sodium channel, including the regions responsible for veratridine and local anaesthetic binding. This finding would justify further investigations on the functional properties of the μ1pore, and would provide the platform for structural studies of the intracellular side of the sodium channel pore.

Table 1
Conductance changes induced by veratridine and PbTx-9, and tetracaine inhibition

	Conductance (μS/pF)			
	Control	+200 μM veratridine + 110 nM PbTx-9	+200 μM veratridine + 110 nM PbTx-9 + 300 nM tetracaine	+200 μM veratridine + 110 nM PbTx-9 + 600 nM tetracaine
HEK-0	0.45 ± 0.09 ($n = 5$)	1.89 ± 0.49 ($n = 5$)	1.85 ± 0.59 ($n = 4$)	
HEK-u1pore	1.76 ± 0.64 ($n = 3$)	4.53 ± 0.86 ($n = 13$)	2.24 ± 0.93 ($n = 6$)	2.07 ± 1.4 ($n = 3$)

Conductance was measured as the slope of the current–voltage relationships as those shown in Fig. 2. Data represent means ± SEM (number of observations).

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