A Superfamily of Voltage-gated Sodium Channels in Bacteria*§

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NaChBac, a six-α-helical transmembrane-spanning protein cloned from Bacillus halodurans, is the first functionally characterized bacterial voltage-gated Na⁺-selective channel (Ren, D., Navarro, B., Xu, H., Yee, L., Shi, Q., and Clapham, D. E. (2001) Science 294, 2572–2575). As a highly expressing ion channel protein, NaChBac is an ideal candidate for high resolution structural determination and structure-function studies. The biological role of NaChBac, however, is still unknown. In this report, another 11 structurally related bacterial proteins are described. Two of these functionally expressed as voltage-dependent Na⁺ channels (NaVPZ from Paracoccus zeaxanthinifaciens and NaVS from Silicibacter pomeroyi). NaVPZ and NaVS share ~40% amino acid sequence identity with NaChBac. When expressed in mammalian cell lines, both NaVPZ and NaVS were Na⁺-selective and voltage-dependent. However, their kinetics and voltage dependence differ significantly. These single six-α-helical transmembrane-spanning subunits constitute a widely distributed superfamily (Na₆Bac) of channels in bacteria, implying a fundamental prokaryotic function. The degree of sequence homology (22–54%) is optimal for future comparisons of Na₆Bac structure and function of similarity and dissimilarity among Na₆Bac proteins. Thus, the Na₆Bac superfamily is fertile ground for crystallographic, electrophysiological, and microbiological studies.

Mammalian voltage-gated sodium (Naᵥ) and calcium (Caᵥ) channels underlie membrane excitability, muscle contraction, and hormone secretion (1). In contrast, the function of prokaryotic voltage-gated ion-selective channels is relatively unknown. Na⁺ channels may drive Na⁺-dependent flagellar motors in certain marine and alkaliphilic species (2–6). In marine vibrio, PomAB and MotXY have been proposed to form a functional membrane-spanning; Na VBac, bacterial voltage-gated sodium channel; 6TM, six-α-helical transmembrane-spanning; Na VPZ, Na VBac from Paracoccus zeaxanthinifaciens; Na VS, Na VBac from Silicibacter pomeroyi; CHO, Chinese hamster ovary.

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§ The on-line version of this article (available at www.jbc.org) contains information on the cloning of additional NaChBac homologs.
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† The abbreviations used are: Naᵥ, voltage-gated sodium channel; Caᵥ, voltage-gated calcium channel; 6TM, six-α-helical transmembrane-spanning; NaᵥBac, bacterial voltage-gated sodium channel; NaVPZ, NaVSP from Paracoccus zeaxanthinifaciens and NaVS from Silicibacter pomeroyi; NaᵥSP, NaᵥBac from S. pomeroyi; CHO, Chinese hamster ovary.
expression clone was used to transform cloned DNA contains additional methionine and valine codons prior to cloned into the pTrcHis2A plasmid using NcoI and XhoI sites. This thrombin recognition site and an XhoI site). The PCR product was AACCGCGTGGCACCAGCTTTTTGGTTTCACCAAG-3

NCBI. A sample of containing ampicillin and grown at 37°C on the World Wide Web at www.tigr.org. homologous to NaChBac was identified, and the preliminary sequence DNA was collected by standard procedures (13). The genomic sequence S. pomeroyi was cloned into a modified pTracer-CMV2 vector containing NaVPZ (Fig. 2A) occurred during the PCR process. Finally, this plasmid DNA was digested with SalI and then self-ligated after the linker sequence was deleted. The resulting expression clone was used to transform E. coli BL-21 (Stratagene).

NaFZ was cloned into a modified pTracer-CMV2 vector (Invitrogen) containing enhanced green fluorescent protein for expression in mammalian cells. Briefly, DNA was amplified by PCR from the E. coli expression clone using the following primers: 5′-AATGGATCCATGCAAAGAATGCAG-3′ (containing a BamHI site) and 5′-ATTGAATTCTCAGACACGCCCACGGCCGCC-3′ (containing an EcoRI site). The PCR product was then cloned into the modified pTracer-CMV2 plasmid (Invitrogen) between the single restriction sites for BamHI and EcoRI. All clones were confirmed by DNA sequencing.

Expression and Purification of Recombinant Proteins—100–300 ml of LB medium containing ampicillin (50 μg/ml) was inoculated from glycerol stocks and grown overnight at 37°C. 100 ml of culture was transferred into 2 liters of Terrific Broth medium (Invitrogen) containing ampicillin and grown at 37°C to an OD_600 of 1.2. Cells were induced with 1 mM 1-β-thiogalactopyranoside and grown at 37°C for 3 h. Cells were then suspended in PBS buffer (pH 8.0) containing protease inhibitors (Protease Inhibitor Mixture; Sigma) and lysed by sonication. The cellular debris was removed by centrifugation and solubilization in 15 mM n-decyl-β-thiomaltoligosaccharide (Anatrace). Centrifugation, the supernatant was loaded onto a Talon Co^2+ affinity column (Clontech). Resin was washed with 20 mM imidazole, and the protein was then eluted in the presence of 400 mM imidazole. Purified protein was resolved by 4–12% SDS-PAGE (Invitrogen) and stained with Coomassie Blue. Molecular weight marker was purchased from Invitrogen (Benchmark™ Prestained Protein Ladder).

Mammalian Electrophysiology—NaFZ and NaFSP as well as other NaChBac homologues were subcloned into an enhanced green fluorescence protein-containing pTracer-CMV2 vector (Invitrogen) for expression into CHO-K1 and HEK293T cells. CHO-K1 and HEK293T cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum at 37°C under 5% CO₂. DNA was transfected using LipofectAMINE™ 2000 (Invitrogen) and plated onto coverslips, and recordings were made after 12 h (NaFZ) or 48 h (NaFSP), respectively. Unless otherwise stated, the pipette solution contained 147 mM Ca²⁺, 120 mM methane sulfonate, 5 mM NaCl, 10 mM EGTA, 2 mM Mg-ATP, and 20 mM HEPES (pH 7.4). Bath solution contained 140 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl, 20 mM HEPES (pH 7.4), and 10 mM glucose. All experiments were conducted at 22 ± 2°C. Unless otherwise indicated, all chemicals were dissolved in water. Nifedipine (dissolved in Me₂SO) was purchased from Sigma. As reported previously (7), unknown agents, presumably leached from the perfusion tubing, caused fast inactivation, and these perfusion systems were subsequently avoided.

RESULTS

Using the whole or partial sequence of NaChBac as the query, we performed standard BLASTP or TBLASTX searches on the GenBank™ data bases from various prokaryotic genomic sequencing projects. Several open reading frames with significant sequence homology (22–54%) to NaChBac were identified in the following species (Fig. 1A): Vibrio vulniﬁcus (22%), Microbulbifer degradans (two genes, 32 and 33%, respectively), Colwellia psychrerythraea (two genes, 35 and 38%, respectively), Magnetooccus sp. (32%), S. pomeroyi (39%), P. zeaxanthinfaciens (39%), Hyphomonas neptuni (33%), Thermobifida fusca (30%), and Oceanobacillus theatensis (54%). Among these species, V. vulniﬁcus (14), M. degradans (15), C. psychrerythraea (16), Magnetooccus sp. (17), S. pomeroyi (18), P. zeaxanthinfaciens (19), and H. neptuni (20) were isolated from deep sea water or water. T. fusca (21), a thermophilic Gram-positive bacteria, was isolated from soil but grows optimally in alkaliphilic conditions. O. theatensis (22) and B. halodurans (23) are alkaliphilic Gram-positive bacteria isolated from deep sea water. Based on the degree of sequence homology, we consider these proteins to be NaChBac homologs (rather than orthologs). Hydrophobicity analysis of these proteins predicted that all have the 6TM architecture. Importantly, threonine, glutamate, and tryptophan residues are conserved in the pore region in all proteins (Fig. 1B). These residues have been shown to be critical for the cationic selectivity of NaChBac (24). As is characteristic for voltage-gated channels (25), positively charged amino acids (Arg) are interspersed every 3 amino acids in the fourth putative transmembrane domain (24). In V. vulniﬁcus, however, the third arginine was not conserved. Based on the sequence homology and the structural similarity to NaChBac, it is likely that these proteins function as voltage-gated channels.

We cloned all 11 sequences (see “Experimental Procedures”) and studied them by expression in mammalian cell lines. As shown below, we were able to measure currents produced by two NaChBac homologs, NaFZ (Fig. 2A) from P. zeaxanthinfaciens (a zeaxanthin-producing marine bacteria (19)) and NaFSP (Fig. 2A) from S. pomeroyi (a dimethylsulfoxonoprotein-degrading marine bacteria (18)). Isolation and sequencing of the gene encoding NaFZ revealed an open reading frame of 262 amino acids with a predicted molecular size of 29 kDa. Similarly, the NaFSP gene encoded an open reading frame of 258 amino acids with a predicted molecular mass of 29 kDa. NaFZ and NaFSP share 39% identity (60% similarity) and 39% identity (59% similarity) with NaChBac, respectively (Fig. 2B). Notably, NaFZ is 65% identical (77% similar) to NaFSP. Upon electrophoresis, both NaFZ and NaFSP proteins

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migrated as a single band (~31 kDa; Fig. 2C), almost identical to the predicted molecular sizes of the His-tagged constructs.

CHO-K1 or HEK293T cell lines were transfected with NaCh-Bac homologs (in pTracer), and whole-cell currents were recorded 12–48 h after transfection (see “Experimental Procedures”). Among 11 NaCh-Bac homologs, only two (NaVPZ and NaVSP) produced detectable currents. Similar current are not present in nontransfected or mock-transfected cells (data not shown). NaVPZ-transfected cells exhibited large (up to 10,000 pA; 10 nA) voltage-activated inward currents (Fig. 3, A–C).
Na\textsubscript{VPZ}-mediated current \((I_{\text{NaVPZ}})\) activated with a time constant \((\tau_{\text{activation}})\) of \(21.5 \pm 1.3\) ms at \(+10\) mV \((n = 19)\), significantly slower than both mammalian Na\textsubscript{V} channels \((\tau_{\text{activation}} < 2\) ms) and \(I_{\text{NaChBac}}\) \((\tau_{\text{activation}} < 13\) ms). Inactivation of \(I_{\text{NaVPZ}}\) was slow \((\tau_{\text{inactivation}} = 102 \pm 4.2\) ms at \(+10\) mV, \(n = 19)\) compared with the typically fast inactivating Na\textsubscript{V} currents \((\tau_{\text{inactivation}} < 10\) ms) but faster than \(I_{\text{NaChBac}}\) \((\tau_{\text{inactivation}} > 160\) ms).

Cation replacement by \(N\)-methyl-D-glucamine NMDG (bath) resulted in complete removal of voltage-dependent \(I_{\text{NaVPZ}}\) inward current (Fig. 3C). Similarly, no significant inward current was seen in isotonic \([\text{Ca}^{2+}]_o\) (monovalent cations replaced with \(105\) mM \([\text{Ca}^{2+}]_o\) ) (Fig. 3, B and C). \(I_{\text{NaVPZ}}\) reversed at \(+75\) mV (Fig. 3C), close to the Nernst potential of Na\textsuperscript{+} under our recording conditions \((E_{\text{Na}} = +72\) mV). These results, together with the large leftward shift of the reversal by external Na\textsuperscript{+} removal \((N\)-methyl-D-glucamine\textsuperscript{+} and isotonic \([\text{Ca}^{2+}]_o\) solution substitution), suggested that Na\textsubscript{VPZ}, like Na\textsubscript{ChBac}, is a Na\textsuperscript{+}-selective channel. We assumed that the outward currents in 0 mM \([\text{Na}^+]_o\) (Fig. 3B) were carried by internal Na\textsuperscript{+}\textsuperscript{−} (8 mM \([\text{Na}^+]_i\)). Due to the very negative \(E_{\text{rev}}\) (Fig. 3C), sizable outward currents were observed at most voltages tested (Fig. 3, B and C).

We evaluated the voltage-dependent activation of \(I_{\text{NaVPZ}}\) by measuring deactivation tail currents (Fig. 4A). A Boltzmann fit of the averaged activation curve yielded a \(V_{1/2}\) of \(-9.5 \pm 0.8\) mV \((n = 9)\) and slope factor \((\kappa)\) of 10.7 \pm 0.7 mV per e-fold change in current (Fig. 4C). Steady-state inactivation of the channel was determined by sequential depolarization to test voltages followed by voltage clamp to the peak of activation at \(+10\) mV (Fig. 4B). Steady-state inactivation was a steep function of voltage, with 50% inactivation at \(-35 \pm 0.4\) mV \((n = 10)\) and slope factor \((\kappa)\) of 6.3 \pm 0.3 mV/e-fold (Fig. 4C). We investigated the time course of \(I_{\text{NaVPZ}}\) inactivation at \(-30\) mV, where activation was minimal. The degree and speed of inactivation was strongly dependent on the duration of the inactivating prepulse \((-30\) mV; \(\tau = 2123 \pm 433\) ms; \(n = 6\); Fig. 4, D and E). \(I_{\text{NaVPZ}}\) recovered slowly with time constant, \(\tau = 839 \pm 90\) ms \((n = 7, \text{HP} = -90\) mV, Fig. 4, F and G).

Na\textsubscript{SP}-transfected cells also yielded voltage-activated inward currents (Fig. 5A), peaking at \(-30\) mV. The \(E_{\text{rev}}\) of Na\textsubscript{SP}-mediated current \((I_{\text{NaSP}})\) was \(+76\) mV. Ion substitution \((\text{Ca}^{2+}\) replacement) experiments confirmed that Na\textsubscript{SP}, like Na\textsubscript{VPZ}, was also a Na\textsuperscript{+}-selective channel (data not shown). \(I_{\text{NaSP}}\) activated and inactivated significantly faster than \(I_{\text{NaVPZ}}\) and \(I_{\text{NaChBac}}\) \((\tau_{\text{activation}} = 3.4 \pm 0.5\) ms at \(+30\) mV, \(n = 17)\); \(\tau_{\text{inactivation}} = 35 \pm 1.5\) ms at \(+30\) mV, \(n = 17)\) but still severalfold slower than Na\textsubscript{V} currents. The Boltzmann fit activation curve yielded a \(V_{1/2}\) of \(+21 \pm 0.4\) mV and \(\kappa\) of 11.8 \pm 0.4 mV/e-fold change \((n = 28)\; \text{Fig. 5E})

Steady-state inactivation was strongly dependent on the voltage \((\kappa = 10.3 \pm 0.5\) mV/e-fold), with half-inactivation at \(-22 \pm 0.8\) mV \((n = 11)\; \text{Fig. 5E}).
NaVPZ and NaVSP were sensitive to high concentrations of nifedipine (30 μM; data not shown).

**DISCUSSION**

Expression of bacterial genes in systems where the protein can be studied (mammalian cells for patch clamp) is crucial to interpreting and extending static structural data through structure-function studies. Such functional expression is also important to understanding their native roles in bacteria. However, successful functional expression of bacterial proteins in mammalian cells is rare. Here, we identified 11 putative 6TM prokaryotic channel subunit genes and were able to functionally express 2 of the 11 in mammalian cells, where their electrophysiological properties could be studied. Both channels were Na⁺-selective and activated by voltage. One conclusion based on comparison of bacterial channels and NaVs is that the major evolutionary pressure for gene duplication and concatenation of subunits was to increase the speed of channel gating. Given the high selectivity of presumed homomeric bacterial Na⁺ channels, the case for pore asymmetry as a means to increase Na⁺ selectivity seems a less likely scenario.

Little is known about the molecular determinants that control mammalian Na₉ activation rates. Mammalian Na₉ channels activate and inactivate within a few milliseconds (<10 ms), roughly 10–100 times faster than NaChBac, the only bacterial voltage-gated channel functionally expressed up to now. Interestingly, NaVSP activation is ~4-fold faster than NaChBac, whereas NaVPZ activation is ~2 times slower than NaChBac, despite 77% sequence homology between NaVSP and NaVPZ. In NaVSP, NaVPZ, and NaChBac, the S4 domain and short S3-S4 linker are highly conserved, suggesting that the structural determinants for the kinetics differences are located elsewhere. Notably, there are several Na₉-SP-specific residues in the putative pore-forming domains (Gly41 in S5, Ile172 in the pore loop, Val119 in the linker between the P loop and S6, and Met305 in S6). These residues may contribute to the relatively fast activation kinetics of Na₉-SP.

Na⁺ channel inactivation mechanisms are better understood than those of activation. Interdomain linkers mediate fast inactivation in Na₉ by “ball and chain” or N type inactivation (26), but these domains are obviously missing in tetramers of...
6TM bacterial channels. Additionally, the removal of segments within the N and C cytoplasmic domains of NaChBac (24) (unpublished data) does not substantially alter its inactivation rate. If the cytoplasmic domains do not participate in inactivation, we can then begin to look at other domains. Studies on 6TM HERG K⁺ channels indicated that the S5-P linker was crucial for its C-type inactivation, probably by providing allosteric coupling between its outer mouth and the voltage sensor (27). NaChBac inactivates with a time constant similar to Na₉PZ, but the NaChBac S5-P linker (from Gln 167 to Ser 180) has low homology to Na₉PZ. NaChBac and Na₉PZ inactivates 5-fold more slowly than Na₉SP. In the pore-S6 linker, Na₉SP lacks the negatively charged glutamate present in both NaChBac and Na₉PZ. Future studies will focus on this and other sequence differences. However, the difficulty of obtaining functional expression of many mutants highlights the need for structural data. By exclusion of alternative mechanisms, we hypothesize that C-type inactivation, in which the Na⁺ pore is shut, is the more likely mechanism for Na₉Bac channel inactivation.

Na⁺ channels have been proposed to play a central role in Na⁺-dependent flagellar mobility in some prokaryotes. Marine Vibrio species utilize their Na⁺-driven polar flagella for swimming (5, 6), and in the alkaliphilic Bacillus species, [Na⁺], determines the activity of the flagellar motor (4, 28). Under alkaliphilic conditions, the H⁺-motive force is weak due to the high intracellular pH (pH 8–9) of these bacteria (4, 29). Therefore, a Na⁺ cycle driven by the Na⁺ channel may have evolved to power the flagellar motor (4, 28, 30–32). Interestingly, bacteria whose flagellar motors are powered by Na⁺ (as opposed to H⁺) express NaChBac homologs. In bacteria, the Na⁺/H⁺ exchanger prevents cytotoxic Na⁺ accumulation and also supports pH homeostasis at elevated pH (4, 31, 33). In low [Na⁺], environments or in the absence of solutes to support Na⁺ uptake through Na⁺-coupled solute transporters, the pH homeostasis function may rely on a Na⁺ channel (4, 31, 33). We propose that sustained voltage-gated Na⁺ channel opening is primarily responsible for this Na⁺ entry (33). It is possible that some mammalian Na⁺ channels play a role in Na⁺ or H⁺ homeostasis. Interestingly, the mammalian persistent and resurgent Na⁺ currents have similar kinetics to Na₉Bac (34, 35). These persistent Na⁺ currents may be mediated...
by subthreshold gating of fast Na\textsubscript{V} channels (36) or perhaps by Na\textsubscript{V}1.8 and Na\textsubscript{V}1.9.

NaChBac selectivity is converted from Na\textsuperscript{+} to Ca\textsuperscript{2+} by replacing an amino acid adjacent to glutamatic acid in the putative pore domain by a negatively charged aspartate (from TLESWAS to TLEDWAS or TLDDWAD) (24). Interestingly, two bacterial strains (C. psychrerythraea and M. degradans) have a putative pore sequence (TFEDWDT) similar to that of the Ca\textsuperscript{2+}-selective NaChBac mutant. We have not been able to functionally express these channel subunits in mammalian cells, but one possibility is that these proteins form heteromeric channels with other related subunits in the same species.

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