TTX-Sensitive and -Resistant Na⁺ Currents, and mRNA for the TTX-Resistant rH1 Channel, Are Expressed in B104 Neuroblastoma Cells

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Gu, Xiang Q., Sulayman Dib-Hajj, Marco A. Rizzo, and Stephen G. Waxman. TTX-sensitive and -resistant Na⁺ currents, and mRNA for the TTX-resistant rH1 channel, are expressed in B104 neuroblastoma cells. J. Neurophysiol. 77: 236–246, 1997. To examine the molecular basis for membrane excitability in a neuroblastoma cell line, we used whole cell patch-clamp methods and reverse transcription-polymerase chain reaction (RT-PCR) to study Na⁺ currents and channels in B104 cells. We distinguished Tetrodotoxin (TTX)-sensitive and -resistant Na⁺ currents and detected the mRNA for the cardiac rH1 channel in B104 cells. Na⁺ currents could be recorded in 65% of cells. In the absence of TTX, mean peak Na⁺ current density was 126 ± 19 pA/pF, corresponding to a channel density of 2.7 ± 0.4/μm (mean ± SE). Time-to-peak (τpeak), activation (τa), and inactivation time constants (τi) for Na⁺ currents in B104 cells were 1.0 ± 0.04, 0.4 ± 0.06, and 0.9 ± 0.04 ms at −10 mV. The peak conductance-voltage relationship had a V1/2 of −39.8 ± 1.5 mV, Vτ50 for steady-state inactivation was −81.6 ± 1.5 mV. TTX-sensitive and -resistant components of the Na⁺ current had half-maximal inhibitions (IC50), respectively, of 1.2 nM and, minimally, 575.5 nM. The TTX-sensitive and -resistant Na⁺ currents were kinetically distinct; time-to-peak, τm, and τi, for TTX-sensitive currents were shorter than for TTX-resistant currents. Steady-state voltage dependence of the two currents was indistinguishable. The presence of TTX-sensitive and -resistant Na⁺ currents, which are pharmacologically and kinetically distinct, led us to search for mRNAs known to be associated with TTX-resistant channels, in addition to the α subunit mRNAs, which have previously been shown to be expressed in these cells. Using RT-PCR and restriction enzyme mapping, we were unable to detect αSNS, but detected mRNA for rH1, which is known to encode a TTX-resistant channel, in B104 cells. B104 neuroblastoma cells thus express TTX-sensitive and -resistant Na⁺ currents. These appear to be encoded by neuronal-type and cardiac Na⁺ channel mRNAs including the RH1 transcript. This cell line may be useful for studies on the rH1 channel, which is known to be mutated in the long-QT syndrome.

INTRODUCTION

A variety of neuroblastoma cell lines express voltage-sensitive Na⁺ channels and have proven to be useful model systems for studies on sodium channel biophysics and pharmacology (Brown et al. 1994; Johansson 1994; Ogata et al. 1990; Reuveny and Narahashi 1991) and action potential electrogenesis (Gu and Waxman 1996). These cells express multiple Na⁺ currents. For example, the action potential upstroke of the LA-N-5 cell is supported by tetrodotoxin (TTX)-sensitive and TTX-resistant Na⁺ currents, which are blocked by nanomolar and micromolar concentrations of TTX, respectively (Weiss and Sidell 1991). Different neuroblastoma cell lines express Na⁺ currents with distinct properties. A range of electrophysiological characteristics have been reported for Na⁺ currents in different neuroblastoma cell lines. For example, V1/2 for inactivation has been reported to be −46 mV in LA-N-5 cells (Weiss and Sidell 1991) and −86 mV in SH-SY5Y cells (Brown et al. 1994). Na⁺ currents in N4TG1 cells (Lang et al. 1993) and N1E-115 cells (Ogata et al. 1990) exhibit different properties, with V1/2 for steady-state inactivation at about −67 mV. The multiplicity of Na⁺ currents that are expressed in various neuroblastoma cell lines suggests that they possess multiple distinct Na⁺ channels, and makes these cell lines attractive models in which to study the molecular basis of channel behavior. However, except for B104 cells in which the expression pattern of Na⁺ channel mRNAs cloned from nervous tissue has been examined (Dib-Hajj et al. 1996), the profile of Na⁺ channel mRNA in neuroblastoma cell lines has not been studied.

The B104 neuroblastoma cell line is a clonal cell line that has been demonstrated to be excitable (Schubert et al. 1974). Action potentials in B104 cells are dependent on Na⁺ currents (Gu and Waxman 1996). In a study using reverse transcription-polymerase chain reaction (RT-PCR) and restriction enzyme mapping to examine the expression of Na⁺ channel mRNAs that are known to be present at high levels in nervous tissue, these cells were shown to express the mRNA for three Na channel α subunits (αII, NaG, and Na6) and the β1 subunit (Naβ1) at high levels (Dib-Hajj et al. 1996). In the present study, we used whole cell patch-clamp recording to examine Na⁺ currents in these cells. We observed TTX-sensitive and -resistant Na⁺ currents that exhibit subtle kinetic differences but are indistinguishable in terms of voltage dependence. On the basis of the electrophysiological and pharmacological results, we used RT-PCR and restriction enzyme mapping to search in B104 cells for mRNAs associated with Na⁺ channels that are known to be TTX resistant. We have found that although B104 cells do not express mRNAs associated with TTX-resistant Na⁺ channels that have been cloned from nervous tissue, these cells express high levels of the mRNA for the cardiac rH1 Na⁺ channel, which is known to be associated with a TTX-resistant current that is mutated in a number of cardiac diseases including the long-QT syndrome in humans.

METHODS

Culture

B104 cells (Schubert et al. 1974) were grown in Dulbecco’s modified Eagle’s medium (500 U/ml) on Corning 75 cm² plastic
flasks and replated on 12-mm circular glass coverslips. Flasks and coverslips were maintained at 37°C in a 5% CO₂-95% air atmosphere and fed every fourth day.

**Patch clamping: solutions**

The extracellular solution contained (in mM) 125.0 NaCl, 10.5 glucose, 5.0 KCl, 1.2 MgSO₄, 1.0 CaCl₂, 1.6 Na₂HPO₄, 0.4 NaH₂PO₄, 32.5 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid HEPES (acid), pH 7.4 adjusted with NaOH. In some experiments, 10 mM tetraethy lammonium chloride (TEA) or 0.1 mM CdCl₂ was added to the extracellular solution to minimize K⁺ and Ca²⁺ currents.

In most experiments, the intracellular solution contained (in mM) 145.0 KCl, 10.0 HEPES sodium salt, 1.0 MgCl₂, 0.2 CaCl₂, 10.0 ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′-tetraacetic acid (EGTA), pH adjusted to 7.4 using tris(hydroxymethyl)aminomethane (Tris). In a few experiments, the intracellular solution contained (in mM) 125.0 N-methyl-d-glucamine (NMDG) and 20.0 TEA instead of 145.0 mM KCl in the above solution. Some studies on kinetics were done using the following intracellular solution (in mM) 10 NaCl, 139 CsCl, 2 MgCl₂, 1 EGTA, and 10 HEPES. These solution changes had no effect on the appearance of the net Na⁺ current.

**Electrophysiological recording**

Recordings were obtained using the whole cell mode of the patch-clamp technique (Hamill et al. 1981) at room temperature (20°C). Patch pipettes were made from thick-walled borosilicate glass pulled with a Flaming Brown puller. Electrodes filled with NMDG or KCl pipette solutions had resistances of 2–6 MΩ. Current recordings (Axopatch-1D amplifier) were low-pass filtered at 5 or 3 kHz with Bessel filters and digitized at 25–100 kHz. Liquid junction potentials were minimized using a 5% agar/200 mM NaCl bridge between external and pipette solution with Ag⁺-AgCl wire as ground. The liquid junction potential was <0.5 mV.

Cell membrane capacity compensation (Cₘ) and series resistance compensation (Rₛ) were used to minimize voltage errors; optimal settings for Cₘ and Rₛ were obtained by minimizing the capacitive transient in response to a 5-mV hyperpolarizing step. Whole cell capacitances were determined in one of two ways that yielded similar results: reading from series resistance setting and whole cell capacity of the cancellation circuitry or the quotient of We used primers designed against highly conserved sequences in the appearance of the net Na⁺ current. Theoretical reversal potentials were calculated according to the Nerst equation. Ion activities were obtained from the ion concentrations multiplied by activity coefficients (Robinson and Stokes 1959), which were 0.888 for [Na⁺] and 0.886 for [K⁺]. The calculated equilibrium potentials were Eᵣ = 63.8 mV and Eₖ = −85.2 mV.

**TTX experiments**

In each experiment, four data points, consisting of peak Na⁺ currents from a single cell, were obtained every 60 s and averaged for each concentration of TTX. Data were obtained 4 min after adding each concentration of TTX. Different sets of experiments then were averaged to measure TTX inhibition.

**Data analysis**

Data analysis was performed on leak-subtracted current traces using pClamp and custom software written by X. Q. Gu. Graphing and curve fitting programs (Origin, MicroCal, MA) were used for analysis of digitized data. Cumulative data are presented and graphed as means ± SE. Significance testing was done using Student’s t-test on distribution of data. All data used for examination of Na⁺ currents met the following criteria: reversal potentials of inward current were within ±10 mV of theoretical equilibrium potential; series resistance (Rₛ) before compensation was <10 MΩ; and uncompensated Rₛ was <2 MΩ (mean = 1.28 MΩ), corresponding to a theoretical limiting time constant of, in the worst case, 70 μs uncompensated. Steady-state inactivation (hₛ) and peak conductance-voltage curves (mᵥ) were fit to a modified Boltzmann equation of the form

\[ h(V) = \frac{1}{1 + \exp(V - V_{1/2})/a} \]

where a represents slope factor.

TTX inhibition curves were fit to a Langmuir binding isotherm of the form

\[ I(x) = AX/(K_d + X) \]

where A represents a constant.

**Reverse transcription**

Total cellular RNA from B104 cells and L₄-L₅ dorsal root ganglia (DRG) from adult Sprague-Dawley rats was isolated by the single step guanidinium isothiocyanate-acid phenol procedure (Chomczynski and Sacchi 1987). RNA concentration was determined by optical density measurements at 230, 260, and 280 nm. The quality of the RNA was assessed by electrophoresis in a 1% agarose-2.2 M formaldehyde gel. First strand cDNA was reverse transcribed in a 50-ml final volume using 2 μg total RNA, 1 mM random hexamer (Boehringer Mannheim), 500 units SuperScript II reverse transcriptase (Life Technologies), and 100 units of RNase Inhibitor (Boehringer Mannheim). The buffer consisted of 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM DTT, and 5 mM dNTP. The reaction was allowed to proceed at 37°C for 90 min, then terminated by heating to 65°C for 10 min. Controls omitted reverse transcriptase.

**PCR**

We used primers designed against highly conserved sequences in domain 1 (D1) to amplify products from multiple α-subunits that might have been present in the cDNA pool. The amplified products from D1 contain the terminal part of the conserved transmembrane segment D1-S3 and extend into the first half of D1-SS1. The center of this region shows significant sequence and length polymorphism (Table 2). The amplified fragment spans the splicing junction of at least 2 introns (George et al. 1993; Gustafson et al. 1993; Sarao et al. 1992); this facilitates distinguishing amplification from contaminating genomic DNA templates. Due to codon degeneracy, three forward primers were designed to ensure efficient priming from all templates that might have been present in the cDNA pool (Table 1); however, any primer may bind to multiple templates. Forward primer F1 (5′ GACCCCA/5TGGAAATTGTTGGA 3′) matches subunits α₁, α₂, α₆, α₁₁, α₁₂ (SkM1), α₁₃ (SKM2), and α₁₁NS. Sequences of individual subunits show one or two mismatches to this primer: T to C at position 16 and A to G at position 6 (α₁NS); C to A at position 6 (α₁₁); A to G at position 18 (α₁₁H) and T to C at position 3 (α₁₁NS). Forward primer F₂ (5′ AATCCCTGGAAATTGTTGGA 3′) matches subunit α₂II. Forward primer F₃ (5′ GACCCCGTGAACTGTTTGA 3′) perfectly matches α₁₁NS but α₁₁H has a single mismatch of C to T at position...
Primer location for the amplification of D1 sequences from Na channel α-subunits

<table>
<thead>
<tr>
<th>Domain 1</th>
<th>αI</th>
<th>αII</th>
<th>αIII</th>
<th>αNa6</th>
<th>αhNE</th>
<th>αμI</th>
<th>αrH1</th>
<th>αSNS</th>
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<td>507</td>
<td>501</td>
<td>602</td>
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<td>Forward</td>
<td>813–832 (F1)</td>
<td>775–784 (F2)</td>
<td>975–994 (F1)</td>
<td>580–599 (F3)</td>
<td>604–623 (F1)</td>
<td>1,020–1,039 (F1)</td>
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<td>Reverse</td>
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<td>1,312–1,335 (R1)</td>
<td>1,512–1,535 (R1)</td>
<td>1,063–1,086 (R1)</td>
<td>1,081–1,104 (R1)</td>
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<td>1,266–1,290 (R1)</td>
<td>1,218–1,241 (R2)</td>
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</table>

Primer locations are in parentheses. * Predicted length of the amplification product in base pairs.

16. The reverse primer R1 (5' CAAGAGGCCAGCTGAAGGT-GTC 3') matches subunits αI, αII, αIII, αNa6, αhNE-Na, αμI, and αrH1. This primer has mismatches compared with four subunits: G to A at position 3, A to G at position 4, and T to G at position 7 (αI); T to C at position 1 and A to G at position 19 (αhNE-Na); G to A at position 3 and A to G at position 7 (αμI); an extra G after position 3, GC to CT at positions 14–15, and A to C at position 21 (αrH1). The lengths of amplified products and restriction enzyme polymorphism from D1 are shown in Table 2. The reverse primer R2 (5' GAGGAATGCCCAGCAAAGGATCC 3') matches subunit αSNS. All subunit sequences are based on Genbank database (accession numbers: αI: X03638; αII: X03639; αIII: Y00766; αNa6: L39018; αhNE-Na: X82835; αμI: M26643; αrH1: M27902 and αSNS: X92184).

Amplification was performed in 60 µl volume using 1 µl of the first strand cDNA, 0.8 µM of each primer, and 1.75 units of Expand Long Template DNA polymerase enzyme mixture (Barnes 1994; Cheng et al. 1994) (Boehringer Mannheim). Three separate amplification reactions were performed to probe for all known α-subunits: F1, F2, and R1 were used to amplify sequences of subunits αI, αII, αIII, αNa6, αhNE-Na, αμI, and αrH1; F3 and R1 were used to specifically amplify αNa6, αrH1 in a second reaction; and F1 and R2 were used in a third reaction to amplify αSNS. Control PCR reactions in which the template was substituted by water, or potential for Na+ current, were included. Amplification was carried out in two stages using a programmable thermal cycler (PTC-100, MJ Research, Cambridge, MA). First, a denaturation step at 94°C for 4 min, an annealing step at 60°C for 2 min, and an elongation step at 72°C for 90 s. Second, a denaturation step at 94°C for 1 min, an annealing step at 60°C for 1 min, and an elongation step at 72°C for 90 s. The second stage was repeated 33 times for a total of 35 cycles, with the elongation step in the last cycle extended to 10 min.

The identity of the α-subunits expressed in the B104 cells and dorsal root ganglia (DRG) were determined by restriction enzyme analysis of their PCR products. For restriction enzyme analysis, typically 1/12 of the PCR reaction was digested for 1 h at the recommended temperature and the products resolved by electrophoresis in a 1.7% agarose gel. A summary of the predicted results of such analyses is presented in Table 2. Fragment sizes were determined by comparison to a 100-bp ladder molecular weight marker (Pharmacia). DNA was visualized by ethidium bromide fluorescence. Gel images were digitized using a GelBase 7500 system (UVP) and printed on a Fargo Primera Pro color printer (Fargo Electronics) in black and white dye sublimation mode.

RESULTS

On entry, 39 of 384 B104 cells met the criteria (reversal potential for Na⁺ current within ±10 mV of theoretical reversal potential; Rₛ before compensation <10 MΩ; uncompensated Rₛ < 2 MΩ with theoretical limiting time constant <70 µs uncompensated) for analysis of kinetics and steady-state properties. These cells exhibited depolarized membrane potentials (∼3.9 ± 1.9 mV; n = 34) and showed

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RT-PCR, reverse transcription-polymerase chain reaction. * BamH1 cuts αhNE and αSNS amplification products. However, sequences of these two subunits are amplified using subunit-specific primers (see Table 1); therefore, the presence of either subunit can be tested in a separate assay.

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TABLE 2. Restriction enzyme polymorphism of Na channel α-subunits of D1 RT-PCR products

<table>
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<tr>
<th>Domain 1</th>
<th>αI</th>
<th>αII</th>
<th>αII</th>
<th>αVI</th>
<th>αhNE</th>
<th>αμI</th>
<th>αrH1</th>
<th>αSNS</th>
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<td>561</td>
<td>507</td>
<td>501</td>
<td>602</td>
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RT-PCR, reverse transcription-polymerase chain reaction. * BamH1 cuts αhNE and αSNS amplification products. However, sequences of these two subunits are amplified using subunit-specific primers (see Table 1); therefore, the presence of either subunit can be tested in a separate assay.
relatively little K⁺ current; although mean \( g_{Na^+} \) was 85.9 ± 12.9 nS (\( n = 39 \)), 21 cells did not display any detectable activatable K⁺ currents (see e.g., Fig. 1A) and the mean \( g_{K^+} \) in cells that expressed activatable K⁺ currents was 1.8 ± 0.5 nS (\( n = 18 \)) at membrane potentials sufficient to activate maximal K⁺ conductances. On the basis of these observations, the calculated \( g_{Na^+}/g_{K^+} \) ratio at membrane potentials sufficient to activate maximal conductances was 48:1. This is likely to be an underestimate, because in most cells, even without the application of K⁺ channel blockers, there was no detectable K⁺ current (Figs. 1A and 2A).

**Na⁺ currents**

Patch-clamp recording revealed Na⁺ currents in 251 of 384 (65%) B104 cells. Within the 39 cells that met our criteria for quantitative analysis, Na⁺ currents ranged in amplitude from −137.0 pA to −118.9 nA. Further analysis is confined to these cells. Mean peak amplitude of Na⁺ currents was 32.5 ± 6.0 nA (\( n = 39 \)), mean whole cell capacity was 30.4 ± 3.4 pF (\( n = 35 \)), and mean peak current density, in cells that expressed Na⁺ currents, was 126.5 ± 18.6 pA/pF (\( n = 35 \)) at 0 to −30 mV.

**Voltage dependence of activation**

Na⁺ current activation was studied by voltage-clamping cells at −80 mV and applying voltage steps of 7.2 ms duration to potentials ranging from −70 to 80 mV (Fig. 1A–D). To ensure that Na⁺ channel inactivation was minimized, voltage steps were preceded by a prepulse potential of −110 mV (198.6 ms duration). Figure 1A shows an example of current traces in response to different voltage steps. The normalized current versus voltage curve is shown in Fig. 1B. Voltage steps more positive than −70 mV activated transient inward currents. At more positive potentials, currents decreased in size and reversed at 58.1 ± 0.8 mV (\( n = 39 \)), which was close to the theoretical Na⁺ equilibrium potential at which Na⁺ channels are closed.

**Fig. 2.** Steady-state Na⁺ current inactivation. Na⁺ currents were recorded by altering 198.6-ms prepulse potential from which currents were activated between −130 and −30 mV and superimposed in A. Alternate voltage steps were shown in protocol. Current was largest at −120 mV, and largest current was used to normalize peak currents (\( I/I_{\text{max}} \)). B: normalized peak currents ( ■) were plotted as a function of prepulse potential and \( h_c \) curve was obtained from curve fitting raw data with a Boltzmann equation. C: averaged peak currents (■) as a function of prepulse potential, together with \( h_c \) curve fit to data; ■, means of 35 cells. \( V_{1/2} \approx −81.8 \text{ mV} \), slope factor = 7.9.
potential (\(E_{na} = 63.8\, \text{mV}\)). From this set of data, peak conductance-voltage (\(m_n\)) curves were constructed. The peak conductance-voltage curve (Fig. 1C) for the cell shown in Fig. 1A had a midpoint at \(-40.6\, \text{mV}\) and a slope factor of 5.7 (see METHODS). For the entire population of B104 cells that met our voltage clamp criteria, the mean normalized peak conductance-voltage relationship had a \(V_{1/2}\) of \(-39.8 \pm 1.5\, \text{mV}\) (\(n = 39\)) and a slope factor of 7.0 \pm 0.4 (\(n = 37\)). The peak conductance-voltage curve in any given cell was stable over the course of an experiment (not shown).

**Steady-state inactivation**

To study the steady-state inactivation of \(\text{Na}^+\) currents, we obtained recordings using a conditioning prepulse protocol (Fig. 2, A–C). Cells were voltage clamped at \(-80\, \text{mV}\). \(\text{Na}^+\) currents were activated by a 7.2-ms voltage step to \(-20\, \text{mV}\), which was preceded by a series of increasingly more positive prepulse potentials ranging from \(-130\) to \(-30\, \text{mV}\) (10-mV increments) with duration of 198.6 ms. Figure 2A shows the current traces obtained from a representative cell. The midpoint of the steady-state inactivation curve of this cell was \(-81.8\, \text{mV}\) and its slope factor was 5.1 (Fig. 2B). Currents were inactivated completely following prepulse potentials of \(-60\, \text{mV}\). Averaging the means for steady-state inactivation curve for 35 cells yielded a \(V_{1/2}\) of \(-81.6 \pm 1.5\, \text{mV}\) (\(n = 35\)) and slope factor of 6.1 \pm 0.2 (\(n = 33\)). Steady-state inactivation curves were stable for each cell over the period of measurement (not shown).

**Kinetic properties of \(\text{Na}^+\) currents**

We also studied \(\text{Na}^+\) current kinetics. Time-to-peak (\(t_{peak}\)) was measured and was plotted as a function of voltage in Fig. 3A. \(\tau_m\) (activation time constant) and \(\tau_h\) (inactivation time constant) were obtained by fitting the current traces in response to each test voltage step with first order exponentials. \(\tau_m \) and \(\tau_h\) are plotted as a function of voltage in Fig. 3, B and C, respectively. \(t_{peak}, \tau_m,\) and \(\tau_h\) at \(-10\, \text{mV}\) were \(1.0 \pm 0.04\, \text{ms}\) (\(n = 38\)), \(0.4 \pm 0.06\, \text{ms}\) (\(n = 34\)), and \(0.9 \pm 0.04\, \text{ms}\) (\(n = 35\)); note that time constants could not be unambiguously resolved in all cells due to unacceptable low signal/noise ratio due to low \(\text{Na}^+\) current density in a few cells. \(\tau_m\) and \(\tau_h\) were stable over the duration of the recordings.

**TTX sensitivity of \(\text{Na}^+\) currents**

Figure 4 shows the average percent of \(\text{Na}^+\) current inhibition in each cell versus TTX concentration. At 1 nM TTX, only 27.9\% of the \(\text{Na}^+\) current was inhibited. Inhibition was not complete (89.1\%) at 1 \(\mu\)M TTX. The dose-response curve shows a plateau between 5 and 100 nM. The doresponse curve was fit by the sum of two Langmuir binding isotherm components, each progressing from 0 inhibition to full inhibition over a three-decade domain as expected, with values for half-maximal TTX inhibition (IC\(_{50}\)) of 1.2 and 575 nM, respectively (Fig. 4).

Figure 5 shows an experiment examining the effect of TTX on \(\text{Na}^+\) current in a single B104 cell. TTX (100 nM) decreased the peak \(\text{Na}^+\) current to 68.5\% of its maximal value (Fig. 5B). This result is consistent with the presence of two approximately equally represented populations of \(\text{Na}^+\) channels with \(K_d\)s close to those predicted (Fig. 4) for the entire B104 population. One micromolar TTX further decreased the peak \(\text{Na}^+\) current, to 9.7\% of its maximal value, in this cell (Fig. 5C). Comparison with Fig. 4 suggests that the residual current, after application of 100 nM TTX, is TTX resistant. The subtracted \(\text{Na}^+\) current (total current - resistant current) should provide a representation of TTX-sensitive current (Fig. 5D). The current-voltage relationships for the \(\text{Na}^+\) current in control solution, residual current in 100 nM TTX, and for the subtracted current for this cell are shown in Fig. 5E. In all three conditions (control; 100 nM TTX; subtracted current), maximum \(\text{Na}^+\) currents were observed at \(-20\, \text{mV}\) and the currents reversed at \(-55\, \text{mV}\) (Fig. 5E), close to the theoretical \(\text{Na}^+\) reversal potential (63.8 \text{mV}). The steady-state inactivation and peak conductance-voltage curves for this cell are shown in Fig. 5, F and G, respectively. This cell exhibited small (<5 \text{mV}) shifts in \(V_{1/2}\) of both steady-state inactivation and peak conductance-voltage when the subtracted current, total peak \(\text{Na}^+\) current, and residual current in 100 nM TTX were compared. These shifts were within the range of stability for \(m_n\) and \(h_n\) in B104 cells. There was not a substantial shift, in any of the six cells studied in this way, in the mean \(V_{1/2}\) for the steady-state inactivation (\(-81.6 \pm 1.4\, \text{mV}\); \(-81.8 \pm 2.3\, \text{mV}\); and \(-80.85 \pm 4.2\, \text{mV}\), \(n = 6, 6,\) and 5) for resistant, total, and subtracted currents, respectively) or in the \(V_{1/2}\) for the nor-
TTX-sensitive and -resistant channels in B104 cells were recorded before and after exposure to different concentrations of TTX. Currents were normalized to those recorded in absence of TTX. Averaged percent of inhibition of current in individual cells from different experiments was plotted as a function of TTX concentration. Error bars show SE. Results are fit by sum of 2 Langmuir binding isotherms, each extending over a 3-decade domain with \( K_e \) of 1.2 and 575 nM, respectively. Numbers of experiments at each concentration were 12, 9, 7, 10, 3, 10, 10, and 2.

The time-to-peak (Fig. 5H) for this cell was shorter for the subtracted current (0.88 ms at -10 mV) compared with the time-to-peak for the total current (1.02 ms at -10 mV) or resistant current (1.23 ms at -10 mV). The mean time-to-peak was 0.98 ± 0.07 ms for the subtracted current compared with 1.10 ± 0.06 ms for the total current and 1.29 ± 0.04 ms at -10 mV for residual current in 100 nM TTX (n = 6). \( \tau_m \) (Fig. 5I) and \( \tau_h \) (Fig. 5J) were shorter for the residual current in 100 nM TTX than for the subtracted current or the total current; for this cell, the values of \( \tau_m \) at -10 mV were 0.31, 0.44, and 0.51 ms, respectively, for subtracted current, total current, and residual current in 100 nM TTX; and the values of \( \tau_h \) at -10 mV were 0.28, 0.75, and 1.06 ms. For the six cells studied in this manner, \( \tau_m \) at -10 mV for subtracted current, total current, and residual current in 100 nM TTX was 0.45 ± 0.09 ms (n = 5), 0.49 ± 0.05 ms (n = 6), and 0.48 ± 0.06 ms (n = 6), and \( \tau_h \) at -10 mV was 0.64 ± 0.31 ms (n = 3), 0.86 ± 0.10 ms (n = 6), and 1.0 ± 0.09 ms (n = 6). Thus it appears that the TTX-resistant component had slower time constants for both activation and inactivation, whereas steady-state properties remained relatively unaffected by TTX.

Because TTX-resistant Na\(^+\) currents have been reported to be blocked by Zn\(^{2+}\) with IC\(_{50}\) of 10–50 \( \mu \)M (Frelin et al. 1986; Ravindran et al. 1991; White et al. 1993), we measured the effect of Zn\(^{2+}\) on the residual Na\(^+\) current after exposure to 100 nM TTX. We found that Zn\(^{2+}\) blocks the TTX-resistant Na\(^+\) current with an IC\(_{50}\) of 47.7 \( \mu \)M, close to the previously reported values. We also found that Cd\(^{2+}\) blocks the TTX-resistant Na\(^+\) current with an IC\(_{50}\) of 64.3 \( \mu \)M, close to previously reported (Frelin et al. 1986) values. However, the TTX-resistant Na\(^+\) current in B104 cells was blocked by La\(^{3+}\) with an IC\(_{50}\) of 414.5 \( \mu \)M, higher than previously reported (White et al. 1993).

**RT-PCR and restriction enzyme analysis**

In a previous study, we used RT-PCR and restriction enzyme mapping to determine whether B104 cells express the mRNAs for neuronal Na\(^+\) channel \( \alpha \) subunits I, II, III, Na6, hNE, and NaG and demonstrated the mRNA for \( \alpha \)III, Na6, and NaG in B104 cells (Dib-Hajj et al. 1996). On the basis of our observation of a TTX-resistant Na\(^+\) current in B104 cells in the present study, we carried out RT-PCR and restriction enzyme analysis to determine whether B104 cells express the mRNA for the recently cloned neuronal \( \alpha \)SNS Na\(^+\) channel (Akopian et al. 1996; Sangameswaran et al. 1996) and the cardiac \( \alpha \)H1 Na\(^+\) channel (Rogart et al. 1989; see also Kallen et al. 1990), both of which are known to be TTX resistant.

The restriction enzyme analysis of amplification products from domain I (D1) is shown in Fig. 6. Amplification product from B104 cells are shown in lanes 2–11 and 13, and for comparison, amplification products from DRG are shown in lanes 14 and 15. Lane 2 contains the amplification products using primers F1/F2 and R1. The appearance of two bands is consistent with the predicted results (Table 2). Subunit \( \alpha \)II1 is not represented in the B104 cell line as judged by the absence of the expected 600-bp amplification product (lane 2); amplification of this subunit was achieved using these primers and skeletal muscle templates (data not shown). Faint bands of lower molecular weight also are observed; these bands are likely to be artifacts or amplification of partially spliced templates. Amplification products of \( \alpha \)II (558 bp), \( \alpha \)II1 and \( \alpha \)III (561 bp) will migrate as a single band, whereas amplification products of \( \alpha \)Na6, \( \alpha \)H1, and \( \alpha \)hNE-Na (507, 518, and 501 bp, respectively) will migrate as a single lower molecular weight band. Lanes 3–8 show the result of cutting this DNA with EcoRV, EcoNI, DraI, SphI, AccI, and BamHI, respectively. The higher molecular weight species in the input DNA (lane 5) appears to be mostly subunit \( \alpha \)III product, suggesting that only a minor fraction is due to \( \alpha \)I and/or \( \alpha \)II products. Subunits \( \alpha \)I (lane 3), \( \alpha \)II (lane 4), and \( \alpha \)II1 (SkM1) (data not shown) are not detectable by this analysis and were not observed by earlier workers (Baines et al. 1992), although we previously presented evidence for the presence of \( \alpha \)I and \( \alpha \)II at low levels (Dib-Hajj et al. 1996). The lower molecular weight band in the input DNA appears to be composed of \( \alpha \)H1 sequences (lane 7). Subunits \( \alpha \)Na6 and \( \alpha \)hNE-Na are not detectable by this analysis and were not observed by earlier workers (Baines et al. 1992), although we previously presented evidence for the presence of \( \alpha \)I and \( \alpha \)II at low levels (Dib-Hajj et al. 1996). The lower molecular weight band in the input DNA appears to be composed of \( \alpha \)H1 sequences (lane 7). Subunits \( \alpha \)Na6 and \( \alpha \)hNE-Na are not detectable by this analysis and were not observed by earlier workers (Baines et al. 1992), although we previously presented evidence for the presence of \( \alpha \)I and \( \alpha \)II at low levels (Dib-Hajj et al. 1996). The lower molecular weight band in the input DNA appears to be composed of \( \alpha \)H1 sequences (lane 7). Subunits \( \alpha \)Na6 and \( \alpha \)hNE-Na are not detectable by this analysis and were not observed by earlier workers (Baines et al. 1992), although we previously presented evidence for the presence of \( \alpha \)I and \( \alpha \)II at low levels (Dib-Hajj et al. 1996). The lower molecular weight band in the input DNA appears to be composed of \( \alpha \)H1 sequences (lane 7).
FIG. 5. TTX-resistant and -sensitive currents are not distinguishable in terms of voltage dependence and show only subtle differences in kinetics. Current traces shown here were obtained by same procedure as in Fig. 1. Cells were voltage clamped at –80 mV, and voltage steps of 7.2 ms were applied from –70 to 80 mV at 10-mV increments. To remove Na+ channel inactivation, 198.6-ms conditioning voltage steps of –110 mV were applied before test voltage steps. Current traces were obtained using this procedure in 0 nM TTX (A), with 100 nM TTX (B), and with 1 μM TTX (C) in extracellular solution, all from same cell. Subtracted current traces, (currents in 0 nM TTX – currents in 100 nM TTX) are shown in D. 

Peak currents were converted to conductances by dividing by driving force Vm – ENa. Values were normalized to largest conductance value [gNa/\(\text{max}\)]. Normalized conductances were plotted as a function of step potentials. Time-to-peak (H), \(t_m\) (I), and \(t_h\) (J) are also shown for total current (■), current in 100 nM TTX (●), and subtracted current (▲).

detected by this analysis (lanes 6 and 8). Using the same set of primers, subunit αhNE-Na, as expected, was detected in dorsal root ganglia (Black et al. 1996).

Lane 9 contains the amplification product using primers F3 and R1. The presence of a single band is consistent with the specificity of this primer pair to αNa6 and αrH1 under the stringent PCR conditions used in this study. The band comigrates with the 500-bp size marker and the lower molecular weight band in lane 2 in agreement with its predicted length of 507–518 bp (see Table 2). This DNA is cut with both SphI (lane 10) and AccI (lane 11), demonstrating the presence of αNa6 and αrH1, respectively. We also have shown the presence of αNa6 by restriction enzyme polymorphism of amplification products from domain 4 and by in situ hybridization (Dib-Hajj et al. 1996).

Lanes 13 and 14 demonstrate the results of amplification using primers F1 and R2, which are expected to amplify αSNS. Lane 13 shows no specific amplification products for αSNS using B104 template, whereas lane 14 shows high levels of αSNS amplification products using DRG template. The amplification product in lane 14 migrates slightly faster than the 500-bp size marker, in agreement with an expected size of 479 bp. This DNA also is cleaved by AflIII to produce the expected 224- and 255-bp products (lane 15). Thus αSNS is clearly present in DRG, but could not be detected in B104 cells.

DISCUSSION

The most novel findings of this study are the demonstration of pharmacologically distinct TTX-sensitive and -resistant Na+ currents that are kinetically different but are indistinguishable in terms of voltage-dependence, in B104 cells; and the demonstration that B104 cells express the mRNA for the rH1 Na+ channel, in addition to NaαIII, NaG, and Na6 α subunits and Na1 mRNA, which are known (Dib-Hajj et al. 1996) to be expressed by these cells. The mean peak conductance-voltage and inactivation curves for Na+ currents in B104 cells (\(V_{1/2}\) at –39.8 and –81.6 mV, respectively) are similar to those in certain neuroblastoma cell lines that have been studied previously (SH-SY5Y) [–37 and –86 mV, respectively (Brown et al. 1994)], but are strikingly different from those reported for some other types of neuroblastoma cells [LA-N-5; –20 and –48 mV, respectively, (Weiss and Sidell 1991)]. The \(V_{1/2}\) of the steady-state inactivation curves of Na+ current of B104 cells is also different from those of N4TG1 mouse neuroblastoma cells (–67 mV) (Lang et al. 1993) and of
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243 cultured mouse neuroblastoma cell N1E-115 (−67 mV) (Ogata et al. 1990). The Na⁺ channel mRNA expression profile in these other neuroblastoma cell lines has not been determined.

**Na⁺ channel density**

Our results permit an estimate of Na⁺ channel density in B104 cells. In stimulation protocols expected to maximize the conductance in the absence of TTX, assuming a single channel conductance of 20 pS (Barres et al. 1989; Howe and Ritchie 1990) and a peak open probability of 0.25 (Barres et al. 1989), the overall Na⁺ channel density in B104 cells can be calculated as 2.7 ± 0.4 channels/µm² (n = 34) with a range from 0.2 to 10.5 channels/µm². This is considered a minimum estimate because Na⁺ channel density was difficult to determine, in part, due to the apparent inability to achieve peak conductance in some cells. We previously observed that even low densities of Na⁺ channels (<3/µm² and <1/µm² in some cells) can support action potentials in B104 cells if they are hyperpolarized to remove inactivation (Gu and Waxman 1996). Low leak and K⁺ conductances contribute to the ability of B104 cells to produce regenerative responses under these conditions.

**TTX-sensitive and -insensitive channels**

The dose-response curve for TTX in B104 cells was broad, extending from 1 to 10,000 nM, with a plateau between 5 and 100 nM. Although it might be argued that this curve represents the effect of TTX on a single type of channel, it extends over a concentration range that is much larger (by 2–3 orders of magnitude) than TTX inhibition curves for either TTX-sensitive or -resistant channels in other cell types (see, e.g., Akopian et al. 1996; Omri and Meiri 1990; Roy and Narahashi 1992). Moreover, the TTX dose-response curve in B104 cells extends over the same concentration domain (0.1–100 nM, where 100% inhibition is observed) as in LA-N-5 neuroblastoma cells where TTX-sensitive and resistant Na⁺ currents, distinguishable in terms of voltage sensitivity, are both expressed (Weiss and Sidell 1991). In both LA-N-5 cells (Weiss and Sidell 1991) and B104 cells (Gu and Waxman 1996), TTX-sensitive and -resistant Na⁺ currents both contribute to action potential generation.

The TTX dose-response curve in B104 cells exhibited a plateau between 5 and 100 nM and was fit by two isotherms with IC₅₀ of 1.2 and 575.5 nM, consistent with the presence of two types of pharmacologically distinct channels: one sensitive to nanomolar concentrations of TTX and the other requiring higher concentrations approaching the micromolar level for block. In this respect, B104 cells are similar to LA-N-5 neuroblastoma cells (Weiss and Sidell 1991) and other cells that express both TTX-sensitive and -resistant channels (Brown et al. 1994; Elliott and Elliott 1993; Hoehn et al. 1993; Honmou et al. 1994; Howe and Ritchie 1990; Ikeda and Schofield 1987; Kostyuk et al. 1981; Ogata and Tateyashii 1992; Roy and Narahashi 1992; Sontheimer and Waxman 1992). The TTX-sensitive currents in B104 cells appeared to display more rapid kinetics than the TTX-resistant currents (Fig. 5). This result is consistent with observations in other cells, which show more rapid kinetics for TTX-sensitive as compared with TTX-resistant currents (Caffrey et al. 1992; Kostyuk et al. 1981; Roy and Narahashi 1992). However, the TTX-sensitive and -resistant components of current in B104 cells could not be distinguished in terms of steady-state voltage-dependence. For the cell shown in Fig. 5, for example, V₁/₂ for the peak conductance-voltage relationship and steady state inactivation of the residual current following 100 nM TTX (−33.8 mV; −81.7 mV) were not significantly different from those properties before TTX exposure (−36.6 mV; −78.1 mV). Furthermore, when the TTX-sensitive components of current were subtracted from the controls, the normalized steady-state voltage relationship did not shift significantly. Although we might have expected different voltage dependence for the TTX-sensitive and -resistant components of current, these results are with-

**FIG. 6.** Analysis of reverse transcription-polymerase chain reaction (RT-PCR) products from B104 cells and DRG. Lanes 1 and 12 contain 100-bp marker, lanes 2–8, 9–11, and 13 contain products from B104 cells and lanes 14 and 15 contain products from DRG. Lane 2 contains product from D1 using primers F1, F2, and R1. Lanes 3–8 contain this RT-PCR product cut with EcoRV (αI-specific), EcoNI (αII-specific), DraI (αIII-specific), SphI (αNa6-specific), AccI (αrH1-specific) and BamHI (αhNE-Na-specific), respectively. Lane 9 contains RT-PCR product from D1 using primers F1 and R2. Lanes 10 and 11 contain this RT-PCR product cut with SphI (αNa6-specific) and AccI (αrH1-specific), respectively. Lane 13 and 14 have amplification products from D1 using B104 and DRG templates, respectively, and primers F1 and R2. Lane 15 contains the DRG amplification product cut with AflII, which is αSNS-specific.
out precedent. TTX-resistant Na⁺ currents in astrocytes exhibit $V_{1/2}$ for steady-state inactivation at $-80$ to $-85$ mV (Barres et al. 1989; Sontheimer et al. 1991a,b; Sontheimer and Waxman 1992), whereas the TTX-sensitive Na⁺ currents in DRG cells have been reported to display $V_{1/2}$ for steady-state inactivation of $-85$ and $-87.5$ mV (Caffrey et al. 1992; Kostyuk et al. 1981). In neurons from entorhinal cortex, TTX-sensitive ($K_d = 6$ nM) and TTX-resistant ($K_d = 146$ nM) Na⁺ currents are both present, but are indistinguishable in terms of kinetics and the $V_{1/2}$ of steady-state activation and inactivation (White et al. 1993). AtT-20 cells express TTX-sensitive and TTX-resistant Na⁺ currents, which show no difference in voltage-dependence of activation and inactivation, although the rate of inactivation of the TTX-resistant current was slower (Flamm et al. 1990).

Which channel produces the TTX-resistant current?

Because B104 cells express multiple Na⁺ currents and multiple Na⁺ channel mRNAs, the expression of these two types of Na⁺ currents can be explained by one of several molecular mechanisms.

One possibility is that TTX sensitivity may be conferred by co-expression of Naβ1 along with an α-subunit and that expression of an α-subunit without Naβ1 accounts for the TTX-resistant current. Consistent with the speculation that Naβ1 confers TTX sensitivity, Naβ1 is expressed at moderate or high levels in stellate astrocytes in which Na⁺ currents are TTX sensitive but is undetectable, or present at very low levels, in flat astrocytes in which Na⁺ currents are TTX insensitive (Oh and Waxman 1994, 1995). However, in cases where Naβ1 has been co-expressed with an α-subunit, there has been a shift in voltage dependence (Isom et al. 1992; McClatchey et al. 1993), which is not predicted by the present results.

A second possibility is suggested by the similar voltage dependence of TTX-sensitive and resistant Na⁺ currents in B104 cells (this study), a result similar to that reported for TTX-sensitive and -resistant currents in entorhinal cortex neurons (White et al. 1993) and AtT-20 cells (Flamm et al. 1990). Point mutations, altering a single amino acid, can confer TTX sensitivity or insensitivity in rat brain (type II) (Noda et al. 1989) and cardiac (RH) (Satlin et al. 1992) sodium channels. Two isoforms of a single Na⁺ channel α-subunit, differing by only one or a few amino acids as a result of RNA editing or alternative splicing, might account for the expression of TTX-sensitive and -resistant currents with indistinguishable voltage dependence in B104 cells. There is evidence for alternative splicing of several α-subunits (Gustafson et al. 1993; Sarao et al. 1992; Schaller et al. 1995; Thackeray and Ganetsky 1994), including αIII, which is known to be expressed in B104 cells (Dib-Hajj et al. 1996).

A third possibility is suggested by our observation of rH1 mRNA in B104 cells. Using RT-PCR and in situ hybridization, we previously detected the mRNA for αIII, NaG, Na6, and Naβ1 at moderate or high levels in B104 cells (Dib-Hajj et al. 1996). Of these three α-subunit mRNAs, only αIII has been re-expressed (in isolation from the β1 subunit) in Xenopus oocytes. The $V_{1/2}$ for peak conductance-voltage for the cloned αIII subunit is $-10.7$ mV, and the $V_{1/2}$ for steady-state inactivation is $-36.1$ mV for 5-s pulses (Joho et al. 1990). Co-expression of Naβ1 shifts voltage dependence for inactivation in a hyperpolarizing direction (Isom et al. 1992; McClatchey et al. 1993), but the observed shifts are smaller than the difference between steady-state inactivation for B104 cells and the cloned αIII subunits. Moreover, the αIII subunit, expressed in Xenopus oocytes, is TTX-sensitive (Joho et al. 1990; Suzuki et al. 1990) and, if expressed in this form in B104 cells, would not account for the TTX-resistant current.

Gautron et al. (1992) suggested that NaG might correspond to a TTX-resistant Na⁺ channel in glial cells. However, NaG is present in both stellate and flat spinal cord astrocytes (Black et al. 1994) and stellate astrocytes express a TTX-sensitive ($K_d = 5.7$ nM) Na⁺ current whereas flat astrocytes express a TTX-resistant ($K_d = 1,007$ nM) Na⁺ current (Sontheimer and Waxman 1992). A TTX-resistant Na⁺ channel [αSNS (Akopian et al. 1996) also termed PN3 (Sangameswaran et al. 1996)] recently has been cloned from dorsal root ganglion. However, when reexpressed in oocytes, this channel was activated at voltages positive to $-30$ mV and displayed a steady-state inactivation $V_{1/2}$ of $-30$ mV (Akopian et al. 1996). Our RT-PCR revealed high levels of αSNS mRNA in DRG, but did not detect αSNS mRNA in B104 cells.

The rH1 channel cloned from rat heart (Rogart et al. 1989), which is homologous to the SkM2 Na channel characteristic of denervated and immature skeletal muscle (Kallen et al. 1990), has electrophysiological and pharmacological properties similar to those of the TTX-resistant Na⁺ current in B104 cells. The $V_{1/2}$ for steady-state inactivation for rH1/SkM2 has been reported to be between $-67$ and $-72$ mV, with relatively rapid kinetics when expressed in oocytes; moreover, the $K_d$ for TTX of SkM2 has been reported to be between 1 and 2 μM when expressed in oocytes (Satlin et al. 1992; White et al. 1991), close to the value of the TTX-resistant current in B104 cells. Our observation of SkM2 in B104 cell has a precedent in another neurotumor, the RT4-Ac cell, which also expresses a TTX-resistant current (Tomozawa and Sueoka 1978) and SkM2 (Donahue et al. 1991).

On the basis of our results, we suggest that rH1 is responsible for the TTX-resistant Na⁺ currents in B104 cells. A TTX-resistant Na⁺ channel highly homologous to rH1 (SkM2) has been cloned from human heart (Gellens et al. 1992). A mutation in this gene is responsible for the long QT syndrome, which is associated with fatal ventricular arrhythmias (Bennett et al. 1995; Wang et al. 1995). Expression of rH1 in B104 cells may permit studies on rH1/SkM2 expressed in a mammalian cell line and provides a new model for the study of the long QT syndrome and of other disorders involving this channel.

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