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Molecular cloning of a putative tetrodotoxin-resistant rat heart Na^+ channel isoform

([^{3}H]saxitoxin receptors/multigene family/denervated skeletal muscle)

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ABSTRACT **Voltage-gated Na^+ channels in mammalian heart differ from those in nerve and skeletal muscle. One major difference is that tetrodotoxin (TTX)-resistant cardiac Na^+ channels are blocked by 1–10 μM TTX, whereas TTX-sensitive nerve Na^+ channels are blocked by nanomolar TTX concentrations. We constructed a cDNA library from 6-day-old rat hearts, where only low-affinity [^{3}H]saxitoxin receptors, corresponding to TTX-resistant Na^+ channels, were detected. We isolated several overlapping cDNA clones encompassing 7542 nucleotides and encoding the entire α subunit of a cardiac-specific Na^+ channel isoform (designated rat heart I) as well as several rat brain I Na^+ channel cDNA clones. The derived amino acid sequence of rat heart I was highly homologous to, but distinct from, previous Na^+ channel clones. RNase protection studies showed that the corresponding mRNA species is abundant in newborn and adult rat hearts, but not detectable in brain or innervated skeletal muscle. The same mRNA species appears upon denervation of skeletal muscle, likely accounting for expression of new TTX-resistant Na^+ channels. Thus, this cardiac-specific Na^+ channel clone appears to encode a distinct TTX-resistant isoform and is another member of the mammalian Na^+ channel multigene family, found in newborn heart and denervated skeletal muscles.**

Voltage-gated Na^+ channels are transmembrane proteins that mediate the early increase in Na^+ flux underlying the initial depolarization of the action potential in many excitable cells (1). It is now clear that mammalian Na^+ channels are encoded by a multigene family. Isolation of separate cDNA clones has led to the identification of three structurally distinct Na^+ channel isoforms in rat brain (2, 3, 16) and at least one other distinct isoform in rat skeletal muscle (4). The relationship of cardiac Na^+ channels to other members of this multigene family is still uncertain.

Na^+ channels in mammalian cardiac membrane have recently been shown to have functional properties quite distinct from Na^+ channels in nerve and skeletal muscle. The most widely known difference is that tetrodotoxin-sensitive (TTX-S) Na^+ channels in nerve and skeletal muscle are blocked by nanomolar concentrations of the neurotoxins, saxitoxin (STX), and tetrodotoxin (TTX), whereas TTX-resistant (TTX-R) Na^+ channels in cardiac membrane require 1–10 μM TTX to block them (5). Cardiac Na^+ channels are also 1000 times more sensitive to inhibition by the antiarrhythmic agent lidocaine (6), which is significant clinically in that most lethal arrhythmias involve cardiac Na^+ channels and cause 15–20% of U.S. deaths (7). Differences between Na^+ channels in heart and other excitable tissues are relevant for a molecular understanding of the basic structure of these channels and the mode of action of antiarrhythmic drugs.

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Knowing that an alternative form of Na^+ channel is expressed in heart, we undertook this study to determine the molecular basis by which this cardiac isoform arises. We show here that in newborn rat heart, where only low-affinity [^{3}H]STX receptors, corresponding to TTX-R Na^+ channels can be detected (8), a separate gene encodes a Na^+ channel isoform, which is distinct from those described in nerve and muscle and is, therefore, another member of this multigene family.† Furthermore, we show that this rat heart I Na^+ channel gene is also expressed in denervated rat skeletal muscle, where low-affinity [^{3}H]STX receptors and TTX-R Na^+ channels also arise (9, 10). Therefore, these studies provide strong presumptive evidence that rat heart I encodes the α subunit of a TTX-R Na^+ channel isoform.

EXPERIMENTAL PROCEDURES AND RESULTS

[^{3}H]STX Receptors in Newborn Rat Heart. To simplify isolation of cDNA clones encoding the physiologically relevant TTX-R cardiac Na^+ channels, we desired a preparation expressing only the corresponding low-affinity [^{3}H]STX receptors. [^{3}H]STX binding measurements [performed as described (8)] in Fig. 1 show that in adult rat heart, both high- and low-affinity [^{3}H]STX receptors exist, corresponding to both TTX-S and TTX-R Na^+ channel subtypes; high-affinity [^{3}H]STX receptors complicate the isolation of cDNAs encoding TTX-R Na^+ channel, as will be detailed below.

While studying the ontogeny of Na^+ channel [^{3}H]STX receptors in rat cardiac muscle, we were fortunate to find that only the TTX-R Na^+ channel subtype was detectable in newborn rats. The results (Fig. 1) reveal that in 6-day-old rat hearts, a single population of low-affinity [^{3}H]STX receptors are found. Furthermore, our previous hybrid melting studies (11) of newborn rat heart RNA and the rat brain IIa NA8.4 cRNA probe (3) had demonstrated a single major peak, suggesting that one single Na^+ channel mRNA species might be present in far greater abundance than others. These results motivated the current cloning strategy to isolate cDNAs encoding a TTX-R cardiac Na^+ channel isoform from a newborn rat heart cDNA library.

Isolation of Cardiac-Specific Na^+ Channel cDNA Clones. Rat brain IIa Na^+ channel cDNA clones for the 5′ terminus [NA5.2, nucleotides (nt) −137–1529] and the 3′ terminus (NA8.4, nt 3361–5868) were provided by Auld *et al.* (3). Previous low-stringency Northern (RNA) blots (8) suggested that the rat brain IIa Na^+ channel cRNA probes NA5.2 and NA8.4 hybridized to a mRNA species of ≈9.5 kilobases (kb)

Abbreviations: STX, saxitoxin; TTX, tetrodotoxin; TTX-R, TTX resistant; TTX-S, TTX sensitive; nt, nucleotide(s); ds, double-stranded.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M27902).

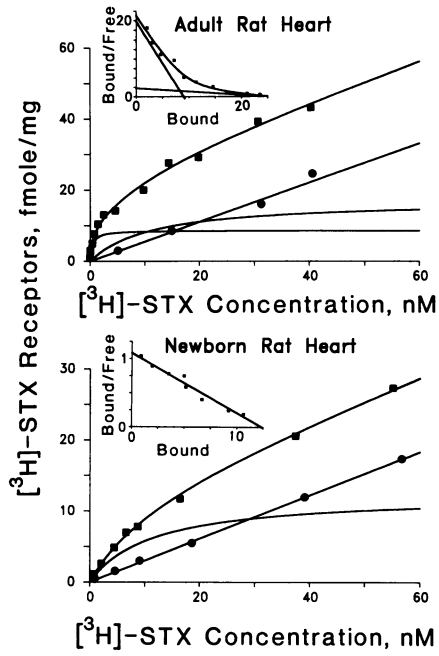

FIG. 1. [^{3}H]STX receptor measurements on adult and 6-day-old rat hearts revealed double and single populations of [^{3}H]STX receptors in adult and newborn rat hearts, respectively. Nonspecific [^{3}H]STX binding was measured in an excess of unlabeled STX (2 μM). Data points are means of three to six samples. In adult rat heart, high- and low-affinity [^{3}H]STX receptors with K_d values of 0.38 and 11.4 nM were present. In 6-day-old rat heart membrane, only low-affinity [^{3}H]STX receptors with K_d value 12.4 nM were present. (*Insets*) Scatchard plots show a curved line for adult heart and a straight line for newborn heart, indicating double and single populations of [^{3}H]STX receptors.

in newborn rat heart. We therefore used these cRNA probes (13) to screen a newborn rat heart cDNA library constructed in collaboration with Stratagene as follows: Total RNA was prepared from 6-day-old rat heart (12), and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (13). Double-stranded (ds) cDNA was synthesized by the hairpin-loop method (13) in two separate reactions, containing 15 μg of poly(A)$^+$ RNA primed with either oligo(dT) or random oligonucleotides. Approximately equal amounts of ds-cDNA from both reactions were combined and size-fractionated by nondenaturing electrophoresis; the fraction corresponding to 3–10 kb was isolated, ligated to dephosphorylated λZap vector arms by *Eco*RI linkers, and packaged *in vitro*. cDNA clones in Bluescript plasmids were excised from the λZap phagemid cloning vector in the *Escherichia coli* strain XL1-Blue as described by the manufacturer (Stratagene). The unamplified library contained $\approx 1 \times 10^6$ recombinants.

The cDNA library (9×10^5 plaques) was screened by hybridization (in buffer I; see Fig. 2), by using cRNA probes (13) derived from the rat brain IIa amino- and carboxyl-terminal Na$^+$ channel clones NA5.2 and NA8.4, respectively (3). Filters were subjected to three low-stringency 30-min washes [45°C in 1× SSC (0.15 M NaCl/0.015 M Na citrate)/0.1% SDS]. Three out of four positive clones initially isolated hybridized selectively to rat brain RNA (data not shown), and only one clone, pRH4-23, hybridized selectively to rat heart RNA. Fig. 2 shows the strong selective hybridization of NA8.4 to brain versus cardiac RNA (Fig. 2*A*) and of pRH4-23 to cardiac versus brain RNA (Fig. 2*B*). Subsequent end-sequence analysis (400–500 nt) of the three brain-specific cDNAs revealed identical sequences with the rat brain I Na$^+$ channel (2).

The same filters (6×10^5 plaques) were rescreened by moderately high-stringency hybridization with cRNA probes derived from cardiac clone pRH4-23 at 55°C and washing at

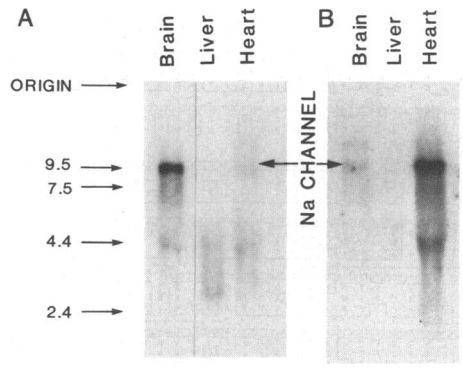

FIG. 2. Northern blot analysis with cRNA probes from rat brain Na$^+$ channel clone NA8.4 (*A*) and rat heart clone pRH4-23 (*B*). Total RNA (20 μg each) from newborn rat brain (lane 1), liver (lane 2), and cardiac muscle (lane 3) was electrophoresed on a 1.2% agarose–formaldehyde gel. RNA M_r standards (BRL) in kb are indicated on left. The hybridization band for Na$^+$ channels occurs at ≈9.5 kb for brain and heart RNA (arrows). Hybridization was at 55°C with 2.5×10^6 cpm/ml of cRNA probe in buffer I [50% (wt/vol) formamide/5× SSC/50 mM $NaPO_4$, pH 6.5/0.1% SDS/5× Denhardt's solution (Denhardt's solution is 0.02% polyvinylpyrrolidone/0.2% Ficoll/0.02% bovine serum albumin)/sheared denatured salmon sperm DNA at 50 μg/ml, wheat germ tRNA at 250 μg/ml (type V; Sigma)]. Wash was at 50°C with buffer II (30% formamide/0.1× SSC/50 mM $NaPO_4$, pH 6.5/0.1% SDS). Autoradiography was for 4 hr.

50°C in 0.5× SSC/0.1% SDS. An additional 23 positive cDNA clones were isolated, ranging from 2.5–5 kb, which also showed strong cardiac RNA-specific hybridization. The 23 cardiac-selective positive clones were further characterized by Southern blot analysis with cRNA probes derived from the 3′ and 5′ brain clones (NA5.2 and NA8.4), followed by end-sequence analysis, identifying the 3′-most and 5′-most clones [pRH12-31 (nt 162–2877) and pRH14-31 (nt 2285–7347, including the 3′ poly(A) tail), respectively].

To obtain a cDNA clone encoding the amino terminus (pRH3-1), we constructed a specific oligonucleotide-primed cDNA library by the Gubler–Hoffman method (14), with the Amersham cDNA synthesis system modified as follows. First-strand synthesis was primed from 2 μg of 6-day-old rat heart poly(A)$^+$ RNA with a mixture of random hexamers (300 ng) and synthetic oligonucleotides (100 ng each) corresponding to three separate positions within the 5′-most clone, pRH12-31 [located 384, 946, and 3461 base pairs (bp) respectively from the 5′ end]. The ds-cDNA was joined to *Eco*RI adaptors (Promega), cloned into *Eco*RI-digested λZap II vector, and packaged *in vitro* (Stratagene). The unamplified library contained $\approx 2 \times 10^6$ recombinants. We screened $\approx 4 \times 10^5$ plaques at the higher stringency conditions described above using a 5′-terminal, 645-nt cRNA probe complementary to clone pRH12-31.

Thus, three overlapping clones spanned the entire newborn rat heart Na$^+$ channel sequence: pRH3-1 (nt −195–1134), pRH4-23 (nt 1014–5005), and pRH14-31 (nt 2285–7347). Because several lines of evidence suggest that more than one Na$^+$ channel isoform may be present in adult heart, we refer to the Na$^+$ channel isoform cloned here as rat heart I.

Nucleotide and Amino Acid Sequence. Sequencing of ds plasmids was done by the dideoxy chain-termination sequencing method (13) using Sequenase (United States Biochemical). Fig. 3 shows the composite cDNA sequence (7542 nt) encoding the rat heart I Na$^+$ channel. The translational initiation site was assigned to the methionine codon at nucleotides 1–3 because this is the first ATG followed by an open reading frame of 2019 amino acid residues (specifying a translated M_r of 227,417); this sequence ends with a translational termination codon (TGA) at nt 6058. Furthermore,

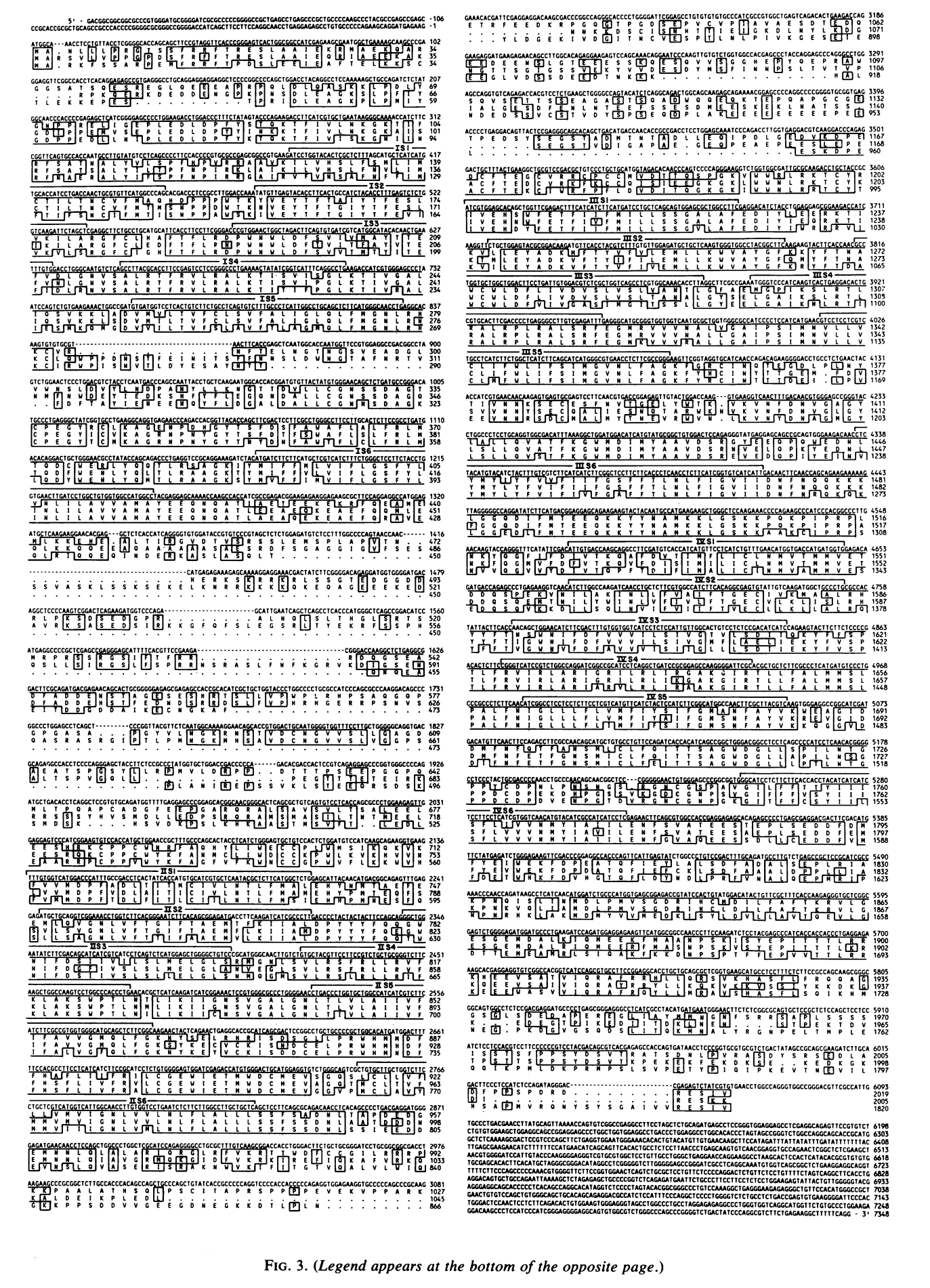

FIG. 3. (*Legend appears at the bottom of the opposite page.*)

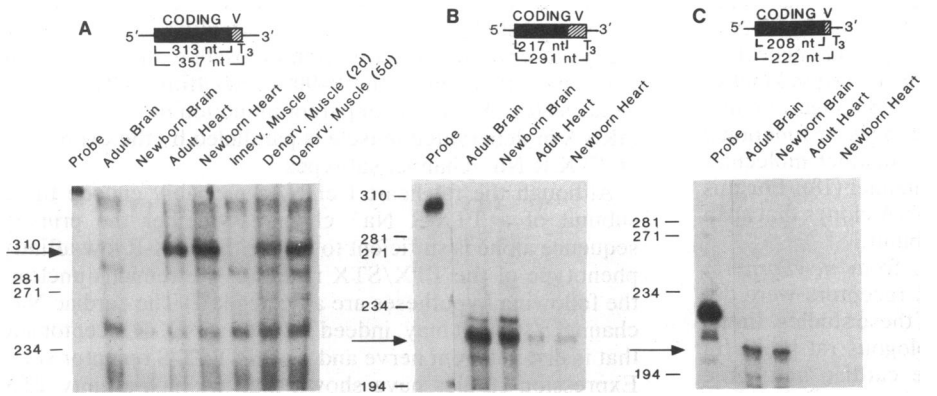

FIG. 4. RNase protection assays. Total RNA (10 μg) from adult and newborn brain and heart as well as innervated (Innerv.) and 2- and 5-day postdenervated (Denerv.) gastrocnemius muscles were hybridized with ^{32}P-antisense cRNA transcripts from rat heart I (nt 5760–6073) (*A*); rat brain I (nt 5810–6027) (*B*); or rat brain IIa (nt 5667–5875) (*C*). RNase-resistant fragments (arrows) were resolved on 6% polyacrylamide gels. A ^{32}P-labeled *Hae* III digest of ϕX174 provided size markers. cRNA probes (above) consisted of a coding portion (black) and an unprotected portion (hatched), consisting of vector (V) transcribed between T_3 promotor and start of coding insert.

the derived amino acid sequence that uses this reading frame is highly homologous with the rat brain Na^+ channels. The coding region is followed by 1290 nt of 3′ untranslated sequence and ends with a poly(A) tract. This 3′ untranslated region is significantly shorter than those found in rat brain I and II (2120 and 2328 nt, respectively) and more comparable to that found in rat brain III (556 nt) (2, 16).

Comparison of Na^+ Channels from Rat Heart, Rat Brain II, and Electroplaque. Fig. 3 aligns the derived amino acid sequences of rat heart I, rat brain II, and *Electrophorus* Na^+ channels (2). The rat heart I Na^+ channel amino acid sequence has an overall identity of 77% vs. rat brain II and 65% vs. *Electrophorus* Na^+ channels. Rat heart I contains four repeated units of homology (designated I–IV), which are highly conserved among the Na^+ channels. In contrast, significantly less homology exists between rat brain II and rat heart I in the regions connecting repeat domains I–IV (<50%) and the amino- and carboxyl-termini (50–70%), except for the short segment between repeats III and IV (92.5%), which has been suggested to encode the highly conserved function of Na^+ channel inactivation (15).

Rat heart I is also similar in hydropathy profile (data not shown) to Na^+ channels previously analyzed, with each internal repeat (Fig. 3) having five hydrophobic segments (S1, S2, S3, S5, and S6) and one positively charged segment (S4) containing arginine or lysine residues at every third position. This scheme is like that found in rat brain I–III Na^+ channels, leading to the suggestion that the positive charges in the S4 segment are part of the voltage sensor. Based upon previous models (2, 15), the amino and carboxyl-terminal residues and the regions connecting repeat domains I–IV all reside on the cytoplasmic side of the membrane. Moderate-sized extracellular connecting segments with low conservation (50–70%) are also found between S5 and S6 in all four repeat domains. Such segments, which show low similarity to previously characterized Na^+ channels, may be important for specifying the distinct functional properties of rat heart I Na^+ channels.

One major difference in the primary structure of previously characterized Na^+ channels is that a large interconnecting loop is found between repeat domains I and II in the rat brain I–III Na^+ channels (2, 16) (337, 326, and 279 amino acids, respectively) as compared with a short loop in the rat skeletal muscle I (4) (127 amino acids) and *Electrophorus* (2) (156 amino acids) Na^+ channels (possibly reflecting the modified muscle tissue origin of the *Electrophorus* electric organ). Interestingly, rat heart I has a large loop connecting repeats I–II (296 amino acids). This large loop has been postulated as a site where modification of channel properties may occur because rat brain I–III α subunits have five major conserved cAMP-dependent phosphorylation sites (16). Only one of these sites (serine at nt 630) is conserved in rat heart I, although there are several other serine residues conserved in rat heart I and brain I–III, which might be phosphorylated less readily by cAMP-dependent protein kinase or by other protein kinases with different specificities. The Na^+ channel is also heavily glycosylated; seven possible sites of N-linked glycosylation in rat brain II are located in repeat domains I and III in the loop between S5 and S6, which therefore are proposed to be extracellular (16). The proposed transmembrane topology of rat heart I is consistent with five potential N-glycosylation sites (aspartic residues at positions 212, 303, 340, 1368, and 1382 in Fig. 3).

RNase Protection Analysis. Total RNAs from various rat tissues were subjected to RNase protection analysis (13) by use of cRNA probes derived from the carboxyl-terminal coding regions of the rat brain I and IIa and rat heart I Na^+ channels (Fig. 4), a relatively divergent region of Na^+ channel sequence. Hence, a protected band in this assay indicates the presence of the corresponding mRNA species.

When the rat heart I probe was used (Fig. 4*A*), protected fragments were seen in the heart, but not in the brain RNA samples, further demonstrating cardiac specificity of rat heart I cDNA. As expected, rat brain I and IIa Na^+ channel mRNAs are expressed in brain (Fig. 4 *B* and *C*). In addition, the rat brain I mRNA species is found in heart (Fig. 4*B*), as also evidenced by our isolation of rat brain I cDNA clones from the newborn rat heart cDNA library.

We also tested for the rat heart I mRNA in both normal and denervated skeletal muscle. Fig. 4*A* shows that this mRNA appears absent in innervated skeletal muscle. With denervation, the rat heart I mRNA species is induced, increasing in abundance from 2 to 5 days after denervation. Further analysis of the expression of the rat heart I Na^+ channel isoform in denervated muscle will be reported elsewhere.

DISCUSSION

Na^+ channels are essential for the conduction of normal cardiac action potentials in the heart and are major target sites for antiarrhythmic agents. Until now, considerable

Fig. 3 (*on opposite page*). Nucleotide and amino acid sequences of Na^+ channel cDNAs. Rat heart I nucleotide sequence (row 1) and optimal alignment of deduced amino acid sequences (one-letter code) for rat heart I (row 2), rat brain II (row 3), and *Electrophorus electricus* (row 4) Na^+ channels. Numbers of the last residues are given on the right. Identical amino acid residues are boxed. Gaps have been inserted into amino acid sequences for maximal sequence homology, and a continuous stretch of gaps was counted as one substitution, regardless of length. The indicated segments S1–S6 in repeat domains I–IV have been determined by comparison with the rat brain II sequence (2).

physiological and pharmacological evidence has indicated substantial differences between cardiac and other Na^+ channels, including (*i*) differential sensitivity to block by TTX of 1000-fold (5), by lidocaine (6), and by polypeptide neurotoxins (17); (*ii*) differences in activation and inactivation kinetics (5) and single-channel conductance (C. Baumgarten and R.B.R., unpublished data). These differences in functional properties are likely to be explained by distinct molecular structures specific to the cardiac Na^+ channel (18). For this report, we isolated three overlapping cDNA clones that span the entire rat heart I Na^+ channel α subunit.

The construction of cDNA libraries from *newborn* rat heart, where only low-affinity [^{3}H]STX receptors were detected, appears critical for the success of these studies. Initial low-stringency screening with a heterologous rat brain IIa Na^+ channel probe detected only one cardiac and three brain-specific cDNA clones. This initial preponderance of brain clones probably reflects their increased homology with the rat brain IIa Na^+ channel probe used for screening, rather than their relative abundance in the rat heart cDNA library because the second screening at moderately high stringency with a rat heart I clone (pRH4-23) yielded 23 additional cardiac-specific clones. In contrast, adult cardiac tissue contains significant (25–50%) high-affinity [^{3}H]STX receptors (Fig. 1), as well as an increased proportion of type I brain-specific Na^+ channel mRNAs (Fig. 4). Hence, had we constructed a cDNA library from adult heart, during initial low-stringency screening, the increased number of brain-specific cDNAs may well have obscured detection of the desired rat heart I clones.

Interestingly, we find here that these brain-specific mRNAs in heart corresponded to the rat brain I Na^+ channel isoform, as corroborated by our RNase protection results. Because electrophysiological studies detect only TTX-R Na^+ channels in cardiac membrane, the presence of high-affinity [^{3}H]STX receptors and now these brain-specific Na^+ channel mRNAs in heart preparations presents an enigma. Their presence could result from nerve contamination of heart preparations (17), but their large proportions make this seem unlikely (18), particularly for brain-specific mRNAs, which are located in sympathetic nerve cell bodies located outside the heart. Future *in situ* hybridization studies may clarify this issue. High-affinity [^{3}H]STX receptors have also been proposed as precursors for low-affinity [^{3}H]STX receptors on TTX-R Na^+ channels (18), which now seems less likely, if, as we propose here, at least two distinct rat heart genes (brain I and heart I) encode the high- and low-affinity [^{3}H]STX receptors, respectively. Other possible explanations include that TTX-S Na^+ channels are: (*i*) located in internal membranes in heart—e.g., they never leave endoplasmic reticulum or Golgi apparatus; (*ii*) inactive—e.g., due to their state of phosphorylation; (*iii*) active, but not under the conditions used in previous electrophysiological investigations.

We have referred to the rat heart I cDNAs as encoding a putative TTX-R cardiac Na^+ channel isoform because the evidence pertaining to this is indirect at present. Expression studies will be required to demonstrate definitively that the rat heart I cDNA sequence encodes the α subunit of a TTX-R Na^+ channel isoform, though strong presumptive evidence suggests that this is so. In newborn rat heart used to construct the libraries, only TTX-R low-affinity TTX-R [^{3}H]STX receptors were detected, corresponding to the isolation of 24 cardiac-specific clones of a total of 27 clones. All 24 cardiac-specific clones shared identical sequence with rat heart I, suggesting the presence of only one cardiac-specific mRNA species. The simplest explanation of these data is that newborn rat heart contains a single predominant Na^+ channel mRNA species (rat heart I), and this mRNA expression parallels the expression of primarily low-affinity [^{3}H]STX receptors and corresponding TTX-R Na^+ channels in the same preparation.

RNase protection assays provided further support that rat heart I encodes a TTX-R Na^+ channel subtype. Whereas innervated skeletal muscle expresses only TTX-S channels, 5 days after denervation, skeletal muscle expresses TTX-R Na^+ channels along with 50–90% low-affinity [^{3}H]STX receptors (10). Again, the apparent induction of the rat heart I mRNA in denervated muscle is paralleled by the expression of TTX-R Na^+ channel subtypes.

Although the rat heart I cDNAs probably encode the α subunit of a TTX-R Na^+ channel, whether the primary sequence alone is sufficient to confer the TTX-R low-affinity phenotype of the TTX/STX receptor site remains unclear; the following hypotheses are advanced: (*i*) The cardiac Na^+ channel α subunit may, indeed, encode a TTX-R receptor site that is distinct from nerve and muscle TTX-S receptor sites. Expression studies have shown that the high-affinity TTX receptor is located on the large α subunit (15). (*ii*) On the other hand, posttranslational modifications may be required for function of the TTX-R receptor site. Such modifications may be secondary to the primary sequence differences of the cardiac α subunit or they may differ in nerve and cardiac cell types. (*iii*) Finally, an accessory protein subunit associated with the cardiac α subunit may result in TTX resistance. The rat heart cDNAs we have isolated will allow us to examine these possibilities by expression of full-length cardiac Na^+ channel cDNAs in heterologous systems and determination of specific molecular structures accounting for the distinctive functional properties of cardiac Na^+ channels.

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