Functional expression of the rat heart I Na^+ channel isoform

Demonstration of properties characteristic of native cardiac Na^+ channels

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We describe the expression of functional Na^+ channel in *Xenopus* oocytes injected with cRNA transcribed from the rat heart I cDNA clone. The expressed rat heart I Na^+ currents show kinetic properties and resistance to tetrodotoxin and saxitoxin which are characteristic of native cardiac Na^+ currents. The primary amino acid sequence of the rat heart I α-subunit is therefore sufficient for expression of tetrodotoxin resistance, and the rat heart I clone is likely to account for the tetrodotoxin-resistant phenotype of cardiac and denervated skeletal muscle.

Na^+ channel; cDNA expression; *Xenopus* oocyte; Na^+ current; Tetrodotoxin; Saxitoxin

1. INTRODUCTION

The voltage-gated Na^+ channel is a transmembrane protein that is essential in the generation of the upstroke of the action potential in most excitable membranes. Early studies assumed that Na^+ channels in nerve, heart, and skeletal muscle were identical, but it is now clear that multiple isoforms arise from a mammalian Na^+ channel multigene family [1-3].

We have recently reported the cloning and characterization of the α-subunit of a rat heart Na^+ channel isoform (RH-I) [3]. The native cardiac Na^+ current is several orders of magnitude less sensitive to the potent neurotoxins, tetrodotoxin (TTX) and saxitoxin (STX), than most nerve and skeletal muscle Na^+ currents (I_{Na}'s) [4]. Although the RH-I clone was derived from neonatal rat myocardium containing only low-affinity [^{3}H]STX receptors [3], definitive identification of RH-I requires expression studies demonstrating similarity of properties with the native TTX-resistant rat heart Na^+ channel.

Recently, Sutton et al. [5] reported that injection of poly(A)$^+$ mRNA prepared from rabbit and rat cardiac muscle resulted in functional expression of a small I_{Na} in *Xenopus* oocytes. Contrary to what might be expected for cardiac Na^+ channels, this I_{Na} was sensitive to TTX (TTX-S). They proposed that the relative low toxin affinity of the native cardiac Na^+ channel might result from a post-translational change in channel structure, or it might require a subunit or a cofactor not present in *Xenopus* oocytes. On the other hand, Krafte et al. [6] expressed I_{Na} in *Xenopus* oocytes injected with guinea pig cardiac poly(A)$^+$ mRNA, showing a TTX sensitivity typical of cardiac Na^+ channels. If RH-I is indeed the cardiac Na^+ channel, then study of the expressed Na^+ current would resolve the question of whether the toxin affinity resides in its primary structure or if some other element is necessary as well.

Noda et al. [7] have recently reported a point mutation of the rat brain II channel (mutant E387Q) that resulted in complete loss of STX/TTX block. Based on the sequence of the RH-I clone, they proposed a structural explanation for the absolute resistance of their mutant and the relative resistance of the cardiac channel. Before their intriguing proposal can be explored, it is necessary to determine whether the RH-I Na^+ channel has the characteristic low toxin affinity of cardiac Na^+ currents.

We report here that injection of cRNA generated from the RH-I clone into *Xenopus* oocytes results in Na^+ currents with kinetics consistent with the native cardiac Na^+ current. Furthermore, the RH-I cardiac Na^+ channel shows about a 100-fold lower sensitivity to block by TTX and STX than the cloned skeletal muscle Na^+ channel μI [2]. While not excluding involvement of other subunits, cofactors, or post-translational modifications in cardiac Na^+ channel function, these results establish that the low affinity for TTX and STX characteristic of the cardiac Na^+ channel resides in the primary structure of the RH-I α-subunit protein.

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Abbreviations: TTX, tetrodotoxin; STX, saxitoxin; I_{Na}, sodium current; TTX-S, TTX-sensitive; TTX-R, TTX-resistant; RH-I, rat heart I Na^+ channel

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2. MATERIALS AND METHODS

The full-length RH-I coding sequence was constructed in the pBluescript SK⁻ vector (Stratagene) as described in Fig. 1. The coding region was excised from pBluescript SK⁻ and cloned into pSP64t [9] using synthetic adaptors (New England Biolabs). This template was linearized with *Eco*R1 and full-length cRNA was transcribed using SP6 RNA polymerase (Promega), primed with the capping analog m^{7}G(5')ppp(5')G. The rat skeletal muscle cDNA pμI-2 (2) was transcribed similarly using T7 RNA polymerase (Stratagene).

Stage IV-VI oocytes were injected with 50 nl of 0.1–1.0 μg of cRNA and incubated for up to 2 weeks at 18°C. Whole cell current measurements of I_{Na} were recorded as described in Fig. 2.

3. RESULTS AND DISCUSSION

3.1. *Electrophysiological identification of the RH-I clone with cardiac Na^+ channels*

A family of transient inward currents elicited by depolarizing steps applied to *Xenopus* oocytes injected with RH-I cRNA is shown in Fig. 2A. The currents in uninjected oocytes (Fig. 2B) fail to express transient inward currents. All current records (Figs 2–4) are uncorrected (i.e. without capacitive and leak currents subtracted) since these measurements were more reliable for determining toxin affinities in Figs 3 and 4. The inward currents in Fig. 2A show voltage-dependent kinetics consistent with the native cardiac I_{Na}. The time to peak I_{Na} and current decay rate in Fig. 2A decrease with increasing depolarization. The peak current-voltage relationship (I–V curve) in Fig. 2C shows a threshold for I_{Na} activation of −40 mV, and a peak inward current elicited at −20 mV. These currents resemble whole-cell I_{Na} recorded from neonatal rat ventricular myocytes (Fig. 2D; [10]).

We compared the properties of the expressed μI skeletal muscle clone to the RH-I cardiac clone. In contrast to RH-I, the typical peak I_{Na} for μI (Fig. 2E) is greater than 1 μA in size and decays slowly, whereas the peak RH-I I_{Na} (Fig. 2A) is less than 200 nA in size (discussed below), and decays rapidly. Studies [11,12] comparing the inactivation kinetics in denervated skeletal muscle membrane of the native TTX-S I_{Na} (associated with the μI cDNA [2] and the native TTX-R I_{Na} (associated with the RH-I cDNA [3,23]) show that they are not measurably different. The μI I_{Na} (Fig. 2E) decays considerably more slowly than native skeletal muscle I_{Na} [2,11], whereas the more rapid decay rate of the RH-I I_{Na} (Fig. 2A) is more comparable to the native TTX-R I_{Na} found in mammalian cardiac and denervated skeletal muscle, albeit still slower (Fig. 2A vs 2D). Thus, the decay rate of RH-I may be influenced by factors other than primary sequence of the RH-I cDNA clone. It is interesting in this regard that low molecular weight fractions of mRNA co-injected in oocytes with the μI [2] and rat brain IIa [15] Na^+ channel cRNAs have been demonstrated to considerably increase the rate of inactivation of both these isoforms. Our ability to express the TTX-R RH-I channel will now allow us

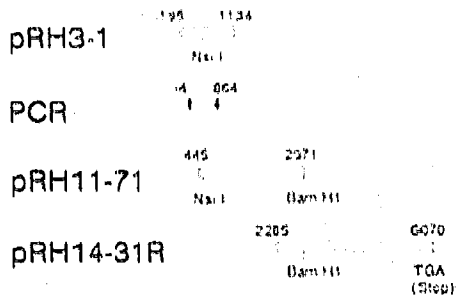

Fig. 1. Construction of the RH-I full-length cDNA clone. The amino-terminal portion was synthesized by the polymerase chain reaction (PCR) [8] using the cDNA clone pRH3-1 [3] as the template. Synthetic oligonucleotides corresponding to positions −4 to +13 and incorporating a *Hin*dIII restriction site on the 5'-end (5'-AAGCTTGAAGATGGCAAACCTCC-3') and an antisense oligonucleotide corresponding to nucleotides 1059–1043 (5'-TTGAGCTCGGTGAAGTT-3') were used as PCR primers. The PCR product was digested with *Hin*dIII and *Nsi*I and ligated to the *Nsi*I site of pRH11-71. This product was digested with *Hin*dIII and *Bam*HI and ligated to the *Bam*HI site of pRH14-31R. The resulting plasmid contained the contiguous coding sequence from −4 to +6072, with the termination codon at nucleotide 6058.

to test how co-injection of low molecular weight cardiac mRNA species with the RH-I cRNA may influence inactivation kinetics of the expressed RH-I I_{Na}.

The RH-I I_{Na} expressed in oocytes is smaller than the I_{Na} typically found with cRNA derived from the μI and the rat brain II and III clones [13–15]. The RH-I isoform may be intrinsically difficult to express for several reasons. (i) It is possible that poorly expressed ion channel isoforms are less readily translated by the oocyte, or require post-translational modifications which are not highly efficient in this heterologous system. Low efficiency of expression has also been found for other cloned ion channel isoforms. The cloned Na^+ channel from electric eel is not expressable in *Xenopus* oocytes [16], and the rat brain I cRNA expresses poorly [13], while expression of the rat brain II, III [13,15] and μI cRNAs results in expression of μA-level currents (Fig. 2E). Similarly, cRNA from the cloned skeletal muscle Ca^{2+} channel fails to express in oocytes [17], whereas cRNA from the cloned cardiac Ca^{2+} channel expresses moderately sized currents [18]. (ii) Untranslated sequences could play a role in stability or translatability of the RH-I cRNA, even though other cloned channel isoforms express well without such sequences. The cDNA construct used for these experiments is devoid of both 5' and 3' untranslated regions (iii) Finally, other factors (e.g. β-subunits) may be required for high level expression of the RH-I Na^+ channel α-subunit, as has recently been demonstrated for expression of the Na^+,K^+-ATPase α-subunit [19].

3.2. *TTX/STX sensitivity of RH-I and μI Na^+ channel clones*

Native cardiac I_{Na} is characteristically resistant to block by TTX and STX, relative to skeletal muscle and

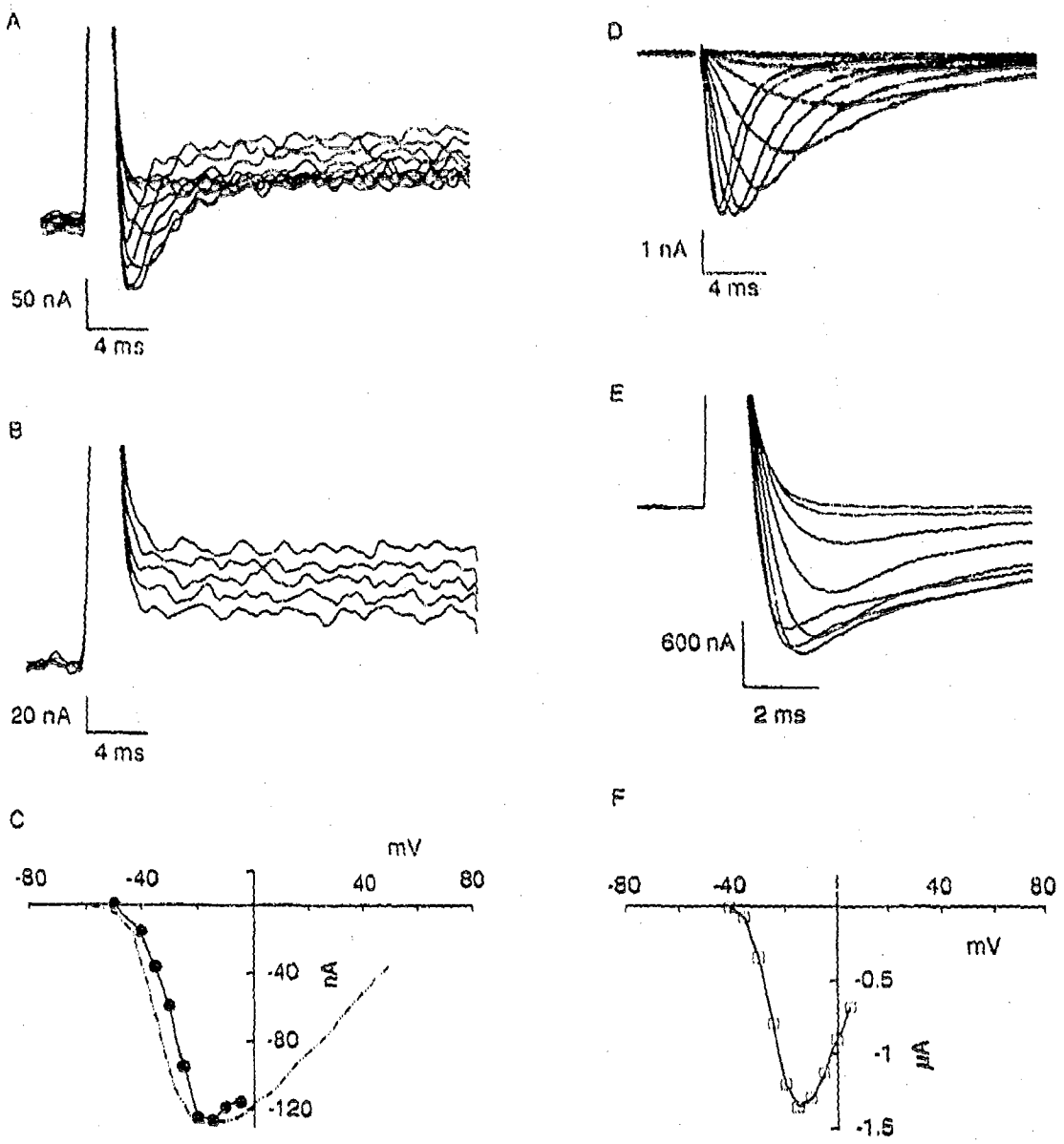

Fig. 2. Expression of I_{Na} in RH-1 mRNA injected oocytes. (A) Inward current elicited by voltage steps from −80 mV to −50 through −5 mV in increments of 5 mV 4 days after injection of RH-1 cRNA; T = 20°C. (B) Uninjected control egg from the same batch as in (A) shows the absence of inward current for the same voltage range in increments of 10 mV; T = 20°C. (C) I–V relationship for RH-1 (solid circles, solid line) and native neonatal rat cardiac I_{Na} (dashed line). RH-1 I_{Na} decays fully within 25 ms. Hence, the I–V curve is the peak minus the steady-state current at the end of a 26 ms depolarization. The I–V curve from the isolated myocyte is normalized to the peak current. (D) Whole-cell currents from an isolated neonatal rat ventricular myocyte. Note similarity in time course of decay of native I_{Na} and RH-1 directed expressed currents (A); T = 14°C. (E) Expression of I_{Na} in μl cRNA injected oocytes. Inward current elicited by voltage steps from −80 mV to −40 mV through 5 mV by 5 mV increments. Note the different current scales in (A) and (E) (F) I–V relationship for peak μl I_{Na}. Methods: Portions of ovaries were removed from adult *Xenopus laevis* (NASCO, Ft. Atkinson, WI and *Xenopus* One, Anne Arbor, MI) and placed in a modified oocyte Ringers solution (OR-2). OR-2 consisted of (in mM): NaCl 86, KCl 2.5, $CaCl_2$ 1.0, $MgCl_2$ 1.0, NaH_2PO_4 10, Hepes 5, pH 7.6 with NaOH. Segments of ovaries containing fifty to several hundred oocytes were incubated for 2 h in Ca^{2+}-free oocyte Ringers with 2 mg/ml collagenase type IA (Sigma Chemicals). One to four hours after collagenase treatment surrounding follicular cells were manually peeled using fine forceps and transferred to OR-2 supplemented with 1 mM Na-pyruvate and 0.1 mg/ml gentamycin (Gibco). Stage IV–VI oocytes were injected with 50 nl of 0.1 to 1.0 μg/μl cRNA and incubated for up to 2 weeks at 18°C. Oocytes were continuously perfused with OR-2 at room temperature (18–22°C) for all electrophysiological recordings. A 2-electrode voltage clamp (OC-725, Warner Instruments) with a virtual ground enabled simultaneous measurement of membrane voltage and current. Both the lowpass filtered current (3 kHz to 10 kHz, 8-pole Bessel, −3 dB, Frequency Devices), and voltage were digitized at 12.5 kHz to 33 kHz (Zenith 386 PC equipped with a Labmaster A/D board). Native currents were recorded from single one-day neonatal ventricular myocytes using the whole-cell mode of the patch-clamp technique. A List EPC-7 recorded current at 33 kHz. The bath consisted of (in mM): NaCl 142, KCl 5, $CaCl_2$ 1.5, $MgCl_2$ 1, Hepes 5, pH 7.4 and the pipette contained: Cs-glutamate 120, $CsCl_2$ 20, NaCl 5, EGTA 5, Mg-ATP 2, Hepes 10, pH 7.4. AXOBASIC 1.0 software was used for data acquisition and analysis. For clarity Figs 2A, 2B, 3A and 3D are the moving average of every 10 points.

nerve I_{Na}'s [4]. We show that the RH1-I I_{Na} is also TTX and STX resistant, compared to similar measurements of toxin sensitivity of the μI Na^+ channel expressed in *Xenopus* oocytes. Fig. 3 compares TTX sensitivity of the RH-I and μI I_{Na}'s. Whereas 50% block of the μI I_{Na} occurs between 10 and 30 nM (Fig. 3B), about 3 μM TTX is required to achieve 50% block of the RH-I I_{Na} (Fig. 3A). The dose-response curves for TTX (Fig. 3C) fit well with a single site curve having K_d values of 40 nM and 1.5 μM for μI and RH-I, respectively. Similarly, Figs 4A–C demonstrate a roughly 100-fold difference in STX sensitivity between the μI (K_d = 0.35 nM) and RH-I (K_d = 44 nM) I_{Na}.

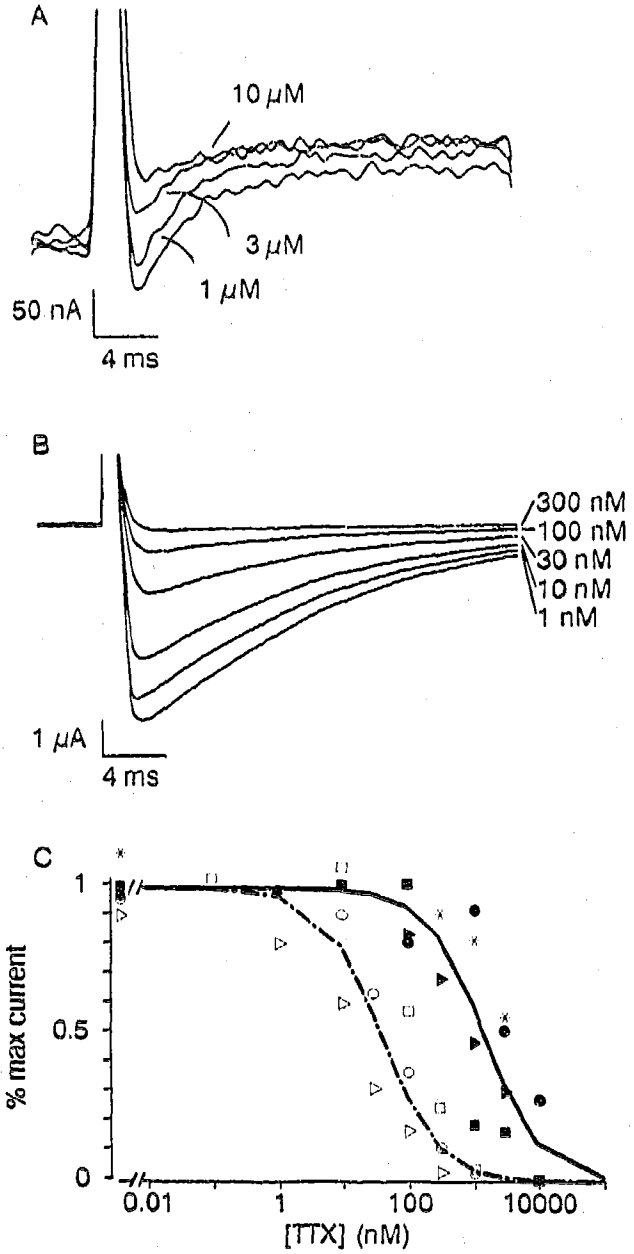

Fig. 3. RH-I (A) and μI (B) expressed I_{Na} have different TTX sensitivities. (A and B) Current was elicited by a voltage step from −80 to −20 mV. TTX was added in increasing doses, with concentrations of 0, 1, 3, and 10 in μM in panel A and 0, 1, 10, 30, 100, and 300 nM in panel B. Note difference in current gain; RH-I current is much smaller. (C) TTX dose-response curves for RH-I (filled symbols) and μI (open symbols) injected oocytes. The smooth curves are fits to the equation $I_{Na}/I_{Control} = (1 + TTX/K_d)^{-1}$ where K_d = 1.52 μM and 40.4 nM for RH-I and μI, respectively. Data are pooled from 4 RH-I (closed symbols and asterisk) and 3 μI (open symbols) injected oocytes.

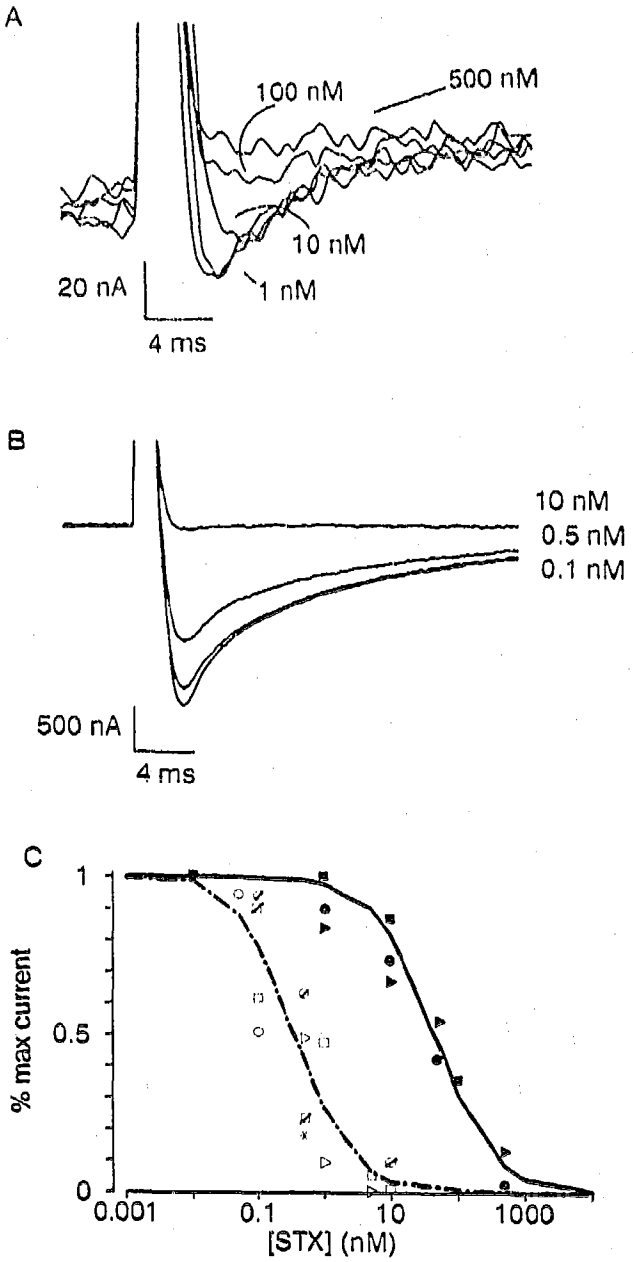

Fig. 4. RHI-I (A) and μI (B) expressed I_{Na} have different STX sensitivities. Current was elicited by a voltage step from −80 to −20 mV. STX was added in increasing doses, with concentrations of 0, 1, 10, 100, and 500 nM in (A) and 0, 0.1, 0.5, and 10 nM in (B). Note difference in current gain; RH-I I_{Na} is smaller than μI I_{Na}. (C) STX dose-response curves for RH-I (filled symbols) and μI (open symbols) injected oocytes. The smooth curves are fits to the equation $I_{Na}/I_{Control} = (1 + STX/K_d)^{-1}$, where K_d = 44 and 0.35 nM for RH-I and μI, respectively. Data are pooled from 3 RH-I and 6 μI injected oocytes.

The TTX/STX-resistant phenotype of the RH-I α-subunit indicates that the toxin resistance of native cardiac Na^+ channels resides in the primary amino acid sequence. The abundance and specificity of the RH-I mRNA, as well as the presence of only low-affinity [^{3}H]-STX receptors in newborn heart [3], suggest that the RH-I Na^+ channel accounts for the major component of the TTX-R I_{Na} in cardiac muscle. However, we cannot rule out the possibility that other cardiac isoforms not yet characterized [20] also contribute to the properties of the cardiac I_{Na}.

Heterologous expression of TTX-R I_{Na} corresponding to RH-I with poly(A)$^+$ mRNA has also been difficult. Injection of oocytes with poly(A)$^+$ mRNA from rabbit, rat and human heart [5,21], and from denervated rat gastrocnemius muscle (unpublished results) resulted in expression of only TTX-S I_{Na}'s, despite the TTX-R I_{Na}'s [4,11] and low affinity [^{3}H]STX receptors in these native preparations [3,22]. Furthermore, Northern blot and RNase protection studies confirmed that the RH-I mRNA species was also abundant in rat heart and denervated gastrocnemius muscle [3,23]. The TTX-S I_{Na} observed probably results from expression of the TTX-S rat brain I and μI mRNA species also present in cardiac [3] and denervated gastrocnemius muscle [2] respectively. We believe that the previous failure to observe TTX-R I_{Na} was due to undetectable or absent expression of the RH-I mRNA species, rather than the proposed requirement for other factors or modifications [5].

3.4. *Hypotheses for amino acids involved in the molecular receptor for TTX/STX*

Previous electrophysiological studies have led to the hypothesis that an essential (−)-charged carboxylic acid group acts as a cationic binding site for the (+)-charged TTX and STX molecules [24]. Mutational analysis of TTX binding in the rat brain II molecule led to a proposal that the TTX binding site resides, at least partially, on the extracellular loop between transmembrane segments S5 and S6 of domain I [7]. A single point mutation of the (−)-charged glutamic acid (residue 387) to a neutral glutamine rendered the channel entirely resistant to TTX, leading Noda et al. [7] to propose that this residue was part of the cationic site involved in high-affinity TTX binding. However, this amino acid is conserved among all Na^+ channels including RH-I. Our demonstration of the partial resistance to TTX of the RH-I I_{Na} supports the idea that the binding site is more complex. Noda et al. [7] suggested from sequence comparison of the RH-I and TTX-S Na^+ channels that the (+)-charged arginine (residue 388) in RH-I, substituting for the neutral asparagine conserved in TTX-S Na channels, partially neutralizes the adjacent (−)-charged glutamic acid (residue 387), resulting in partial TTX-resistance of RH-I. One possible test of this suggestion would be to determine whether replacement of this arginine with an asparagine residue in the RH-I Na channel increases the TTX sensitivity of this RH-I mutant. Having demonstrated here an expression system for the RH-I Na^+ channel clone, along with its property of TTX resistance, we are now in a position to characterize this proposed mutant and other structural features of the molecular receptor conferring different STX/TTX sensitivities of various Na^+ channel isoforms. Furthermore, we have established the identification of the RH-I Na^+ channel with the TTX-R cardiac-type I_{Na} (and with the TTX-R I_{Na} in mammalian denervated skeletal muscle). Hence, we can study structural aspects of the Na^+ channel that account for functional physiological and pharmacological variations between the RH-I and other Na^+ channel isoforms.

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