0026-895X/91/050604-05$03.00/0

MOLECULAR PHARMACOLOGY, 39:604-608

ACCELERATED COMMUNICATION

SkM2, a Na^+ Channel cDNA Clone From Denervated Skeletal Muscle, Encodes a Tetrodotoxin-Insensitive Na^+ Channel

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Received December 10, 1990; Accepted February 4, 1991

SUMMARY

Approximately one third of the Na^+ channels expressed in denervated or developing skeletal muscle are tetrodotoxin (TTX) insensitive, with a K_d for channel blockade of approximately 1 μM, similar to that found for cardiac Na^+ channels. We have recently reported the cloning of a putative Na^+ channel subtype that is characteristic of denervated and developing skeletal muscle (SkM2), the deduced amino acid sequence of which is identical to that of a Na^+ channel cDNA isolated from heart. We have now examined the functional properties of SkM2 Na^+ channels after expression in *Xenopus* oocytes. We found that the efficiency of expression of constructs containing the SkM2 clone was strongly dependent on the amount of 5′-untranslated region (5′UTR) included. Constructs containing a 206-nucleotide 5′UTR were expressed poorly, whereas constructs from which most of the 5′UTR was removed were expressed well. The channels showed rapid voltage-dependent activation and inactivation. In addition, SkM2 Na^+ channels were insensitive to low concentrations of TTX but were ultimately blocked by this toxin, with a K_d of 1.9 μM. The TTX block exhibited use dependence. Finally, SkM2 Na^+ channels were not blocked by 100 nM μ-conotoxin, which blocks Na^+ channels in innervated skeletal muscle in the low nanomolar concentration range. These data indicate that SkM2 Na^+ channels are the TTX-insensitive Na^+ channels found in denervated or developing skeletal muscle and are identical to the TTX-insensitive Na^+ channels from heart.

Unlike voltage-dependent K^+ channels, which exhibit great diversity in both gating and permeability properties, voltage-gated Na^+ channels show far less functional variability from cell type to cell type (1). This is may be due to the fact that Na^+ channels have essentially one role to play in cells, producing the rapid depolarization of an action potential. Although Na^+ channels from nerve, skeletal muscle, and cardiac tissue exhibit similar gating and permeability properties, they are separate members of a multigene family; different channel subtypes can be distinguished by their sensitivity to pharmacological agents that modulate channel activity. For example, μ-CTX, a small peptide toxin isolated from the piscovorous marine snail *Conus geographicus*, blocks Na^+ channels from innervated skeletal muscle, but not those from nerve (2). In addition, TTX blocks sodium channels in nerve and innervated skeletal muscle with nanomolar affinity, whereas heart and denervated or developing skeletal muscle contain TTX-insensitive Na^+ channels that are only blocked at micromolar concentrations of this toxin (3–5).

This work was supported by funds from the Lucille Markey Charitable Trust (M.M.W.), the Muscular Dystrophy Association (R.L.B.), and National Institutes of Health Grants NS 23885 (M.M.W.) and NS 181013 (R.L.B.).

The recent cloning of Na^+ channels from a variety of tissues, including brain (6–8), heart (9), and skeletal muscle (10, 11) now makes it possible, in principle, to determine the structural features that underlie various aspects of Na^+ channel function and to understand the molecular basis for the pharmacological differences between Na^+ channels from different tissues. A Na^+ channel has been cloned from rat skeletal muscle (called μ1 or SkM1) that is expressed in both innervated and denervated skeletal muscle, but not in heart. μ1 Na^+ channels expressed in *Xenopus* oocytes are blocked by nanomolar concentrations of TTX and μ-CTX (10), hallmarks of the Na^+ channels found in innervated adult skeletal muscle. We have recently reported the cloning of a second Na^+ channel from rat skeletal muscle (11), whose deduced amino acid sequence is identical to that of a putative Na^+ channel cDNA isolated from cardiac tissue (9). This skeletal muscle cDNA clone, termed SkM2, is not expressed in innervated skeletal muscle but is prominent in developing muscle, denervated adult skeletal muscle, and cardiac tissue. Based upon the tissue distribution of the expression of SkM2 transcripts, we proposed that SkM2 is the TTX-insensitive Na^+ channel expressed in both skeletal and cardiac tissue. In this report, we present data showing that this is in fact the case.

ABBREVIATIONS: CTX, conotoxin; TTX, tetrodotoxin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 5′UTR, 5′-untranslated region; kb, kilobases; 3′UTR, 3′-untranslated region.

Materials and Methods

Plasmids. The full-length SkM2 cDNA was constructed by ligation of the appropriate restriction fragments of three partial cDNA clones, pSkM2-A (nucleotides −206–2844), pSkM2-B (nucleotides 1894–5249), and pSkM2-C (nucleotides 5177–6869) (11) (the initiation codon starts at position 1 and the termination codon starts at position 6052). The full-length cDNA was subcloned into pSP64T (12) containing a custom polylinker with a *Not*I site inserted into the unique *Bgl*II site in the vector. This construction is termed SkM2 and contains 206 nucleotides of 5′UTR, the entire coding region, and 816 nucleotides of 3′UTR. The 5′UTR truncation constructions were made from SkM2, using the exonuclease III/mung bean nuclease method (13). Two such constructions were used in this study, D3-2, which contains 17 nucleotides of 5′UTR, and D3-48, which contains 4 nucleotides of 5′UTR.

***In vitro* transcription.** Plasmids were linearized by digestion with *Ase*I. Full-length trasnscripts were generated using SP6 RNA polymerase, as described (14). *In vitro* transcribed RNA was resuspended in sterile distilled water, at a concentration of 0.15 mg/ml, and stored at −70° until used.

Translation in oocytes. Adult female *Xenopus laevis* (Xenopus One, Ann Arbor, MI) were anesthetized by immersion in 0.17% tricaine, and a portion of the ovarian lobe was removed. The isolated tissue was dissociated using collagenase (type IA; Sigma), and the adhering follicular layer was removed manually. Isolated, follicle-free stage V and VI oocytes were microinjected with 50 nl of RNA solution. Injected oocytes were maintained in SOS (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.6), supplemented with 2.5 mM sodium pyruvate and 50 μg/ml gentamycin, at 19° for 47–72 hr.

Electrophysiology. Oocytes were voltage clamped using a standard two-microelectrode voltage clamp (OC-725; Warner Instruments, Hamden, CT), with pulse generation and data acquisition under control of a DEC 11/73-based data acquisition system (INDEC Systems, Sunnyvale, CA). Microelectrodes were filled with 3 M KCl and had resistances of 0.5–1.5 MΩ. The recording chamber was continually perfused with SOS; the exchange time of the chamber was approximately 5 sec. In all cases, the holding potential was −100 mV. Current traces were not corrected for leak or capacity currents. For data analysis, the data were fit to the appropriate equation using a Levenberg-Marquart algorithm in a commercially available Apple Macintosh-based analysis program (IGOR; WaveMetrics, Lake Oswego, OR).

Results

A full-length clone for the SkM2 Na^+ channel was constructed from several partial clones and subcloned into pSP64T, a transcription vector designed for the production of *in vitro* transcripts that are translated efficiently in *Xenopus* oocytes (12). Three constructs with different 5′UTRs were made (Fig. 1A); SkM2, which contains the 206-nucleotide 5′UTR reported for this cDNA, D3-2, which contains a 17-nucleotide 5′UTR, and D3-48, which contains a 4-nucleotide 5′UTR. Both SkM2 and D3-2 contain an out-of-frame ATG, which starts at position −8, whereas D3-48 does not. Capped RNAs were synthesized *in vitro* using SP6 RNA polymerase. The RNA produced from each construct was analyzed by Northern blots, with a probe covering nucleotides 167–2824 in the SkM2 sequence. Each construct gave rise to two products that hybridized with this probe, one 5.5 kb in length and the other of 7.2 kb (which corresponds to the expected full-length transcript from these constructs). The ratio of the 5.5-kb to the 7.2-kb product was approximately 3:1 for each construct. A probe for the 3′UTR of SkM2 (nucleotides 6516–6869) detected only the 7.2-kb product, suggesting the presence of a termination signal for SP6 RNA polymerase in the coding region of SkM2.

Each RNA preparation was injected into the cytoplasm of

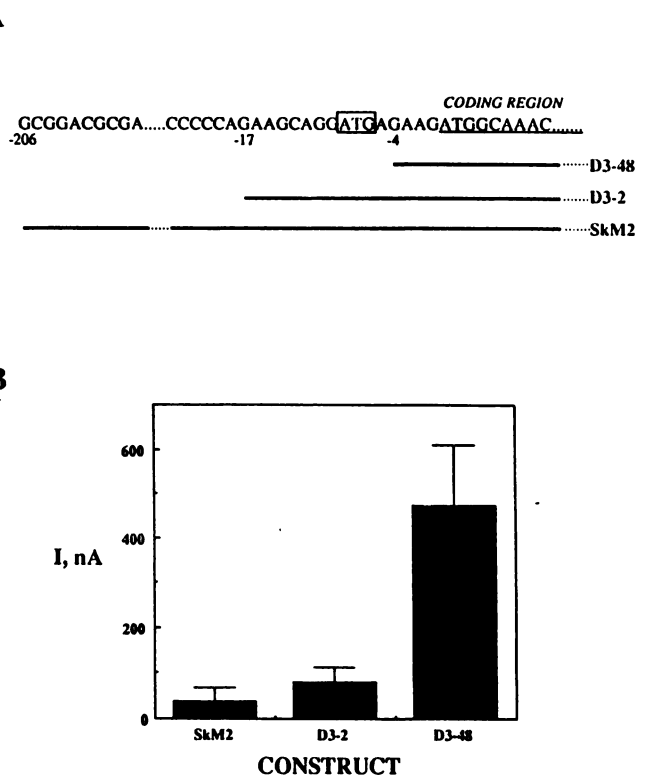

Fig. 1. A, The three SkM2 Na^+ channel constructs are shown. All three contain the entire 6051-nucleotide coding region, 816 nucleotides of 3′UTR and a variable 5′UTR ranging from 4 (D3-48) to 206 (SkM2) nucleotides. The beginning of the coding region is *underlined*, and the out-of-frame ATG starting at position −8 is *boxed*. B, Peak inward currents elicited by a 27-msec pulse from −100 mV to −20 mV were measured 48 hr after injection of RNA transcribed *in vitro* from the indicated construct. Each value represents the mean ± standard error from four to six determinations from each of two or three separate transcription reactions for each construct. Note the marked increase in the size of the current as the 5′UTR is truncated.

immature *Xenopus* oocytes. All three constructs gave rise to voltage-dependent, transient, inward currents that could be elicited by voltage jumps from a holding potential of −100 mV to −20 mV. However, the size of the currents was strongly affected by the particular 5′UTR. Injection of RNA containing the entire 206-nucleotide 5′UTR (SkM2) resulted in the expression of small inward currents, whereas RNAs with shorter 5′UTRs gave rise to larger currents. RNA made from the construct containing a 4-nucleotide 5′UTR (D3-48) gave rise to currents that were 8–10 times larger than those from the clone with the 206-nucleotide 5′UTR (Fig. 1B). All subsequent studies were done using the D3-48 construct.

Transient inward currents could be detected in response to depolarizations from −100 mV to voltages more positive than −50 mV, reaching a maximum at approximately −20 mV (Fig. 2, A and B). The currents activate and inactivate rapidly, with a voltage-dependent inactivation time constant, τ_h, ranging from 5.2 ± 0.1 msec at −40 mV to 2.7 ± 0.21 msec at −10 mV (mean ± standard error; $n = 7$ oocytes; data not shown). These properties of τ_h are in contrast to those found for the μ1 Na^+ channel expressed in oocytes, where τ_h is relatively voltage independent and much slower (τ_h approximately 12 msec) (10), but are similar to those for Na^+ currents recorded from denervated skeletal muscle in the presence of 100 nM TTX (3). The steady state voltage dependence of inactivation was determined by measurement of the current at −20 mV after a 50-msec

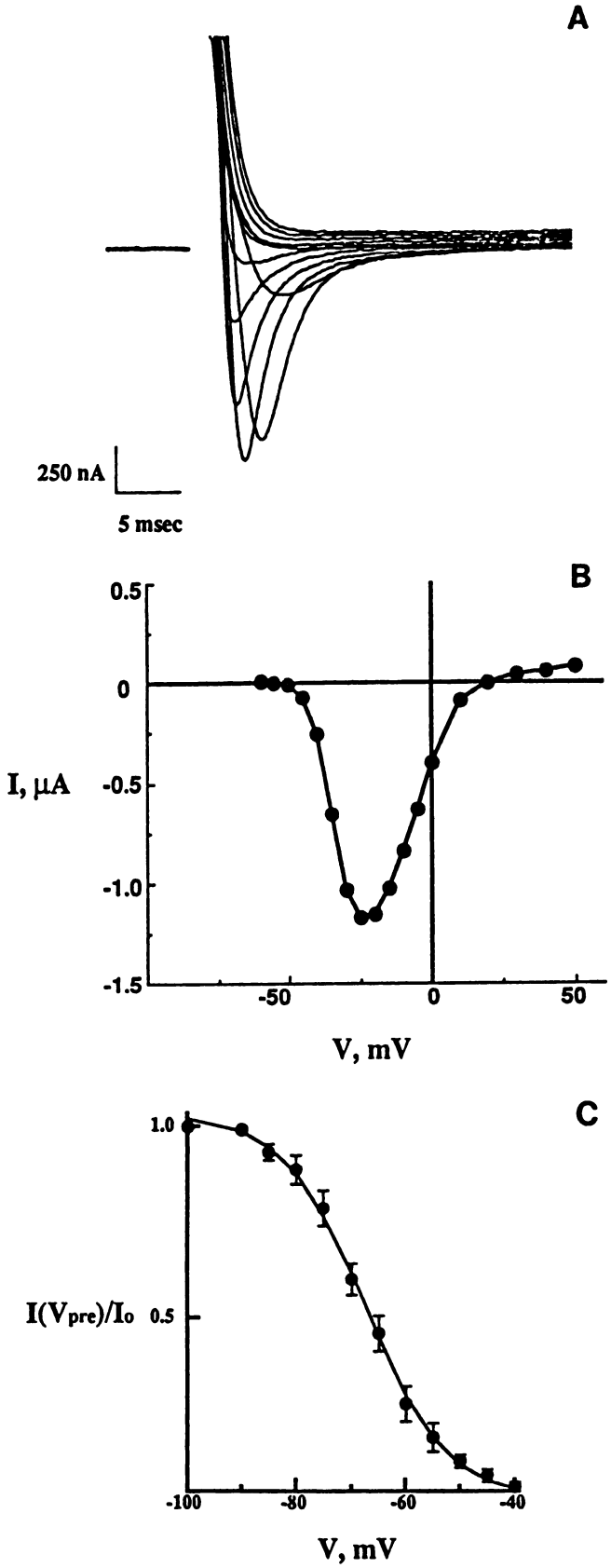

Fig. 2. A, Currents elicited, from an oocyte injected 72 hr previously with RNA made from construct D3-48, by a series of 27-msec pulses from a holding potential of −100 mV to voltages ranging from −60 to +50 mV are shown. The maintained outward current is the leakage current observed for large depolarizations. Note the rapid time course of activation and inactivation of the Na^+ currents. B, The peak currents from the cell shown in A are plotted as a function of the test potential. The maximum inward current is seen at approximately −20 mV. C, The steady state voltage dependence of inactivation was determined by measurement of the peak inward current recorded at −20 mV after a 50-msec prepulse from −100 mV to the indicated potential. Each *point* represents the mean ± standard error of determinations from five oocytes. The *solid curve* is the relation $I(V_{pre})/I_o = 1/(1 + \exp((V_{pre} - V_{1/2})/k))$, with $V_{1/2} = -67$ mV and $k = 7.4$ mV.

prepulse to various voltages (Fig. 2C). The data are well fit by a Boltzmann distribution with a midpoint of $V_{1/2} = -67$ mV and a slope factor of $k = 7.4$ mV. Both the peak of the *I-V* curve and the midpoint of the steady state inactivation curve are approximately 20 mV more positive than those for currents recorded from denervated skeletal muscle in the presence of 100 nM TTX (3).

On the basis of tissue distribution of SkM2 mRNA, we previously suggested that SkM2 cDNA encodes the TTX-insensitive Na^+ channel found in developing muscle and after denervation of skeletal muscle, as well as the channel present in heart (11). These TTX-insensitive Na^+ channels are blocked by TTX with a K_d on the order of 1 μM (3, 4), in contrast to TTX-sensitive channels in muscle and μ1 Na^+ channels expressed in oocytes, which are blocked with a K_d of approximately 5 nM (3, 5, 10). Fig. 3A shows that SkM2 Na^+ channels expressed in oocytes are resistant to 100 nM TTX and are only partially blocked, in a reversible fashion, by 2 μM TTX. The blockade by TTX follows a single binding site isotherm with a K_d of 1.9 μM (Fig. 3B), as is expected for a TTX-insensitive Na^+ channel.

One of the hallmarks of TTX-insensitive Na^+ channels is that, unlike TTX-sensitive channels, the block by TTX exhibits use dependence (4). This use dependence is due to the fact that the rate of recovery from inactivation for TTX-blocked channels is much slower than for unblocked channels. Use dependence manifests itself as a dimunition in the size of the Na^+ current in response to a train of identical voltage steps, if the frequency of stimulation is high enough to allow the unblocked, but not the blocked, channels sufficient time to recover from inactivation during the interval between pulses in the train. Fig. 4 shows the relative amplitudes (i.e., normalized to that of the first pulse in the train) for currents elicited by a train of 10 voltage steps from −100 mV to −20 mV, in the absence and presence of 2 μM TTX, at two different stimulation rates. Na^+ currents in the absence of TTX are essentially constant for all 10 steps at both 0.5- and 2.5-Hz stimulation rates, as is the case for Na^+ currents in the presence of 2 μM TTX at 0.5-Hz stimulation. However, when the stimulation rate is increased to 2.5 Hz, the relative amplitude of the Na^+ current in the presence of 2 μM TTX decreases during the first five steps of the train, reaching a plateau level of half the initial amplitude. In both the absence and presence of TTX, the 2-sec interval between voltage steps at 0.5-Hz stimulation is long enough to allow complete recovery from inactivation. However, the 300-msec interval between the end of one pulse and the beginning of the next at 2.5 Hz is sufficient to allow complete recovery from inactivation in the absence of TTX but not in its presence.

To further determine the pharmacological profile of SkM2 Na^+ channels, we examined the effect of μ-CTX. This toxin blocks Na^+ channels in innervated skeletal muscle as well as μ1 Na^+ channels expressed in oocytes, with a K_d of 10–20 nM, but not Na^+ channels from brain (2, 10, 15). Gonoi *et al.* (16) have reported the existence of two pharmacologically distinct

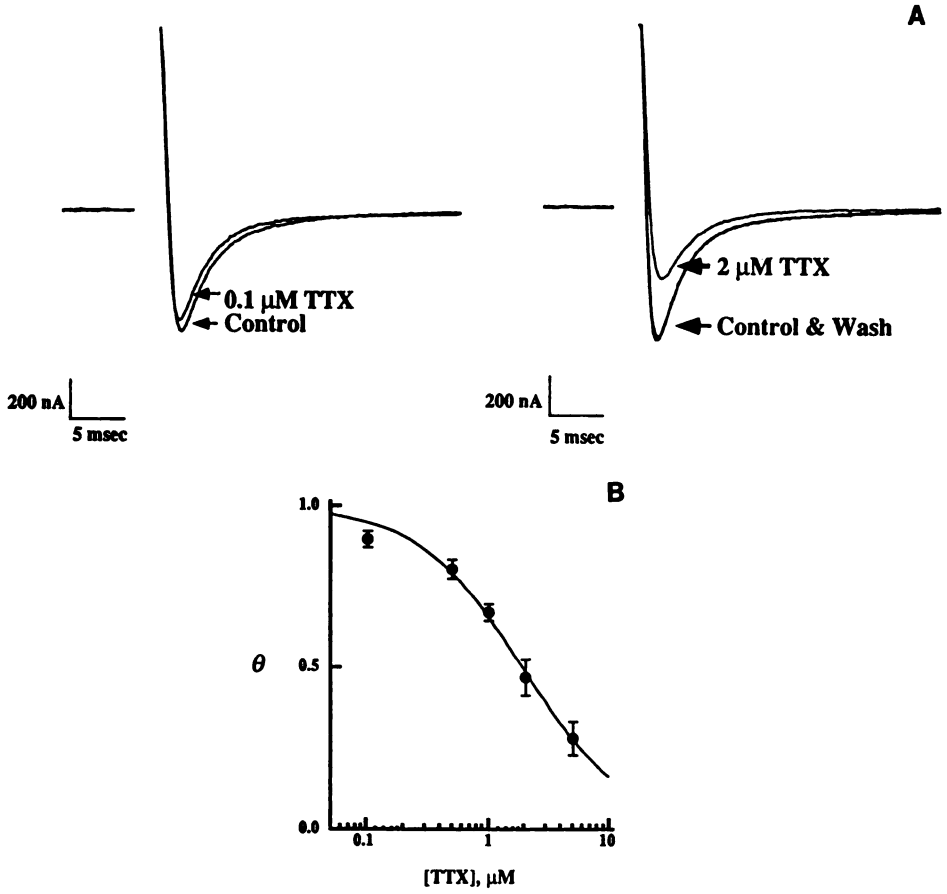

Fig. 3. A, Currents elicited by a 27-msec pulse from −100 mV to −20 mV, in the absence and presence of 100 nM TTX (*left*) and in the absence of, presence of, and 30 sec after washing out of 2 μM TTX (*right*), are shown. Note the full reversibility of the TTX block and the relative insensitivity to TTX. B, The concentration dependence of the TTX block is shown. The fractional current elicited at −20 mV remaining in the presence of TTX, θ, is plotted as a function of TTX concentration. Each application of TTX was bracketed by control current measurements, to assure full reversibility. Each *point* represents the mean ± standard error of determinations from three to five oocytes. The *solid curve* is the relation $\theta = 1/(1 + ([TTX]/K_d))$, with $K_d = 1.9$ μM.

classes of Na^+ channels in myoballs prepared from developing muscle cells, each accounting for approximately 50% of the current. One class is blocked by μ-CTX with a K_d of 20 nM and is TTX sensitive, whereas the other class is resistant to 2.5 μM μ-CTX and is TTX insensitive ($K_d = 1.3$ μM). Fig. 5 shows that 100 nM μ-CTX has little, if any, effect on SkM2 Na^+ channels expressed in oocytes. The insensitivity of SkM2 Na^+ channels to both TTX and μ-CTX provides strong evidence that these channels are, in fact, the "classical" TTX-insensitive Na^+ channel expressed in denervated and developing skeletal muscle, as well as in heart.

Discussion

This represents the first report of the expression of TTX-insensitive Na^+ channels in *Xenopus* oocytes. Methfessel *et al.* (17) were unable to detect TTX-insensitive Na^+ current in oocytes injected with poly(A)$^+$ RNA isolated from denervated skeletal muscle, which should have contained RNAs for both μ1 and SkM2 Na^+ channel subtypes (12). Sutton *et al.* (18) reported that Na^+ currents expressed after injection of heart RNA were TTX sensitive and suggested that *Xenopus* oocytes were incapable of carrying out a particular posttranslational modification required for acquisition of TTX insensitivity. Our data show that this is not the case. The size of the currents obtained by Sutton *et al.* (18) were extremely small and could have been due to contamination by RNA from nerves innervating the heart.

The data presented in Fig. 1 suggest that the failure of these groups to observe TTX-insensitive Na^+ currents may be due to the fact that the SkM2 channels translate inefficiently in

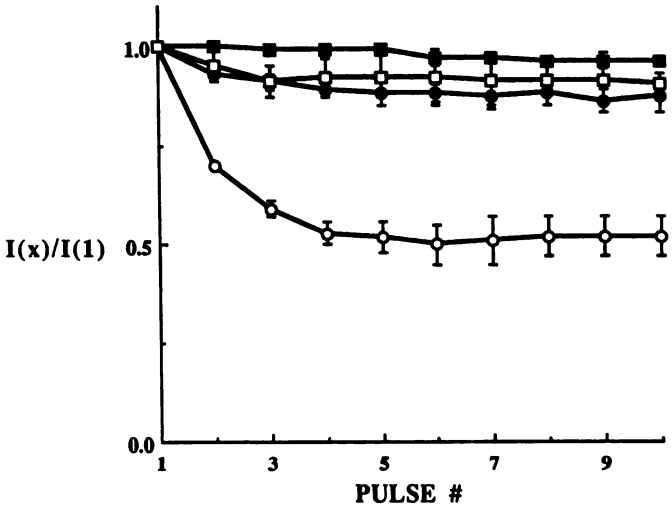

Fig. 4. TTX blockade shows use dependence. Na^+ currents were elicited by a train of 10 voltage steps from −100 mV to −20 mV, in the absence (●, ■) or presence (○, □) of 2 μM TTX, at a stimulus rate of either 0.5 (■, □) or 2.5 (●, ○) Hz. Data are presented as the current at a given pulse, $I(x)$, normalized to that in response to the first pulse, $I(1)$. Each *point* represents the average ± standard error for determinations on three different oocytes, and each oocyte was tested at both the 0.5- and 2.5-Hz stimulation rates. The fractional current remaining in the presence of TTX, θ, in response to the first pulse of the train for the data in this figure was 0.5 ± 0.04 (mean ± standard error).

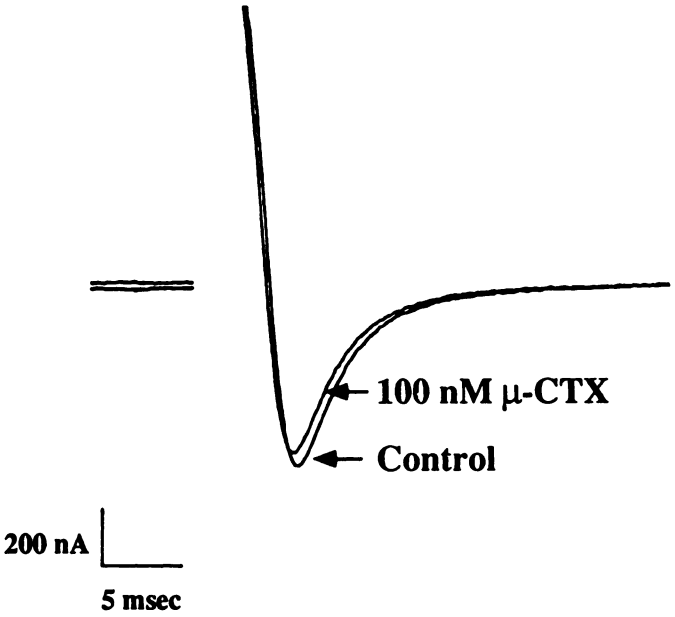

Fig. 5. TTX-insensitive Na^+ channels are also insensitive to μ-CTX. Currents elicited by a 27-msec pulse from −100 mV to −20 mV, in the absence and presence of 100 nM μ-CTX, are shown. At this concentration, the TTX-sensitive Na^+ current from muscle would be inhibited by greater than 80%.

oocytes unless almost all of the 5′UTR is removed. This dependence on the length of the 5′UTR has been seen for every transcription reaction that we have carried out. The small currents observed with the construct containing the full 5′UTR are not due to a difficulty with *in vitro* synthesis of full-length RNA, because the amount of full-length RNA produced from each construct was comparable. Rather, it appears that some sequence within the 5′UTR inhibits translation of the SkM2 message in the oocyte. This inhibitory effect may be related to the existence of the out-of-frame ATG located at position −8 or to the presence of a short open reading frame located from residues −175 to −146. In any case, one would expect that poly(A)$^+$ RNAs isolated from denervated skeletal muscle and heart would contain all of the features deleterious to *in ovo* translation that are found in the 5′UTR of the cDNA clone. It is not known whether these as yet uncharacterized inhibitory sequences affect translation in muscle and heart. Kobilka *et al.* (19) have reported a similar finding for the expression of the human β_2 adrenergic receptor in oocytes, where truncation of the 5′UTR from 210 to 40 nucleotides led to a 10-fold increase in receptor expression.

The structural basis of the TTX insensitivity is unclear. Noda *et al.* (20) have reported that a single point mutation (Glu-387 to Gln) in the rat brain type II Na^+ channel confers TTX insensitivity to the channels. These authors suggested that Glu-387 is the site of *O*-methylation of Na^+ channels by trimethyloxonium, which markedly reduces TTX sensitivity (21). Although this glutamate residue is conserved in all Na^+ channels cloned to date, including SkM2 and the cardiac Na^+ channel, the asparagine residue next to this glutamate (found in all other Na^+ channels) is replaced by arginine in SkM2 and the cardiac clones, which could also have the effect of neutralizing the negative charge of glutamate. However, the arginine to asparagine change may have a different effect on the local structure of the TTX binding site than does the glutamate to glutamine mutation, and this may explain why the mutant studied by Noda *et al.* (20) was unaffected by TTX concentrations as high as 10 μM, whereas the SkM2 channel would be 85% blocked at this concentration. If this is true, then replacement of this arginine residue in SkM2 with asparagine should increase the TTX sensitivity of the SkM2 channel. The availability of an expression system for the TTX-insensitive Na^+ channel will now allow this and other issues relating to the kinetic and pharmacological differences between muscle Na^+ channel subtypes to be resolved.

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