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# Tension generation and increase in voltage-activated $Na^+$ current by crotamine

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**Abstract**

We performed the present experiments to study the action of crotamine, a toxin isolated from the venom of the South American rattlesnake, *Crotalus durissus terrificus*, on macroscopic $Na^+$ currents in frog skeletal muscle by using the loose patch clamp technique. Crotamine at 50 $\mu$M increased the peak $Na^+$ current by 50% ($P < 0.05$). In addition, the voltage dependence of inactivation was shifted by +8 mV. Other parameters of $Na^+$ currents (reversal potential, voltage-dependence of activation and time courses of inactivation, of activation and of removal of inactivation) were not significantly affected. We suggest that crotamine inhibits the direct transition of channels from closed to inactivated states, thereby forcing their transition through the open states. 

*Keywords:* $Na^+$ channel; Crotamine; Myotoxin; *Crotalus durissus terrificus*; Skeletal muscle, frog; Patch clamp, loose; Myonecrosis

## 1. Introduction

$Na^+$ channels are the main targets for many toxins of widely diverse structure that exist in the venom of arthropods, coelenterates, microorganisms, fish, plants, but generally not in snake venoms (reviewed by Adams and Swanson, 1996). One reported exception for this rule is crotamine, which has been suggested to increase the $Na^+$ permeability of skeletal muscle membranes. Crotamine induces a depolarization that is $Na^+$-dependent and blocked by tetrodotoxin, and potentiates the depolarizing effects of veratrine, batrachotoxin and grayanotoxin I (Chang et al., 1983; Cheymol et al., 1964, 1971a,b; Hong and Chang, 1983, 1989; Vital Brazil and Fontana, 1993).

Crotamine, which is obtained from the venom of the South American rattlesnake (*Crotalus durissus terrificus*), is a single-chain polypeptide consisting of 42 amino-acid residues but lacks alanine, valine and threonine (Laure, 1975). It shares high primary sequence homology with other myonecrosis-causing toxins (Smith and Schmidt, 1990) such as peptide C (Maeda et al., 1978); myotoxin a (Fox et al., 1979) and myotoxins I and II (Bieber et al., 1987). Crotamine and the myotoxins have a well characterized myonecrotic effect (Ownby et al., 1988; Cameron and Tu, 1978). Crotamine induces contracture of mammalian skeletal muscle, both in situ and in vitro (Cheymol et al., 1971b; Chang and Tseng, 1978). Chang and Tseng (1978) and Vital Brazil and Fontana (1993), using rat neuromuscular preparations, have suggested that the membrane depolarization induced by crotamine is the cause of the contracture. However, the primary mechanism by which crotamine acts has not yet been fully clarified.

The purpose of the present study was to investigate further the effects of crotamine by directly assessing skeletal muscle $Na^+$ channel function under voltage-clamp conditions. An improved loose patch-clamp technique was used as a convenient method to study macroscopic $Na^+$ currents ($I_{Na}$) in intact muscle fibers.

Some of these data have been presented in abstract form (XIIth International Biophysical Congress, 1996).

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## 2. Materials and methods

### 2.1. Muscle preparation

The present study was carried out on semitendinous muscle of bullfrogs (*Rana catesbeiana*) of either sex weighing about 50–70 g. The animals were killed by destruction of the central nervous system. The muscle was dissected and pinned down through the tendons in a Petri dish covered with Sylgard and filled with Ringer solution. Connective tissue was removed from the muscle fiber as much as possible by careful manual dissection, without any enzymatic treatment. Thin bundles of muscle fibers were affixed slightly stretched in an experimental chamber at 10–12°C for electrophysiological measurements.

### 2.2. Isolated semitendinous preparation

The muscles were dissected as described above and fixed to a mount in a cuvette containing 2.2 ml normal Ringer solution kept at 25°C.

The contractions were induced by field electric stimulation (0.05 Hz trains of 100 Hz, 1.43 V/cm, 0.1 s duration), and tension development was recorded by using an isometric force transducer–chart recorder–oscilloscope set-up.

### 2.3. Loose patch voltage clamp

The method used here is similar to that described by Stühmer et al. (1983), with the modifications reported by Araújo et al. (1993) to eliminate 'rim currents'. Pipettes were drawn in two stages from soft glass (1.5 mm diameter) capillaries on a vertical puller and heat-polished with a homemade microforge under visual control. Tip diameters were measured with an inverted microscope and ranged between 21.2 and 33.2 $\mu$m. When filled with Ringer solution, the pipettes had resistances ($R_P$) ranging from 220 to 370 k$\Omega$, and the seal resistances ($R_S$) ranged between 0.52 and 1.8 M$\Omega$. Seal factors, $A = R_S/(R_S + R_P)$, were kept above 0.5. The loose patch amplifier (Dagan 8900 with a loose patch clamp probe model 8970 and a 10 M$\Omega$ feedback resistor) balances an impedance bridge, formed by $R_P$, $R_S$ and two resistances that are adjusted to balance out the voltage divider formed by $R_P$ and $R_S$ (for details, see Stühmer et al., 1983). This arrangement compensates for $R_P$ and $R_S$ and keeps the voltage at the pipette tip under control. To avoid 'rim currents', the fibers were depolarized with 27 mM $K^+$ (see composition of external solution), which depolarizes the fibers to $-43$ mV, thereby inactivating $Na^+$ channels (Araújo et al., 1993). By hyperpolarizing the patch of membrane under potential control with $-50$ mV for at least 10 min, the inactivation of the $Na^+$ channels at the tip of the pipette is selectively removed.

Ionic currents were recorded through a low-pass filter (cut-off frequency of 10 kHz) and sampled at 30 kHz with a 12-bit Analog-to-Digital converter (Engenharia Eletro-Eletrônica, São Paulo). Data acquisition and analysis were performed with an IBM compatible AT486 computer. A suite of programs, developed and written in Pascal language by one of us (Dr. P.S.L. Beirão), was used for data acquisition and analysis. The linear leakage and capacitative currents were subtracted by use of the P/4 protocol (Bezanilla and Armstrong, 1977).

### 2.4. Solutions

The Ringer solution had the following composition (in mM): 115 NaCl; 2.5 KCl; 1.8 $CaCl_2$ and 5.0 Hepes. The pipettes were filled with Ringer solution plus 10 mM 4-aminopyridine, to block $K^+$ channels (Hille, 1992). Except for control experiments, crotamine was added to the pipette solution, in most experiments, at a saturating concentration of 50 $\mu$M. The bathing solution contained (in mM): 88 sodium acetate; 16 potassium acetate; 11 KCl; 1.8 calcium propionate and 5.0 Hepes. The pH of all solutions was 7.2.

### 2.5. Chemicals

All chemicals and drugs used in this study were of the best quality available (ACS grade). Crotamine was kindly provided by Dr. Eduardo B. Oliveira from the Department of Immunology of the Faculdade de Medicina de Ribeirão Preto, who tested all batches of crotamine for the presence of alanine, valine and threonine as a criterion of purity, in addition to electrophoretic and chromatographic profiles.

## 3. Results

### 3.1. Effect on resting tension

When crotamine (10 $\mu$M) was added to the frog isolated semitendinous muscle preparation, the resting tension increased considerably (Fig. 1). The resting tension ranged from 0.10 to 0.22 g without crotamine ($n = 4$) and from 0.67 to 0.91 g with crotamine ($n = 2$). This effect was not reversed even after 40 min of repetitive washing of the muscle bundle, indicating that the observed effect is practically irreversible, as has been described by others using different preparations (Chang and Tseng, 1978).

### 3.2. Action of crotamine on the $Na^+$ current

As previously reported (Chang et al., 1983), crotamine induces a large depolarization in a dose-dependent manner, with $EC_{50}$ of 0.15 $\mu$g/ml for rat diaphragm preparation. Under our experimental conditions, only a small (353 to 855 $\mu m^2$) patch of the sarcolemmal membrane is exposed to crotamine, and the membrane potential of this patch is fully controlled, thereby maintaining its 'resting' potential

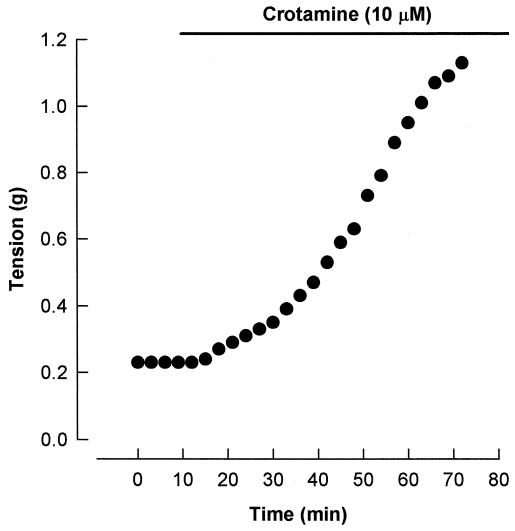


Fig. 1. Typical changes induced by crotamine on muscle resting tension. The toxin was applied directly to the bath to achieve a final concentration of 10 $\mu$M. After 10 min there was a monotonic increase in tension. Each point represents the resting tension recorded every 3 min.

near $-93$ mV (Araújo et al., 1993) and preventing the depolarization produced by crotamine. Our main question was to verify whether the $Na^+$ currents ($I_{Na}$) generated by $Na^+$ channels exposed to saturating concentrations of crotamine have an altered behavior that could account for the tension generation. Fig. 2A shows superimposed current traces obtained with 10 ms step depolarizations from a holding potential of $-93$ mV to a test potential of $-20$ mV. The amplitude of $I_{Na}$ was significantly affected by crotamine (50 $\mu$M). As shown in Fig. 2A, the maximal peak current increased by 50% after exposure to the toxin ($n = 9$; $P < 0.05$). From the plot of the mean Peak $Na^+$ current $\times$ voltage ($I$–$V$) relationship (Fig. 2B), it was found that after exposure to crotamine: (1) the maximal mean peak $Na^+$ current was increased; (2) the toxin-induced increase in peak current amplitude was much larger at small depolarizations compared to the increase in current at voltages near the level for current reversal; (3) there was no apparent change in the activation voltage, voltage of peak current, and reversal potential; (4) the maximum $Na^+$ conductance, as calculated by the positive limb of the $I$–$V$ curve, increased by 58%. In Fig. 2B peak $Na^+$ currents are plotted as a function of clamp potential for twelve patches (control) and for nine patches (experimental). All curves were fitted to the experimental points by using the following equation:

$$I_{Na} = g_{Na(max)} \times (V_m - V_r) / \left(1 + \exp\left((V_{1/2} - V_m)/\kappa\right)\right)$$

where $g_{Na(max)}$ is the maximum conductance for $Na^+$; $V_m$ is the membrane potential; $V_r$ is the reversal potential; $V_{1/2}$ is the potential at which half of the channels are activated, and $\kappa$ is the Boltzmann constant. The mean peak current density was $-2.84$ for control and $-5.85$ mA/cm$^2$ after 50 $\mu$M crotamine. The current had a threshold at about $-60$ mV, reached a maximum at about $-20$ mV, and declined towards a reversal potential ($\sim +60$ mV) under both conditions.

### 3.3. Voltage-dependence of steady-state inactivation

To test the possible effect of crotamine on the steady-state inactivation of the $Na^+$ channels, we applied a standard $h_\infty$ - pulse protocol ($h_\infty$ is the steady-state inacti-

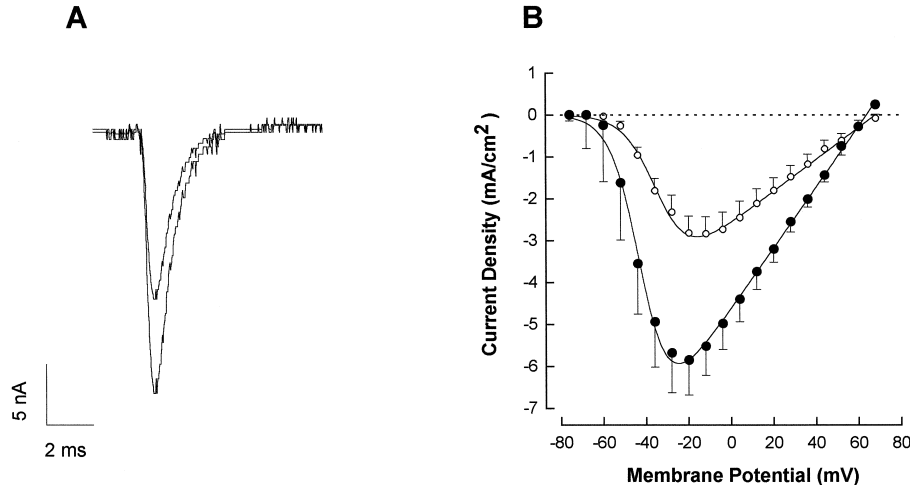


Fig. 2. Effect of crotamine on macroscopic $Na^+$ currents and on the current–voltage ($I$–$V$) relationship. (A) Representative $Na^+$ current traces recorded with 10-ms voltage steps to $-20$ mV from a steady-state holding potential of $-93$ mV, using the loose patch-clamp technique. The control $Na^+$ current record is superimposed on the current record in the presence of 50 $\mu$M crotamine. The peak $Na^+$ current increased by 50%. (B) $I$–$V$ of $Na^+$ current during 10-ms voltage steps from a steady holding potential of $-93$ mV to various test potentials. Open circles, control ($n = 12$). Filled circles, in the presence of 50 $\mu$M crotamine ($n = 9$). Crotamine increased the $Na^+$ current at almost all potentials measured ($P < 0.05$, $t$-test) without causing any significant changes in the reversal potential ($P > 0.05$, t-test). However, the maximum $Na^+$ conductance, as calculated by the positive limb of the $I$–$V$ curve, increased by 58% ($P < 0.05$, $n = 9$).

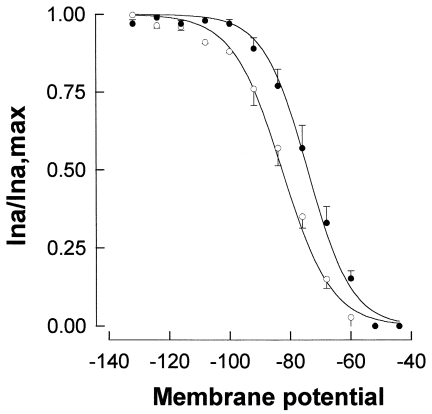


Fig. 3. The voltage-dependence of steady-state inactivation was not altered by crotamine. Open circles show the control values ($n = 9$) and filled circles show the values measured in the presence of crotamine (50 $\mu$M, $n = 6$). The steady-state of inactivation ($I_{Na}/I_{Na(max)}$) was measured by using a well-known procedure: A 100-ms pre-pulse to potentials in the range $-132$ to $-44$ mV was immediately followed by a 10-ms test pulse to the potential which evoked the maximum current. Steady-state inactivation was calculated by dividing the current achieved following a given pre-pulse by the maximum test pulse current achieved. The pre-pulse potential at which inactivation was 0.5 is termed $V_h$, and the slope factor of the inactivation curve is $\kappa_h$. The smooth curves represent fits to the Boltzmann distribution with $V_h = -82.1$ and $\kappa_h = 8.6$ mV for control and $V_h = -74.1$ and $\kappa_h = 7.8$ mV in the presence of 50 $\mu$M crotamine. Crotamine at 50 $\mu$M changed the membrane potential at which 50% of the $Na^+$ channels were inactivated ($V_h$) by $+8$ mV ($P < 0.05$, t-test). All parameters are means.

vation parameter, as defined by Hodgkin and Huxley (1952)). A-100 ms duration conditioning pulse to $-132$ mV was first applied to render all channels available, by removing fast inactivation. This was followed by a 100-ms pulse to variable pre-potentials before the application of a test pulse to $-12$ mV, to investigate the remaining $Na^+$ channels that were not inactivated by the pre-potential. The resulting peak currents were normalized in relation to non-inactivated currents (pre-potential of 0 mV) and plotted against the pre-potential amplitude. A Boltzmann distribution was used to determined the membrane potential at which inactivation was 0.5 ($V_h$) and the slope factor of the inactivation curve ($\kappa_h$) from a nonlinear least squares fit of the data obtained by using the equation below:

$$I_{Na}/I_{Na(max)} = 1/\left(1 + \exp\left((V_m - V_h)/\kappa_h\right)\right);$$

where $V_m$ is the membrane potential.

The Boltzmann fit to the experimental data in Fig. 3 yielded $V_h$ at $-82.1$ mV (before crotamine, open circles, $n = 9$) and $-74.1$ mV (after crotamine 50 $\mu$M, closed circles, $n = 6$). The corresponding slope factors were 8.6 and 7.8 mV. As shown in Fig. 3, the voltage dependence of the steady-state inactivation in the presence of crotamine (closed circles) was about 8 mV more positive than that of control (open circles), which was significant ($P < 0.05$, *t*-test).

### 3.4. Voltage dependence of $Na^+$ conductance

The voltage dependence of the normalized $Na^+$ conductance of the control preparation and in the presence of crotamine (50 $\mu$M) is illustrated in Fig. 4. The $Na^+$ conductance ($g_{Na}$) was calculated according to the Hodgkin–Huxley formalism (1952) by means of the equation:

$$g_{Na} = I_{Na}/(V_m - V_{Na})$$

where $I_{Na}$ is the current amplitude, $V_m$ the patch potential and $V_{Na}$ the reversal potential taken from the $I$–$V$ relationships. The $g_{Na}$ values were normalized as a ratio to their maximum for each patch and then averaged. The smooth curves in Fig. 4 are the fits of the mean data points to a Boltzmann distribution of the form:

$$g_{Na}/g_{Na(max)} = \left\{1 + \exp\left[(V_g - V_m)/\kappa_g\right]\right\}^{-1}$$

where $V_g$ is the potential at which 50% of the maximal conductance is achieved and $\kappa_g$ is the slope factor. For direct comparison, the $g_{Na}/g_{Na(max)}$ curve of the control (open circles, $n = 8$) and in the presence of 50 $\mu$M crotamine (closed circles, $n = 11$) is depicted. As seen in Fig. 4, the voltage-dependence of the control $Na^+$ conductance was identical to that in the presence of crotamine ($P > 0.05$, *t*-test).

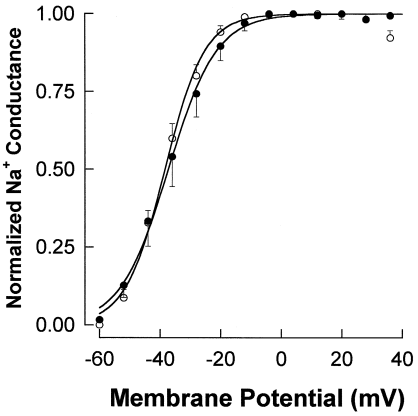


Fig. 4. The voltage dependence of $Na^+$ condutance was not changed by crotamine. Open circles represent the control ($n = 8$) and the filled circles values measured in the presence of 50 $\mu$M crotamine ($n = 11$). The peak $Na^+$ conductance ($g_{Na}$) at each potential was calculated, as a chord conductance, from the corresponding peak current (for details see Section 3). The test potential at which $g_{Na}$ was half of its maximum value ($g_{Na(max)}$) is termed $V_g$, and the slope factor of the normalized conductance–voltage relationship is termed $\kappa_g$. $V_g$ and $\kappa_g$ were determined from a nonlinear least-squares fit to the experimental data of a rising sigmoidal relationship; their mean values were $-38.4$ mV and 6.6 mV for the control and $-37.3$ mV and 7.9 mV for crotamine (50 $\mu$M), respectively. The holding potential was $-93$ mV.

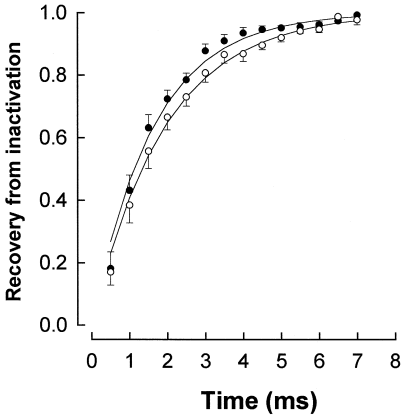


Fig. 5. Recovery from inactivation. The kinetics of recovery from inactivation over a period of 7.0 ms were determined. A 100-ms pre-pulse to −44 mV that recruited $Na^+$ channels to the inactivated state was followed by a hyperpolarized conditioning voltage step to −124 mV which varied in duration between 0.5 ms and 7.0 ms. Since $V_m = -44$ mV, at the very end of pre-pulse all of the channels will be in the inactivated state. The fraction of channels that have reprimed during time $t$ at −124 mV, therefore, is calculated by dividing the peak current achieved during voltage step to −12 mV. Open circles, control ($n = 7$) and filled circles, crotamine (50 μM, $n = 8$). There was no major modification in the repriming kinetics of $Na^+$ channels ($P > 0.05$, $t$-test).

### 3.5. Time constant for inactivation was not changed by crotamine

The time constant for $Na^+$ channel inactivation was measured directly from the decay phase of $I_{Na}$ at potentials more positive than the critical activation level for $Na^+$ channels. As shown in Fig. 5, the time constants for inactivation were not significantly ($P > 0.05$; $t$-test) changed by crotamine (closed circles, $n = 8$) when compared to those of controls (open circles, $n = 6$).

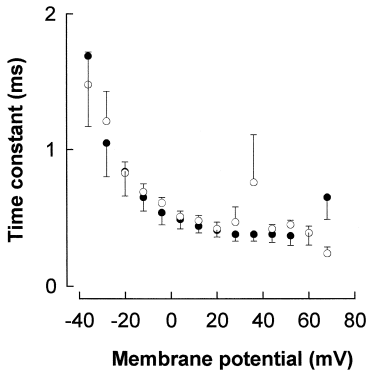


Fig. 6. Voltage-dependence of the time constant for inactivation. The voltage-dependence of the kinetics of $Na^+$ current inactivation in the range −40 to +64 mV was evaluated. The results represent the mean + S.E.M. for control (open circles, $n = 6$) and the mean − S.E.M for 50 μM crotamine (filled circles, $n = 8$). There was no significant change in the voltage-dependence of the time constant for inactivation of $Na^+$ channels ($P > 0.05$, $t$-test).

### 3.6. Recovery from inactivation

The effects of crotamine on the recovery rate from inactivation are shown in Fig. 6. The recovery rates under control conditions (open circles, $n = 7$) and during exposure to crotamine (closed circles, $n = 8$) were identical ($P > 0.05$; $t$-test). The smooth curves in Fig. 6 are the best fits to the mean data points obtained by using the equation:

$$f = [1 - \exp(-t/\tau)]$$

where $\tau$ is the time constant of recovery.

## 4. Discussion

The present study demonstrates that crotamine significantly augmented the voltage-activated $Na^+$ current and shifted the voltage dependence of $Na^+$ current inactivation by +8 mV without causing other apparent changes in the kinetic parameters of frog skeletal muscle.

Modulation of the $Na^+$ current has been shown in several preparations, and usually involves a decrease in the current. Recently Wieland et al. (1996) have shown that intracellular injection of arachidonic acid markedly increases the $Na^+$ current of cultured skeletal muscle cells, without causing any alteration of the $Na^+$ current kinetics and voltage dependence. These authors have extended their observations to cloned $Na^+$ channels expressed in the human kidney epithelium cell line (HEK293t). They were able to measure a rapid augmentation of $Na^+$ currents (average of 190% increase) upon addition of arachidonic acid to the interior of cells expressing tetrodotoxin-sensitive human skeletal muscle (hSkM1) $Na^+$ channels, but not in cells expressing human heart (hH1) $Na^+$ channels, which are likely to be identical to the tetrodotoxin-insensitive human skeletal muscle isoform (hSkM2). This different effect points to a mechanism that affects the two clones differently, and can be reproduced in heterologous cells. These results show that the mechanism for augmentation is not restricted to the cellular environment of differentiated skeletal muscle fibers and must at some point distinguish between the two channel isoforms. They suggested that arachidonic acid shifts channels from a voltage-insensitive to a voltage-sensitive pool. The presence of this voltage-insensitive pool, which may comprise latent channels or channels not inserted into the cell membrane, has not been yet demonstrated (Wieland et al., 1996).

We considered two possible explanations for the increase in $Na^+$ currents induced by crotamine: an increase in the number of functioning $Na^+$ channels and an alteration of the $Na^+$ channel kinetics.

Not all $Na^+$ channels in the membrane can be recruited by depolarization because of the phenomena of slow and fast inactivations. Under our conditions, the hyperpolarizing pre-pulse of −132 mV and 100-ms duration that always preceded the test pulse is more than enough to

remove the fast inactivation of all channels. Slow inactivation would require much longer pre-pulses (Almers et al., 1983), but at the holding potential that was used throughout this work ($\sim -93$ mV), the percentage of inactivated $Na^+$ channels is estimated to be less than 5% and therefore cannot account for the much larger effect of crotamine. There have been reports that new $Na^+$ channels can be inserted in the plasma membrane under certain circumstances (Wieland et al., 1996). We cannot rule out this possibility, although the fact that our experiments were carried out at low temperatures ($\sim 12°C$) renders this possibility quite unlikely.

Alterations of $Na^+$ channel kinetics can generate increased peak currents. The best known way to achieve this is to increase the time the channel spends in the open state (by slowing down the rate of inactivation), thereby increasing the number of channels that will be open simultaneously (Patlak, 1991). We can exclude this possibility, because there was no alteration of the time constant for inactivation or its voltage dependence by crotamine (Fig. 6). Another possibility is that crotamine increased the number of channels that pass through the open state upon membrane depolarization. There is a consensus in the literature that a significant fraction of $Na^+$ channels goes directly into an inactivated state, without passing through the open state. Aldrich et al. (1983) estimated that 53% of the $Na^+$ channels in N1E115 neuroblastoma cells move between resting and inactivated states upon depolarization. In GH3 cells the estimated proportion was 35% for pulses to $-40$ mV (Vandenberg and Horn, 1984). The definitive proof for this hypothesis requires single-channel recording, and is currently being investigated in our laboratory.

Is it possible to explain the toxic effects of crotamine in vivo and in vitro as a consequence of the alteration of $Na^+$ channel kinetics such that transition through the open state is favored? We believe that the answer is yes. Membrane depolarization is attributed to the opening of $Na^+$ channels (Hodgkin and Huxley, 1952). Even small increases in $Na^+$ conductance lead to a significant depolarization, because of positive feedback so that depolarization activates more $Na^+$ channels. The depolarization is limited, however, by inactivation of the channels, which is also caused by depolarization. The typical contractures that are caused by crotamine can also be accounted for by $Na^+$ channel modification. It has been shown recently (for review see Barchi, 1995) that paramyotonia congenita, an inherited muscle disease that causes similar contractures and difficulties to relax skeletal muscle, is caused by a genetic alteration of muscle $Na^+$ channels so that they remain in the open state longer. Cannon et al. (1993) have put forward the concept of dynamic availability of $Na^+$ channels to explain the delicate balance of $Na^+$ channel kinetics that allows muscles to repolarize after the activation of an action potential without repetitive firing or prolonged inactivation. These authors proposed a computational model that could reproduce the effects of myotonia and paralysis caused by defective $Na^+$ channels. One of the most prominent immediate effects of crotamine poisoning is hypertonia of muscles that oppose gravity, which can easily be explained by an increased dynamic availability of $Na^+$ channels according to the model of Cannon et al. (1993). Extensor muscles are more prone to these effects because they are stimulated more.

The other prominent effect of crotamine, i.e., myonecrosis, has been attributed to increased membrane muscle permeability to $Na^+$, thereby causing an osmotic imbalance that can progressively lead to myonecrosis (Ownby et al., 1988). In fact, the ultrastructural changes of muscle fibers chronically exposed to crotamine bear a striking resemblance to those of fibers under osmotic shock. Other authors have suggested that crotamine may act by altering the function of the sarcoplasmic reticulum (Utaisincharoen et al., 1991 and Ohkura et al., 1995). Our data cannot confirm or exclude this possibility.

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