

## Use of selective toxins to separate surface and tubular sodium currents in frog skeletal muscle fibers

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**Abstract.** The interaction between toxin  $\gamma$  from the venom of the scorpion *Tityus serrulatus* and sodium channels in skeletal muscle membranes from the frog *Caudiverbera caudiverbera* was studied. Sodium current from cut sartorius muscle fibers is a complex signal in which early and late components are difficult to separate. External application of Tityus  $\gamma$  toxin initially blocked the early component in a voltage-dependent manner. Longer exposure to the toxin induced a complete blockade of the two components of the inward current. Application of tetrodotoxin to fibers pretreated with Tityus toxin at submaximal concentrations allowed the observation of the two distinct components of the inward current. Binding of <sup>125</sup>I-labelled toxin to highly purified membrane fractions from the same muscle was used to establish the presence of high affinity receptors both in the transverse-tubular and in the surface membrane.

**Key words:** Tityus toxin, Na<sup>+</sup>-channels — Tubular Na<sup>+</sup>-current — Skeletal muscle

### Introduction

Voltage-clamp measurements of membrane currents in skeletal muscle fibers using either double sucrose gaps (Caille et al. 1978) or triple vaseline gaps (Hille and Campbell 1976) have demonstrated that the inward current has distinct early and late components. The late component of the sodium current has been suggested to represent tubular membrane current (Adrian and Peachey 1973; Caille et al. 1978; Vergara et al. 1978; Heiny and Vergara 1982).

Recent biochemical and electrophysiological studies using neurotoxins, which specifically interact with voltage-sensitive Na<sup>+</sup>-channels, have demonstrated differential effects of these toxins on surface and tubular currents. For example, the derivative I of en-tetrodotoxin (en-TTX), at low concentrations, blocks the surface sodium current with no change in the late sodium current (Jaimovich et al. 1983). On the other hand, derivative II of en-TTX blocks tubular currents with no effects on the early surface current. Toxin II (Css II) from scorpion *Centruroides suffusus* selectively acts on surface Na<sup>+</sup>-channels and has no effect on either tubular current or muscle contraction in fibers from *Rana esculenta* (Jaimovich et al. 1982). Toxin  $\gamma$ , a component of the venom from the scorpion *Tityus serrulatus*, was reported to preferentially bind to receptors on the surface membrane

of skeletal muscles from rabbit or frog. Likewise, this toxin reduces the amplitude of the early inward current without affecting the late sodium current in single twitch fibers from *Rana esculenta* (Barhanin et al. 1984).

In this work we have studied the differential effects of TTX and Tityus  $\gamma$  toxin on the surface and tubular components of the sodium currents in muscle fibers from the frog, *Caudiverbera caudiverbera*. Two distinct inward current components are present in the current records from these fibers. The effects of the toxin on the currents together with the binding of Tityus  $\gamma$  toxin to a highly purified transverse tubular membrane preparation were examined.

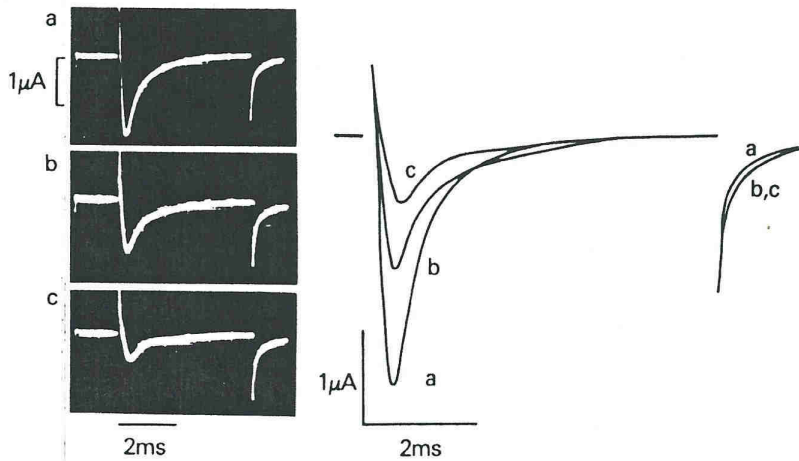
### Methods

Voltage-clamp experiments were carried out on single fibers from the sartorius muscle of the Chilean frog *Caudiverbera caudiverbera*. The dissection of single fibers, experimental chamber, voltage-clamp amplifier were as described previously (Arispe et al. 1984). The solution applied to the external surface of the segment of the fiber in pool A had the following composition (in mM): 115 NaCl, 2.5 KCl, 1.8 CaCl<sub>2</sub>, 15 TrisCl at pH 7.2.

The solution used in pools C and E to block K<sup>+</sup>-channels by diffusion of ions from the cut ends had the following composition (in mM): 120 tetraethylammonium aspartate, 2 MgSO<sub>4</sub>, 2 ATP Na<sub>2</sub>, 10 monosodium glutamate, 5 MOPS, 1 EGTA at pH 6.8. Neurotoxins were added directly to the external solution (pool A). TTX was obtained from Sigma Chemical and Tityus  $\gamma$  toxin was kindly provided by Dr. M. Lazdunski.

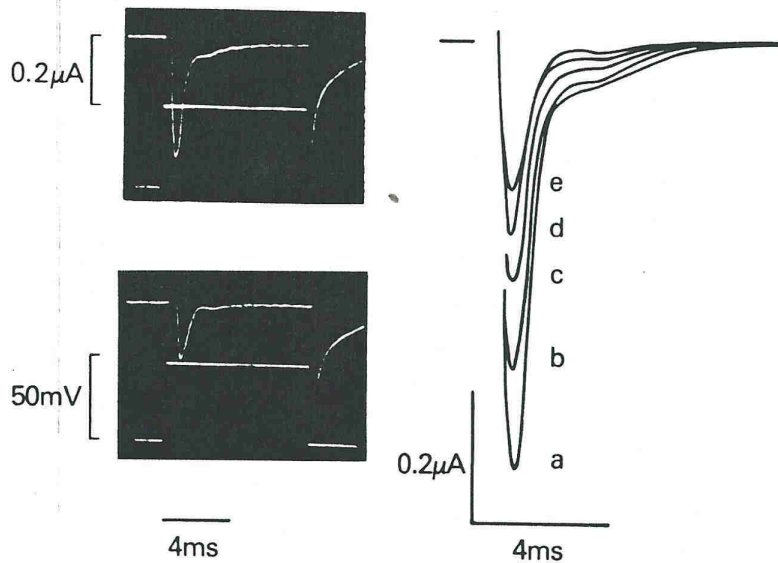
**Iodination of Tityus toxin.** The procedure used to iodinate Tityus  $\gamma$  toxin has been described elsewhere (Barhanin et al. 1982). Briefly, 25  $\mu$ l of 170  $\mu$ M solution of Tityus toxin were incubated in a buffer solution containing 200 mM NaH<sub>2</sub>PO<sub>4</sub> buffer at pH 7.2 in the presence of Na<sup>125</sup>I (100  $\mu$ Ci at a specific activity about 17 C/mg NaI) and lactoperoxidase beads plus 1% D-glucose. After 15–30 min incubation at room temperature, the mixture was centrifuged at 1,500  $\times$  g for 10 min. The supernatant was applied to a sephadex column (SP-C25) pre-equilibrated with buffer solution containing (mM): 150 NaCl, 3 CaCl<sub>2</sub>, 20 TrisCl (pH = 8.7) and 0.1% bovine serum albumin. After three washes of the column with the buffer, the NaCl concentration was increased to 350 mM. The supernatant was added and the toxin was collected in the eluent flowing at a rate of 0.3–0.4 ml/min.

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**Fig. 1**

Blockade of the inward currents induced by a high dose (10 nM) of Tityus toxin. Traces on the right side were made from records taken at (a) 0, (b) 1.5 and (c) 3 min in the presence of the toxin. Fiber diameter = 80  $\mu\text{m}$ ; Gap width in pool A = 100  $\mu\text{m}$



**Fig. 2**

Blockade of inward currents induced by a low dose (1 nM) of Tityus toxin. Membrane currents in response to voltage clamp pulses taking the membrane potential  $-40$  mV. Traces on the right side were made from records taken at (a) 0, (b) 1.5, (c) 12, (d) 18 and (e) 24 min in the presence of the toxin. Diameter = 80  $\mu\text{m}$ ; Gap width in pool A = 100  $\mu\text{m}$

**Membrane preparation.** Transverse tubular membrane were prepared and characterized as described elsewhere (Hidalgo et al. 1986). This preparation generates inside-out sealed vesicles. To expose the binding sites present on the external side of the tubular membrane it was necessary to treat the membrane fraction with saponin (0.2 mg/ml). The binding assay was carried out in a solution of the following composition (mM): 120 cholineCl, 2.5 KCl, 1.8  $\text{CaCl}_2$ , 10 TrisCl at pH 7.4 and 0.1% bovine serum albumin (BSA). Membrane bound toxin was separated by fast filtration under reduced pressure. The filters used (Millipore HA 0.45  $\mu\text{m}$ ) were rinsed twice with a  $\text{Ca}^{2+}$ -free solution similar to the solution used in the binding assay. Radioactivity in the filters was measured in a liquid scintillation counter.

## Results

### *Effect of Tityus $\gamma$ toxin on inward currents*

Application of depolarizing voltage clamp pulses to a segment of a muscle fiber only occasionally revealed the presence of distinct early and late components of the inward currents. However, the inactivation of the sodium current

showed two time constants which is consistent with the presence of two components of the inward current. The late component of the sodium current will not be apparent if masked by a much larger early component. Shown in Fig. 1 are three records of the inward currents in response to depolarizing pulses to  $-48$  mV before (a), 1.5 (b) and 3 min (c) after application of Tityus  $\gamma$  toxin to the segment of the fiber in pool A. It may be seen that the early component of the inward current is clearly reduced while the late component appears enhanced (record b).

Maintained exposure to the toxin even at low concentration (1 nM) gave rise to a gradual decrease of the early component. Initially the late component was unmasked and, with time in the presence of the toxin, a decrease in both early and late components was apparent (Fig. 2). This blockade of the  $\text{Na}^+$ -currents by Tityus  $\gamma$  toxin was almost irreversible. However, removal of the toxin prevented any further progress of the blockade. It was found that brief exposures of the muscle to higher concentrations of Tityus toxin was sufficient to clearly show the presence of the two components of the inward current.

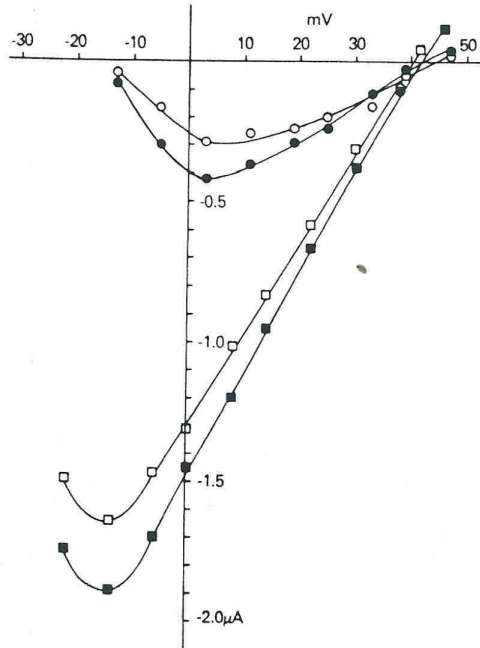
It has been reported that the extent of the blockade of the inward  $\text{Na}^+$  currents by Tityus  $\gamma$  toxin depends on



membrane potential (Barhanin et al. 1984). The effects of membrane potential on the blockade induced by Tityus  $\gamma$  toxin (5 nM) are illustrated in Fig. 3. The current-voltage relationships were measured at two holding potentials.

At a holding potential of  $-76$  mV, the control current for the early component (filled squares) was 13% larger than after exposure to the drug (empty squares). At  $-51$  mV the blocking effect of Tityus  $\gamma$  toxin was more pronounced. The peak currents of the early component obtained with the drug (empty circles) were 31% smaller than the control records (filled circles).

It should be noticed that in the case of the current-voltage curve obtained at a holding potential of  $-76$  mV the



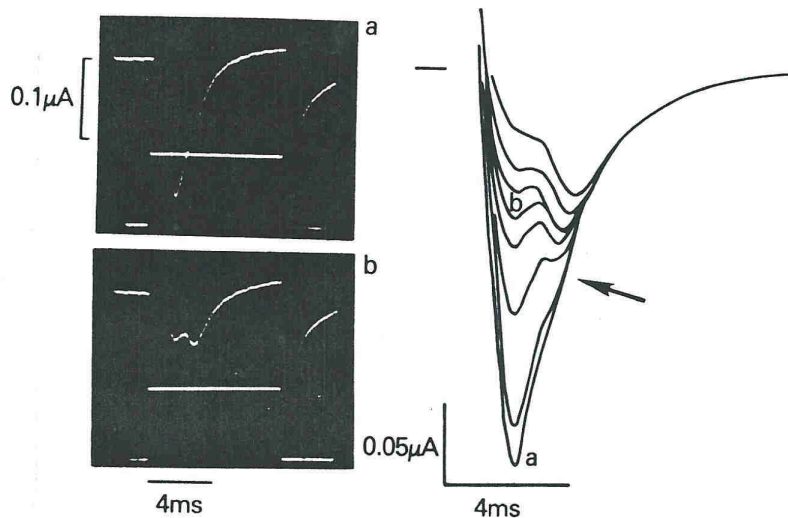
**Fig. 3.** Current-voltage relationships at different holding potentials in presence of (1 nM) Tityus toxin. Holding potential  $-50$  mV: (○) with and (●) without toxin. Holding potential  $-76$  mV: (□) with and (■) without toxin. Diameter =  $80 \mu\text{m}$ ; Gap width in pool A =  $100 \mu\text{m}$ .

maximum peak inward current was shifted towards positive potentials with respect to the curve obtained at  $-51$  mV. This apparent shift of the I-V curve is presumably due to the potential difference between the solution in pool A and the potential on the external side of the sarcolemma caused by the resistance in series. Using a value of  $3 \text{ cm}^2$  for the series resistance (Collins et al. 1982) the curves at  $-76$  mV can be displaced  $25$  mV in the depolarizing direction. This displacement of the points along the potential axis brings both maxima to nearly the same absolute membrane potential.

#### *Effect of TTX on fibers pretreated with Tityus toxin*

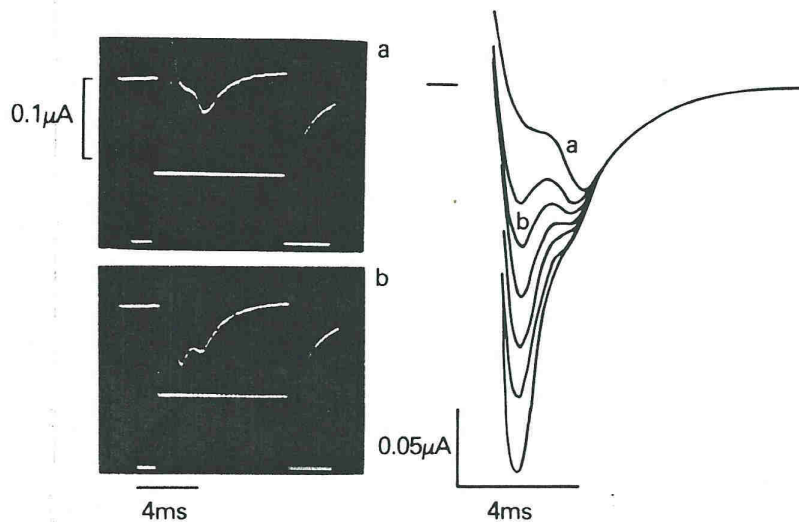
The addition of tetrodotoxin (5 nM) to fibers in which the fast inward current was partially blocked by Tityus  $\gamma$  toxin, showed a clear separation of the two components of the inward current. Figure 4 illustrates the onset of the blockade by TTX in one of these fibers. The depolarizing pulse to  $-40$  mV produced inward currents in which the early and late component of the  $\text{Na}^+$ -current were mixed and difficult to separate from each other (upper record on the left side of Fig. 4). The time course of the blockade by TTX revealed that the early component was reduced first and this made the second component more evident. Later, the blockade was similar for the two components. However, since the time to peak for each component was different in this experiment, the two inward currents are clearly separated (bottom record of Fig. 4). After 78 s in TTX, the early current had almost completely disappeared from the record leaving a fraction of late current. After 78 s in the presence of TTX the early current had almost completely disappeared from the record leaving a fraction of late current. The superimposed traces on the right side of Fig. 4 show more records taken at various intervals between 30 and 78 s after the addition of TTX. TTX blocked first the early component of the inward currents and, during the blockade, the shape of the currents remained almost unchanged.

The blocking action of TTX eventually led to almost complete abolition of what was left of the early component after exposure to Tityus  $\gamma$  toxin. TTX was then removed and records made during the 15 min of the recovery of the currents (Fig. 5).



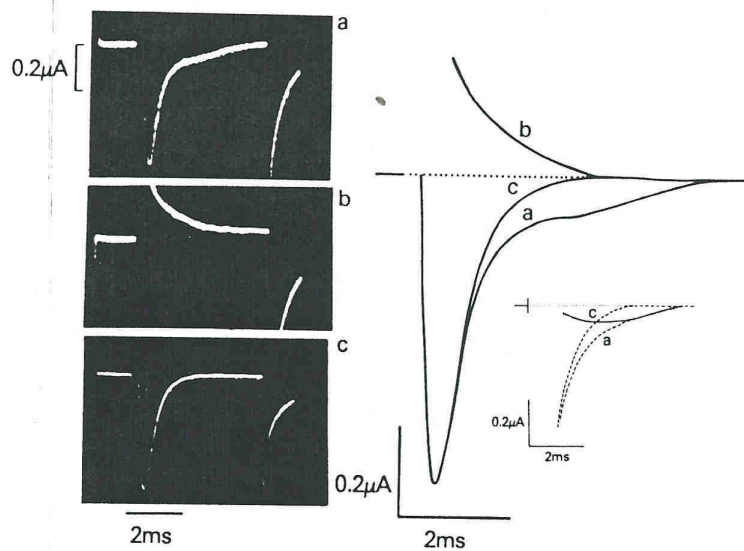
**Fig. 4**

Time course of the onset of the TTX-induced blockade of the early and late inward currents. *Left side:* (a) control; (b) record taken 57 s after the application of TTX in a fiber pretreated with Tityus toxin (10 nM). *Right side:* superimposed traces of records taken at: 30, 50, 57, 63, 68, 72 and 78 s in the presence of TTX. Diameter =  $100 \mu\text{m}$ ; Gap width in pool A =  $100 \mu\text{m}$ .



**Fig. 5**

Recovery from the TTX-induced blockade of the two components of the inward currents. Same fiber as in Fig. 4. *Left side:* (a) control record taken immediately after the removal of the TTX. *Right side:* Traces from records made (a) 3, 4, (b) 4.5, 5, 6 and 10 min after the removal of the toxin



**Fig. 6**

Separation of the early and late component of the inward currents. *Left side:* (a) record made immediately before the application of TTX; (b) after 6 min in TTX; (c) 10 min after the removal of the toxin. *Right side:* traces of the records shown on the left. *Inset:* continuous trace represents the difference of trace (a) minus trace (b). Diameter = 80  $\mu\text{m}$ . Gap width in pool A = 100  $\mu\text{m}$

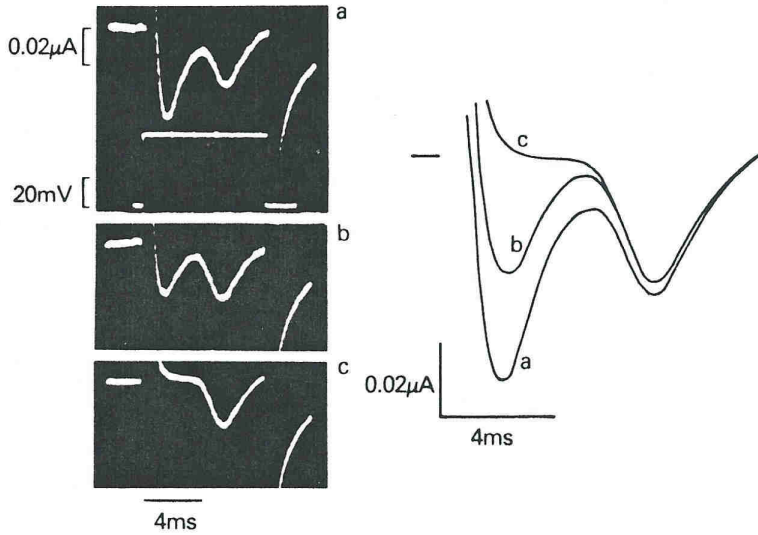
The upper record on the left of Fig. 5 was made after 1.3 min of exposure to TTX. Bottom record of the figure shows an intermediate stage of the recovery phase taken 4.5 min after the removal of the toxin. The superimposed traces on the right represent records made 3 (upper trace from record a) 4, 4.5, 5 (from record b), 6, 10 and 15 min (bottom trace) after the removal of the toxin. In all experiments recovery from the TTX blockade was slower than the onset. After the removal of the drug, the early component reappeared within 0.5 min, and the recovery was complete 15 min after the wash out of the toxin.

To separate the main components of the inward current, blockade was allowed to proceed to completion. Then, after removal of the toxin, the recovery of the currents was followed until the early component reached its control size. Subtracting from the control record with both components present, the record with the second component blocked, we obtained the time course of the second component (Fig. 6). Records on the left side of Fig. 6 were made before the

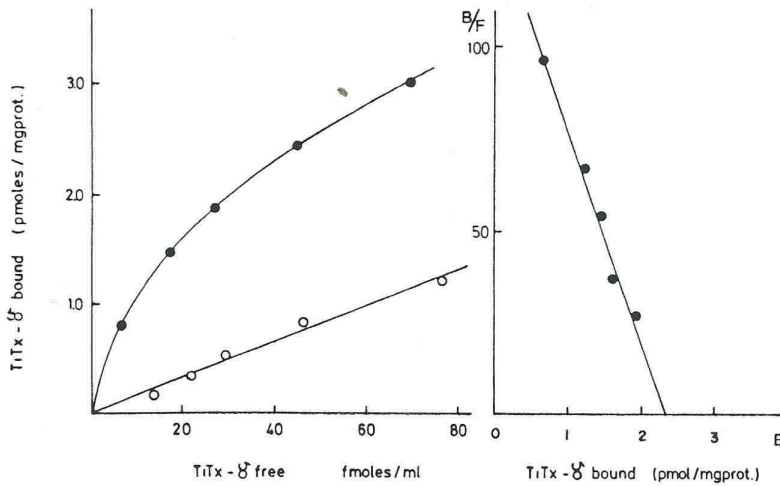
application of TTX (record a), in the presence of TTX when complete blockade of the  $\text{Na}^+$  currents had been achieved (record b), and 10 min after the removal of the toxin, with only the early  $\text{Na}^+$  current recovered (record c). The superimposed traces on the right were drawn from the records on the left to show full recovery of the early component and the characteristic single time constant decay to base line of the early component. The inset shows the isolated late component (continuous line) which was obtained subtracting from the record with both components (record a) the record with only the early current (record c).

In some experiments, after sequential exposure to Tityus  $\gamma$  toxin and TTX, inactivation of the early inward current proceeded almost to completion before the late current component appeared. After treatment with TTX, two distinct current peaks were seen in the control record (Fig. 7, record a) and in records taken 1 and 2 min (record c) after exposure to TTX. The bottom record shows an almost complete blockade of the early current leaving the late current





**Fig. 7**  
Late component of the inward current in isolation. Fiber previously treated with 10 nM Tityus toxin and then 5 nM TTX. Records on the left were taken at 0 (a), 1 (b) and 2 (c) min after addition of the TTX. Superimposed traces of the records are shown on the right



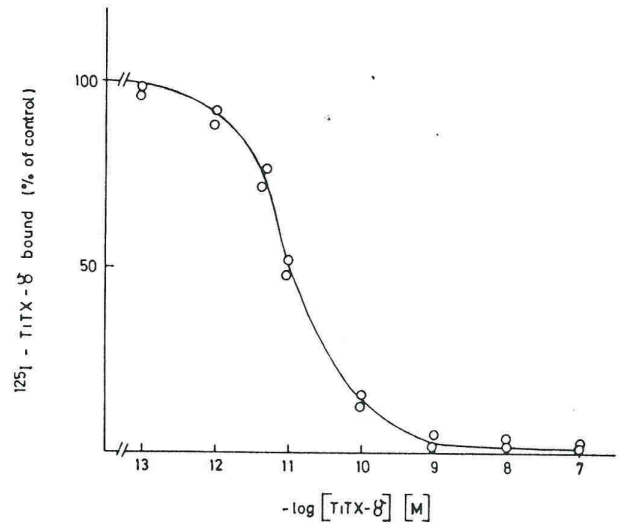
**Fig. 8**  
Equilibrium binding of <sup>125</sup>I-Tityus toxin. T-tubule membranes from frog muscle (17.4 μg of protein per cm<sup>3</sup>) were incubated in a medium containing increasing concentrations of the iodinated toxin for 40 min at 22°C. Left: Total binding of radioactive toxin (●) and non-specific binding measured in the presence of an excess of cold Tityus γ toxin (○). Right: Scatchard plot for the specific binding, namely, total binding minus non-specific binding

component in isolation. The superimposed traces on the right illustrate this effect more clearly.

These results suggest (Fig. 1 and 2) that Tityus γ toxin reacted with both surface sarcolemma and transverse tubular membrane Na<sup>+</sup>-channels.

To further characterize the interaction of Tityus γ toxin with muscle membranes, we measured the binding of the iodinated toxin to a highly purified membrane fraction of transverse tubules. Figure 8 shows the binding isotherm for Tityus γ toxin to saponin-treated membranes. The size of the non-specific Tityus γ toxin binding is very small in this membrane preparation (Fig. 8). Thus, the Scatchard analysis (Fig. 8, right side) exhibits a single straight line indicative of a single binding site with a  $K_D$  of 17 pM and a maximal binding capacity of 2.3 pmol/mg protein.

The binding after incubation of the membrane preparation with a low concentration of the radioactive toxin and increasing concentrations of the non-radioactive toxin also generated linear Scatchard plots with a single receptor for the toxin and a  $K_D$  of about 10 pM. The data are presented in Fig. 9.



**Fig. 9.** Displacement of bound <sup>125</sup>I-toxin by cold Tityus toxin. Binding measurements were performed as described in the Methods section. Conditions as for Fig. 8

## Discussion

The existence of a regenerative,  $[Na^+]$ -dependent, action potential at level of the membrane of the transverse tubule has been demonstrated in several ways (for review see Caille et al. 1985). The actual demonstration of the tubular sodium current in intact muscle fibers under voltage clamp conditions using the sucrose gap technique was attempted by Caille et al. (1975, 1978) and Mandrino (1979) and on cut muscle fibers by Hille and Campbell (1976) using vaseline gaps. The shape and size of the late inward current varied from a clear shoulder in the inactivating phase of the inward current record (Caille et al. 1978) to a minute signal which could be clearly distinguished as a second time constant in the declining phase of the early inward current (Hille and Campbell 1976). Muscle fibers from the toad *Bufo marinus* generated records where the inward current could be separated into two components with different kinetic and voltage dependence (Arispe, Argibay, and Rojas, unpublished observations).

In cut muscle fibers, under voltage clamp conditions, the tubular potential may be different to the potential imposed at the surface (Vergara and Bezanilla 1981; Heiny and Vergara 1982). Therefore, measurement of the kinetic parameters of the tubular component of the inward  $Na^+$  current will not be accurate unless the T-tubule membrane potential is estimated.

We found that external application of Tityus  $\gamma$  toxin reduced both the early and the late components of the inward current (Fig. 1) and, as reported earlier (Barhanin et al. 1984), the blockade depends on membrane potential. The extent of the blockade was significant only at holding potentials less negative than  $-80$  mV.

The observation that both early and late inward currents are reversibly blocked by TTX at low concentrations can be used to identify the currents as  $Na^+$ -currents. The rate of the onset of the blockade by TTX and the rate of recovery following removal of the toxin are clearly different for the early and the late currents (Figs. 4 and 5). These results suggest that the channels responsible for these current are located in different anatomical regions of the fiber, the late current being less accessible to TTX (Allen et al. 1980; Taylor et al. 1980). Although  $Na^+$ -channels in surface and tubular membranes have different receptors for toxins (Jaimovich et al. 1976, 1982; Jaimovich 1986), the kinetic data cannot be used to assign a given anatomical location to the components of the  $Na^+$  currents. The observation that both surface and tubular membranes have different lipid composition (Hidalgo 1986) may explain the different properties observed for the early and late sodium currents.

The differential blockade of these two currents by Tityus  $\gamma$  toxin and TTX was used study the late inward current in isolation. Tail currents were recorded under conditions where the amplitude of both inward currents were similar. The relative value of the time constants for activation of the two components appear to be different, and the activation of the late component also appears to be slower.

The results obtained with the radioactive toxin showed binding to both surface and transverse tubular membrane fragments. Previous attempts to demonstrate binding of Tityus  $\gamma$  toxin to a transverse tubular membrane fraction failed to show Tityus  $\gamma$  toxin receptors (Barhanin et al. 1984). There are two possible explanations for this negative result. Firstly, Tityus  $\gamma$  toxin receptors seem to be rather labile and

aged membrane preparations tend to lose their binding capacity (data not shown). The negative result obtained by Barhanin et al. (1984) may reflect an ill preserved membrane fraction. Secondly, a more likely explanation is that T-tubule membranes are composed of vesicles mostly sealed with inside-out configuration (Hidalgo et al. 1986; Jaimovich 1986). The extent of sealed vesicles varies from one preparation to the other. As the receptor for Tityus  $\gamma$  toxin is located in the external face of the membrane, it is likely that Barhanin et al. (1984) had highly sealed inside-out vesicles in their preparation. The permeabilization of the vesicles by saponin as used in the present work allows access to the binding sites and explains the different results obtained here.

While transverse tubular and surface membrane receptors have similar  $K_D$  for Tityus  $\gamma$  toxin, the toxin exhibits differential capacity to block the two components of the  $Na^+$ -currents. There are three possible explanation for these findings. Firstly, there may be a diffusion barrier for the toxin to reach the sodium channels in the T-tubule. The fact that a blockade of T-tubule sodium channels is obtained after prolonged exposure supports this idea. A second explanation is that the inhibitory effect of Tityus  $\gamma$  toxin in T-tubules is not readily seen under our conditions because a more negative membrane potential is actually present across this membrane. The third possibility is that Tityus  $\gamma$  toxin may have a dual effect on T-tubule sodium channels depending on concentration and/or membrane potential. As suggested by the result shown in Fig. 1, one effect of Tityus  $\gamma$  toxin would be an enhancement of the late  $Na^+$  current. A second effect would be a blockade of the  $Na^+$ -channel present in the T-tubule.

It is interesting to note that, unlike Tityus  $\gamma$  toxin, TTX derivatives have the capacity to block both components of the  $Na^+$  currents. However the affinities for the two receptors is different (Jaimovich et al. 1983; Jaimovich 1986).

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