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## Saxitoxin Blocks L-type $I_{Ca}$

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## ABSTRACT

Saxitoxin (STX) and tetrodotoxin (TTX) are frequently used to selectively block sodium channels. In this study we provide evidence that commercial STX also inhibits L-type  $\text{Ca}^{2+}$  currents ( $I_{\text{Ca,L}}$ ) in adult mouse ventricular myocytes (VM) and tsA-201 cells which were transiently cotransfected with three calcium channel subunits. We measured inhibition of sodium currents ( $I_{\text{Na}}$ ) in mouse VM, of  $I_{\text{Ca,L}}$  in mouse VM and tsA-201 cells, and  $[\text{Ca}^{2+}]_i$  transients in single mouse VM. STX or TTX was abruptly applied before the test voltage pulse using a rapid solution switcher device. STX (10  $\mu\text{M}$ , Calbiochem) and TTX (60  $\mu\text{M}$ , Sigma) completely blocked  $I_{\text{Na}}$  in mouse VM. However, STX at 10  $\mu\text{M}$  also reduced  $I_{\text{Ca,L}}$  in mouse VM by 39% ( $p < 0.0001$ ,  $n = 14$ ), while TTX at 60  $\mu\text{M}$  had no effect on  $I_{\text{Ca,L}}$ . STX (10  $\mu\text{M}$ , Calbiochem) reduced the amplitude of the  $[\text{Ca}^{2+}]_i$  transients in mouse VM by 36% ( $p < 0.0001$ ,  $n = 10$ ). In contrast, TTX (60  $\mu\text{M}$ , Sigma) only reduced the amplitude of the  $[\text{Ca}^{2+}]_i$  transients by 9% ( $p = 0.003$ ,  $n = 5$ ). STX (10  $\mu\text{M}$ ) obtained from Sigma Company showed a similar inhibitory effect on  $I_{\text{Ca,L}}$  (33%,  $p < 0.0001$ ,  $n = 5$ ) in mouse VM. STX (Calbiochem) inhibited the calcium currents of tsA-201 cells in a dose-dependent manner. This inhibition was voltage-independent. The current-voltage relationship of calcium currents in tsA-201 cells was not altered by STX. These results indicate that STX partially blocks L-type  $\text{Ca}^{2+}$  channels, and thus provide further evidence that its effects are not specific for  $\text{Na}^+$  channels.

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Saxitoxin (STX) and tetrodotoxin (TTX) have been well-known sodium channel blockers. They are frequently used to selectively block sodium currents in studies of sodium channels and other membrane currents such as  $I_{Ca,L}$  (Jones and Marks, 1989; Sheets et al., 1996; Penzotti et al., 1998; Vites and Wasserstrom, 1996; Su et al., 2001). In preliminary experiments we observed a greater depression of the  $[Ca^{2+}]_i$  transient in mouse ventricular myocytes after exposure to STX than to TTX. In ventricular myocytes the major pathway of calcium influx during excitation-contraction coupling is L-type calcium channel (Adachi-Akahane et al., 1997; Yao et al., 1998). Jones and Marks (1989) have shown that a commercial preparation of STX could block N-type calcium channel currents in bullfrog sympathetic neurons; however, possible effects of STX on L-type  $Ca^{2+}$  currents have not been investigated. Therefore, we have carried out additional experiments on the effects of STX and TTX on  $[Ca^{2+}]_i$  transients in mouse ventricular myocytes, and examined whether STX reduces currents of native L-type calcium channels in ventricular myocytes and L-type calcium channels heterologously expressed in tsA201 cells.

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## METHODS

### Dissociation of adult mouse ventricular myocytes

Single mouse ventricular myocytes were isolated as described previously (Yao et al., 1998). After retrograde perfusion with modified Tyrode's solution ( $\text{Ca}^{2+}$ -free) for 5 min, the heart was digested for 7-12 min with 0.9 mg/ml collagenase D (Boehringer Mannheim Biochemicals) in modified Tyrode's solution containing 25  $\mu\text{M}$   $\text{CaCl}_2$ . The modified Tyrode's solution (pH 7.4) contained the following (mM): 126 NaCl, 4.4 KCl, 1.0  $\text{MgCl}_2$ , 18  $\text{NaHCO}_3$ , 11 glucose, 4 HEPES, 30 butanedione monoxime (BDM), and 0.13 U/ml insulin, and was gassed with 5%  $\text{CO}_2$ /95%  $\text{O}_2$ . The digested left ventricle was cut into small pieces in modified Tyrode's solution containing 100  $\mu\text{M}$   $\text{Ca}^{2+}$ . These pieces were gently agitated to release single myocytes and then incubated in the same solution with 2% albumin at 30°C for 20 min. The cell suspension was centrifuged at 300 rpm for 3 min and the pellet of cells was resuspended in modified Tyrode's solution containing 200  $\mu\text{M}$   $\text{Ca}^{2+}$  and 2% albumin and allowed to settle for another 20 min at 30 °C. Cells were then suspended in culture media composed of 5% fetal bovine serum, 47.5% MEM (GIBCOBRL, Gaithersburg, MD), 47.5% modified Tyrode's solution, 10 mM pyruvic acid, 4.0 HEPES, and 6.1 mM glucose and finally maintained in a 5%  $\text{CO}_2$  atmosphere at 30°C until use. All experiments in this study were performed at 23-25 °C.

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### **Transient expression of L-type calcium channels in tsA-201 cells**

We maintained tsA-201 cells (large T antigen-transformed human embryonic kidney cells) at 37°C with Dulbecco's modified Eagle's medium (DMEM, GIBCOBRL, Gaithersburg, MD) plus 10% fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO<sub>2</sub> atmosphere (Sheets et al., 1996). TsA-201 cells were fused as described previously (Sheets et al., 1996). Fused tsA-201 cells were transiently cotransfected by calcium phosphate-mediated transfection with 5 µg each of three calcium channel subunits (rabbit  $\alpha_{1C}$ , rat  $\beta_{2a}$  and  $\alpha_2/\delta$ ) per 10 cm plate (Perez-Reyes et al., 1992; Wei et al., 2000). Rabbit  $\alpha_{1C}$  was subcloned into pRc/CMV (Invitrogen, San Diego, CA), rat  $\beta_{2a}$  into p91023(B), and rat  $\alpha_2/\delta$  into pZEM229 (ZymoGenetics, Seattle, WA). As tsA-201 cells normally have no detectable Ca<sup>2+</sup> currents, the presence of L-type Ca<sup>2+</sup> currents provided confirmation of transfection.

For electrophysiological study, the fused tsA-210 cells (2-3 days after transfection) were harvested from the culture dish by trypsinization, washed once with DMEM plus 10% FBS, and maintained in this medium at room temperature for later use. Only cells that showed a current amplitude >300 pA were used for drug studies.

### **Electrophysiology**

The set-up for voltage clamp has been previously described in detail (Su et al, 1998). Cells were voltage clamped at room temperature with single suction pipettes which were made from borosilicate glass tubing (Corning 7052, 1.65 mm o.d., 1.2 mm i.d., A-M System Inc., Everett, WA) and had initial resistances of 1.0-2.5 M $\Omega$  when filled with pipette solution

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containing (in mM): 15 NaCl, 100 CsCl, 30 tetraethylammonium chloride, 5 MgATP, 10 EGTA, 10 Hepes, 5.5 dextrose (pH 7.1 adjusted with CsOH). To measure  $[Ca^{2+}]_i$  transients in voltage-clamped myocytes (Su et al., 2001), EGTA was omitted from pipette solution. Inhibition of both sodium currents ( $I_{Na}$ ) in mouse VM and L-type calcium currents ( $I_{Ca,L}$ ) in mouse VM and tsA-201 cells was measured by a whole-cell voltage-clamp technique using an Axopatch 200A amplifier (Axon instrument). Series resistance compensation (70-80%) was used in all experiments. Currents were filtered at 2-5 kHz and digitized at 5-10 kHz. Cell capacitance was also measured to calculate the current density.

Mouse VM were perfused with an external solution containing (mM): 126 NaCl, 1.0  $MgCl_2$ , 1.08  $CaCl_2$ , 4.4 CsCl, 11 dextrose, 24 Hepes (pH 7.4 adjusted with NaOH). TsA-201 cells were superfused with an external solution containing (mM): 126 NaCl, 5  $CaCl_2$ , 4.4 CsCl, 11 dextrose, 24 Hepes (pH 7.4 adjusted with NaOH).

### Measurement of $[Ca^{2+}]_i$

The  $[Ca^{2+}]_i$  was measured as previously described (Yao et al, 1998, Su et al., 2001). Myocytes were loaded with fluo-3 by exposure to 1  $\mu M$  fluo-3 AM (Molecular Probes) at 30°C for 30 min. Fluo 3-loaded myocytes were placed in a chamber mounted on an inverted microscope. Once myocytes had settled to the bottom, they were superfused with a Hepes-buffered solution containing (in mM): 126 NaCl, 4.4 KCl, 1.0  $MgCl_2$ , 1.08  $CaCl_2$ , 11 dextrose, 0.5 probenecid, 24 Hepes (pH 7.4 adjusted with NaOH to give a final external  $Na^+$  concentration of 140 mM).

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Myocytes were held at -80 mV at room temperature and clamped to 0 mV for 200 msec to trigger  $[Ca^{2+}]_i$  transients. Eight conditioning pulses (200 ms, -80 to 0 mV, 0.25 Hz) were applied before test pulse to provide a steady state loading of SR with  $Ca^{2+}$ .

Fluo-3 loaded myocytes were illuminated by 485 nm excitation light via an epifluorescence attachment (510 nm dichroic mirror, Omega) and a x40 Fluor oil objective lens. The resulting fluorescence signals at 530 nm (DF30, Omega) were detected with a photomultiplier (SFX-2, Solamere Technology Group, Salt Lake City, USA). Fluo-3 fluorescence was transformed to  $[Ca^{2+}]_i$  by a "pseudo-ratio method" (Cheng et al., 1993):  $[Ca^{2+}]_i = Kd(F/F_0)/(Kd/[Ca^{2+}]_{i_{rest}} + 1 - (F/F_0))$ , where Kd is the dissociation constant for fluo-3 (493 nmol/L at 25°C), F the fluorescence intensity,  $F_0$  the intensity at rest, and  $[Ca^{2+}]_{i_{rest}}$  the  $[Ca^{2+}]_i$  at rest and assumed to be 80 nM under our experimental conditions (Yao et al., 1998).

### **Rapid application of drugs**

To observe the effects of STX (Calbiochem #B19491; Sigma #39H1139) and TTX (Sigma #99H0832) on  $[Ca^{2+}]_i$  transients, sodium currents and/or L-type calcium currents, we rapidly applied those drugs to clamped single cells. Rapid applications of STX and TTX were accomplished with a fast solution switcher device (Yao et al., 1997) which minimizes the amounts of test solution required, and eliminates uncertainty regarding the precise time of exposure.



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### **Measurement of cation content**

Cation content in the commercially prepared STX solution was determined by inductively coupled plasma (ICP) emission spectroscopy (D'souza et al., 1999) by use of an ICPS-7500 instrument (Shimadzu Corporation, Kyoto, Japan). All glassware that was used was soaked in concentrated nitric acid and rinsed in cheletel neuro pure water to remove any metal contaminants.

### **Data Analysis**

All recordings were digitized online with a DigiData 1200 interface (Axon Instruments, Inc.) and stored on disk. The digitized data were analyzed with pCLAMP6 (Axon Instrument, Inc.) and ORIGIN (Microcal Software, Inc.). Results were expressed as means  $\pm$  SEM, and statistical differences were determined by unpaired or paired *t*-tests. The "n" indicates the number of myocytes or cells studied. One to three myocytes were studied from each heart dissociation. Differences were considered statistically significant at  $p < 0.05$ .

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## RESULTS

### Effects of STX and TTX on $[Ca^{2+}]_i$ transients in mouse ventricular myocytes

To induce a stable  $[Ca^{2+}]_i$  transient magnitude, a pre-pulse protocol (eight pulses at 0.25 Hz, -80 to 0 mV, for 200 ms) was applied before each test pulse. The left panel of Fig 1A shows the last of a series of eight conditioning voltage-clamp pulses (*trace a*) and its corresponding membrane currents (*trace b*) as well as the triggered  $[Ca^{2+}]_i$  transient (*trace d*). The test pulse was initiated 4 s after the start of the last conditioning pulse. To determine the effects of STX or TTX on  $[Ca^{2+}]_i$  transients, the clamped myocyte was superfused with STX (10  $\mu$ M) or TTX (60  $\mu$ M) for 2 s immediately before and during the test pulse by using a rapid solution switcher (*trace c* in Fig 1A). In our experiments,  $K^+$  currents were blocked by replacing intracellular and extracellular  $K^+$  with  $Cs^+$  and including tetraethylammonium chloride (TEA) in the pipette solution. Therefore, the inward currents in a 200 ms test pulse (from -80 to 0 mV) in the presence of STX or TTX should be largely ascribed to calcium currents. Fig 1A illustrates that the peak inward currents ( $I_{Na}$  plus  $I_{Ca}$ ) activated during the 200 ms pulse from -80 to 0 mV were dramatically reduced by rapid application of STX (Calbiochem) at 10  $\mu$ M. The  $[Ca^{2+}]_i$  transient amplitude was also significantly decreased (36%) by STX. In contrast, previous studies in our laboratory (Fig 1B) showed that the  $[Ca^{2+}]_i$  transient was only slightly decreased (9%) by rapid application of TTX (Sigma Co.) at 60  $\mu$ M. We also found that the mean of the peak inward currents during the test pulse (-80 to 0 mV) in the presence of STX (Calbiochem) were smaller than those measured in the presence of TTX (Sigma Co.) ( $624 \pm 88$  pA (n=10) vs  $947 \pm 110$  pA

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(n=7),  $P < 0.05$ ). These results suggested that STX may inhibit L-type calcium channel currents in mouse ventricular myocytes. We therefore carried out additional experiments to examine the effects of the commercial STX preparations on L-type calcium channel currents.

### **Effects of STX and TTX on $I_{Ca,L}$ in mouse ventricular myocytes**

As shown in panel "a" of Fig 2, mouse ventricular myocytes were held at -80 mV, depolarized to -40 mV for 200 ms to inactivate  $I_{Na}$ , and then depolarized to 0 mV to activate  $I_{Ca,L}$ . Application of STX (10  $\mu$ M, Calbiochem) to the same cell appeared to completely block  $I_{Na}$  and inhibited  $I_{Ca,L}$  by 39% ( $P < 0.0001$ , n=14) (panel "b" of Fig 2). Both  $I_{Na}$  and  $I_{Ca,L}$  recovered upon washout of STX (panel "c" of Fig 2). STX (10  $\mu$ M) purchased from Sigma Company also showed a similar inhibitory effect on  $I_{Ca,L}$  in mouse ventricular myocytes (current densities were reduced by 33%, n=5,  $P < 0.001$ , data not shown). This inhibitory effect of STX from Sigma was also reversible upon washout. In contrast, TTX (60  $\mu$ M, from Sigma Co.) blocked  $I_{Na}$ , but did not influence  $I_{Ca,L}$  (Fig 3). These results provide another line of evidence for the inhibitory effect of commercial STX preparations on L-type calcium channel currents in mouse ventricular myocytes, and are consistent with STX-induced reduction in the amplitude of  $[Ca^{2+}]_i$  transients.

To exclude the possibility that the STX-induced reduction of the inward current during a voltage pulse (0 mV) was not due, in part, to block of residual  $I_{Na}$  resulting from incomplete inactivation of Na channels during the pre-pulse to -40 mV, the effect of STX on  $I_{Ca,L}$  was

observed in the absence of extracellular  $\text{Na}^+$ . The same voltage protocol as described in panel a of Fig 2 was used in this experiment. The  $I_{\text{Ca,L}}$  was recorded before and after STX application.

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STX (10  $\mu\text{M}$ , Calbiochem) showed the same inhibitory effect on  $I_{\text{Ca,L}}$  (decreased by 39%,  $n=5$ ) when extracellular  $\text{Na}^+$  was replaced by tetramethylammonium chloride (TMA). We also examined the effect of acetic acid (vehicle solvent in the commercial STX preparation) on the inhibitory effect of STX on  $I_{\text{Ca,L}}$ . Acetic acid at 1 mM, which was the concentration of acetic acid in the working solution of STX (10  $\mu\text{M}$ ), did not reduce the amplitude of  $I_{\text{Ca,L}}$  (data not shown).

### Effects of STX on $I_{\text{Ca,L}}$ in tsA-201 cells

To confirm further that residual  $I_{\text{Na}}$  did not appreciably contaminate measurements of  $I_{\text{Ca,L}}$ , we examined the effect of STX on calcium currents mediated by L-type calcium channels expressed in fused tsA-201 cells. TsA-201 cells were transiently cotransfected with three calcium channel subunits (see Methods Section). As shown in Fig 4, STX inhibited calcium currents in a dose-dependent manner. Note the absence of significant inward current due to  $I_{\text{Na}}$  when stepping from  $-80$  mV to  $-40$  mV. Current traces (recorded from the same cell) in panels A, B, and C of Fig 4 exhibited a very similar amplitude of control currents, indicating that the effects of STX on calcium currents of L-type calcium channels expressed in tsA-201 cells were completely reversible, consistent with recovery of L-type calcium channel currents after wash-out of STX in mouse ventricular myocytes. As shown in Fig 4D, the maximal effect of STX on the L-type Ca current in tsA-201 cells was a 49% block, and the "Kd" (concentration required

for ½ of this effect) for this effect was 0.3 μM. The current-voltage relationship (I-V curve) of calcium currents in tsA-201 cells was not altered by STX (Fig 5C), indicating that the voltage-  
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dependence of the activation of L-type calcium channel is not influenced by STX. This inhibition was also voltage-independent (Fig. 5D).

### **Content of metal ions in the commercial STX solution**

Our experimental results described above shows evidence for the inhibitory effect of STX (Calbiochem or Sigma Co.) on L-type calcium channels. It is important to exclude the possibility that a metal ion contaminant of the commercially prepared STX solution is responsible for this inhibitory effect. We therefore measured the content of metal ions in the commercial STX solution (Calbiochem) by ICP emission analysis. Concentrations of metal ions ( $\text{Ni}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Cd}^{2+}$  and  $\text{La}^{3+}$ ) in the STX preparation from Calbiochem were all under detection limits (400 nM) of ICP emission analysis. These results, as well as the rapid reversibility of the effects of STX on  $I_{\text{Ca,L}}$ , suggest that metal ions are not responsible for the inhibitory effect of the commercial STX on L-type calcium channels.

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## DISCUSSION

It is well recognized that STX can bind to and block Na channels in cardiac myocytes as well as in neuronal cells, although the Kds for these effects differ (Hille, 2001; Guo et al, 1987). In the present study we have provided evidence demonstrating that STX also partially blocks L-type calcium channels. First, STX reduced the amplitude of  $[Ca^{2+}]_i$  transients in mouse ventricular myocytes more markedly than did TTX. The apparent slowing of the decline of the  $[Ca^{2+}]_i$  transient after STX probably is due to the reduction in its amplitude (Bers & Berlin, 1995) rather than an effect of STX on sarcoplasmic reticulum  $Ca^{2+}$ -ATPase. Second, STX from two different commercial suppliers significantly inhibited calcium currents of native L-type calcium channel in mouse ventricular myocytes. Third, the inhibitory effect of the STX on  $I_{Ca,L}$  is not due to the solvent, acetic acid, nor to divalent cation contamination by  $Ni^{2+}$ ,  $Co^{2+}$ ,  $Mn^{2+}$ ,  $Cd^{2+}$  or the trivalent cation  $La^{3+}$  in the STX preparation. Fourth, STX inhibited calcium currents mediated by L-type calcium channels expressed in tsA-201 cells which have little or no fast  $Na^+$  currents (Sheets et al, 1996). The I-V relationship of calcium currents in tsA-201 cells was not altered by STX, indicating that there was no surface charge effect on I-V relationships and there is no voltage-dependence of the block of L-type calcium channel by STX. The lack of a shift in the I-V relationship further suggests that the block of  $I_{Ca,L}$  is not caused by metal ion contamination in the commercial STX preparation, because a shift in the I-V relationship is expected if metal ions are responsible for the block of L-type calcium channels (Hille, 2001; Hobai et al., 2000).

The inhibitory effect of STX on  $I_{Ca,L}$  was rapid in onset, particularly at a high concentration of STX (10  $\mu$ M). Block of  $I_{Ca,L}$  was also rapidly reversible, with currents recovered

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within 40 sec after washout. These basic features of STX-mediated blockade of  $I_{Ca,L}$  suggest that STX acts on the calcium channel at a site that has access from the external surface, and may have some similarities to the binding site for STX in the external vestibule of the  $Na^+$  channel ion permeation pathway (Lipkind and Fozzard, 1994).

The maximal effect of STX on the L-type Ca channel was a 49% inhibition, and the "Kd" for this effect was 300 nM (Fig 4D). The concentration of STX at which a half-maximal effect of STX on the L-type Ca channel was seen is similar to that reported by Jones and Marks (1989) in bullfrog neuronal N-type Ca channels (400 nM). These concentrations are much higher than for the effect of STX on neuronal Na channels ( $K_d < 20$  nM), but somewhat less than for STX inhibition of the cardiac Na channel ( $\sim 5000$  nM) which is known to be STX resistant (Hille, 1991). Interestingly, Jones and Marks (1989) also found that STX produced an incomplete block of bullfrog neuronal Ca channels (66% at 1  $\mu$ M). Also, a mutated  $\mu$ -conotoxin has been reported to only partially reduce the skeletal muscle Na current due to a partial block at the single channel level (French et al, 1996; Becker et al, 1992). The incomplete block of STX of L-type Ca channel currents may be due also to only a partial inhibition by STX of single L-type  $Ca^{2+}$  channels. A channel blocker acting at a single site would be expected to completely inhibit the channel at a sufficiently high concentration. The fact that this is not observed with STX inhibition of  $Ca^{2+}$  channels may indicate that the mechanism is not a simple pore block. A

recent report by Wang et al (2003) has shown that STX can decrease hERG K<sup>+</sup> currents by stabilizing closed channel states, manifest by a shift in the voltage dependence of channel opening to more

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depolarized membrane potentials. However, in our experiments STX had no effect on the voltage dependence of activation of the L-type Ca<sup>2+</sup> channel expressed in tsA-201 cells. Our data cannot rule out the possibility that STX decreased I<sub>Ca,L</sub> by producing a negative (leftward) shift of the steady state inactivation (SSI) curve resulting in inactivation of I<sub>Ca,L</sub> at our holding potential of -40 mV. However, such an effect is unlikely because it would require that STX cause a marked shift in the SSI curve while at the same time not producing block of the channel pore. Furthermore, in Na channels STX has been demonstrated to shift gating charge by only -4.9 mV (Heggeness and Starkus, 1986).

It has been shown that the binding interaction between the Na channel and STX is different from that between the Na channel and TTX (Kirsch and Hartmann, 1994; Penzotti et al., 1998). Based on the different effect of STX and TTX on L-type calcium channel, it is plausible that L-type calcium channels may bear a binding site for STX, but not for TTX. A different channel mediated current, the TTX-blockable calcium current (*I*<sub>Ca,(TTX)</sub>), has been reported in human atrial (Lemaire et al., 1995) and ventricular (Gaughan et al., 1999) myocytes, guinea-pig (Cole et al., 1997) and rat (Aggarwal et al., 1997) ventricular cells, and in neural preparations (Meves and Vogel, 1973; Akaike and Takahashi, 1992). The identity of the channel which conducts *I*<sub>Ca,(TTX)</sub> is still controversial; however it is possible that *I*<sub>Ca,(TTX)</sub> is mediated by a



subtype of a sodium channel and not a calcium channel (Aggarwal et al., 1997). In any event, our studies in tsA201 cells expressing  $I_{Ca,L}$  eliminate the  $I_{Ca,(TTX)}$  as a contributor to our results.

In our study, we also noted minor but significant variations in the sensitivity to STX depending upon the tissue studied. STX at 10  $\mu$ M reduced L-type calcium currents by  $39\pm 3\%$  JPET/2003/56564

( $n=14$ ) in mouse ventricular myocytes, vs  $53\pm 4\%$  ( $n=6$ ) in transfected tsA-201 cells ( $p<0.05$ ) and  $58\pm 6\%$  ( $n=4$ ) in rabbit myocytes ( $p<0.01$ ). Consequently, the degree of block of the L-type Ca channel produced by STX in cardiac myocytes from different species may vary.

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## REFERENCES

- Adachi-Akahane S, Lu L, Li Z, Frank JS, Philipson KD and Morad M (1997) Calcium signaling in transgenic mice overexpressing cardiac Na<sup>+</sup>-Ca<sup>2+</sup> exchanger. *J Gen Physiol* **109**:717-729.
- Aggarwal R, Shorofsky SR, Goldman L and Balke CW (1997) Tetrodotoxin-blockable calcium currents in rat ventricular myocytes; a third type of cardiac cell sodium current. *J Physiol* **505**:353-369.
- Akaike N and Takahashi K (1992) Tetrodotoxin-sensitive calcium-conducting channels in the rat hippocampal CA1 region. *J Physiol* **450**:529-546.
- Bers DM and Berlin JR (1995) Kinetics of [Ca<sup>2+</sup>]<sub>i</sub> decline in cardiac myocytes depend on peak [Ca<sup>2+</sup>]<sub>i</sub>. *Am J Physiol* **268**:C271-C277.
- Cheng H, Lederer WJ and Cannell MB (1993) Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* **262**:740-744.
- Cole WC, Chartier D, Martin M and Leblanc N (1997) Ca<sup>2+</sup> permeation through Na<sup>+</sup> channels in guinea-pig ventricular myocytes. *Am J Physiol* **273**:H128-137.

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D'souza VM, and Holz RC (1999) The methionyl aminopeptidase from escherichia coli can function as an iron (II). *Biochemistry* **38**:11079-11085.

French RJ, Prusak-Sochaczewski E, Zamponi GW, Becker S, Kularatna AS and Horn R (1996) Interactions between a pore-blocking peptide and the voltage sensor of the sodium channel: An electrostatic approach to channel geometry. *Neuron* **16**:407-413.

Gaughan JP, Frukawa S, and Houser SR (1999) Tetrodotoxin-sensitive calcium current induces contraction in failed human ventricular myocytes. *Circulation* **100**(suppl):I-422.

Guo XT, Nehara A, Ravindran A, Bryant SH, Hall S, Moczydlowski E (1987) Kinetic basis for insensitivity to tetrodotoxin and saxitoxin in sodium channels of canine heart and denervated rat skeletal muscle. *Biochem* **26**:7546-7556.

Heggeness ST and Starkus JG (1986) Saxitoxin and tetrodotoxin. Electrostatic effects on sodium channel gating current in crayfish axons. *Biophys J* **49**:629-643.

Hille B (2001) *Ionic Channels of Excitable Membranes* (3rd edition). Sinauer Associates Inc., Sunderland, MA.

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Hobai IA, Hancox JC and Levi AJ (2000) Inhibition by nickel of the L-type Ca channel in guinea pig ventricular myocytes and effect of internal cAMP. *Am J Physiol* **279**:H692-H701.

Jones SW and Marks TN (1989) Calcium currents in bullfrog sympathetic neurons. *J Gen Physiol* **94**:151-167.

Kirsch GE, Alam M and Hartmann HA (1994) Differential effects of sulfhydryl reagents on saxitoxin and tetrodotoxin block of voltage-dependent Na channels. *Biophys J* **67**:2305-2315.

Lemaire S, Piot C, Seguin J, Nargeot J and Richard S (1995) Tetrodotoxin-sensitive Ca<sup>2+</sup> and Ba<sup>2+</sup> currents in human atrial cells. *Receptors and Channels* **3**:71-81.

Lipkind GM and Fozzard HA (1994) A structural model of the tetrodotoxin and saxitoxin binding site of the Na<sup>+</sup> channel. *Biophys J* **66**:1-13.

Meves H and Vogel W (1973) Calcium inward currents in internally perfused giant axons. *J Physiol* **235**:225-265.

Penzotti JL, Fozzard HA, Lipkind GM and Dudley SC Jr (1998) Differences in saxitoxin and tetrodotoxin binding revealed by mutagenesis of the Na<sup>+</sup> channel outer vestibule. *Biophys J* **75**:2647-2657.

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Perez-Reyes E, Castellano A, Kim HS, Bertrand P, Baggstrom E, Lacerda AE, Wei XY, and Birnbaumer L (1992) Cloning and expression of a cardiac/brain beta subunit of the L-type calcium channel. *J Biol Chem* **267**:1792-1797.

Sheets MF, Kyle JW, Krueger S and Hanck DA (1996) Optimization of a mammalian expression system for the measurement of sodium channel gating currents. *Am J Physiol* **271**:C1001-C1006.

Su Z, Zou A, Nonaka A, Zubair I, Sanguinetti MC and Barry WH (1998) Influence of prior Na<sup>+</sup> pump activity on pump and Na<sup>+</sup>/Ca<sup>2+</sup> exchange currents in mouse ventricular myocytes. *Am J Physiol* **275**:H1808-H1817.

Su Z, Sugishita K, Ritter M, Li F, Spitzer KW and Barry WH (2001) The sodium pump modulates the influence of  $I_{Na}$  on  $[Ca^{2+}]_i$  transients in mouse ventricular myocytes. *Biophys J* **80**:1230-1237.

Vites AM and Wasserstrom JA (1996) Fast sodium influx provides an initial step to trigger contractions in cat ventricle. *Am J Physiol* **271**:H674-H686.

Wang J, Salata JJ and Bennett PB (2003) Saxitoxin is a gating modifier of hERG K<sup>+</sup> channels. *J*

*Gen Physiol* **121**:583-598.

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Wei S, Colecraft HM, Demaria CD, Peterson BZ, Zhang R, Konout TA, Rogers TB and Yue

DT. 2000. Ca<sup>2+</sup> channel modulation by recombinant auxiliary  $\beta$  subunits expressed in young adult heart cells. *Circ Res* **86**:175-184.

Yao A, Spitzer KW, Ito N, Zaniboni M, Lorell BH and Barry WH (1997) The restriction of

diffusion of cations at the external surface of cardiac myocytes varies between species. *Cell Calcium* **22**:431-438.

Yao A., Su Z, Nonaka A, Zubair I, Lu L , Philipson KD, Bridge JHB and Barry WH (1998)

Effects of overexpression of the Na<sup>+</sup>-Ca<sup>2+</sup> exchanger on [Ca<sup>2+</sup>]<sub>i</sub> transients in murine ventricular myocytes. *Circ Res* **82**:657-665.

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## Figure legends

Figure 1. A, Influence of STX (Calbiochem) on  $[Ca^{2+}]_i$  transients in mouse ventricular myocytes. Left panel: Trace a shows the last two of a series of eight voltage clamp protocols in which the cell was held at  $-80$  mV and depolarized to  $0$  mV for  $200$  msec; trace b, membrane currents ( $I_{Na}$  and  $I_{Ca,L}$ ) in which  $K^+$  currents were blocked by TEA and Cs; trace c, rapid application of STX ( $10 \mu M$ ) was accomplished by a double-barreled solution switcher; trace d,  $[Ca^{2+}]_i$  transients. Average values of the peak  $[Ca^{2+}]_i$  are presented as mean  $\pm$  SEM of 10 cells from four hearts and shown in the bar graph on the right. B, Similar recordings showing the effects of TTX in an identical protocol as in Figure 1A. (B reprinted from Su et al., 2001, with permission of publisher).

Figure 2. Effects of STX from Calbiochem on membrane currents ( $I_{Na}$  and  $I_{Ca,L}$ ) in mouse ventricular myocytes. Panel a: a control run of the voltage protocol in which the cell was held at  $-80$  mV and pulsed to  $-40$  mV to activate  $I_{Na}$ , and then to  $0$  mV to activate  $I_{Ca,L}$ . Potassium currents were blocked by replacing the extracellular and intracellular  $K^+$  with  $Cs^+$ . Panel b: the same voltage protocol as in "a" was applied to the same cell in the presence of STX ( $10 \mu M$ ). Note the  $I_{Na}$  was completely blocked and the  $I_{Ca,L}$  was partially inhibited. Panel c: Recovery of both  $I_{Na}$  and  $I_{Ca,L}$  upon washout of STX. Panel d: an overlapped plot of L-type  $Ca^{2+}$  current tracings in the absence and presence of STX.



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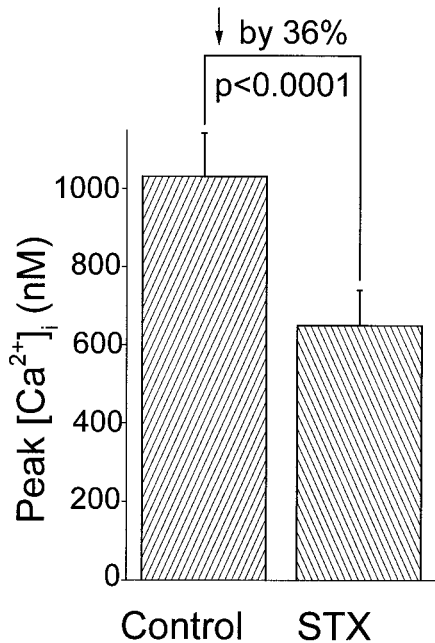
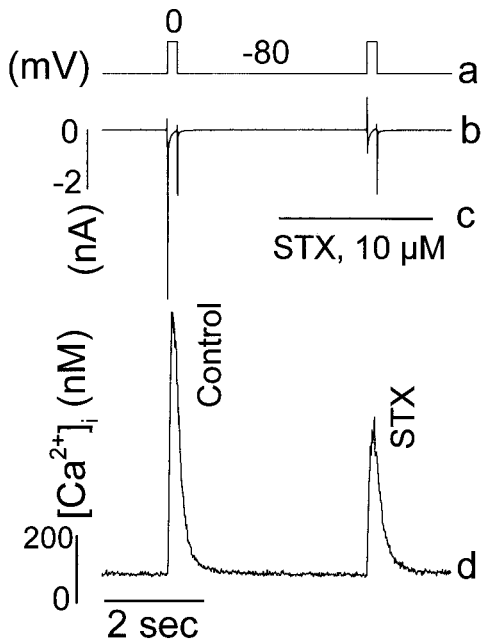
Figure 3. Effects of TTX (Sigma) on membrane currents ( $I_{Na}$  and  $I_{Ca,L}$ ) in mouse ventricular myocytes. Left panel: a control run of the voltage protocol as described in Figure 2. Right panel: the same voltage protocol was applied to the same cell in the presence of TTX (60  $\mu$ M). TTX blocked  $I_{Na}$ , but did not influence  $I_{Ca,L}$ . Inset on the right panel is an overlapped plot of L-type calcium currents in the absence and presence of TTX. Similar results were observed in 8 cells from three hearts.

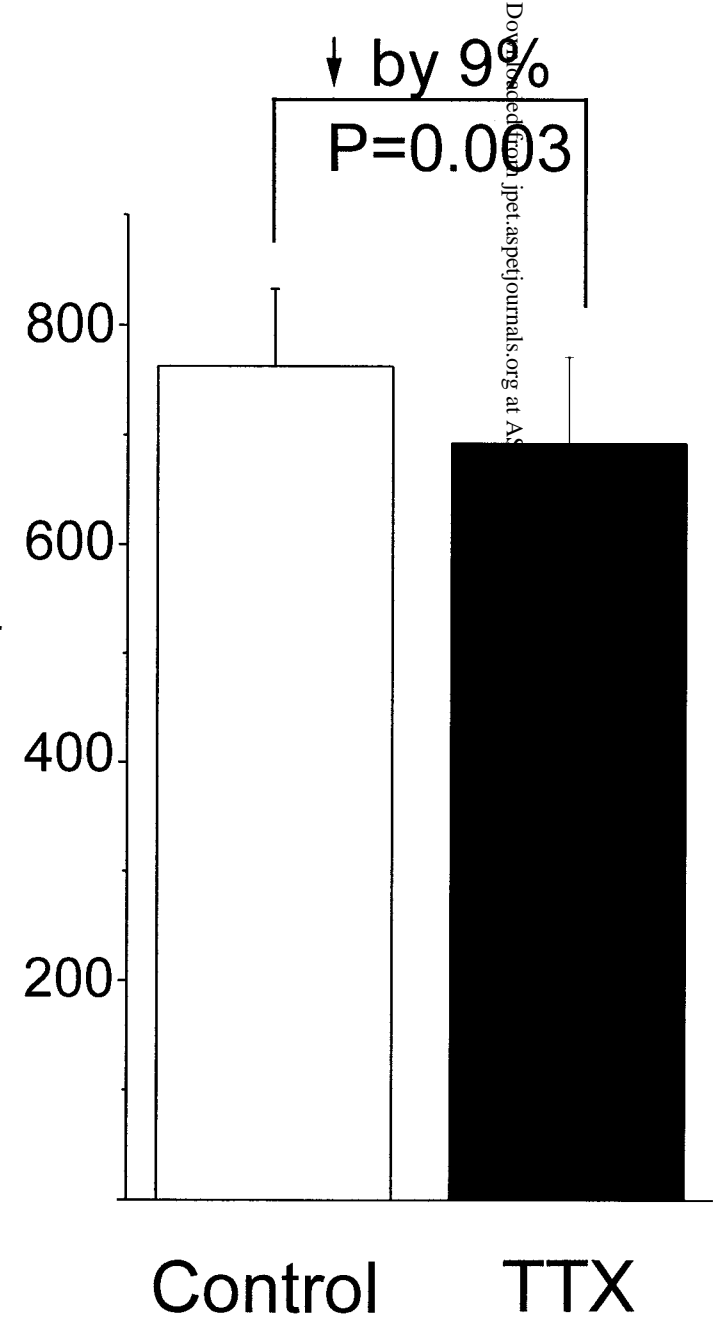
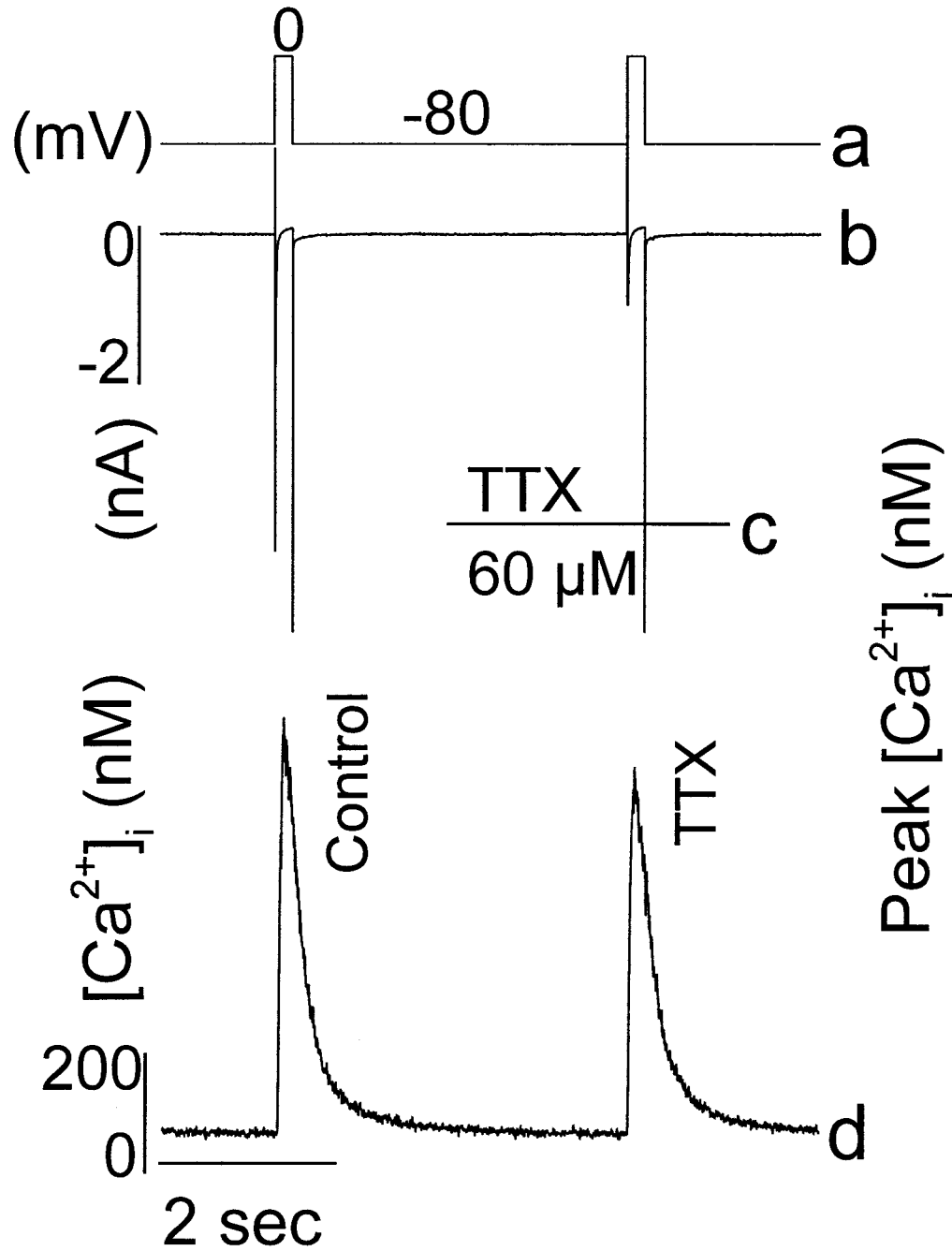
Figure 4. Dose-dependent effects of STX (Calbiochem) on L-type calcium channel currents in tsA-201 cells. The same voltage protocol as used in mouse VM (Figure 2) was employed to examine the effects of STX on calcium currents mediated by L-type calcium channels expressed in tsA-201 cells. Panels A, B, and C are representative traces showing the effects (in the same cell) of STX at 0.3, 1, and 3  $\mu$ M, respectively. Panel D shows the dose-dependent effect of STX on L-type calcium currents on a log scale. The data were fit using SAS and a non-linear least squares analysis of the equation "fractional block = maximum block x (dose / (Kd + dose))." The Kd is 0.3  $\mu$ M, and the maximum block 0.49. Results are expressed as means  $\pm$  SEM, n=5.

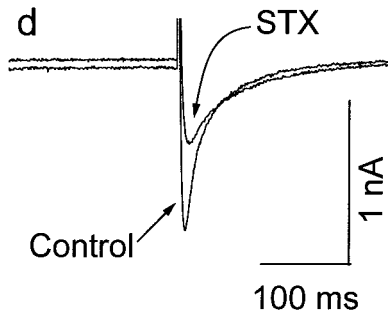
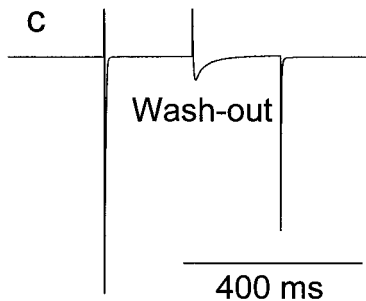
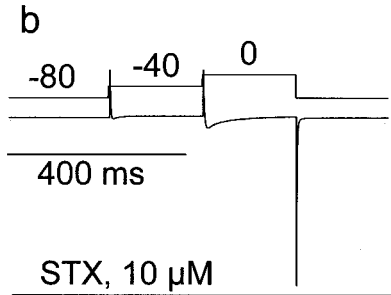
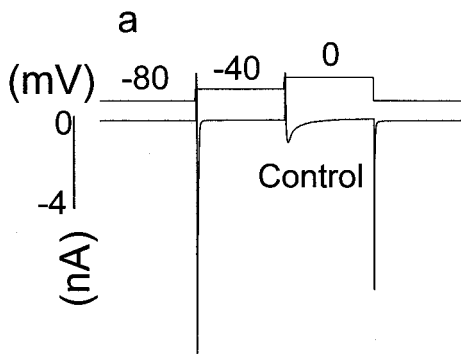
Figure 5. Voltage-independent effects of STX on L-type calcium channels expressed in tsA-201 cells. L-type calcium channel currents were activated by depolarizing the cell membrane from -30 to 70 mV at a 10 mV increment. Panel A, representative traces showing the control currents (in the absence of STX). Panel B, representative traces showing the currents in the same cell in the presence of 3  $\mu$ M STX. Pooled data (means  $\pm$  SEM, n=5) is shown in Panel C and indicates

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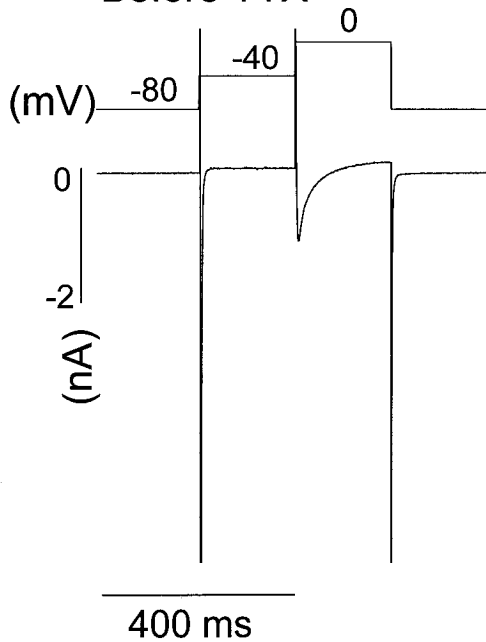
that the I-V relation is not altered by STX. Panel D shows the ratio of  $I_{Ca,L}$  in STX to control demonstrating lack of a voltage-dependent reduction by STX of peak  $I_{Ca,L}$ .







Before TTX



After TTX

