Do Heavy Metal Contaminants Mediate the Response of T-type Ca\(^{2+}\) Channel Clones to Na\(^{+}\) Channel Toxins?

Because of the relatively low affinity of the responses of T-type Ca\(^{2+}\) channels to TTX and STX, one possible confounding factor might be the undesired presence of heavy metals in the aliquots of toxins from various commercial sources. Heavy metals block many types of channels including T-type Ca\(^{2+}\) channels (Jeong et al., 2003). We initially contacted the vendors regarding this issue and the Program Research Leader from the Institute for Marine Biosciences (NRC-IMB, Halifax, Nova Scotia, Canada) responded by saying that he was “doubtful that there could be significant concentrations of heavy metals” and that the “STX CRM is produced specifically for instrumental analytical chemistry” and is thus highly pure. We nevertheless tested the effects of EDTA, a chelator that binds many heavy metals with high affinity (e.g., Fe\(^{2+}\), Fe\(^{3+}\), Pb\(^{2+}\), Zn\(^{2+}\), Mn\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\)) on the toxin responses of I\(_{Ca\,(TTX)}\). We chose a concentration of 100 µM EDTA that would have a very small impact on free Ca\(^{2+}\) concentrations (~0.1 mM reduction) and a negligible effect on free Mg\(^{2+}\) levels in our external solution. This would lower free heavy metal concentrations to levels ~10 nM (e.g., Mn\(^{2+}\)) or lower assuming that the concentration of the contaminant is 1 µM (based on the assumption that 5% of the toxin sample on a weight basis is composed of heavy metal contaminants). The buffering effect of EDTA on Ni\(^{2+}\) was compensated for by increasing the total Ni\(^{2+}\) concentration by an appropriate amount (see Materials and methods for a detailed description) in order to keep free Ni\(^{2+}\) concentration constant as

**Figure S1.** Effects of low [Na\(^{+}\)]\(_{o}\) on low voltage–activated I\(_{Ca\,(TTX)}\) in dog atrium. (A) Current traces were recorded from a typical cell and were elicited by voltage steps from –90 to –30 mV. Currents were recorded in the absence (Ctl) or presence of 50 µM, 200 µM, 1 mM of [Na\(^{+}\)]\(_{o}\), or after addition of 30 µM TTX in the presence of 1 mM [Na\(^{+}\)]\(_{o}\). This experiment was performed at room temperature. External Na\(^{+}\) at concentrations of 50 or 200 µM had no effect on the low voltage–activated I\(_{Ca\,(TTX)}\). The faster current component recorded in the presence of higher [Na\(^{+}\)]\(_{o}\) can be abolished by TTX or washout of [Na\(^{+}\)]\(_{o}\), a result consistent with Na\(^{+}\) permeation through classical Na\(^{+}\) channels. (B) Bar graph summarizing the effects of external sodium on peak inward current recorded at –30 mV from HP = –90 mV. Peak inward current is expressed as mean ± SEM % relative to the control value (filled column, 0 mM [Na\(^{+}\)]\(_{o}\)). Not all Na\(^{+}\) concentrations could be tested in the same cell; each bar represents the mean of 6–11 experiments. W, washout. *, significantly different from control with P < 0.05.

**SUPPLEMENTAL MATERIAL**

**ONLINE SUPPLEMENTAL MATERIAL**

Dog Atrial Cells Lack I\(_{Ca\,(TTX)}\)

Studies have provided evidence in cardiac myocytes for a Ca\(^{2+}\) entry pathway that is sensitive to block by TTX (so-called I\(_{Ca\,(TTX)}\); Lemaire et al., 1995; Aggarwal et al., 1997; Cole et al., 1997; Santana et al., 1998; Sha et al., 2003). Fig. S1 summarizes the results of typical experiments designed to explore the possibility that a component of the low voltage–activated inward Ca\(^{2+}\) current (LVA) might be attributed to Ca\(^{2+}\) permeation through a TTX-sensitive cation channel. In guinea pig ventricular myocytes, Cole et al. (1997) suggested that Ca\(^{2+}\) could permeate TTX-sensitive Na\(^{+}\) channels in the absence of Na\(^{+}\) in the superfusate. In addition to a modulation of this TTX-sensitive Ca\(^{2+}\) current by veratridine (10 or 50 µM), this group also showed that micromolar concentrations of extracellular Na\(^{+}\) were able to partially suppress this current with 10 mM Ca\(^{2+}\) as the charge carrier. Fig. S1 A shows that LVA was influenced little, if any, by exposing the myocyte to [Na\(^{+}\)]\(_{o}\) ~ 200 µM. However, cell exposure to 1 mM [Na\(^{+}\)]\(_{o}\) led to significant enhancement of the inward current, an effect that was abolished by 30 µM TTX. Mean data for these experiments are shown in Fig. S1 B. While 50 and 200 µM [Na\(^{+}\)]\(_{o}\) failed to affect the inward current, the magnitude of the peak inward current nearly doubled and more than quadrupled in the presence of 1 and 4 mM [Na\(^{+}\)]\(_{o}\), respectively. These results suggest that the LVA is carried by T-type Ca\(^{2+}\) channels and does not appear to be contaminated by Ca\(^{2+}\) influx through either classical Na\(^{+}\) channels (Cole et al., 1997) or a separate class of Ca\(^{2+}\) channels that are sensitive to TTX (Aggarwal et al., 1997; Sha et al., 2003).
calculated by WinMAXC software (version 2.5, Chris Patton, http://www.stanford.edu/~cpatton/downloads). Fig. S2 shows mean data comparing the effects of STX and TTX in the absence (black columns) or presence (light gray columns) of EDTA on T-type $\text{Ca}^{2+}$ currents elicited by the expression of all three $\text{Ca}_3$ subunits. Since TTX produced no significant effect on $\text{Ca}_3.1$ and $\text{Ca}_3.2$ (Figs. 6 and 7), the effects of EDTA in the presence of this toxin were not tested on these clones. Please note that each column represent the mean ± SEM % inhibition of $I_{\text{CaT}}$ evaluated with or without EDTA; each column had its own control so that the effects of the toxin would not be due to EDTA per se. For all three $\text{Ca}_3$ subunits, EDTA exerted no significant effect on any of the responses of $I_{\text{CaT}}$ to STX or TTX described in this report. These results argue against a possible contribution of heavy metal contaminants and suggest that the differential Na$^+$ channel toxins on T-type $\text{Ca}^{2+}$ channels are authentic.

**REFERENCES**


**Figure S2.** Effects of EDTA on the responses of cloned T-type $\text{Ca}^{2+}$ channels to Na$^+$ channel toxins. The standard protocol used to test the effects of toxins on $I_{\text{CaT}}$ recorded from transfected HEK-293 cells was identical for all the $\text{Ca}_3$ subtypes and consisted of 100-ms steps to $-40 \text{ mV}$ from HP = $-90 \text{ mV}$. For all column pairs (black and light gray), the data were collected from different cells. As explained in the text, for each column showing the effects of TTX or STX, with or without Ni$^{2+}$, the control (no toxin) and test (toxin alone or toxin + Ni$^{2+}$) solutions both lacked EDTA (black) or both contained 100 µM EDTA (light gray). Each column represents a mean ± SEM % block of $I_{\text{CaT}}$ ($n = 4–8$) by toxin alone or toxin in the presence of Ni$^{2+}$. The numbers in parentheses reflect the number of experiments. (A–C) Pooled data obtained from HEK-293 cells transfected respectively with $\text{Ca}_3.1$, $\text{Ca}_3.2$, or $\text{Ca}_3.3$ as indicated. Unpaired Student’s t tests for each column pair in all three panels revealed no significant differences between control and 100 µM EDTA groups (P > 0.05).