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Block of High-Threshold Calcium Channels by the Synthetic Polyamines sFTX-3.3 and FTX-3.3

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SUMMARY

A polyamine component of *Agelenopsis aperta* spider venom designated FTX is reported to be a selective antagonist of P-type calcium channels in the mammalian brain. Consequently, this component has frequently been used as a pharmacological tool to determine the presence, distribution, and function of P-type channels in physiological systems. We describe antagonism of calcium channels by the synthesized polyamine FTX-3.3, which has the proposed structure of natural FTX. We also examined a corresponding polyamine amide, sFTX-3.3. These polyamines are critically evaluated for antagonism of three high-threshold calcium channel subtypes in rat neurons through the use of the whole-cell patch-clamp technique. FTX-3.3 (IC_{50} = ~0.13 mM) is approximately twice as potent as sFTX-3.3 (IC_{50} = ~0.24 mM) against P-type channels and ~3-fold more potent against N-type channels (FTX-3.3, IC_{50} = ~0.24 mM; sFTX-3.3, IC_{50} = ~0.70 mM). Both polyamines also block L-type calcium channels with similar potencies. sFTX-3.3 (1 mM) and FTX-3.3 (0.5 mM) typically block 50% and 65% of Bay K8644-enhanced L-type current, respectively. Antagonism of each calcium channel subtype is voltage dependent, with less inhibition of Ba^{2+} currents at more-positive potentials. These data show that both sFTX-3.3 and FTX-3.3 antagonize P-, N-, and L-type calcium channels in mammalian Purkinje and superior cervical ganglia neurons with similar IC_{50} values.

Arthropod and molluscan venoms have yielded numerous toxins that have been useful for pharmacological classification of calcium channel subtypes in nerve and muscle cells (1, 2). The availability of selective toxins has facilitated investigations into the functional roles of specific calcium channel subtypes in cellular processes, such as the release of neurotransmitters from nerve terminals and secretion of hormones from endocrine cells (3–7).

One of the first substances used in the study of P-type calcium channels was a polyamine-containing fraction of venom from the American funnel web spider (*Agelenopsis aperta*), designated FTX. Llinás *et al.* (8, 9) determined that FTX blocked a component of calcium channel current resistant to block by dihydropyridines and ω-CgTX-GVIA. This current was classified as P-type because of this unique pharmacology and characteristic association with cerebellar Purkinje neurons. Since then, the peptide toxins ω-Aga-IVA and -IVB have been purified from the same venom (10, 11) and shown to be selective, high affinity antagonists of P-type calcium channels (2, 11, 12).

The early use of FTX to detect the presence of P-type channels in various preparations involved either whole *A. aperta* venom or partially purified polyamine fractions (8, 9, 13–18). Chemical analyses of the FTX fraction have led to the suggestion that the active constituent is an arginine polyamine with a molecular mass of ~200-400 Da. Cherksey *et al.* (13) proposed a putative structure for this constituent, which has been named FTX-3.3. The nomenclature refers to the number of carbon atoms separating the nitrogen atoms along the polyamine chain. The polyamine moiety of FTX-3.3 contains two alkyl units, each consisting of three methylenes (3.3).

In studies of structure-activity relationships, a series of sFTX analogs were produced. These molecules were easier to synthesize than FTX-3.3 and displayed FTX-like effects in some experimental systems (9). One of the most active analogs is sFTX-3.3 [*N*-(7-amino-4-azaheptyl)-L-argininamide], which is also known as arginine polyamine. sFTX-3.3 is essentially the same as FTX-3.3 except that it contains a carbonyl group. FTX and its synthetic analogs were subsequently used for purification and electrophysiological

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ABBREVIATIONS: CgTX, conotoxin; Aga, agatoxin; SCG, superior cervical ganglion; sFTX, synthetic FTX; HVA, high voltage-activated; DRG, dorsal root ganglion; I_{Ba}, calcium channel currents; V_H, holding potential; V_T, test potential; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

characterization of a central nervous system protein proposed to be the P-type calcium channel (13).

The use of FTX fractions or sFTX-3.3 to determine the presence, distribution, and functional role of P-type or P-like calcium channels in physiological systems (9, 17–21) has yielded results that are at times inconsistent with data obtained using peptide toxins specific for P-type channels. For example, Sutton *et al.* (22) reported that sFTX-3.3 blocked ≤60% of calcium channel currents in rat DRG neurons, despite the fact that only 25% of the total current is considered P-type (10, 12). In another study, Brown *et al.* (23) showed that 80% of HVA current in rat neocortical neurons was FTX sensitive, whereas nifedipine-, ω-CgTX-GVIA-, and ω-Aga-IVA-sensitive components accounted for ~30% each. These data suggest that FTX blocks multiple subtypes of HVA current.

The aim of this study was to critically evaluate the effects of both the recently synthesized "natural" FTX (i.e., FTX-3.3) (24) and the carbonyl-containing analog sFTX-3.3 on P-, N-, and L-type calcium channel currents in mammalian neurons that have previously been well defined pharmacologically (2, 10, 12). Accordingly, the effects of these polyamines on calcium channel currents of rat Purkinje and SCG neurons were examined using conventional whole-cell patch-clamp techniques. The results indicate that both polyamines antagonize all three high-threshold calcium channels with little selectivity.

Materials and Methods

Synthesis of sFTX-3.3 and FTX-3.3. Details of the synthesis of polyamines FTX-3.3 and sFTX-3.3 from tribenzyloxycaronyl-L-arginine have been reported previously (24–26). The identities of purified synthetic FTX-3.3 and sFTX-3.3 were confirmed by ^{1}H and ^{13}C NMR spectroscopy, and their chemical structures are shown in Fig. 1.

Cell preparation. Purkinje neurons were acutely dissociated from the cerebellar vermes of 8–12-day-old Sprague-Dawley rats (male or female) using a protocol based on that of Mintz *et al.* (12). Brain tissue was isolated and dissected in an ice-cold solution containing 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 10 mM HEPES, 10 mM glucose, and 0.001% phenol red, pH adjusted to 7.4 with 1 M NaOH. The cerebellar vermis was cut into small pieces (~1 mm^3), transferred into the same high-Na^+/high-K^+ solution containing 3 mg/ml protease XXIII (Sigma Chemical, St. Louis, MO), and then incubated at 37° for 7–8 min. After being rinsed in high-Na^+/high-K^+ solution at 37°, the tissue was transferred to a minimal essential medium (GIBCO, Grand Island, NY) supplemented with 10 mM glucose, 15 mM HEPES, 1 mg/ml bovine serum albumin (Sigma), and 1 mg/ml trypsin inhibitor (Sigma), pH adjusted to 7.4 with 1 M NaOH. Cells were gently triturated by 5–10 passages through the tips of fire-polished Pasteur pipettes and then maintained in the same solution at ~4°. Purkinje neurons were identified by their morphology: large cell bodies (15–25-μm diameter) with a single dendritic stump. The identification of the cells by their distinct morphology has been confirmed previously by labeling of Purkinje neurons with propidium iodide (27). Cells remained viable for whole-cell current recording for ~3-5 hr. There were no significant changes in the time course and inhibition of evoked calcium channel currents in control conditions during this period.

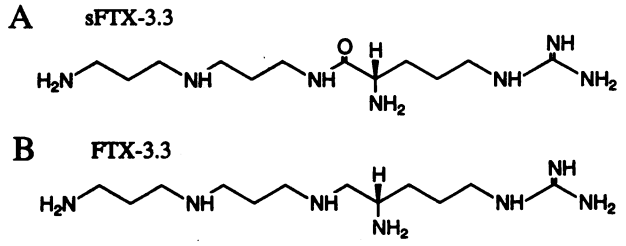

Fig. 1. Structures of synthetic polyamine toxins sFTX-3.3 (A) and FTX-3.3 (B).

Rat SCG neurons were prepared using a protocol modified from that of Bernheim *et al.* (28). Four ganglia were dissected from 16–24-day-old Sprague-Dawley rats in ice-cold Liebovitz's medium (GIBCO). Each ganglion was cut four to six times and incubated in Ca^{2+}-free Tyrode's solution containing 150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES plus 0.5 mM EDTA, 2 mM cysteine, and 25 units/ml Papain (Worthington Biochemicals, Freehold, NJ), pH adjusted to 7.4 with 1 M NaOH, for 20 min at 37°. The ganglia were transferred to Ca^{2+}-free Tyrode's solution containing 2 mg/ml collagenase (Type I, Sigma) and 16 mg/ml dispase (grade II, Boehringer-Mannheim, Indianapolis, IN) for 35 min at 37°. Cells were acutely dissociated by light trituration of the ganglia several times during this incubation and then maintained in the same solution at ~4°. Cells remained viable for whole-cell current recording for ~4-6 hr. Again, there were no changes in the time course and inhibition of evoked calcium channel currents during this period.

Electrophysiological recording. Ionic currents were recorded using conventional whole-cell patch-clamp techniques (29). Patch pipettes with a resistance of 1–3 MΩ were made from Boralex glass (Dynalab, Rochester, NY) and filled with an internal solution of 108 mM cesium methanesulfonate, 4 mM $MgCl_2$, 9 mM EGTA, 9 mM HEPES, 4 mM Mg-ATP, 14 mM creatine phosphate (Tris salt), and 0.3 mM GTP (Tris salt), pH adjusted to 7.4 with CsOH. Patch pipettes were coated with Sylgard (Dow-Corning, Midland, MI), and their tips were heat polished. Pipette-membrane seals were established with cells bathed in Tyrode's solution plus 2 mM $CaCl_2$ and 4 mM $BaCl_2$, and calcium channel currents were recorded with Tyrode's solution replaced with an external solution containing 5 mM $BaCl_2$, 160 mM tetraethylammonium chloride, and 10 mM HEPES, pH adjusted to 7.4 with tetraethylammonium hydroxide. The charge carrier in all experiments was 5 mM Ba^{2+}. The external solution could be changed in <1 sec by moving the cell between continuously flowing solutions from 140-μm-diameter capillary tubes. Cytochrome *c* (1 mg/ml) was included in all external solutions to prevent loss of toxins to the walls of perfusion tubing and the recording chamber. Synthetic ω-CgTX-GVIA was purchased from Bachem California (Torrance, CA), and Bay K8644 was a kind gift from Dr. A. Scriabine (Miles Laboratories, New Haven, CT).

Whole-cell currents were recorded using an Axopatch 200A integrating patch-clamp amplifier (Axon Instruments, Burlingame, CA). Currents were low-pass Bessel filtered at 1–2 kHz and then digitized every 50–100 μsec using the Basic-Fastlab system (INDEC Systems, Sunnyvale, CA). All reported potentials have been corrected for a liquid junction potential of −10 mV between the internal pipette solution and the Tyrode's solution in which the pipette current was zeroed before sealing onto a cell. Calcium channel currents were corrected for leak and capacitative currents by subtracting a scaled current elicited by a 10-mV hyperpolarization from the cell V_H of −80 mV. All experiments were performed at room temperature (21–24°). Time zero in all plots was the time at which evoked currents stabilized after establishing a whole-cell recording.

Results

Actions of sFTX-3.3 and FTX-3.3 on I_{Ba} in Purkinje neurons. High-threshold I_{Ba} was evoked in acutely dissociated rat Purkinje neurons by holding cells at a potential of −80 mV (V_H) and then depolarizing to a V_T of −10 mV for 30 msec every 4–6 sec (Fig. 2, A and B). Exposure of these cells to ω-Aga-IVA at concentrations of ≥50 nM caused 93 ± 3% (values are given as mean ± standard deviation unless oth-

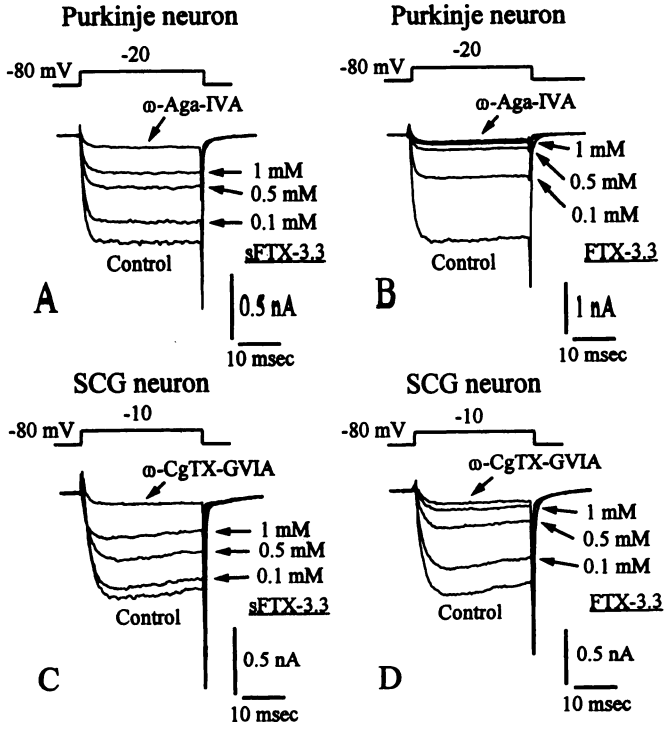

Fig. 2. sFTX-3.3 and FTX-3.3 block high-threshold I_{Ba} in rat Purkinje and SCG neurons in a concentration-dependent manner. I_{Ba} was evoked in Purkinje neurons by 30-msec depolarizations from −80 mV to a V_T of −20 mV. SCG neurons were held at −80 mV, and currents were evoked by 30-msec depolarizations to −10 mV (V_T). A, Block of I_{Ba} in a Purkinje neuron by sFTX-3.3. Application of 0.1, 0.5, and 1 mM sFTX-3.3 inhibited control current by 18%, 50%, and 64%, respectively. ω-Aga-IVA (200 nM) blocked the control current by 89%. B, I_{Ba} inhibition in a Purkinje neuron by FTX-3.3. The application of 0.1, 0.5, and 1 mM FTX-3.3 to another cell inhibited control current by 60%, 86%, and 91%, respectively. ω-Aga-IVA (200 nM) blocked total I_{Ba} by 94%. C, Block of I_{Ba} in SCG neuron by sFTX-3.3. The application of 0.1, 0.5, and 1 mM sFTX-3.3 inhibited control current by 10%, 35%, and 57%, respectively. ω-CgTX-GVIA (1 μM) blocked I_{Ba} by 89%. D, Block of I_{Ba} in a SCG neuron by FTX. The application of 0.1, 0.5, and 1 mM FTX-3.3 to this cell inhibited control current by 26%, 64%, and 81%. Subsequent exposure to 1 μM ω-CgTX-GVIA blocked control current by 88%.

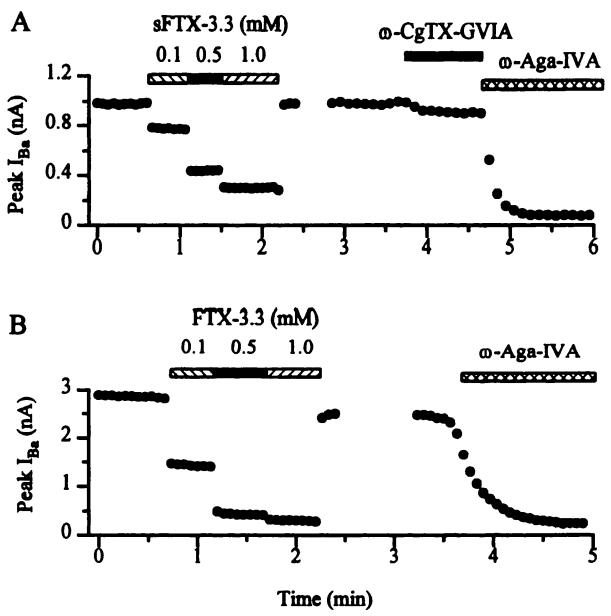

Fig. 3. Block of P-type calcium channels in rat Purkinje neurons by sFTX-3.3 and FTX-3.3 is rapid, reversible, and concentration dependent. A, Trend of the peak amplitude of inward I_{Ba} evoked in a Purkinje neuron by 30-msec depolarizations from −80 mV to −20 mV (V_T) every 4–6 sec. The application of 0.1, 0.5, and 1 mM sFTX-3.3 caused rapid inhibition of I_{Ba} by 21%, 56%, and 69%, respectively. The effects of sFTX-3.3 were rapidly reversed on washing in toxin-free solution. Subsequent exposure of the cell to 1 μM ω-CgTX-GVIA resulted in ~7% block of total I_{Ba}, whereas 200 nM ω-Aga-IVA blocked ~89% of the total I_{Ba}. B, Trend of the peak amplitude of inward I_{Ba} evoked in a Purkinje neuron using the same stimulus protocol as above. The application of 0.1, 0.5, and 1 mM FTX-3.3 to this cell resulted in rapid inhibition of currents by 50%, 85%, and 90%, respectively. Exposure of this cell to 100 nM ω-Aga-IVA caused ~90% inhibition of total inward I_{Ba}.

erwise indicated; 14 neurons) inhibition of I_{Ba}. Typically, the remaining current was abolished by exposure of the cells to ω-CgTX-GVIA and dihydropyridines (data not shown). These observations are consistent with previous reports of calcium channel block in Purkinje neurons (10, 12).

Both sFTX-3.3 and FTX-3.3 rapidly suppressed I_{Ba} in Purkinje neurons (Figs. 2, A and B, and 3, A and B). Inhibition was concentration dependent in both cases, taking <4 sec to reach equilibrium, and was fully and rapidly reversible on washing. For example, Fig. 3, A and B, shows trends of the peak amplitude of I_{Ba} evoked by holding Purkinje neurons at a V_H of −80 mV and stepping to a V_T of −20 mV for 30 msec every 4–6 sec. Sequential application of 0.1, 0.5, and 1 mM sFTX-3.3 to one cell reduced the peak amplitude of I_{Ba} by 21%, 56%, and 69%, respectively (Fig. 3A). However, application of 0.1, 0.5, and 1 mM FTX-3.3 to another cell caused 50%, 85%, and 90% reduction in the amplitude of evoked I_{Ba}, respectively (Fig. 3B). The amplitude of I_{Ba} returned within 5 sec to control levels on switching to toxin-free solution. The subsequent application of 100–200 nM ω-Aga-IVA resulted in ~90% inhibition of the total I_{Ba} in both cases (Fig. 3, A and B).

The concentration dependence of Purkinje neuron calcium channel block by sFTX-3.3 and FTX-3.3 is shown in Fig. 4A. Concentrations of the polyamines required to reach 100% block were not tested because of the large quantities of material required, but 1 mM sFTX-3.3 and FTX-3.3 was typically sufficient to block ≤90% of the total I_{Ba}. The amplitudes of evoked I_{Ba} (V_H = −80 mV, V_T = −20 mV) in the presence of polyamine were measured relative to the amplitudes of current in polyamine-free conditions and plotted versus polyamine concentration. The relationship between relative mean current amplitude and the concentration of sFTX-3.3 was best fit with a logistic curve (*dotted line*) with a Hill slope of 1.2 and half-maximal block achieved with a concentration of ~0.24 mM (14 neurons). Similarly, the relationship between relative current amplitude and the concentration of FTX-3.3 was best fit with a logistic curve (*solid line*) with a Hill slope of 1.1 and half-maximal block achieved with a concentration of ~0.13 mM (14 neurons). Interestingly, FTX-3.3 is approximately twice as potent as sFTX-3.3 at blocking HVA calcium channels in Purkinje neurons. It is also noteworthy that neither polyamine had any obvious effects on the time course of the evoked calcium channel currents or the rate of decay of tail currents (Fig. 2, A and B). This suggests that the rates of HVA calcium channel activation and deactivation in Purkinje neurons are unaffected by these synthetic polyamines.

Actions of sFTX-3.3 and FTX-3.3 on I_{Ba} in SCG neurons. HVA currents were evoked in SCG neurons by holding

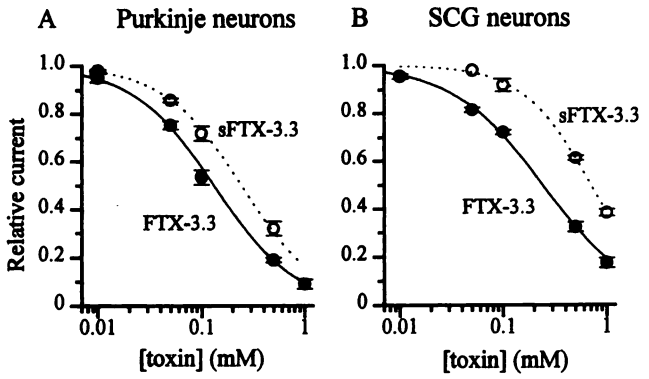

Fig. 4. Concentration-dependent block of I_{Ba} in Purkinje and SCG neurons by sFTX-3.3 and FTX-3.3. Neurons were held at a potential of −80 mV, and I_{Ba} was evoked by depolarizations to either −20 mV (Purkinje neurons) or −10 mV (SCG neurons). *Data points*, mean amplitude of evoked currents in the presence of polyamine relative to the mean amplitude of currents evoked in control conditions (mean ± standard error) plotted versus polyamine concentration. Data points for block of current in Purkinje and SCG neurons by both toxins were best fit with a curve described by the logistic equation $[(I_{max} - I_{min})/(1 + ([toxin]/C)^{nH} + I_{min}]$, where maximal current (I_{max}) was assumed to be 1.0, minimum current after block (I_{min}) was assumed to be 0, C is the concentration of toxin that gives 50% block of current, and n_H is the Hill slope. A, FTX-3.3 and sFTX-3.3 inhibited I_{Ba} in Purkinje neurons with Hill slopes of 1.1 and 1.2, respectively, whereas they block 50% of the current at concentrations of 0.13 and 0.25 mM. Each value was derived from the average amplitude of four to six evoked currents measured only when steady state block was achieved (6–14 cells for FTX-3.3 and 4–14 cells for sFTX-3.3). B, FTX-3.3 and sFTX-3.3 inhibited I_{Ba} in SCG neurons with Hill slopes of 1.0 and 1.3, respectively, whereas they block 50% of the current at concentrations of 0.24 and 0.70 mM (5–10 cells for FTX-3.3 and 4 cells for sFTX-3.3).

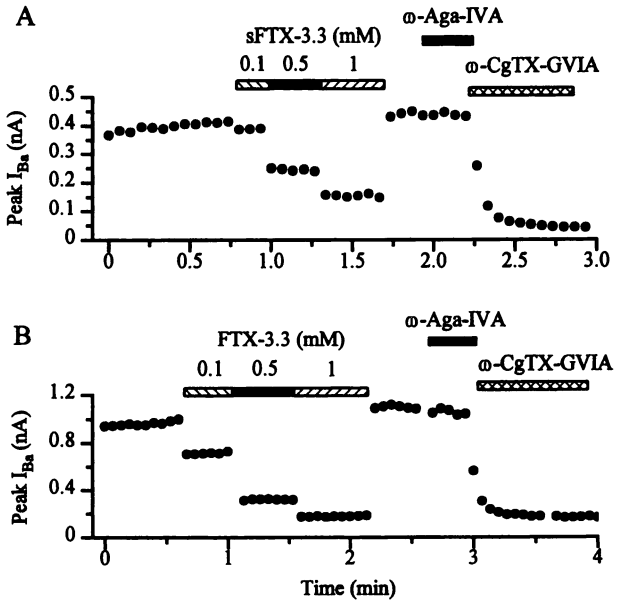

Fig. 5. Block of N-type calcium channels in rat SCG neurons by sFTX-3.3 and FTX-3.3 is rapid, reversible, and concentration dependent. A, Trend of the peak amplitude of inward I_{Ba} evoked in SCG neurons by 30-msec depolarizations from −80 mV to −10 mV (V_T) every 4 sec. Exposure of the SCG neuron to 0.1, 0.5, and 1 mM sFTX-3.3 resulted in inhibition of I_{Ba} by 10%, 41%, and 64%, respectively. The effects of sFTX-3.3 were rapidly reversed on washing. Subsequent exposure of the cell to 400 nM ω-aga-IVA had no effect, whereas 1 μM ω-CgTX-GVIA blocked ~90% of the total evoked current. B, Trend of the peak amplitude of inward I_{Ba} evoked in a SCG neuron using the same stimulus protocol as above. Application of 0.1, 0.5, and 1 mM FTX-3.3 caused inhibition of currents by 27%, 65%, and 80%, respectively. This inhibition was rapidly reversed on washing. The application of 400 nM ω-Aga-IVA had no effect, whereas 1 μM ω-CgTX-GVIA blocked ~86% of the total evoked I_{Ba}.

cells at a potential of −80 mV and depolarizing to a V_T of −10 mV for 30 msec every 4–6 sec (Fig. 2, C and D). Exposure of SCG neurons to 1 μM ω-CgTX-GVIA resulted in 90 ± 3% (12 neurons) block of evoked I_{Ba}. This is consistent with recent reports of N-type calcium channel block by ω-CgTX-GVIA (12, 30). Calcium channel currents evoked in SCG neurons were not sensitive to ω-Aga-IVA at concentrations of ≤400 nM (Fig. 5, A and B), but after exposure to ω-CgTX-GVIA, the remaining current was typically blocked by dihydropyridine antagonists (data not shown).

Evoked I_{Ba} through HVA calcium channels in SCG neurons were suppressed by sFTX-3.3 and FTX-3.3 in a concentration-dependent manner (Figs. 2, C and D, and 5, A and B). The inhibition was rapid, taking <4 sec to reach equilibrium, and was completely reversed on washing. For example, Fig. 5A shows that the amplitude of evoked I_{Ba} in one SCG neuron (V_H = −80 mV, V_T = −10 mV) was suppressed by 10%, 41%, and 64% when the cell was exposed to 0.1, 0.5, and 1 mM sFTX-3.3, respectively, whereas I_{Ba} in another SCG neuron was suppressed by 27%, 65%, and 80% on exposure to 0.1, 0.5, and 1 mM FTX-3.3, respectively. The effects of both polyamines reversed rapidly on switching to polyamine-free solution, after which treatment with 1 μM ω-CgTX-GVIA blocked ~90% of the total I_{Ba} in both cells. These data indicate that the majority of current blocked by FTX-3.3 and sFTX 3.3 is N-type.

As in the case with Purkinje neurons, saturating effects of sFTX-3.3 and FTX-3.3 were not achieved because of the large quantities of material that were needed, although at concentrations of 1 mM, both polyamines typically blocked 65–85% of evoked current. The amplitudes of evoked I_{Ba} (V_H = −80 mV, V_T = −10 mV) in the presence of polyamine were measured relative to the amplitudes of current in polyamine-free conditions and plotted versus concentration (Fig. 4B). Assuming that 100% block was maximal, the relationship between relative mean current amplitude and the concentration of sFTX-3.3 was best fit with a logistic curve (*dotted line*) with a Hill slope of 1.3 and half-maximal block occurring with a concentration of ~0.70 mM (five neurons). The same relationship for FTX-3.3 was best fit with a logistic curve (*solid line*) with a Hill slope of 1.0 and half-maximal block occurring with a concentration of ~0.24 mM (10 neurons). In this case, FTX-3.3 blocked HVA calcium channel currents in SCG neurons with ~3-fold higher potency than sFTX-3.3, which is similar to the relative potency of these polyamines for inhibition of I_{Ba} in Purkinje neurons. Again, no effects of the polyamines were observed on the rates of decay of evoked currents or tail currents, suggesting that inactivation and deactivation of HVA calcium channels in SCG neurons are unaffected by these synthetic polyamines (Fig. 2, A and B).

Voltage-dependent block of I_{Ba} in Purkinje and SCG neurons by sFTX-3.3 and FTX-3.3. Both P- and N-type channels were blocked by sFTX-3.3 and FTX-3.3 in a voltage-dependent manner. This phenomenon was examined by investigating the actions of these toxins on the current-voltage relationship for calcium channels in Purkinje and SCG neurons (Fig. 6, A–D). Purkinje neurons, in which >90% of the

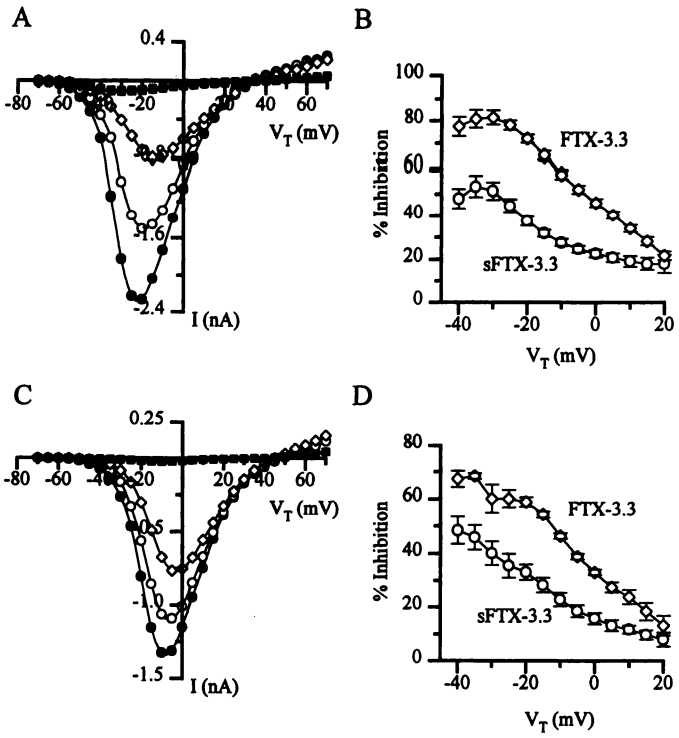

Fig. 6. sFTX-3.3 and FTX-3.3 modify the current-voltage relationship for high-threshold I_{Ba} in Purkinje neurons (A and B) and SCG neurons (C and D). HVA calcium channel currents were evoked by depolarizing cells from a V_H of −80 mV to V_T values ranging from −70 mV to +70 mV in 5-mV increments. The peak amplitudes of evoked currents were plotted as a function of the V_T for control conditions (●) and in the presence of 0.1 mM sFTX-3.3 (○), 0.1 mM FTX-3.3 (◇), or 50 μM Cd^{2+} (■). A, Block of I_{Ba} in Purkinje neurons by sFTX-3.3 and FTX-3.3 is more pronounced at more-negative V_T values. In this cell, 0.1 mM sFTX-3.3 and FTX-3.3 caused the current-voltage curve to shift +3 and +8 mV, respectively. B, Percentage of inhibition of evoked I_{Ba} in five Purkinje neurons by 0.1 mM sFTX-3.3 (○) and FTX-3.3 (◇) plotted as a function of the V_T. C, Both polyamines blocked I_{Ba} in SCG neurons with similar voltage dependence. Block of I_{Ba} is more pronounced at more-negative V_T values. In this cell, 0.1 mM sFTX-3.3 (○) and FTX-3.3 (◇) caused the current-voltage curve to shift +3 and +6 mV, respectively. D, Percentage of inhibition of I_{Ba} in five SCG neurons by 0.1 mM sFTX-3.3 (○) and FTX-3.3 (◇) plotted as a function of the V_T.

high-threshold current is carried through P-type calcium channels, were held at a potential of −80 mV and stepped to V_T values ranging from −70 mV to +70 mV in increments of 5 mV. The peak amplitude of evoked I_{Ba} was plotted as a function of the V_T (Fig. 6, A and C). Block of I_{Ba} by both polyamines was more pronounced at negative potentials than at positive potentials. For example, the application of 0.1 mM sFTX-3.3 to Purkinje neurons caused a 53 ± 11% (six neurons) reduction in the peak amplitude of evoked currents at −35 mV (Fig. 6B), whereas evoked currents at +20 mV were reduced by only 18 ± 10% (six neurons). The peak of the current-voltage curve was shifted ~+3 mV. Also, exposure of Purkinje neurons to 0.1 mM FTX-3.3 reduced the peak amplitude of evoked currents at −35 mV by 81 ± 9% (five neurons), whereas currents at +20 mV were reduced by only 22 ± 4% (five neurons) with the peak of the current-voltage curve shifted ~+10 mV. Application of 50 μM Cd^{2+} to Purkinje neurons caused complete inhibition of evoked inward currents (Fig. 6A).

The effects of sFTX-3.3 and FTX-3.3 on the current-voltage relationship for SCG neuron calcium channels were examined using the same protocol as described above. Again, block of SCG neuron calcium channels by these polyamines was voltage dependent (Fig. 6C). Exposure to 0.1 mM sFTX-3.3 reduced the peak amplitude of evoked currents at −35 mV (Fig. 6D) by 46 ± 9% (four neurons), whereas currents were reduced by only 8 ± 5% (four neurons) at +20 mV. The peak of the current-voltage curve was shifted ~+3 mV. Exposure of SCG neurons to 0.1 mM FTX-3.3 resulted in a 68 ± 2% (four neurons) reduction in the amplitude of evoked currents at −35 mV, whereas currents at +20 mV were reduced by only 13% ± 7% (four neurons) with the peak of the current-voltage curve shifted ~+5 mV. As with Purkinje neurons, the application of 50 μM Cd^{2+} to SCG neurons caused complete inhibition of evoked inward currents (Fig. 6B).

Block of Bay K8644-enhanced I_{Ba} by sFTX-3.3 and FTX-3.3. The effects of sFTX-3.3 and FTX-3.3 on L-type current in SCG neurons were examined in more detail because these polyamines were observed to block I_{Ba} that remained after exposure to saturating concentrations of ω-CgTX-GVIA. Previous studies have established that ~5% of the total high-threshold I_{Ba} in SCG neurons is carried through L-type calcium channels (12). This portion of I_{Ba} was isolated by exposing cells to 1 μM ω-CgTX-GVIA, which selectively blocks N-type calcium channels carrying ~95% of the total I_{Ba}. No sensitivity of these cells to ω-Aga-IVA (≤400 nM) was observed. The amplitude of evoked I_{Ba} (V_H = −80 mV, V_T = −10 mV) increased ≤100% upon subsequent exposure to 3 μM Bay K8644, a dihydropyridine calcium channel agonist that selectively increases the mean open time of L-type calcium channels (Fig. 7, A and B) (32, 33). In addition, tail currents that were elicited when cells were repolarized from −10 mV to −50 mV increased in amplitude, and the time course of their decay was dramatically slowed (Fig. 7, A and B). This effect is unique to L-type current in that Bay K8644 selectively slows the rate of L-type channel deactivation (32, 33).

Bay K8644-enhanced I_{Ba} in SCG neurons was blocked by both sFTX-3.3 and FTX-3.3 in a concentration-dependent manner (Fig. 7, A and B). Fig. 7A shows that exposure of five SCG neurons to 0.1, 0.5, and 1 mM sFTX-3.3 resulted in a reduction in the peak amplitude of Bay K8644-enhanced I_{Ba} (V_H = −80 mV, V_T = −10 mV) by 14 ± 5%, 35 ± 11%, and 50 ± 15%, respectively. In comparison, the application of 0.1, 0.5, and 1 mM FTX-3.3 to another five SCG neurons (stimulus protocol as described above) resulted in block of Bay K8644-enhanced currents by 33 ± 6%, 65 ± 9%, and 87 ± 3%, respectively (Fig. 7B). There was a small amount of cell-to-cell variation observed for the HVA calcium channel block by the polyamines. Although these data suggest block of L-type calcium channels by sFTX-3.3 and FTX-3.3, the current evoked in the presence of Bay K8644 by a depolarization to −10 mV may not be purely L-type.

Bay K8644-enhanced I_{Ba} in SCG neurons was blocked in a voltage-dependent manner by sFTX-3.3 and FTX-3.3. SCG neurons (in the presence of 1 μM ω-CgTX-GVIA and 3 μM Bay K8644) were held at −80 mV and depolarized to V_T values in 10-mV increments. The peak amplitude of evoked Bay K8644-enhanced I_{Ba} was plotted as a function of the V_T. Fig. 7C shows the effects of 1 mM sFTX-3.3 on the current-voltage relationship for Bay K8644-enhanced I_{Ba} in one SCG neuron. The peak of the current-voltage curve was shifted ~+5 mV, whereas the block of current seemed to be voltage dependent. Application of 1 mM sFTX-3.3 inhibited 71% of the peak Bay

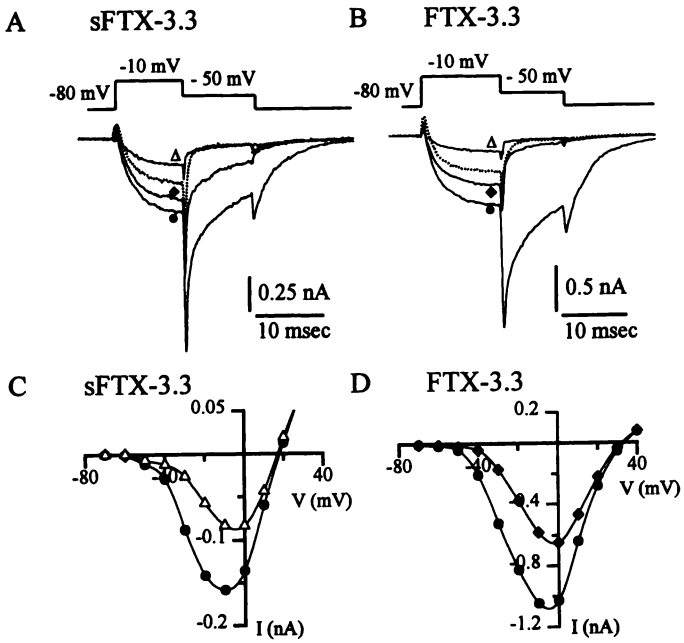

Fig. 7. sFTX-3.3 and FTX-3.3 block Bay K8644-enhanced I_{Ba} in SCG neurons in a concentration- and voltage-dependent manner. Currents were elicited by 10-msec depolarizations from −80 mV to a V_T of −10 mV. Neurons were exposed to 1 μM ω-CgTX-GVIA throughout. A, Application of 3 μM Bay K8644 (○) to one SCG neuron enhanced the amplitude of evoked I_{Ba} at −10 mV and slowed the tail current at −50 mV. *Dotted line*, control current. The application of 0.1 mM (♦) and 1 mM (△) sFTX-3.3 blocked Bay K8644-enhanced current at −10 mV by 18% and 63%, respectively. Tail currents at −50 mV and −80 mV were also suppressed by 0.1 mM sFTX-3.3. B, Another SCG neuron was exposed to 3 μM Bay K8644 (●). *Dotted line*, control current. The application of 0.1 mM (♦) and 1 mM (△) FTX-3.3 inhibited evoked current at −10 mV by 28% and 76%, respectively. Tail currents at −80 mV were almost completely suppressed. Both polyamines modify the current-voltage relationship of Bay K8644-enhanced I_{Ba} in SCG neurons. C, Currents were elicited by depolarizations from −80 mV to a V_T, in increments of 10 mV. Neurons were exposed to 1 μM ω-CgTX-GVIA and 3 μM Bay K8644 throughout. Current amplitudes are plotted as a function of the V_T in control conditions (●) and in the presence of 1 mM sFTX-3.3 (△). sFTX-3.3 shifted the peak of the current-voltage curve by +5 mV, and block was more pronounced at more-negative potentials (see text). D, Current-voltage curves for an SCG neuron in control conditions (●) and in the presence of 0.1 mM FTX-3.3 (♦) were constructed as described above. FTX-3.3 shifted the peak of the current-voltage curve by +5 mV, and block was again more pronounced at more-negative potentials (see text).

K8644-enhanced I_{Ba} at −30 mV, whereas only 25% of the current was inhibited at +10 mV. Fig. 7D shows the effects of 0.1 mM FTX-3.3 on the current-voltage relationship of Bay K8644-enhanced I_{Ba} in another SCG neuron. FTX-3.3 block also seemed to be voltage dependent; 67% of the I_{Ba} was inhibited at −30 mV, whereas only 25% was blocked at +10 mV. Again, it should be noted that a small proportion of the evoked current may be the result of activation of non-P-, -N-, and -L-type calcium channels. Unfortunately, quantification of voltage-dependent antagonism of L-type calcium channels in SCG neurons is complicated because both the polyamines and Bay K8644 exert voltage-dependent actions. For example, enhancement of L-type current by Bay K8644 is more pronounced at negative potentials (2), whereas the polyamines inhibit more current at negative potentials.

A less ambiguous test of L-type calcium channel block in SCG neurons is to observe the effects of the polyamines on Bay K8644-enhanced tail currents. The tail current observed when the cell membrane was stepped from −50 mV to −80 mV includes only Bay K8644-enhanced L-type current (2). Both sFTX-3.3 and FTX-3.3 inhibited the tail currents dramatically at the concentrations applied (0.1–1 mM). In fact, the application of sFTX-3.3 and FTX-3.3 at concentrations of >0.5 mM almost completely inhibited the tail current observed when the cell membrane was stepped from −50 mV to −80 mV. This is consistent with the polyamines blocking L-type calcium channels with greater potency at more-negative potentials.

Discussion

Under the conditions of our experiments, both FTX-3.3 and sFTX-3.3 blocked P-, N-, and L-type I_{Ba} recorded from acutely dissociated rat Purkinje and SCG neurons. Both polyamines blocked P-type channels in Purkinje neurons in a concentration-dependent manner, with FTX-3.3 (IC_{50} = ~0.13 mM) exhibiting approximately twice the potency as sFTX-3.3 (IC_{50} = ~0.24 mM). Likewise, FTX-3.3 and sFTX-3.3 blocked N-type channels in SCG neurons, in which FTX-3.3 (IC_{50} = ~0.24 mM) was ~3-fold more potent than sFTX-3.3 (IC_{50} = ~0.70 mM). These results clearly show that both synthetic polyamines block N-type channels at concentrations similar to those that are effective against P-type channels.

The slightly lower potency of I_{Ba} block in SCG neurons compared with that in Purkinje neurons by these polyamines may be accounted for, at least in part, by the voltage dependence of their effects. I_{Ba} was evoked in SCG neurons by depolarizations to −10 mV compared with depolarizations to −20 mV in Purkinje neurons. Our evidence clearly indicates that FTX-3.3 and sFTX-3.3 block I_{Ba} in these neurons with higher potency at more-negative potentials.

FTX-3.3 and sFTX-3.3 also blocked L-type currents enhanced by Bay K8644 in SCG neurons. At concentrations of 1 mM, FTX-3.3 and sFTX-3.3 blocked Bay K8644-enhanced I_{Ba} by ~85% and ~50%, respectively. Also, Bay K8644-enhanced tail currents that were evoked by stepping the membrane potential from −50 mV to −80 mV were dramatically suppressed by 0.1 mM FTX-3.3 and sFTX-3.3. Thus, both polyamines block L-type calcium channels in SCG neurons with similar potency as N- and P-type calcium channels.

Concentrations of sFTX-3.3 similar to those used in this study (0.5–5 mM) typically have been used in both electrophysiological and biochemical studies of cells to determine the presence or physiological role of calcium channels with P-type channel pharmacology. For example, 1–3 mM sFTX-3.3 was used to block a component of whole-cell calcium channel current in bovine adrenal chromaffin cells (17, 18). From this, it was concluded that these cells possess a component of current carried through channels with P-type pharmacology. In another study, Lundy *et al.* (21) used sFTX-3.3 as a selective P-type channel antagonist. They found that evoked $^{45}Ca^{2+}$ uptake into rat brain synaptosomes was inhibited with the use of 1 and 5 mM sFTX-3.3 by 50% and 80%, respectively (21). Also, it was assumed that P-type calcium channels were present in *Torpedo* synaptosomes because of the inhibitory effects of diluted FTX fractions of *A. aperta* venom and 0.35–3 mM sFTX-3.3 on evoked acetylcholine release (19).

The block of calcium channel currents in Purkinje neurons by FTX-3.3 and sFTX-3.3 that we report here is consistent with data presented by Llinás *et al.* (8, 9, 13), who showed that a purified fraction of *A. aperta* venom containing FTX blocked >90% of the calcium channel current in Purkinje neurons. On the basis of these experiments, FTX was proposed to be a selective P-type channel blocker. However, because the predominant calcium channel current in Purkinje neurons is P-type, such evidence alone is not sufficient to determine subtype selectivity.

Our results showing block of a variety of high-threshold calcium channels by sFTX-3.3 and FTX-3.3 are generally consistent with the observations of Scott *et al.* (34), who found that sFTX-3.3 blocked overlapping components of calcium channel currents in cultured rat DRG neurons and inhibited the binding of [I^{125}]-ω-CgTX-GVIA to rat cortical membranes. Scott *et al.* reported an ~80-fold higher potency for sFTX-3.3 for antagonism of I_{Ba} in DRG neurons than the potency we report for Purkinje and SCG neurons in this study. This discrepancy could be related to differences in cellular preparation; we used acutely dissociated neurons from 8–20-day-old rats compared with the cultured DRG neurons from 2–3-day-old rats used by Scott *et al.* (34). The discrepancy also might be accounted for, in part, by the lower concentration of divalent cations (2 mM Ca^{2+}) used as the charge carrier in the previous study. Increasing the divalent cation concentration (5 mM Ba^{2+} in this study) is known to attenuate the effects of sFTX-3.3 on calcium channels (34).

Other studies of neurons containing substantial components of N- and L-type current have revealed that FTX fractions and sFTX-3.3 block a majority of the total calcium channel current (22, 23, 34). For example, Brown *et al.* (23) determined that nifedipine, ω-CgTX-GVIA, and ω-Aga-IVA block nonoverlapping components of P-, N-, and L-type I_{Ba} in rat neocortical neurons (each accounting for ~30% of the total I_{Ba}), whereas an FTX fraction (1:1000 dilution) and sFTX-3.3 (1 mM) blocked 60–80% of total I_{Ba}. The implication from these data was that the polyamines block multiple components of HVA current in these cells. The proportion of total I_{Ba} blocked by the FTX fraction and sFTX-3.3 in neocortical neurons (23) is similar to the proportion that we find to be sensitive to these polyamines in Purkinje and SCG neurons.

A notable correlation exists between the slightly higher potency of FTX-3.3 compared with sFTX-3.3 and the absence of the carbonyl group present in the latter polyamine (Fig. 1). The addition of a single carbonyl moiety reduces the potency of sFTX-3.3 by ~2-3-fold compared with that of FTX-3.3. Polyamines carry a positive charge on each nitrogen atom at physiological pH, and it has been suggested that these protonated amino groups may be the means by which polyamines bind to membrane proteins or other cellular macromolecules to exert their effects (35). The addition of a single carbonyl group in the structure of FTX-3.3 to produce sFTX-3.3 reduces the basicity of the molecule by decreasing the number of nitrogen atoms that can be protonated and consequently may account for the decreased potency of sFTX-3.3 for blocking calcium channels.

Although there may be a lack of calcium channel subtype selectivity by FTX-3.3 and sFTX-3.3 at the concentrations used here, single-channel studies suggest that different groups of P-type channels can be distinguished by their different sensitivities to low concentrations of the polyamines (IC_{50} = 55 pM to 125 nM) (36). We did not find any components of I_{Ba} in rat Purkinje neurons that were blocked by comparable concentrations of either polyamine. However, it should be noted there were some major differences between this study and that of Dupere *et al.* (36). In our study, whole-cell recordings of I_{Ba} in freshly dissociated Purkinje neurons from 8–12-day-old rats were analyzed, whereas Dupere *et al.* recorded single-channel currents (cell-attached patch conformation) from Purkinje neurons in cerebellar slices of mature rats (35–42 days old). It is possible that P-type channels develop a high affinity FTX-3.3 binding site in mature rats compared with young rats (which were used in the current study). Alternatively, channels with high affinity to the polyamines may run down rapidly, a problem inherent in the whole-cell recording technique, and therefore not contribute to the currents recorded in this study. It is interesting that Usherwood (37) noted similar dramatic differences in the concentrations of a polyamine spider venom toxin (argiotoxin$_{636}$) required to block locust muscle excitatory glutamate receptors. Usherwood reported that low concentrations of argiotoxin$_{636}$ (10 fM to 10 pM) block currents at the single-channel level, whereas much higher concentrations (50 nM to 1 μM) were required to block glutamatergic responses at neuromuscular junctions in the whole-nerve/muscle preparation. Further studies are clearly required to elucidate the basis for such potency differences.

Our results show that both FTX-3.3 and sFTX-3.3 rapidly and reversibly block P-, N-, and L-type calcium channels in rat neurons at concentrations similar to those used in previous studies to identify the presence of P-type calcium channels. Thus, neither FTX-3.3 nor sFTX-3.3 is a likely candidate to account for the highly potent, selective P-type calcium channel block reported to be caused by polyamine fractions of *A. aperta* venom (8, 9, 13, 38), and further studies are required to determine the true structural identity of such a ligand.

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