**Introduction**

Botulinum toxins (BoNT/A – G) are neurotoxins produced by the bacterium *Clostridium botulinum* and one of the most potent naturally occurring poisons (1, 2). Recently, botulinum neurotoxins are attracting considerable attention because they could be useful in the treatment of neuronal hyperexcitability, including epilepsy (3, 4). In vitro studies have provided the first characterization of the effects of BoNT/A and BoNT/E on the central nervous system (CNS) neurons in vivo. It has been shown that intrahippocampal injections of BoNT/E resulted in significant inhibition of seizure activity in experimental models of epilepsy (5, 6). BoNT/A and BoNT/E cleave synaptosomal-associated protein of 25 kDa (SNAP-25), BoNT/C cleaves both syntaxin and SNAP-25, and all other BoNTs act on vesicle-associated membrane protein/synaptobrevin (7). BoNT/A and BoNT/E cleave the same substrate SNAP-25, but they cause synaptic blockade with very different properties. Indeed, neuroparalysis triggered by BoNT/E is short-lived, while the blockade induced by BoNT/A lasts for much longer (8, 9).

Epilepsy is the most common serious neurological

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**Abstract.** Recent studies have demonstrated that the botulinum neurotoxins inhibit the release of acetylcholine, glutamate, GABA, and glycine in central nerve system (CNS) neurons. The Na⁺ current (\(I_{\text{Na}}\)) is of major interest because it acts as the trigger for many cellular functions such as transmission, secretion, contraction, and sensation. Thus, these observations raise the possibility that A type neurotoxin might also alter the \(I_{\text{Na}}\) of neuronal excitable membrane. To test our idea, we examined the effects of A type neurotoxins on \(I_{\text{Na}}\) of central and peripheral neurons. The neurotoxins in femtomolar to picomolar concentrations produced substantial decreases of the neuronal \(I_{\text{Na}}\), but interestingly the current inhibition was saturated at about maximum 50% level of control \(I_{\text{Na}}\). The inhibitory pattern in the concentration–response curve for the neurotoxins differed from tetrodotoxin (TTX), local anesthetic, and antiepileptic drugs that completely inhibited \(I_{\text{Na}}\) in a concentration-dependent manner. We concluded that A type neurotoxins inhibited membrane Na⁺-channel activity in CNS neurons and that \(I_{\text{Na}}\) of both TTX-sensitive and -insensitive peripheral dorsal ganglion cells were also inhibited similarly to a maximum 40% of the control by the neurotoxins. The results suggest evidently that A2NTX could be also used as a powerful drug in treating epilepsy and several types of pain.

**Keywords:** Na⁺ channel, hippocampal CA1 neuron, dorsal root ganglion (DRG) neuron, botulinum toxin, A2 type neurotoxin (A2NTX)
disorder worldwide, affecting about 50 million people (10). Epilepsy can be defined as a paroxysmal disturbance of the CNS, which is associated with excessive synchronous and self-limiting neuronal discharge (11). It is a chronic and often progressive brain disorder, characterized by the periodic and unpredictable occurrence of seizures (10). In the absence of a specific etiological understanding, chronic administration of antiepileptic drugs (AEDs) to suppress seizures is the treatment for epilepsy. However, about 30% of epilepsy patients do not respond to the usual AEDs despite adequate drug treatment (12). Most epileptic seizures are due to discharges generated in cortical and hippocampal structures, although subcortical structures are also involved in some seizure types. The aberrant excitability associated with an epileptic discharge will necessarily be mediated by voltage-gated Na⁺ channels, Ca²⁺ channels, GABA_A receptor, and more recently glutamatergic AMPA and NMDA receptors. Because, the pivotal role is played by these ion channels in the physiology of all forms of epilepsy, they are obvious AED targets. Na⁺ channels are molecular targets of many of the most widely used classical and newer AEDs, like carbamazepine, because they mediate regenerative inward currents that are responsible for the initial depolarization of action potentials in brain neurons. AEDs suppress abnormal neuronal excitability associated with seizures by means of complex voltage- and frequency-dependent inhibition of ionic currents through Na⁺ channels (13). Na⁺ channels mediate regenerative inward currents that are responsible for the initial depolarization of action potentials in brain neurons. In vitro studies have demonstrated that BoNTs can affect the release of several neurotransmitters from CNS neurons (14, 15). Recent studies have provided the first characterization of the effects of BoNT/E on CNS neurons in vivo. It has been shown that BoNT/E injected into the rat hippocampus resulted in significant inhibition of seizure activity in experimental models of epilepsy produced by intrahippocampal kainic acid (3 – 5), suggesting a potential therapeutic use of BoNTs in the CNS. The toxin also reduced behavioral limbic seizures resulting from systemic kainic acid injection (5, 16).

The action of BoNTs in the peripheral nervous system (PNS) has been extensively documented and knowledge gained in this field laid the foundation for the use of BoNTs in human disorders characterized by hyperfunction of peripheral nerve terminals. However, much less is known about the action of BoNTs on the CNS. Especially, the effect of BoNTs on Na⁺ channels that are more closely linked to epilepsy has not yet been reported. In the present study, therefore, to understand the antiepileptic function of type A BoNTs in the CNS we examined how A2 type neurotoxin (A2NTX) and A1LL (BoTX) affect tetrodotoxin (TTX)-sensitive (TTX-S) or TTX-resistant (TTX-R) Na⁺ channels as compared with AEDs, using mechanically dissociated rat hippocampal CA1 and dorsal root ganglion (DRG) neurons using the whole-cell patch recording mode under the voltage-clamp condition.

Materials and Methods

Cell preparation

All experiments were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals Approved by The Japanese Pharmacological Society and approved by the Local Animal Experiment Committee in Kumamoto Health Science University.

In the experiment, 32 Wistar rats were used in total. CA1 pyramidal neurons were isolated from the dorsal site of the rat hippocampus. Wistar rats (12 – 18 days postnatal; Kyudo, Kumamoto) were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and then decapitated. Hippocampi were removed and cut into fine transverse slices (400 μm) with a vibrating slice cutter (VT1200S; Leica, Nussloch, Germany) in a well-oxygenated ice-cold incubation solution. Cut slices containing the CA1 region were recovered in incubation solution saturated well with 95% O₂ and 5% CO₂ at room temperature (21°C – 24°C) for at least 1 h before mechanical dissociation. Mechanically dissociated CA1 neurons were prepared as reported previously (17, 18). In brief, a slice was transferred into a 15-mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) containing normal external solution and the CA1 region was identified under a binocular microscope (SMZ645; Nikon, Tokyo). Mechanical dissociation was accomplished with a fire-polished glass pipette coupled to a vibration device (SI-10 Cell Isolator; K.T. Labs, Tokyo). The tip of the glass pipette was lightly placed on the surface of the CA1 region and vibrated horizontally (0.3- to 0.5-mm displacement) at about 50 – 60 Hz. Within 30 min, the neurons attached to the bottom of the Petri dish and were used for electrophysiological recordings. These dissociated neurons maintained their original morphological features with proximal dendrites of 50 – 100 μm.

DRG were dissected from specimens of Wistar rats. The ganglion mass was dissolved in a well-oxygenated incubation solution containing 0.3% (w/v) collagenase and 0.05% (w/v) trypsin at pH 7.4 for 20 min at 37°C. The ganglion mass was recovered in normal external solution and the surface of the CA1 region and vibrated horizontally (0.3- to 0.5-mm displacement) at about 50 – 60 Hz. Within 30 min, the neurons attached to the bottom of the Petri dish and were used for electrophysiological recordings. These dissociated neurons maintained their original morphological features with proximal dendrites of 50 – 100 μm.

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ocular observation. Details of the dissociation technique have been previously reported (19, 20). After these dissociated neurons had adhered to the Petri dish base (usually within 20 min), electrophysiological measurements were started.

**Electrical measurements**

The voltage-dependent Na+ currents ($I_{Na}$) were recorded in the whole-cell configuration by the use of a conventional patch-clamp technique (21, 22). Neurons were viewed under phase contrast using an inverted microscope (DMIRB, Leica). Macroscopic $I_{Na}$ was measured using a patch-clamp amplifier (Multiclamp 700B; Molecular Devices, Sunnyvale, CA, USA). Patch pipettes were made from borosilicate capillary glass (1.5-mm outer diameter, 0.9-mm inner diameter, G-1.5; Narishige, Tokyo) in two stages on a vertical pipette puller (PP-830, Narishige, Tokyo). The resistance of the recording pipettes filled with internal (patch pipette) solution was 3 – 5 MΩ.

Neuronal electrical activities were continuously monitored on an oscilloscope (DCS-7040; Kenwood, Tokyo) and by a pen recorder (RECTI-HORIT-8K; Sanei, Tokyo). All membrane currents were filtered at 2 kHz with a low-pass filter (E-3201A Decade Filter; NF Electronic Instruments, Tokyo) and stored on a computer using pCLAMP 10.2 (Axon Instruments, Foster City, CA, USA). Hyperpolarizing step pulses (10 mV, 30-ms duration) were used to monitor the access resistance. If the access resistance of neurons changed by more than 20%, the recording was rejected. Neurons were voltage-clamped at a holding potential ($V_h$) of −60 mV throughout the experiments. All experiments were carried out at room temperature (21°C – 24°C) to allow comparison with our previous reports (21, 23).

**Solutions**

Various external solutions were used in the present study. The ionic composition of the incubation solution consisted 124 mM NaCl, 5 mM KCl, 1.2 mM KH$_2$PO$_4$, 24 mM NaHCO$_3$, 2.4 mM CaCl$_2$, 1.3 mM MgSO$_4$, and 10 mM glucose saturated with 95% O$_2$ and 5% CO$_2$. The low Na+ external solution used for recording $I_{Na}$ contained 60 mM NaCl, 100 mM choline-Cl, 5 mM CsCl, 2.5 mM CaCl$_2$, 10 mM glucose, and 10 mM HEPES. Low Na+ external solution used for recording $I_{Na}$ contained 60 mM NaCl, 100 mM choline-Cl, 5 mM CsCl, 2.5 mM CaCl$_2$, 10 mM glucose, and 10 mM HEPES. The low Na+ external solution also contained a Ca$^{2+}$ channel blocker, 10 μM La. All external solutions were adjusted to a pH of 7.4 using Tris-base. The composition of the internal (patch pipette) solution was 110 mM CsF, 30 mM NaF, 5 mM TEA-Cl, 2 mM EGTA, 10 mM HEPES, and 2 mM ATP. The pH of the internal solution was adjusted to 7.2 with Tris-base.

**Preparation of A2NTX**

Botulinum neurotoxins type A (150 kDa, NTX) were prepared using a modification of a previously reported method (24). C. botulinum type A strains 62A and Chiba-H, which belong to subtypes A1 and A2, respectively, were cultured in PYG medium containing 2% peptone, 0.5% yeast extract, 0.5% glucose, and 0.025% sodium thioglycollate by allowing them to stand at 30°C for 3 days. M toxin was purified from the culture fluid by acid precipitation, protamine treatment, ion-exchange chromatography, and gel filtration. Each subtype of M toxin was adsorbed onto a DEAE Sepharose column equilibrated with 10 mM phosphate buffer and eluted with a 0 – 0.3 M NaCl gradient buffer for the separation of NTX from the non-toxic component. The NTXs were stored at −70°C until use. The commercial progenitor LL toxin was purchased (BOTOX®, Allergan, Inc., Irvine, USA: here after A1LL).

**Drugs**

The drugs and chemicals used in the present study were procaine, flunarizine dihydrochloride, phenytoin, lamotrigine, tetraethylammonium (TEA), EGTA, bicineulline, and Mg-ATP purchased from Sigma (St. Louis, MO, USA). TTX was from Wako Pure Chemicals (Osaka). Both A1LL and A2NTX were generous gift from The Chemo-Sero Therapeutic Research Institute (Kumamoto). All test solutions containing drugs were applied by a ‘Y-tube system’ for rapid solution exchange within 20 ms (20, 25).

**Data analyses**

The data were calculated using Clampfit 10.2 (Axon Instruments). Dose–inhibition curves were fitted with Origin Software (OriginLab, Northampton, MA, USA) to a sigmoidal dose–response equation for IC$_{50}$ value determination. Numerical values are provided as the means ± standard errors of the means (S.E.M.) after normalization of the respective control values. Significant differences in mean amplitude were tested by the Student’s paired two-tailed t-test. Statistical significance was defined as a value of $P < 0.05$.

**Results**

**Effect of TTX and A2NTX on TTX-S $I_{Na}$**

TTX-sensitive voltage-dependent Na+ current (TTX-S $I_{Na}$) was recorded in single CA1 neurons using a whole-cell patch recording configuration under voltage-clamp condition. The $I_{Na}$ was separated by use of ion substitution and pharmacological treatments. For the $I_{Na}$ record-
ing, a non-selective Ca\textsuperscript{2+}-channel blocker, 10 µM La, was always added to the external test solution, while a K\textsuperscript{+}-channel blocker, 5 mM TEA–Cl, was added to the internal perfusion solution.

Figure 1Aa shows a typical example of the inhibitory action of TTX on TTX-S \(I_{Na}\). Application of TTX blocked TTX-S \(I_{Na}\) evoked by a depolarizing step from a \(V_{H}\) of –60 to –10 mV in a dose-dependent manner. In the presence of TTX, \(I_{Na}\) was inhibited within 45 s after adding TTX and completely recovered in several minutes by washing out the drug (Fig 1: Aa and B, filled circles).

Figure 1Ab shows a typical current trace of 10 pM A2NTX action on TTX-S \(I_{Na}\). The effect was observed for about 13 min after adding A2NTX. Initially, A2NTX slightly enhanced the amplitude of \(I_{Na}\) (transient, \(t\)) and then gradually suppressed \(I_{Na}\) to a steady-state level (steady, \(s\)) about 11 – 13 min after the application. Eight of ten tested neurons produced almost the same results. However, it was observed that two neurons reached a steady-state level about 15 – 20 min after the toxin application. The \(I_{Na}\) amplitude in the steady-state period decreased to about 40% of the control values. This inhibitory effect was hardly recovered by washing out A2NTX (Fig. 1: Ab and B, open circles).

Figure 2A shows typical current–voltage (\(I–V\)) relationships for the TTX-S \(I_{Na}\) before and during the application of 10 nM TTX or 10 pM A2NTX. When depolarizing pulses of 10-ms duration were applied from a \(V_{H}\) of –60 to +40 mV in 10-mV increments at every 15 s, \(I_{Na}\) was elicited. The threshold potential for the \(I_{Na}\) was around –50 mV and peaked around –20 mV (Fig. 2A, open circles). The decreases of \(I_{Na}\) amplitude by either TTX (gray circles) or A2NTX (filled circles) did not shift the threshold potential, peak current potential, and reversal potential (\(E_{Na}\)) for TTX-S \(I_{Na}\). All six or seven neurons tested had the same results, respectively.

The steady-state inactivation and activation curves for TTX-S \(I_{Na}\) were investigated by using conventional double-pulse and test-pulse protocols (insets) with and without 10 nM TTX or 10 pM A2NTX (Fig. 2B). For the steady-state inactivation curves, the Boltzman fit was used as follows: \(I / I_{max} = 1 / \{1 + \text{exponential} [(V_{1/2} – V) / k]\}\), where \(I_{max}\) is the peak current, \(I\) is the current generated from the step depolarization to –10 mV from each pre-pulse step, \(V\) is each individual pre-pulse, \(V_{1/2}\) is the half-activation value, and \(k\) is the slope constant. Also, the activation curves for the fast-inactivating current was used as follows: \(G / G_{max} = 1 / \{1 + \text{exponential} [(V_{1/2} – V) / k]\}\), where \(G_{max}\) is the peak conductance value, \(G\) is the conductance for each voltage step, \(V\) is the ending voltage level at each step, \(V_{1/2}\) is the half-activation value, and \(k\) is the slope constant.

TTX shifted the steady-state inactivation curve of TTX-S \(I_{Na}\) toward hyperpolarized membrane potentials (Fig. 2B, gray circles). TTX shifted the \(V_{0.5}\) of the inactivation curve by 8.47 mV from –46.68 ± 0.68 to –57.16 ±...
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1.12 mV (n = 6), indicating that the inactivation of Na\(^+\) channel was enhanced in the presence of TTX. On the other hand, TTX showed little influence on the activation curve (\(V_{0.5}\) from \(-31.01 \pm 0.45\) to \(-30.93 \pm 0.73\) mV) (n = 6) (Fig. 2B, gray squares).

The effect of A2NTX was also tested 10 – 13 min after the toxin application at which time the inhibitory effect of A2NTX had reached a steady-state as seen in Fig. 1B. A2NTX also shifted the steady-state inactivation curve of TTX-S \(I_{Na}\) toward hyperpolarized membrane potentials like TTX did (Fig. 2B). A2NTX shifted the \(V_{0.5}\) of the inactivation curve by 6.39 mV from \(-48.65 \pm 0.84\) to \(-55.04 \pm 0.69\) mV (n = 6), indicating that the inactivation of Na\(^+\) channel was enhanced in the presence of A2NTX. However, A2NTX had no effect on the activation curve (\(V_{0.5}\) from \(-30.70 \pm 0.85\) to \(-30.92 \pm 0.33\) mV, n = 6) (Fig. 2B, filled squares).

The results show that the inactivation of TTX-S Na\(^+\) channels was similarly promoted by both TTX and A2NTX.

Effects of AEDs, TTX, and procaine on TTX-S \(I_{Na}\)

Previous reports show that some AEDs inhibit voltage-dependent Na\(^+\) channels (26). Thus, we investigated the action of three kinds of AEDs on TTX-S Na\(^+\) channels.

Application of AEDs of various concentrations significantly inhibited TTX-S \(I_{Na}\) in a concentration-dependent manner. Figure 3 summarizes the concentration-dependent inhibition of three kinds of AEDs on TTX-S \(I_{Na}\) where the order of inhibitory potency was flunarizine > phenytoin > lamotrigine. The IC\(_{50}\) values are 20 \(\mu\)M for flunarizine, 100 \(\mu\)M for phenytoin, and 210 \(\mu\)M for lamotrigine. The inhibitory effects of phenytoin and lamotrigine on TTX-S \(I_{Na}\) were completely reversible, whereas that of flunarizine was partially recovered (data not shown). Application of A2NTX, A1LL, or procaine on single CA1 neurons also blocked TTX-S \(I_{Na}\) in a concentration-dependent manner (Fig. 3). Especially, it was observed that the inhibitory effect of A1LL was almost saturated at concentrations more than 0.1 pM. In this figure, it is evident that both A2NTX and A1LL (BoNT/A) inhibit TTX-S \(I_{Na}\) at extremely low concentrations as compared with AEDs, TTX, and the local anesthetic procaine. In addition, the inhibitory effects of two toxins were almost saturated at less than 50% inhibition level.
Effects of A2NTX on TTX-S and TTX-R $I_{Na}$ in rat DRG neurons

Next we studied whether A2NTX can equally modulate TTX-S and TTX-R $I_{Na}$ in rat isolated DRG neurons like other AEDs that block both TTX-S $I_{Na}$ and TTX-R $I_{Na}$ in DRG neurons (26, 27). Therefore, the effects of TTX on $I_{Na}$ of TTX-S and TTX-R DRG neurons were first examined (Fig. 4A). TTX-S and TTX-R $I_{Na}$ were elicited by 20-ms depolarizing pulse of +10 mV from $V_{th}$ −60 mV. This range was chosen because the threshold for the TTX-S $I_{Na}$ was at around −50 mV and peaked around 0 mV. On the other hand, the threshold for the TTX-R $I_{Na}$ was around −25 to −20 mV and reached its peak when the membrane was depolarized to about 0 to 5 mV (see to Fig. 4D, open circles) as reported by Ogata et al. (28). Application of 300 nM TTX immediately inhibited TTX-S $I_{Na}$ and the maximal inhibition appeared about 15 s after adding TTX (Fig. 4A, left panel). In TTX-R small cells, however, TTX had no effect on the current amplitude and kinetics of TTX-R $I_{Na}$, even at a high concentration of 1 μM TTX at which concentration TTX-S $I_{Na}$ was inhibited almost completely (Fig. 4A).

Figure 4B shows a typical time course of the inhibitory effect of 1 pM A2NTX on the peak amplitude of TTX-R $I_{Na}$. Initially, A2NTX slightly enhanced the amplitude of TTX-R $I_{Na}$ and then gradually suppressed it to a steady-state level 8 – 10 min after the application, as found in CA1 pyramidal neurons. In some neurons, however, it reached to a steady-state level at 13 – 15 min after the application. This effect of A2NTX on TTX-R $I_{Na}$ at the transient ($t$) and steady-state ($s$) periods during the application of A2NTX are shown in the inset of Fig. 4B. In the steady-state period, amplitude was markedly decreased to 65% of the control values. This inhibitory effect was slightly reversible in some cells (30%) but the majority of cells showed no recovery at all. Tested nine neurons showed almost the same results. As shown in Fig. 4C, application of various concentrations of A2NTX (0.1 – 10 pM) significantly and equally reduced the current amplitude of both TTX-S and TTX-R $I_{Na}$ in a dose-dependent manner ($n = 7 – 10$). The reduction of TTX-R $I_{Na}$ amplitude by 1 pM A2NTX was not followed by a shift of either the threshold potential or the potential of peak inward currents in the $I$–$V$ curves (Fig. 4D). The result shows that the 7 tested neurons have the same results. These results indicate that A2NTX inhibits not only CNS Na$^+$ channels but also peripheral TTX-S and TTX-R Na$^+$ channels and that the effects of A2NTX are somewhat stronger on CNS neurons than on peripheral ganglion cells.

Discussion

While they are wonderful drugs, BoNTs are the most poisonous toxins known and the causative agent of botulism. Based on their exquisitely powerful neuroparalytic activity, BoNTs have gained tremendous popularity in the past few years as they became the first biological toxin [BoNT serotype A (BoNT/A)] to receive USA Federal Drug Administration (FDA) approval for the treatment of human disease, most notably for dystonias, blepharospasm, cervical torticollis, neurophic pain, chronic pain, and strabismus, though it has been elevated to the practical rank of “panacea” for a rapidly growing list of conditions involving muscle spasm, not the least of which is its cosmeceutical blockbuster role (29).
Inhibition of Na\(^+\) Currents by A2NTX

However, it is poorly understood how the widely used BoNTs affects neurons. In the present study, therefore, to understand the antiepileptic function and pain modulation of BoNT/A in the CNS, we have examined how A2NTX and A1LL (BoTX/A) affect TTX-S and TTX-R \(I_{\text{Na}}\), and compared the results with already reported AEDs using mechanically dissociated rat hippocampal CA1 and DRG neurons. In the present study, A2NTXs inhibited equally both TTX-S and TTX-R \(I_{\text{Na}}\) in a concentration-dependent manner. BoNT/A acted through the same mechanism on \(I_{\text{Na}}\). However, how the action mechanisms of BoNT/A on \(I_{\text{Na}}\) was not resolved clearly.

To begin to investigate the action mechanism of BoNT/A, we compared its affects with TTX. TTX is known to block \(I_{\text{Na}}\) by binding in the outer vestibule of the channel to be driven in to its inactivation state (30).

In the present study, both TTX and A2NTX had no effect voltage-activation curve while both drugs similarly shifted the steady-state inactivation curve to more negative potential. This suggests some similarity in their actions. However, they differed markedly in their potencies (with BoNT/A 100 – 1000 times more potent), their time course of blocking (TTX fast, A2NTX slow with transient increase), and recovery (A2NTX partly irreversible). Furthermore, A2NTX inhibited equally TTX-sensitive and TTX-resistant currents, indicating BoNT/A cannot simply be binding to the TTX site. The slow onset and partial recovery may suggest any intracellular binding site of the Na\(^+\) channel. This is consistent with the reported ability of BoNT/A to cross the plasma membrane and interfere with vesicle exocytosis (31, 32). Interestingly, the transient facilitation by BoNT/A is similar.
to the time course of translocation of the BoNT/A light chain by the heavy chain (33). BoNT/A have two chains linked by a disulfide bond: an N-terminal light chain of approximately 50 kDa and a heavy chain of approximately 100 kDa. Early studies have shown that BoNT heavy chain forms channels in lipid bilayers (34, 35) and PC12 cells (36). A recent study also shows that the heavy chain of BoNT/A acts as a channel and transmembrane chaperon that enables the translocation of the catalytic light chain into the cytosol (37). Translocation of the BoNT/A light chain by the heavy chain was observed as a transient increase in channel conductance (33), which would lead to membrane depolarization followed by Na⁺ and Ca²⁺ ion entry into the plasma membrane and presynaptic membrane and in turn explain the transient increase in the amplitude of \( I_{Na} \) observed in the present study or in the release of fast transmitters observed in our previous study (see Figs. 1B and 4B) (14). The light chain of BoNTs is normally delivered by their partner heavy chain, but alternative means of delivery, such as liposomes or molecular biology constructs, are also effective (38, 39). These results suggest that BoNT/A may move directly through the cell membrane mediated by their translocation domain heavy chain and can work in the nerve cell interior. Clearly, however, more research is needed to determine molecular and biophysiological mechanisms of action on \( I_{Na} \).

As summarized in Fig. 3, A2NTX, A1LL, phenytoin, lamotrigine, and flunarizine inhibited TTX-S \( I_{Na} \) channels. Also, A2NTX inhibited the TTX-R Na⁺ channels at lower concentrations than previously reported phenytoin and lamotrigine (40, 41). Approximately 1% of the population worldwide suffers from epilepsy characterized by recurrent behavioral seizures. The aberrant excitability associated with an epileptic discharge will be necessarily mediated by Na⁺ channels, Ca²⁺ channels, GABA_A, AMPA, and NMDA glutamate receptors (42). Because of the pivotal role played by these ion channels in the physiology of all forms of epilepsy, they are obvious AED targets since AEDs, including blockers of voltage-gated sodium and calcium channels, agonists of GABA_A receptors, and, more recently, antagonists of AMPA and NMDA glutamate receptors (42). A recent therapeutic approach focuses on the delivery of BoNTs directly into the epileptic seizure focus in the brain and this approach is currently being investigated using animal models (3, 6, 42). Costantin et al. (5) have demonstrated antiepileptic effects of centrally administered BoNT/E in the treatment of focal onset, pharmacologically-resistant epilepsies such as mesial temporal lobe epilepsy. BoNTs also display an anticonvulsant action on chronic seizures in a mouse model of mesial temporal lobe epilepsy (6). According to our recent observation, A2NTX and A1LL are delivered by retro- and anterograde axonal transport (unpublished data). Therefore, there is a renewed interest in novel antiepileptic drugs with long-lasting action that could be delivered directly into the epileptic focus. In this context, intrahippocampal infusion of BoNTs proved very effective in the control of acute limbic seizures (5). Therefore, these previous reports and our results suggest that A2NTX could be used for as a useful drug to treat epilepsy.

AEDs have also been proposed for pain treatment. In pain, Na⁺ channels play a major functional role. Considerable data support the hypothesis that hyperexcitability and spontaneous action potential firings in peripheral sensory neurons mediated by Na⁺ channels contribute to the pathophysiology of chronic pain and neuropathic pain. Especially, TTX-R Na⁺ channels are involved in the development of certain types of neuropathic pains. Most AEDs that are used to alleviate the neuropathic pain are Na⁺-channel blockers (43). AEDs act at several sites that may be relevant to pain, but the precise mechanism of their analgesic effect remains unclear. The AEDs such as phenytoin, carbamazepine, and flunarizine have a long history of use in treatment of chronic pain conditions, particularly neuropathic pain (44). According to several recent reports, these AEDs have been reported to block not only TTX-S \( I_{Na} \) but also TTX-R \( I_{Na} \) in the brain neurons (45) and DRG neurons (27, 41). Luvisetto et al. (46) have also demonstrated antinociceptive effects of centrally administered BoNT/A in a mouse model of inflammatory pain. Incidental reports of painful muscle spasms being relieved by locally administered BoNTs have spurred interest in their use. Of particular interest is BoNT/A as a treatment for chronic pain syndromes of muscular origin. Observations in patients with cervical dystonia treated with BoNT/A reported significant pain relief with associated motor benefit (47, 48). These previous reports may support that BoNT/A also can become a good analgesic for several types of pain.

In summary, the present results have shown that BoNTs such as A2NTX and A1LL inhibited TTX-S and TTX-R \( I_{Na} \) in a concentration-dependent manner. In addition, the inhibitory effect of A2NTX on \( I_{Na} \) was stronger than that of AEDs. These results suggest that A2NTX might be used for as a powerful drug in epilepsy and several types of pain.

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