Neurotoxin A2NTX Blocks Fast Inhibitory and Excitatory Transmitter Release From Presynaptic Terminals

Toshitaka Yamaga1,2, Shuji Aou1, Min-Chul Shin2, Masahito Wakita2, and Norio Akaike2,*

1Department of Brain Science and Engineering, Kyushu Institute of Technology, 2-4 Hibikino, Wakamatsu, Kitakyushu 808-0196, Japan
2Research Division for Life Sciences, Kumamoto Health Science University, Kumamoto 861-5598, Japan

Received July 12, 2011; Accepted November 9, 2011

Abstract. Our recent study showed a possibility that newly developed A2 type botulinum toxin (A2NTX) inhibits both spontaneous and evoked transmitter release from inhibitory (glycinergic or GABAergic) and excitatory (glutamatergic) nerve terminals using rat spinal sacral dorsal commissural nucleus neurons. In the present study, to determine the modulatory effect of A2NTX on glycinergic and glutamatergic release probabilities, we tested the effects of A2NTX on a single inhibitory or excitatory nerve ending adherent to a dissociated neuron that was activated by paired-pulse stimuli by using the focal electrical stimulation technique. The results of the present paired-pulse experiments showed clearly that A2NTX enhanced paired-pulse facilitation of evoked glycinergic inhibitory postsynaptic currents and glutamatergic excitatory postsynaptic currents and increased the failure rate (Rf) of the first postsynaptic currents (P1) and both the responses. These effects of A2NTX on the amplitude and Rf of the P1 and the second postsynaptic currents (P2) and paired-pulse ratio were rescued by application of 4-aminophthalimide. In summary, the present results showed that A2NTX acts purely presynaptically and inhibits the release machinery of transmitters such as glycine and glutamate, and the transmitter release machinery became less sensitive to intracellular free-Ca2+ in A2NTX poisoned nerve terminals.

Keywords: A2NTX, botulinum toxin, glycinergic evoked inhibitory postsynaptic current (eIPSC), glutamatergic evoked excitatory postsynaptic current (eEPSC), paired-pulse ratio
ter release from the GABAergic presynaptic terminal (8, 9) and glutamatergic one (10 – 12). Therefore, to know the modulatory effect of A2NTX on glycinergic and glutamatergic release probabilities, we made whole-cell recordings from the mechanically isolated rat spinal SDCN neurons and hippocampal CA3 pyramidal neurons where a single inhibitory or excitatory nerve ending (bouton) adherent to a dissociated neuron was activated by paired-pulse stimuli with the focal electrical stimulation technique.. In our recent report (4), A2NTX inhibited the glycinergic eIPSCs with an IC50 of 0.016 pM and the glutamatergic eEPSCs with an IC50 of 0.14 pM. Therefore, in the present study, we used equivalent concentrations of 0.01 and 0.1 pM A2NTX, which are close to the IC50 for glycinergic and glutamatergic transmission, respectively.

Material and Methods

Preparation of ‘synaptic bouton preparations’

Wistar rats (12 – 18-day-old) were decapitated under deep pentobarbital anesthesia (50 mg·kg−1, i.p.). A segment of the lumbar sacral spinal cord (L6 – S2) was quickly removed and immersed in an ice-cold incubation medium (see below), saturated with 95% O2 and 5% CO2. Slices at a thickness of 400 μm containing SDCN were prepared with a vibrating microtome (VT 1200S; Leica, Nussloch, Germany). The SDCN slices were then incubated in a medium oxygenated with 95% O2 and 5% CO2 at room temperature (21°C – 24°C) for at least 1 h before mechanical dissociation. For dissociation, slices were transferred into a 35-mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) containing the standard external solution (see below), and the region of the SDCN was identified under a binocular microscope (SMZ645; Nikon, Tokyo). Similarly, the brain slice preparation containing the region of hippocampal CA3 neurons was prepared (7). Full details of the mechanical dissociation procedure have been described previously (4, 5, 7, 13). Mechanically dissociated SDCN or CA3 neurons were allowed to settle and adhere to the bottom of the dish for at least 15 min before recordings. All experiments were performed in accordance with the Guiding Principles for Care and Use of Laboratory Animals in The Japanese Pharmacological Society and approved by the Local Animal Experiment Committee in Kumamoto Health Science University.

Electrical measurements

All electrical measurements were obtained from the soma membrane of isolated SDCN or CA3 neurons in conventional whole-cell patch recording configuration under voltage clamp condition at a holding potential (VH) of 0 mV for glycinergic IPSCs in dissociated SDCN neurons or −65 mV for glutamatergic EPSCs in dissociated CA3 neurons (Multiclamp 700B; Molecular Devices, Sunnyvale, CA, USA) at room temperature (21°C – 24°C), respectively. 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). The resistance of the recording pipettes filled with internal (patch pipette) solution was 3 – 6 MΩ. Isolated neurons were observed under phase contrast on an inverted microscope (Diapont; Nikon, Tokyo). Neurons were continuously monitored on an oscilloscope (DCS-7040; Kenwood, Melrose, MA, USA) and a pen recorder (RECTI-HORIT-8K; Sanei, Tokyo). All membrane currents were filtered at 3 kHz (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 (5 mV, 30-ms duration) were used to monitor the access resistance, and if neurons changed by more than 20%, the recording was rejected.

Paired-pulse focal electrical stimulation of a single glycinergic or glutamatergic bouton using θ glass pipette

Focal electrical stimulation of a single bouton adherent to a mechanically dissociated neuron has been described previously (5, 14). The stimulating pipette was made from a glass tube and filled with normal external test solution. The stimulating electrode was placed closer to the postsynaptic soma membrane of a single SDCN or CA3 neuron while recording. The stimulating pipette was then carefully moved along the surface membrane of soma or dendrites. In individual neurons, subthreshold test current pulses (100 μs duration, 0.1 – 0.3 mA intensity and 30 – 60 ms interval for eIPSCs; 100 μs duration, 0.05 – 0.08 mA intensity and 20 – 30 ms interval for eEPSCs) were applied across the a glass stimulating pipette at a frequency of 1 stim/10 s from an isolator (SS-202J; Nihon Kohden, Tokyo). As the stimulation pipette was moved, glycine-gated outward currents (eIPSCs) and glutamate-gated inward currents (eEPSCs) appeared in all-or-none fashion (14), indicating that the stimulating pipette was positioned just above a single glycinergic or glutamatergic bouton.

Solutions

The ionic composition of the incubation medium consisted of 124 mM NaCl, 5 mM KCl, 1.2 mM KH2PO4, 24 mM NaHCO3, 2.4 mM CaCl2, 1.3 mM MgSO4, and 10 mM glucose saturated with 95% O2 and 5% CO2. The pH was adjusted to 7.4. The standard external solution used for recordings contained 150 mM NaCl, 5 mM KCl,
2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES. All external solutions were adjusted to a pH of 7.4 using Tris-base. The composition of the internal pipette solution for voltage clamp experiments was 5 mM CsCl, 135 mM CsF, 5 mM tetraethylammonium (TEA)-Cl, 2 mM ethylene glycol tetraacetic acid (EGTA), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The pH of the pipette solution was adjusted to 7.2 with Tris-base.

Glycinergic eIPSCs were isolated from GABAergic and glutamatergic ones by using bicuculline, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), d-(2R)-amino-5-phosphono pentan-oate (d-AP5), and intracellular perfusion of ATP-free internal solution at a holding potential (V̄_{H}) of 0 mV that is close to the reversal potential of glutamatergic responses (4). In neurons perfused with ATP-free internal solution, GABA responses could be completely eliminated within 10 – 15 min (15, 16). On the other hand, glutamatergic eEPSCs were isolated from both glycinergic and GABAergic eIPSCs by pharmacologically using strychnine and bicuculline and by maintaining V̄_{H} of −65 mV that is close to Cl⁻ equilibrium potential (ECl).

**Preparation of A2NTX**

A2NTX was cultured and purified using a modification of a previously reported method (17). *C. botulinum* type A stains were cultured in PYG medium containing 2% peptone, 0.5% yeast extract, 0.5% glucose, and 0.025% sodium thioglycolate by allowing them to stand at 30°C for 3 days. The culture fluid was purified to M toxin, which is composed of light and heavy chains of NTX and nontoxic non-HA (NTNH) subunits, by acid precipitation, protamine treatment, ion exchange chromatography, and gel filtration. The M toxin was adsorbed on to a DEAE Sepharose column equilibrated with 10 mM phosphate buffer, and eluted with a 0 – 0.3 M NaCl gradient buffer for A2NTX and nontoxic component separation. The A2NTX was stored at −70°C until use.

**Drugs**

The drugs and chemicals used in the present study were tetraethylammonium (TEA)-Cl, strychnine, CNQX, d-AP5, EGTA, and bicuculline purchased from Sigma (St. Louis, MO, USA). All test solutions containing drugs were applied by a ‘Y-tube system’ for rapid solution exchange within 20 ms (18). A2NTX is a generous gift from The Chemosero Therapeutic Research Institute (Kumamoto).

**Data analyses**

The amplitude, Rf, and PPR of eIPSCs and eEPSCs were analyzed with pCLAMP 10.2 (7). The data were analyzed during A2NTX application over a period of 0 – 5 and 10 – 15 min for eIPSCs, and 5 – 10 and 25 – 30 min for eEPSCs. The effects of drugs on the amplitude and Rf for eIPSCs and eEPSCs were normalized as relative changes from their respective controls. Numerical values are reported as the mean ± standard error of the mean (± S.E.M.) of these normalized values. The significance of drugs was tested by analysis of variance (ANOVA) and post hoc Dunnnett’s test. Possible differences in the current amplitude and Rf distribution were tested by Student’s paired two-tailed t-test. Values of *P* < 0.05 were considered significant.

**Results**

**Effects of A2NTX on the glycinergic eIPSCs**

All the following experiments were carried out with the ‘synaptic bouton preparation’. In this study, the synaptic bouton preparations were obtained from the spinal SDCN region. When focal paired-pulse electrical stimuli were applied to a single glycinergic bouton at each 10 s (0.1 Hz), glycinergic eIPSCs appeared where the first eIPSC (P₁) was followed by the second eIPSC (P₂). The paired-pulse stimulation of presynaptic bouton induced facilitation of eIPSCs in the majority of the boutons used. Therefore, the following experiments were made on glycinergic boutons that induced paired-pulse facilitation (PPF), which is an increase in the second postsynaptic response (P₂) after the first one (P₁). In the presence of 0.01 pM A2NTX, the eIPSC amplitude of P₁ and P₂ gradually decreased to 0.55 ± 0.12 (*P* < 0.01, *n* = 7) and 0.64 ± 0.12 (*P* < 0.01, *n* = 7) of the control at 10 – 15 min after the toxin application (Fig. 1B). However, 0.01 pM A2NTX gradually increased the eIPSC Rf of P₁ to 2.05 ± 0.13 of the control at 10 – 15 min after (*P* < 0.001, *n* = 7) (Fig. 1Ba). The Rf of P₂ also increased to 1.78 ± 0.11 of control (*P* < 0.01, *n* = 7) (Fig. 1Bb). Additionally, application of 30 μM 4-aminophthalimide (4-AP), a nonselective K⁺-channel blocker, at 15 min after addition of A2NTX slightly restored the eIPSC amplitude of P₁ and P₂ (not significant, *n* = 4) and markedly decreased the Rf of P₁ and P₂ (*P* < 0.01, *n* = 4 and *P* < 0.05, *n* = 4, respectively), which were smaller than the control one, respectively.

Figure 2A shows the ratio of *P*₂/*P*₁ (PPR) of eIPSC amplitude. The ratio of *P*₂/*P*₁ ranged between 0.23 and 7.31, and the average value of PPR of control was 1.53 ± 0.09 (*n* = 5). A2NTX gradually increased the PPR to 2.37 ± 0.19 at 10 – 15 min after the toxin application (*P* < 0.05, *n* = 7) (Fig. 2B). The increments of PPR were quickly restored by application of 30 μM 4-AP.
Effect of A2NTX on the glutamatergic eEPSCs

The effect of A2NTX (0.1 pM) on the glutamatergic $P_1$ and $P_2$ eEPSCs were examined using the synaptic bouton preparation dissociated from the CA3 region. All neurons used (6/6) demonstrated PPF in response to focal electrical stimulation of a single glutamatergic bouton. As shown in Fig. 3A, application of A2NTX slowly decreased the eEPSC amplitude of $P_1$ (after 25 – 30 min; 0.3 ± 0.09 of control, $P < 0.05$, $n = 4$) and $P_2$ (after 25 – 30 min; 0.38 ± 0.09 of control, $P < 0.05$, $n = 4$). A2NTX, however, increased the eEPSC Rf of $P_1$ (after 25 – 30 min; 2.97 ± 0.76 of control, $P < 0.05$, $n = 4$) (Fig. 3Ba) and $P_2$ (after 25 – 30 min; 1.33 ± 0.76 of control, $n = 4$) (Fig. 3Bb). These inhibitory effects on eEPSCs appeared more slowly than that on eIPSCs. A similar observation was reported in our previous paper (4). Application of 30 μM 4-AP at 25 min after addition of A2NTX markedly increased the amplitude of $P_1$ and $P_2$ and rescued the Rf of $P_1$ and $P_2$.

Fig. 4A presents PPR of eIPSCs with and without 0.1 pM A2NTX, where the neurotoxin increased the PPR in a time-dependent manner. The average PPR of the control was $1.82 ± 0.18$ ($n = 7$). The PPR increased to $2.53 ± 0.7$ ($P < 0.05$, $n = 4$) at 25 min after adding A2NTX, and it was significantly greater than the control. The results of PPR are summarized in Fig. 4B. In addition, the application of 30 μM 4-AP rescued not only $P_1$,
Presynaptic Inhibition by A2NTX

P2 amplitude and Rf but also PPR of eEPSCs (Fig. 4).

Figure 5A shows how 0.01pM A2NTX affected the appearance of glycinergic P1 and P2 eIPSCs evoked by focal paired-pulse stimulation. The neurotoxin decreased significantly the appearance at one time of both P1 and P2 responses. In the case of glutamatergic eEPSCs, the dis-

Fig. 4. Effect of A2NTX on paired-pulse ratio (PPR) of glutamatergic eEPSCs. A: Time course of glutamatergic PPR before and during the presence of 0.1 pM A2NTX or 30 μM 4-AP. B: The relative PPR obtained from 4 – 6 boutons. *P < 0.05.

Fig. 5. Summary data for changes of failure rate (Rf) on inhibitory and excitatory synaptic responses. A: Rf of glycinergic 4 – 7 boutons to the first P1 (diagonal line), second P2 (gray), and both P1 and P2 (black) pulse at every 10-s stimulation. White space shows no failure responses in both P1 and P2 pulse. Vh was −65 mV. B: Rf of glutamatergic 4 – 6 boutons. Vh was −65 mV.
appearance of either $P_1$ or $P_2$, and the both $P_1$ and $P_2$ of eEPSCs responses increased after adding 0.1 pM A2NTX (Fig. 5B). In addition, the Rf of glutamatergic $P_1$, $P_2$, and both the eEPSCs in the control were smaller than those of glycineric eIPSCs.

Discussion

Paired-pulse depression (PPD) is a common form of short-term synaptic plasticity of GABAergic inhibitory terminals in cultured rat hippocampal neuron (9) and in rat collicular cultures (8). On the other hand, PPF is well known at many excitatory synapses of CNS (12, 19, 20), though PPF shifted to PPD as stimulation frequency was increased from 0.05 to 0.1 and 1 Hz at rat hippocampal unitary CA3-CA3 synapses (11) or extracellular Ca$^{2+}$ concentration was increased from 0.5 to 2 to 5 mM (9). In the present study, paired-pulse stimulation was made every 10 s (0.1 Hz) in external solution containing 2 mM Ca$^{2+}$. In all 7 cells, PPR was characterized by facilitation (PPF). According to He et al. (10), PPD in cultured rat hippocampal neurons was involved in other mechanisms such as inactivation of presynaptic voltage-dependent Na$^+$ channels which influences coupling of action potential to transmitter release. To know the relation of PPR with glycine and glutamate release probability, therefore, PPF was used as an important indication.

In our previous report using the same ‘synaptic bouton’ preparation, A2NTX decreased the frequency of glycineric, GABAergic, and glutamatergic sIPSCs and sEPSCs, and the neurotoxin decreased the glycineric and GABAergic sIPSC amplitude but not glutamatergic amplitude. The neurotoxin also decreased the current amplitude of both eIPSCs and eEPSCs and increased Rf and completely ceased the appearance of these currents with time. Therefore, our previous results suggest that the decrease of the amplitude of spontaneous and evoked postsynaptic currents could be pre- and/or postsynaptic (4). However, the present paired-pulse experiments showed clearly that A2NTX enhanced PPF of glycineric eIPSCs and glutamatergic eEPSCs as shown in Figs. 2 and 4 and increased the Rf of $P_1$ and both the responses (Fig. 5). A-type botulinum toxin truncates SNAP-25 in synaptic terminal and impedes the release of various neurotransmitters such as glycine, glutamate, and so on. (2, 3, 21). Therefore, these data suggest that A2NTX acts purely presynaptically and inhibits the release machinery of transmitters such as glycine and glutamate, as suggested previously (4). However, the defect in filling process of the inhibitory and excitatory neurotransmitters into the presynaptic vesicles also results in the decrease of amplitude of these postsynaptic currents.

4-AP, a nonselective K$^+$-channel blocker, raises the intracellular Ca$^{2+}$ concentration via the activation of voltage-dependent Ca$^{2+}$ channels and rescues the transmission inhibited by A2NTX poisoning (4). Treatment with excess [Ca$^{2+}$], A23187 (Ca-ioniophore), and 4-AP restored the evoked acetylcholine release of skeletal muscle poisoned by A-type neurotoxin (22). These reports well confirm our present results that 4-AP rescued the amplitude and Rf of $P_1$ and $P_2$ postsynaptic currents and PPR, where the transmitter release machinery became less sensitive to intracellular free-Ca$^{2+}$ in A2NTX-poisoned nerve terminals (23, 24).

Acknowledgments

This work was supported by Grants-in-Aid from Kumamoto Health Science University for N. Akaike and M.C. Shin.

References

81

Presynaptic Inhibition by A2NTX


