Short Communication

Intra-axonal Ca^{2+} mobilization contributes to triphenyltin-induced facilitation in glycinergic transmission of rat spinal neurons

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Running title: Triphenyltin and intra-axonal Ca^{2+} mobilization

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Highlights

• TPT increased the frequency of spontaneous glycinergic postsynaptic currents (sIPSC).

• 2-APB (IP3 receptor inhibitor) decreased the sIPSC frequency facilitated by TPT.

• Dantrolene (ryanodine receptor inhibitor) masked the TPT-induced facilitation.

• Thapsigargin (ER Ca^{2+}-pump inhibitor) was more potent than dantrolene.

• TPT is concluded to mobilize intra-axonal Ca^{2+}, resulting in the facilitation of sIPSC.

Abstract

Triphenyltin (TPT) is an organotin compound causing environmental hazard to many wild creatures. Our previous findings show that TPT increases of the frequency of spontaneous glycinergic inhibitory postsynaptic currents (sIPSCs) in rat spinal neurons without changing the amplitude and *1/e* decay time. In our study, the effects of 2-aminoethoxydiphenyl borate (2-APB), dantrolene sodium, and thapsigargin on sIPSC frequency were examined to reveal the contribution of intra-axonal Ca^{2+} mobilization by adding TPT. 2-APB considerably attenuated the TPT-induced facilitation of sIPSC frequency while dantrolene almost completely masked the TPT effects, suggesting that the TPT-induced synaptic facilitation results from the activation of both IP_3 and ryanodine receptors on endoplasmic reticulum (ER) membrane, though inositol triphosphate (IP_3) receptor is less sensitive to TPT. Thapsigargin itself significantly increased the sIPSC frequency without affecting the current amplitude and decay time. Successive addition of TPT could not further increase the sIPSC frequency in the presence of thapsigargin, indicating that thapsigargin completely masked the facilitatory action of TPT. Results suggest that TPT activates the IP_3 and ryanodine receptors while TPT inhibits the Ca^{2+}-pump of ER membranes, resulting in the elevation of intra-axonal Ca^{2+} levels, leading to the increase of spontaneous glycine release from synaptic vesicles.

Key words: triphenyltin; glycinergic spontaneous transmission; intra-axonal Ca stores; synaptic button preparation

1. Introduction

Triphenyltin (TPT) was a biocide (fungicide and antifoulant) used for agricultural and industrial purposes (Yi et al., 2012). Although the use of TPT is illegal in developing countries, its environmental impacts on wild animals are observed (Wu et al., 2010). Because the pollution of seacoast area by TPT is reported in Asia (Meng et al., 2005; Ho and Leung, 2014), its health impact is still concerned in Asian countries. The risk assessment of organotin intake from foods has been carried out in Taiwan, indicating that phenyltin levels in seafood (fish, shellfish, sea vegetable) are higher than butyltin levels (Lee et al., 2016).

TPT poisoning patients have some neurological disorders such as diplopia, drowsiness, giddiness, and vertigo without significant changes in imaging by magnetic resonance and single-photon emission tomography (Lin et al., 1998). In our previous studies, TPT at nanomolar levels facilitates spontaneous and/or miniature synaptic transmissions mediated with glutamate, glycine and GABA (Wakita et al., 2015; Noma et al., 2018). TPT also increases voltage-gated Na^+ current and decreases voltage-gated K^+ current of rat brain neurons (Oyama and Akaike, 1990), suggesting the expression of neuronal excitability.

The TPT-induced facilitation of the frequency of glycinergic spontaneous inhibitory postsynaptic currents (sIPSCs) is independent from Ca^{2+} influx through voltage-gated Ca^{2+} channels but depends on intracellular Ca^{2+} mobilization (Noma et al., 2018). TPT elevates intracellular Ca^{2+} levels of neuronal cells under external Ca^{2+}-free conditions (Oyama et al., 1992). In the Ca^{2+}-free external solution, TPT still increases the glycinergic sIPSC frequency (Noma et al., 2018). Thus, intracellular Ca^{2+} is considered to be an essential factor in the synaptic facilitation by TPT. Endoplasmic reticulum (ER) is also present in neurons and axonal terminals. Ca^{2+} release channels and Ca^{2+} pumps residing in intra-axonal ER membranes control many neuronal functions including synaptic transmissions (Verkhratsky et al., 2005). Disruptions of ER cause Ca^{2+} dyshomeostasis, leading to various forms of neuropathology. Because it is necessary to examine if TPT affects Ca^{2+} releasing channels and Ca^{2+} pumps of ER membranes in nerve endings in

order to characterize the TPT-induced facilitation of synaptic transmission, we examined the effects of 2-aminoethoxydiphenyl borate (2-APB; a blocker of IP_3 receptor), dantrolene sodium (a blocker of ryanodine receptor), and thapsigargin (an inhibitor of Ca^{2+} pump in ER membranes) on the glycinergic sIPSCs without and with TPT.

2. Materials and methods

2.1. Chemicals

TPT was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). TPT was initially dissolved in dimethyl sulfoxide (DMSO) and 300 μM TPT was then added to the external solution to achieve 300 nM TPT. DMSO at 0.1 % did not affect parameters measured in this study.

2.2. Cell preparation – 'synaptic bouton' preparation

The experiments using experimental animals were approved by the Ethics Committee of Kumamoto Kinoh Hospital. Details of the "synaptic bouton" preparation were previously described (Murakami et al., 2002; Akaike and Moorhouse, 2003).

Briefly, Wistar rats (11–23 days old, either sex) were sacrified under anesthesia (intraperitoneal injection of thiopental 50 mg/kg). The spinal cord was removed and immersed in ice-cold oxygenated incubation medium (124 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1.2 mMKH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, and 10 mM glucose) that was saturated with 95% O_2 and 5% CO_2 to adjust pH to 7.4. Spinal cord slices (400 μm thick) were made using a vibrating microtome (Leica VR 1200S, Nussloch, Germany). The glass pipette attached to a vibration device (K.T. Labs S1-10 Cell Isolator, Tokyo, Japan) was horizontally vibrated at 50 Hz on the surface of the slice containing spinal sacral commissural nucleus (SDCN) region. After mechanical dissociation of SDCN neurons, they adhered to culture dish bottom.

2.3. Electrophysiological measurements and data analysis

Glycinergic spontaneous inhibitory postsynaptic currents (sIPSCs) from "synapse bouton preparation" of mechanically isolated spinal SDCN neurons (Murakami et al., 2002; Akaike and Moorhouse, 2003; Noma et al., 2018) were recorded at a holding potential (V_H) of 0 mV and

analyzed under conventional whole-cell patch-clamp mode (Multiclamp 700B; Molecular Devices, Sunnyvale, CA). All experiments were performed at room temperature (21–24 ºC). The resistances of the recording pipettes filled with the internal solution were 3–6 MΩ. All membrane currents were acquired with 20 kHz sampling rate and stored on a computer. Compositions of the solutions for recording the synaptic currents are listed in Table 1. Drugs were applied using a 'Y-tube system' which rapidly changes the external solution around the neurons within 20 ms (Murase et al., 1990).

(Table 1 near here)

In each experiment, currents were analyzed in pre-set epochs before and during test conditions using the MiniAnalysis Program (Synaptosoft, NJ, USA) and Origin Pro 7.5 software (OriginLab Corporation, Northampton, MA, USA). The time-to-peak amplitude and time course of *1/e* decay of individual sIPSCs were analyzed using pCLAMP software (Axon Instruments). Data were tested using one-way ANOVA followed by a post hoc Bonferroni test for multiple comparisons using absolute values. P values of less than 0.05 were considered statistically significant. Numerical data are described as means ± S.E.M. of normalized values.

3. Results

3.1. Effects of 2-aminoethoxydiphenyl borate (2-APB) and dantrolene sodium on sIPSCs

TPT at concentrations of 30, 100 and 300 nM significantly increased the frequency of glycinergic sIPSCs to about 1.4, 2.6 and 10.3 times of control without affecting the current amplitude and *1/e* decay time, respectively (Noma et al., 2018). The effect of 300 nM TPT on the glycinergic frequency is shown in Figure 1A. To see if IP_3 receptor on ER membranes is involved in the TPT-induced increase of sIPSC frequency, the effect of 2-APB, a membrane-permeable blocker for IP_3 receptor (Maruyama et al., 1997; Verkhratsky, 2005), on sIPSC frequency was examined. As shown in Figure 2A, the application of 20 μM 2-APB itself significantly decreased the frequency to 0.61 times ($p < 0.05$, $n = 5$) without changing the amplitude and decay time of sIPSCs. Under the presence of 2-APB, the successive application of 300nM TPT still induced

significant increase of sIPSC frequency to 5.0 times ($p < 0.001$, $n = 5$) (Figure 2Ba) without affecting the amplitude and decay time (Fig. 2Bb, c). But the enhancement ratio was smaller than 10.3 times in control without 2-APB.

(Figures 1 and 2 near here)

Dantrolene inhibits Ca^{2+} release from ER by antagonizing ryanodine receptors (Zucchi and Ronca-Testoni, 1997). Effect of dantrolene was examined to see if the ryanodine receptor contributes to the TPT-induced increase of sIPSC frequency. The application of 20 μM dantrolene (Zhao et al., 2001) slightly, but not significantly, decreased the frequency without affecting the amplitude and decay time of sIPSCs. The successive application of 300 nM TPT induced a little increase ($p < 0.05$, $n = 5$) of sIPSC frequency without affecting the amplitude and decay time (Figure 2C, D). The enhancement ratio decreased from 10.3 time of control to only 1.68 time in the presence of dantrolene. Results on the effects of 2-APB and dantrolene sodium on the frequency in the presence of 300 nM TPT are summarized in Figure 1B.

3.2. Effect of thapsigargin

Thapsigargin is a non-competitive inhibitor of ER membrane Ca^{2+} ATPase (Ca^{2+} pump), causing the elevation of intracellular Ca^{2+} levels by Ca^{2+} release from ER (Thastrup et al., 1990). As shown in Figure 2E and F, the application of 1 μM thapsigargin itself markedly increased the frequency of sIPSCs without changing the amplitude and decay time. In the presence of 1 μM thapsigargin, further increase in sIPSC frequency was not induced by successive application of 300 nM TPT (Figure 2E and F). TPT also did not affect the current amplitude and decay time of sIPSCs in the presence of thapsigargin (Figure 2F). Results show that thapsigargin fully masked the facilitatory effects of TPT on sIPSC frequency.

4. Discussion

It is recognized that synaptic transmitter release is triggered by elevations in intracellular Ca^{2+} concentration near the sites of vesicle fusion (Malenka et al., 1988; Schneggenburger and Neher, 2000). In this study it is likely that the Ca^{2+} release from channels of calcium stores

activated by $InsP_3$ and ryanodine receptors is somewhat attribute to spontaneous release of glycine, because the application of both 2-APB and dantrolene slightly reduced the glycinergic sIPSC frequency (Figures 1 and 2). However, it is unlikely that TPT increases glycinergic transmission via activation of $InsP_3$ and ryanodine receptors because TPT significantly increased the sIPSC frequency even in the presence of 2-APB or dantrolene (Figure 2). In the presence of thapsigargin (Figure 2), however, TPT did not increase the frequency, suggesting that TPT utilizes Ca^{2+} in ER to release the transmitter. TPT at micromolar concentrations was reported to inhibit Ca^{2+}-ATPase and Ca^{2+} uptake of sarcoplasmic reticulum isolated from rabbit skeletal muscles (Kang et al., 1997). Therefore, the action site and mechanism of TPT to elevate intracellular Ca^{2+} level may be similar to that of thapsigargin. Thus, TPT is supposed to facilitate synaptic transmission via the elevation of intracellular Ca^{2+} level through Ca^{2+} release from ER. There is also a possibility that TPT depletes Ca^{2+} in ER, leading to activation of stromal interaction molecule 1 that causes the elevation of intracellular Ca^{2+} concentration via store-operated Ca^{2+} influx (Liou et al., 2005; de Juan-Sanz et al., 2018).

TPT is considered to mobilize intracellular Ca^{2+} by affecting ER in neurons from present and previous results (Oyama et al., 1992; Noma et al., 2018). It is unlikely that the action of TPT is limited in the facilitation of synaptic transmission. Excessive facilitation may deplete transmitter in synaptic vesicles, resulting in attenuation of synaptic transmission. Furthermore, the continuous application of TPT depletes Ca^{2+} from ER that activates the store-operated Ca^{2+} influx (Liou et al., 2005), probably leading to sustained increase in intracellular Ca^{2+} concentration. Intracellular Ca^{2+} is a messenger in cellular signaling, and the excessive elevation of intracellular Ca^{2+} level causes cell death (Brini et al., 2014). Therefore, TPT is thought to cause extensive damages to brain neurons.

Acknowledgments

This study is supported by Grant-in-Aids from both Kumamoto Kinoh Hospital and Kitamoto Hospital for N. Akaike.

Conflict of interest

The authors declare no conflicts of interest in this study.

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Figure legends

Figure 1. TPT-induced change in glycinergic sIPSC frequency in absence (A) and presence (B) of respective inhibitors. Column and bar show mean ± S.E.M. from 5 neurons. *** $p < 0.001$. It is noted that 2-ABP decreased the basal level of frequency of sIPSCs while it was not the case for dantrolene as shown in Figure 2.

Figure 2. Effect of 300 nM TPT on glycinergic sIPSC frequency in the presence and absence of respective inhibitors. (A) Representable current traces of sIPSCs in the presence of 20 μM 2-APB and 2-APB + 300 nM TPT. (B) Effects of TPT on the relative frequency (a), relative amplitude (b) and *1/e* decay time (ms) (c) of sIPSCs with and without 2-APB. (C) Effect of 300 nM TPT on sIPSCs with and without 20 μM dantrolene sodium. (E) Effects of TPT on the relative frequency (a), relative amplitude (b) and *1/e* decay time (ms) (c) of sIPSCs with and without dantrolene. (E) Effect of 300 nM TPT on the sIPSCs with and without 1 μM thapsigargin. (A) Representative current recordings. (B) Effects of TPT on the relative frequency (a), relative amplitude (b) and *1/e* decay time (ms) (c) of sIPSC. All events of a and b were normalized to each control value. Data was obtained from 5 neurons. *** $p < 0.001$, ** $p < 0.01$. *ns*, no significant.

Table 1. Solutions for recording spontaneous currents

Recording Currents	Glycinergic Currents	
	External Solution	Internal Pipette Solution
Composition	150 mM NaCl	5 mM CsCl
	5 mM KCl	135 mM Cs-methanesulfonate
	2 mM $CaCl_2$	5 mM TEA-Cl
	1 mM $MgCl_2$	10 mM EGTA
	10 mM Glucose	10 mM HEPES
	10 mM HEPES	4 mM ATP-Mg
	pH 7.4 Adjusted with Tris base	pH 7.2 Adjusted with Tris base

ATP-Mg; adenosine 5´-triphosphate magnesium salt

EGTA; ethyleneglycol-bis-(α-aminoethylether)-N,N,N´,N´-tetraacetic acid

HEPES; N-2-hydroxyethylpiperazine-N´-2-ethanesulphonic acid

TEA-Cl; tetraethylammonium chloride

Tris base; tris(hydroxymethyl)aminomethane

Figure 1

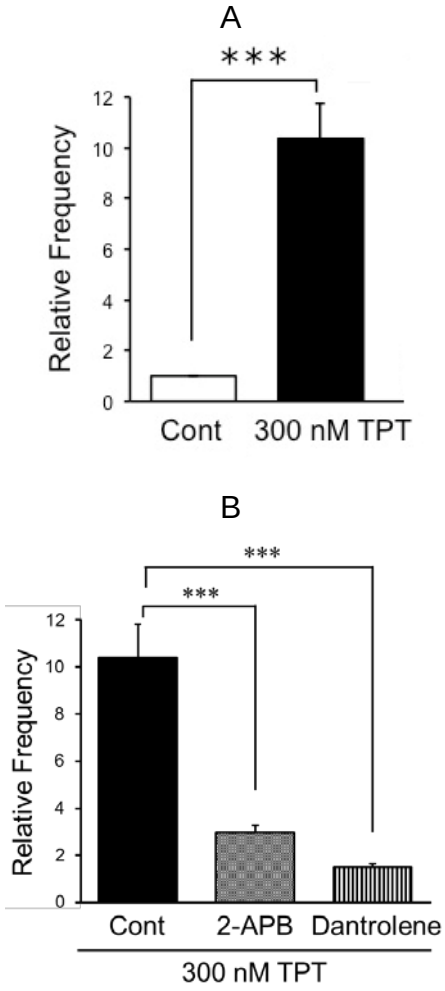
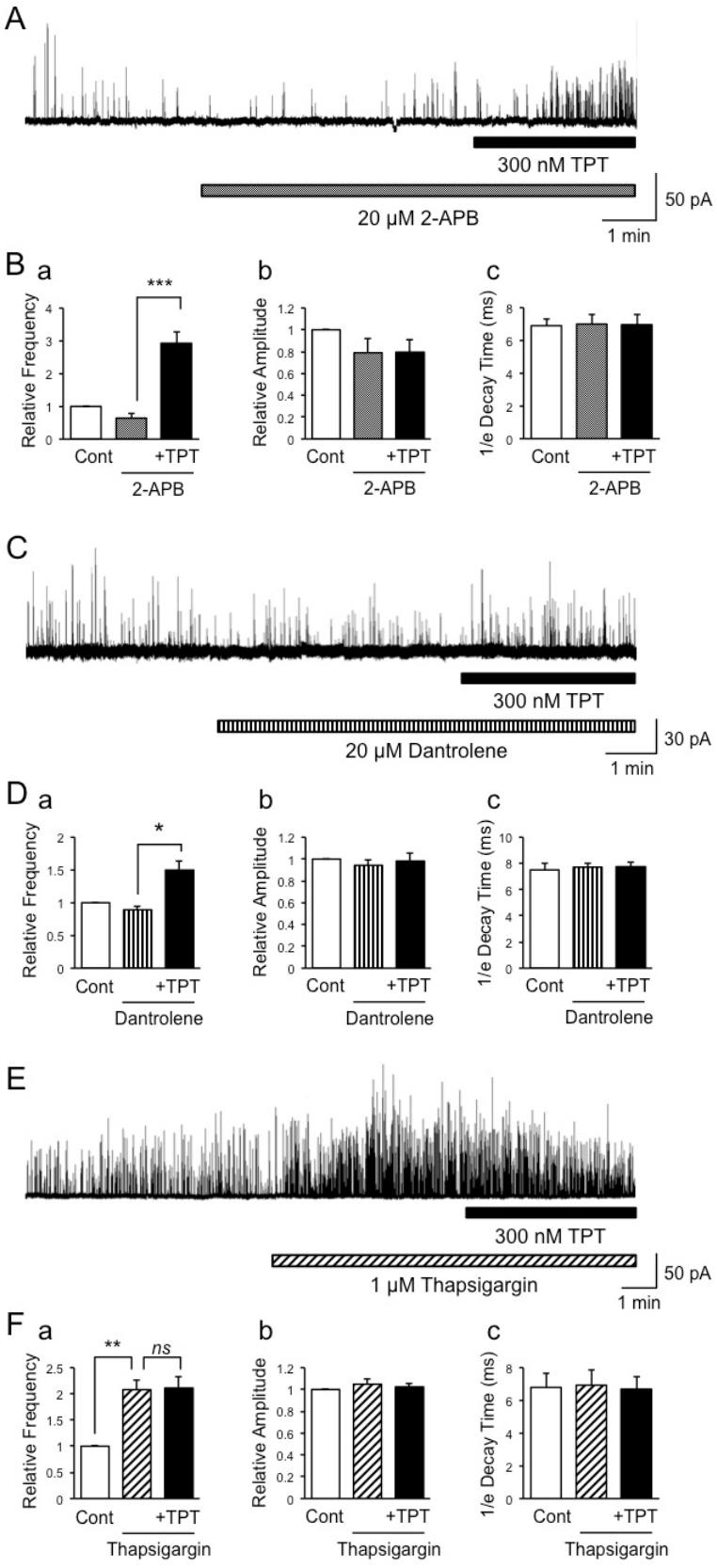

A
***
Relative Frequency
12
10
8
6
4
2
0
Cont
300 nM TPT
B
***
***
Relative Frequency
12
10
8
6
4
2
0
Cont
2-APB
Dantrolene
300 nM TPT

Figure 2


A
300 nM TPT
20 μM 2-APB
50 pA
1 min
B a
***
Relative Frequency
Cont
+TPT
2-APB
b
Relative Amplitude
Cont
+TPT
2-APB
c
1/e Decay Time (ms)
Cont
+TPT
2-APB
C
300 nM TPT
20 μM Dantrolene
30 pA
1 min
D a
*
Relative Frequency
Cont
+TPT
Dantrolene
b
Relative Amplitude
Cont
+TPT
Dantrolene
c
1/e Decay Time (ms)
Cont
+TPT
Dantrolene
E
300 nM TPT
1 μM Thapsigargin
50 pA
1 min
F a
**
ns
Relative Frequency
Cont
+TPT
Thapsigargin
b
Relative Amplitude
Cont
+TPT
Thapsigargin
c
1/e Decay Time (ms)
Cont
+TPT
Thapsigargin