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Enzyme-luminescence method: Tool for real-time monitoring of natural neurotoxins in vitro and L-glutamate release from primary cortical neurons

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ABSTRACT

Novel enzyme-luminescence method is used for the rapid and sensitive in vitro detection of natural neurotoxins (e.g., shellfish and mushroom toxins) using model brain cells. Paralytic shellfish poisons gonyautoxins (e.g., GTX2,3 and GTX1,4) were detected at 1 nM level by their inhibition of glutamate release from C6 glioma cells upon drug stimulation (IC_{50}: GTX2,3 = 30 nM and GTX1,4 = 8 nM). Activation of glutamate release from C6 cells by ibotenic acid (a mushroom toxin) was also evaluated (EC_{50} = 10 nM). The method was tested for real-time detection of glutamate release from primary rat cortical neurons. Dose-dependent effects of KCl (0–200 mM) and NMDA on glutamate release from primary cortical neurons were studied. The effects of different culture conditions on K^+-depolarization-induced glutamate release were also investigated. The method may be applicable to screening of drugs and toxins, and finding glutamatergic neurons in brain slices without in situ staining.

1. Introduction

Marine poisons (e.g., paralytic shellfish poisons) and mushroom toxins (e.g., ibotenic acid) are extremely neurotoxic compounds due to their potent toxicity to the nervous systems of humans. A number of paralytic shellfish poisons (PSPs) are known to be responsible for the inhibition of glutamate release from cells. Ibotenic acid, isolated from Amanita muscaria and Amanita pantherina also stimulates glutamatergic neurons or glial cells through *N*-methyl-D-aspartic acid (NMDA) receptor-mediated elevation of intracellular Ca^{2+}, which can lead to exocytosis of L-glutamate release from cells [1–3]. Glutamate, a principal excitatory neurotransmitter [4,5–7], is responsible for several neurological disorders, including strokes, epilepsy, schizophrenia, Alzheimer's disease, and Parkinson's disease [8,9]. Therefore, the natural toxins such as marine poisons and mushroom toxins can be detected indirectly by in vitro monitoring of extracellular L-glutamate release. The chemical structures of gonyautoxins (e.g., GTX1, GTX2, GTX3, GTX4) and ibotenic acid (a mushroom toxin) are shown in Scheme 1.

Voltage-dependent Na^+ channel is critical for depolarization and conduction in most excitable cells. Paralytic shellfish toxins, a family of potent neurotoxins block the voltage-gated Na^+ channel (VGSC). A schematic illustration of the induction mechanism of the activator (e.g., veratridine (VTRD) which activates voltage-dependent Na^+ channels) and the inhibition mechanism of shellfish toxins (e.g., GTX2,3, GTX1,4 which block $Na_v1.4$) of L-glutamate release from a glutamatergic nerve cell or a glial cell is depicted by numbers in squares in Scheme 2. The activation of the NMDA receptor is also important in the release of neurotransmitters, such as L-glutamate [1–3,10–12]. A schematic representation of the action mechanisms of the NMDA receptor agonists (e.g., ibotenic acid and NMDA) is depicted by numbers in circles in Scheme 2. NMDA and ibotenic acid are specific agonists for the NMDA receptors and they help to increase intracellular concentration of Ca^{2+} ions as well as the release of L-glutamate from cells. However, only a very few studies have been conducted on the effects of ibotenic acid on NMDA receptors on the release of glutamate [1–3]. The effects of NMDA are normally determined using fluorescence microscopy with a calcium indicator [10]. However, this technique is labor intensive as the indicator reagent should be loaded into the cell bodies, and any excess of the reagent should be removed by washing each time.

Several analytical techniques have been proposed to detect natural neurotoxins, such as HPLC with pre- and post-column derivatization [13,14], thin-layer chromatography (TLC) [15], fluorometry [16], cell bioassay [17], flow cytometry [18],

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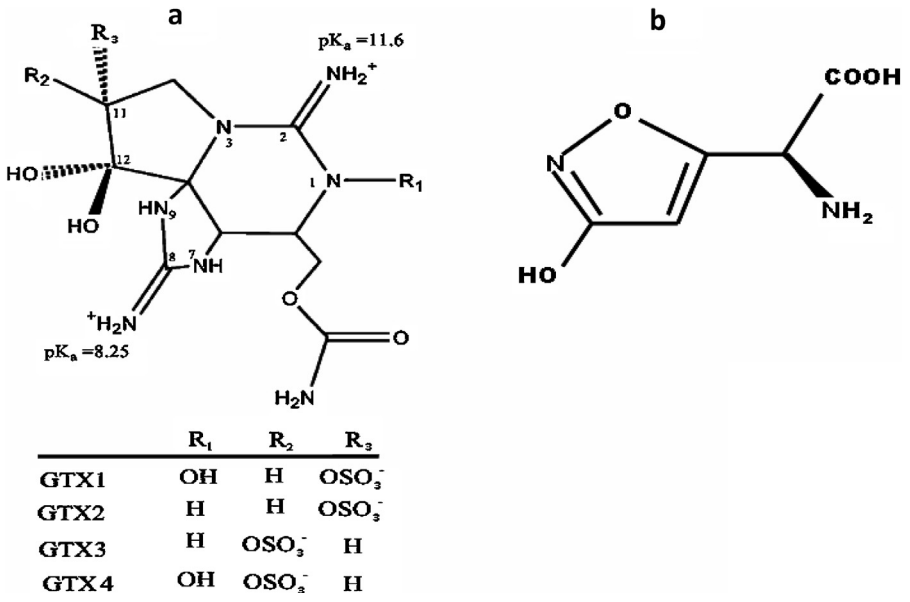
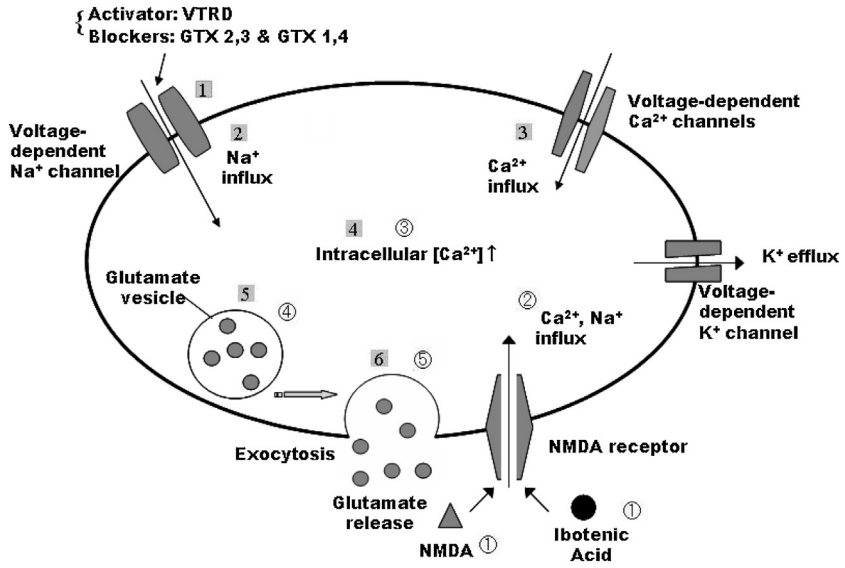


Scheme 1. Chemical structures of (a) paralytic shellfish poisons (e.g., GTX1, GTX2, GTX3, GTX4), and (b) ibotenic acid.



Scheme 2. Schematic representation of mechanisms of the release of L-glutamate from C6 cells by gonyautoxins, a Na^+ channel activator, and NMDA receptor agonists. Veratridine activates voltage-dependent Na^+ channels while ibotenic acid and NMDA activate the NMDA receptor, thereby helping trigger exocytosis of neurotransmitters, such as L-glutamate from the cells through membrane depolarization and the increase of the intracellular Ca^{2+} concentration. Shellfish toxins (e.g., GTX2,3 and GTX1,4) block voltage-dependent Na^+ channels inhibiting the release of L-glutamate.

neurophysiological methods [19], capillary electrophoresis [20,21], hemolysis [22], electrochemical methods [23], electrophoretic method [24], and ELISA [25,26]. An ELISA test kit for detecting saxitoxin in shellfish is also commercially available (RIDASCREEN® Saxitoxin and RIDASCREEN® FAST Saxitoxin; r-Biopharm AG). The pros and cons of these methods is outlined in Table 1. Although all

Table 1
Pros and cons of conventional and enzyme luminescence methods.

Method of analysis	Pros and cons
1. Conventional techniques: HPLC, thin-layer chromatography (TLC), fluorometry, cell bioassay, flow cytometry, neurophysiological methods, capillary electrophoresis, hemolysis, Elisa, electrochemical methods, and electrophoretic method	Not simple and easy to operate, lower sample throughput, labor intensive nature, highly selective and sensitive, real-time monitoring is limited due to the long time required for analysis, staining of the cells with dye is required for some methods, relatively poor spatial resolution
2. Enzyme-luminescence technique	Simple and easy to use, highly selective and sensitive, high sample throughput, good for real-time detection, lower labor costs, applicable for screening of drugs and toxins, has potential in finding glutamatergic neurons without immunostaining, may be suitable to investigate the effects of various growth factors and chemicals on neuronal differentiation, neurotransmitter dynamics, neurodegeneration, and synaptogenesis