Exocytosis determination of SH-SY5Y single cell stimulated by different stimulants on indium tin oxide (ITO) micro-pore electrode

Hui ZHAO, 1,2 Fan ZHANG, 1 Ling LI, 1 Pingang HE, 1 Yuzhi FANG 1

1. Department of Chemistry, East China Normal University, Shanghai, China
2. Department of Chemistry, Zhoukou Normal University, Zhoukou, China

Key words: amperometric monitoring; microelectrodes; mechanism; response time

ABSTRACT: The human neuroblastoma SH-SY5Y cell line has been used as a model to study mechanisms of neurotransmitter release. In order to study the mechanism of SH-SY5Y single cell exocytosis stimulated by different stimulants, including high K+; 3-(1-nitroso-2-pyrrolidinyl) pyridine and nicotine, a type of indium tin oxide (ITO) micro-pore electrode was used to obtain the corresponding amperometric response time. When the cell is stimulated by 0.1 M K+, almost immediate exocytosis could be detected, due to the rapid depolarization of cell membrane. However, the stimulations with 1 mM nicotine and 3-(1-nitroso-2-pyrrolidinyl) pyridine result in a short delay between stimulation and exocytosis, which can be correlated with the time needed for binding of the stimulant to the nicotinic acetylcholine (ACh) receptor and the induction of post-binding phenomena. Thus, the response time of SH-SY5Y single cell exocytosis is significantly affected by the exocytosis mechanisms.

Introduction

The study of communication between cellular organisms has always been an area of great interest in biological and medical science (Wang et al., 2009; Bukoreshtliev et al., 2013). As one of the communication methods among cells, exocytosis plays an important role in biological processes (Burgyne and Morgan, 2003). According to the pathway, exocytosis can be divided into constitutive exocytosis and regulated exocytosis (Gerber and Sudhof, 2002). In regulated exocytosis, following appropriate stimulation, intracellular vesicles containing neurotransmitters fuse with cell membrane and release their contents into extracellular space (Pickett and Edwardson, 2006). The steps of regulated exocytosis are controlled by multiple biological and physicochemical factors (Amatore et al., 2000; Amatore et al., 2006; Amatore et al., 2007).

Quantitative and kinetic detection of these chemical messengers with developing methods is a significant task for understanding the mechanisms and functions of chemical communications and for revealing life activities, and it has attracted a tremendous amount of attention throughout the last few decades, in both the physical and the life science, to better understand the molecular basis for the physiological and behavioral aspects of organisms (Ge et al., 2010; Omiatek et al., 2010; Watson et al., 2011). Moreover, more and more researchers focus on studying exocytosis in single cells (Jin et al., 2008; Spegel et al., 2008; Zhang et al., 2008; Liu et al., 2011; Marquis et al., 2011; Bae et al., 2012; Ghosh et al., 2013). In fact, the interpretation of statistical results from a collection of cells, even of the same type, is hindered by individual variations in size, shape, biological activity and physiological state.

The pioneering work on monitoring exocytosis in single cells was done by Wightman and his colleagues with carbon fiber electrodes (Kawagoe et al., 1991; Wightman et al., 1995; Troyer and
Electrochemical oxidation of catecholamine released from single cell resulted in a series amperometric spikes which corresponded to the single exocytotic events (Mosharov and Sulzer, 2005; Wang et al., 2009). The carbon fiber electrode exhibits numerous advantages, but it could just collect the signals from a small part of the cell surface and thus, these signals are not representative of the unequal secretory activity of the cell surface. In recent years, ITO electrodes have fascinated researchers (Jin et al., 2009; Choi et al., 2010; Meunier et al., 2011; Shi et al., 2011) because they overcome shortcomings of carbon fiber electrodes, for instance, small active region, difficulty in touching cells appropriately, moreover, the exocytosis determination on ITO electrodes is feasible, accurate and reproducible (Amatore et al., 2006; Zhao et al., 2012).

Cells in the human neuroblastoma SH-SY5Y cell line express several neuronal properties (Vaughan et al., 1995; Webster et al., 2001) and have been widely used as a cellular model to investigate the intracellular mechanisms mediating the actions of drugs on human neurons (Vaughan et al., 1995; Ault and Werling, 2000; Roberts et al., 2001). The SH-SY5Y cell line has also been used as an in vitro model of dopaminergic neurons for testing the effects of parkinsonian neurotoxins (Mclaughlin et al., 2006; Heraud et al., 2008; Das and Tizabi, 2009; Yamakawa et al., 2010; Ham et al., 2012; Brown et al., 2013).

The SH-SY5Y cells with multiple receptors are electrically excitable (Webster et al., 2001). They synthesize, store and release norepinephrine from large, dense cored vesicles in a quantal manner and can be evoked by reagents such as high K+, nicotine, bradykinin, dimethylphenylpiperazinium iodide, carbachol, muscarinic agonists, veratridine, polygodial and the calcium ionophore A23187 (Vaughan et al., 1993a; Vaughan et al., 1993b; Vaughan et al., 1995; Andres et al., 1997; Goodall et al., 1997; Webster et al., 2001; Agis-Torres et al., 2002). SH-SY5Y cells exhibit norepinephrine-containing large dense-cored vesicles (LDCVs) and Ca2+-dependent release of norepinephrine (Goodall et al., 1997).

Previous studies involving mechanisms of the SH-SY5Y cells exocytosis have been carried out using radiochemical methods (Murphy et al., 2001; Webster et al., 2001; Amano et al., 2006; Mathieu et al., 2010) which require sampling from a population of cells and can not provide the time resolution needed to follow the dynamics of exocytosis at single cells. In this study, ITO micro-pore electrode is used to monitor the exocytosis by SH-SY5Y single cells, which were stimulated by different stimulants (high K+ solution, nicotine and 3-(1-nitroso-2-pyrrolidinyl) pyridine). Importantly, different stimulants have led to different exocytosis response times in our study, which should correspond to different mechanisms of action.

Material and methods

Chemicals and materials

All chemicals used for standards and buffers were obtained from Sigma Chemical Co. (St. Louis, MO, USA) or Aladdin Chemistry Co. Ltd (Shanghai, China), unless otherwise stated. Cell culture chemicals and media were obtained from Gibco (Grand Island, USA). The ITO glass and photoresist (RZJ-304-25mpa.s) were purchased from Kaivo Electronic Co. Ltd (Zhuhai, China) and Ruihong Corp (Suzhou, China), respectively. Device fabrication and characterization.

The ITO micro-pore electrode was developed as follows: first, the insulating positive photoresist was spin-coated on the cleaned ITO surface with ~5 µm in thickness, and then the photoresist was exposed to UV light. The micro-pore (40 µm in diameter) was formed after development. Second, the substrate was baked at 130 °C for 3 min to remove the solvent. An electrolytic well was fixed on ITO micro-pore electrode, which could be assembled and removed easily, and could also be used repeatedly.

The ITO micro-pore electrode was electrochemically characterized by measurements in a buffered saline solution (150 mM NaCl, 4.2 mM KCl, 2 mM CaCl2, 0.7 mM MgCl2, 1 mM NaH2PO4, 10 mM HEPES, pH=7.4) and a 0.5 mM norepinephrine solution in buffered saline, at room temperature. Cyclic voltammetry proceeded in the potential range of -0.2-1.0 V (vs. Ag/AgCl (saturated KCl) reference electrode) with scan rate of 50 mV/s (CH Instrument 660C, Chen Hua Instruments Co., China).
The stability of ITO micro-pore electrode was also determined by immersing it in a cell culture medium under a 5% CO2 atmosphere at 37 °C for 3 h. Then, the medium was removed and replaced by 500~600 μL of buffered saline solution. At the potential of 0.78 V vs. Ag/AgCl (saturated KCl) reference electrode, the working electrode was scanned 30 times continuously in the current-time mode with EPC10 USB double patch clamp (model HAKA EPC-10, Germany). The interval was set at 0.1 ms and the period was set at 3 min.

Cell culture

SH-SY5Y cells were generously provided by Prof. Chonggang Yuan (School of Life Science, East China Normal University). The cells were cultured at 37 °C in 5% CO2 incubator, in Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum and 1% of a penicillin/streptomycin solution. The culture medium was renewed every 3 days. After dissociation in 0.05% trypsin-EDTA and centrifugation, cells were resuspended and seeded on the ITO micro-pore electrode. Due to the small dimension of micro-pore electrode, only one or two individual cells could attach on its surface. After 30-60 min, the medium was removed and the cells were washed with 0.1 M phosphate buffered saline (PBS, pH=7.4). Then, 500-600 µL of buffered saline solution were put into the electrolytic well. The cells were prepared to be stimulated by injecting different stimulants in its vicinity through a micropipette.

Electrochemical monitoring of SH-SY5Y single cell exocytosis

Figure 1 shows the photograph of single cell on an ITO micro-pore electrode. The distance between the microcapillary and the cell was controlled by the observation under an inverted microscope (IX51, Olympus, Japan). The amperometric monitoring was performed on the stage of the inverted microscope. A glass microcapillary (20-50 μm in diameter) was placed at a distance of 50~100 μm from the cell with the help of a micromanipulator (MHW-103, Narishige, Japan), through which the stimulants (i.e., high K+ solution, 3-(1-nitroso-2-pyrrolidinyl) pyridine and nicotine) were injected (IM-9B, Narishige, Japan) towards the cell. Norepinephrine released from SH-SY5Y single cell was monitored by a double patch clamp and the adjustable response time was 0.1 ms. The amperometric data were collected with an applied potential +0.78 V vs. Ag/AgCl (saturated KCl) reference electrode. The apparatus was grounded and shielded within a Faraday cage to minimize the electrical noise.

Results

Characterization of ITO micro-pore electrode

An ITO micro-pore electrode in contact with a single SH-SY5Y cell is shown in Fig. 1. The electrochemical characterization of ITO micro-pore electrode is presented in Fig. 2. Obviously, in buffered saline solution, the electrode shows no response; while, in 0.5 mM norepinephrine (in buffered saline solution), the electrode presents a pair of redox peaks, indicating that norepinephrine can be electrochemically oxidized on the ITO micro-pore electrode.
physiological activities.

**FIGURE 2.** Cyclic voltammetric responses of ITO micro-pore electrode in (a) buffered saline solution and (b) 0.5 mM norepinephrine in buffered saline solution.

**Amperometric monitoring of SH-SY5Y single cell exocytosis**

A high quality electrode should have a keenly specific response only to the analytes. In order to testify the specificity of ITO micro-pore electrode in exocytosis monitoring of SH-SY5Y single cell, the amperometric determination was performed in different conditions (Fig. 3). Clearly, without the presence of 0.1 M K+, no matter whether the cell is in the determination system, the amperometric curve is smooth (Fig. 3(a) and (c)). When the solution contains K+ but there are no cells, the electrode still shows no amperometric response (Fig. 3(b)). The signals are only produced by the stimulation of 0.1 M K+ to the cell on the electrode surface (Fig. 3(d)). The results show that the exocytosis of SH-SY5Y single cell would not happen without the stimulation of 0.1 M K+. When the cell is stimulated by 3-(1-nitroso-2-pyrrolidinyl) pyridine and nicotine, similar results are also obtained (data not shown).

**Response time of SH-SY5Y single cell stimulated by different stimulants**

The SH-SY5Y cells provide a convenient model system for investigating the exocytosis mechanisms, by which the second messengers regulate neurotransmitter secretion. In order to study the exocytosis mechanism of SH-SY5Y single cell, three stimulants are utilized in this work, including high K+, 3-(1-nitroso-2-pyrrolidinyl) pyridine and nicotine, and the corresponding response time is ~2 s, ~50 s and ~50 s, as shown in Fig. 4.

**FIGURE 3.** The amperometric curves of ITO micro-pore electrode in different conditions: (a) culture medium; (b) culture medium containing 0.1 M K+; (c) SH-SY5Y single cell on the electrode without the existence of K+; (d) SH-SY5Y single cell on the electrode stimulated by 0.1 M K+.

**FIGURE 4.** Current-time traces of SH-SY5Y single cell exocytosis stimulated by (a) 0.1 M K+; (b) 1 mM 3-(1-nitroso-2-pyrrolidinyl) pyridine; (c) 1 mM nicotine. The injecting time of stimulants into the culture medium is indicated by triangles.

**Discussion**

Ca2+ can accelerate the process of exocytosis (Prada et al., 2007; Sugita, 2008). The entry of Ca2+ into the cytoplasm and subsequent exocytosis can be accomplished in the following ways (Stallcup, 1979): the first and also simplest mechanism is the depolarization of cell membrane, which triggers the influx of extracellular Ca2+ through voltage-sensitive Ca2+ channels (VSCCs) (Kukkonen et al., 1997; Dajas-Bailador et al., 2002). Depolarization may be achieved by electrical stimulation or by elevated extracellular K+ levels, and the response time will last for a short period. Exocytosis of SH-
SY5Y cells is Ca2+-dependent. The opening of Ca2+ channel allows a rapid increase in the intracellular Ca2+ concentration, which can evoke the mobilization of vesicles for exocytosis, and the exocytosis occurs usually in several seconds; the second mechanism involves the binding of the stimulant to the nicotinic acetylcholine (ACh) receptor and the post-binding phenomena that lead to the opening of ion channels, resulting in depolarization of the membrane and influx of Ca2+ through VSCCs, therefore, the response time will be longer than that of the first mechanism. ACh receptor is one of the many receptors expressed by SH-SY5Y cells, and it can regulate several neuronal processes through Ca2+-dependent mechanisms. The versatility of ACh receptor-mediated responses presumably reflects the spatial and temporal characteristics of local changes in intracellular Ca2+ arising from a variety of sources. The mechanism of exocytosis stimulated by 3-(1-nitroso-2-pyrrolidinyl) pyridine and nicotine belongs to the second type. The binding of these two stimulants to the ACh receptor and the receptor conformation on ligand binding makes that the consequent increase of Ca2+ concentration does not occur at a sufficient rate to evoke exocytosis before the VSCCs are opened, leading to the longer response time (Zerby and Ewing, 1996; Dani, 2001; Dajas-Bailador et al., 2002). In conclusion, the exocytosis stimulated by high K+, 3-(1-nitroso-2-pyrrolidinyl) pyridine and nicotine occurs in different time, which is decided by the corresponding mechanisms: the stimulation with high K+ produces almost immediate exocytosis, because the cell membrane can be depolarized faster; in contrast, the exocytosis stimulated by nicotine and 3-(1-nitroso-2-pyrrolidinyl) pyridine responds after a longer time, because this mechanism needs the binding of stimulants to ACh receptor and the receptor conformation on ligand binding. The response time of exocytosis may be an important indicator in intercellular communication by providing another level of information in the processing of neuronal signals. Summarizing, the use of ITO micro-pore electrode allows the study of exocytosis by single cells after stimulation as well as the measurement of subsequent events in a time-resolved manner. It also can be used together with fluorescent or chemiluminescent methods in the study of cell physiological phenomena. Such technique is envisaged to provide new insights into the mechanisms and kinetics of exocytosis.

Acknowledgments

We thank the National Nature Science Foundation of China (Grant No. 21075042) and Professors Lian-Wei Wang (Department of Electronic Engineering, ECNU) and Chong-Gang Yuan (School of Life Science, ECNU, for their generous help.

Reference


from the human neuroblastoma, SH-SY5Y. *Journal of Neurochemistry* 60: 1365-1371.


