Biosensors and Bioelectronics 72 (2015) 10–17

Contents lists available at ScienceDirect

Biosensors and Bioelectronics

journal homepage: www.elsevier.com/locate/bios

An improved functional assay for rapid detection of marine toxins, saxitoxin and brevetoxin using a portable cardiomyocyte-based potential biosensor

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ARTICLE INFO

Article history:
Received 26 January 2015
Received in revised form
31 March 2015
Accepted 12 April 2015
Available online 13 April 2015

Keywords:
Saxitoxin (STX)
Brevetoxin (PbTX-2)
Cardiomyocyte-based potential biosensor
Multisite electrical activity of cardiomyocyte network
Extracelluar field potential (EFP) signals

ABSTRACT

Saxitoxin (STX) and brevetoxin (PbTX-2), which are produced by marine dinoflagellates, are highly-toxic marine toxins targeting separate sites of the α subunit of voltage-dependent sodium channels (VDSCs). In this work, a portable cardiomyocyte-based potential biosensor is designed for rapid detection of STX and PbTX-2. This potential biosensor is constructed by cardiomyocyte and microelectrode array (MEA) with a label-free and real-time wireless 8-channel recording system which can dynamically monitor the multisite electrical activity of cardiomyocyte network. The recording signal parameters, spike amplitude, firing rate and 50% of spike potential duration (SPD_{50}) extracted from extracelluar field potential (EFP) signals of the potential biosensor is analyzed to quantitatively evaluate toxicological risk of STX and PbTX-2. Firing rate of biosensor signals presents high sensitivity to STX with the detection limit of 0.35 ng/ml within 5 min. SPD_{50} shows high sensitivity to PbTX-2 with the detection limit of 1.55 ng/ml within 5 min. Based on the multi-parameter analysis, cardiomyocyte-based potential biosensor will be a promising tool for rapid detection of these two toxins.

1. Introduction

Marine toxins, mainly produced by microalgae, pose a serious threat to human health and environmental safety around the world. Numerous marine toxins, such as saxitoxin (STX) and brevetoxin (PbTX-2), have been demonstrated to disrupt sodium channel function, either by inhibition of sodium current through the channels, or by modifying the activation and inactivation gating processes (Al-Sabi et al., 2006; Gerssen et al., 2010). They are likely to be involved in some ion channel diseases, such as cardiovascular diseases and neurodegenerative diseases. STX and PbTX-2, which are produced by the marine dinoflagellates (Bottein Dechraoui and Ramsdell, 2003; Lefebvre et al., 2008) are highly toxic on the whole ecological system. Through food and water ingestion, human may experience exposure to these toxins and passively take them, resulting in fatal health problems (Assessment, 2005; Hinder et al., 2011; Vale et al., 2008). STX and PbTX-2 exert their biological effects through interactions with sites 1 and 5 of the α subunit of the voltage-dependent sodium channel (VDSC), respectively (Catterall and Gainer, 1985; Poli et al., 1986). VDSC is responsible for sodium current and depolarization phase of action potential in excitable cells, such as cardiomyocytes and neurons. STX is a potent and selective inhibitor of VDSC, which produces a blockade of action potentials (Lipkind and Fozzard, 1994), and may affect cardiac and neural electrophysiological activity. In contrast to STX, PbTX-2 is known as a VDSC activator, which triggers repetitive action potential discharges (Templeton et al., 1989), and leads action potential depression and complete blockade (Huang et al., 1984).

Mouse-bioassay (MBA) is a standard approach for detecting STX and PbTX-2. Despite this method is reliable for regulatory purposes and easy to operate, it is labor-intensive, insensitive and ethically problematic (Okumura et al., 2005). In order to overcome these limitations, alternative methods based on chemical (Oshima, 1995), immunological (Usleber et al., 2001) and cellular (Krishnan et al., 2001; Ruberu et al., 2003; Vilariño et al., 2010) methods have been employed. Among the commonly used analytical methods, liquid chromatography/mass spectroscopy (LC/MS) method has been more accepted in recent years due to its qualitative and

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http://dx.doi.org/10.1016/j.bios.2015.04.028
0956-5663/

quantitative analysis (Humpage et al., 2010). However, LC/MS needed expensive instrumentation and a skilled professional with detailed technical knowledge (Humpage et al., 2010). Immunoassays (Usleber et al., 2001), including radioimmunoassay and ELISA, have reasonably high specificity and sensitivity, but the specific monoclonal antibodies are relatively expensive and extremely difficult to obtain. Unlike analytical methods and immunoassays, functional cell-based assays, which respond to biologically active analytes rather than particular anticipated chemical structures, attract more and more attention recently. Many cell lines have been used to assess the toxic effects of STX and PbTX-2, such as N2A neuroblastoma cells expressing neuronal VDSCs (Manger et al., 1994) and human embryonic kidney cells (HEK cells) transfected with heart or skeletal muscle VDSCs (David et al., 2003; Fairey et al., 2001). These cell-based assays have used endpoints for cytotoxicity, which lose much dynamic and real-time information in toxin action.

Cell-based biosensor, as a new functional cell-based assay, can detect various environmental toxins by non-invasively monitoring physiological parameter changes of the cellular internal and external environment in dynamic and real-time way with high sensitivity and excellent selectivity (Gavello et al., 2012; Liu et al., 2007). Previous work in our group has established two type of cell-based impedance biosensors using cardiomyocyte and neuroblastoma cell for dynamically detection of STX with high sensitivity (Wang et al., 2015; Zou et al., 2015). However, both cell-based impedance biosensors for STX detection were time-consuming at least 24 h after toxin addition due to the detection cellular physiological parameters (cell growth or cardiomyocyte beating). Some details about both biosensors are described in Supplementary materials.

In this study, a portable cardiomyocyte-based potential biosensor is designed with a wearable wireless 8-channel system, which was established by cardiomyocytes and microelectrode array (MEA). Cardiomyocytes grown on MEA have emerged as potential biosensors for rapid detection of ion channel compounds (Cerignoli et al., 2012; Jahnke et al., 2013; Yeung et al., 2009). Theoretically, cardiomyocyte-based potential biosensor can also rapidly detect the toxins that affect the Na^+ channels, such as STX and PbTX-2. Furthermore, the regular extracellular field potential (EFP) signals of cardiomyocyte-based potential biosensors can be also used to quantitatively analyze toxicity. Detailed work will be described in the following sections.

2. Experimental and methods

2.1. Construction of cardiomyocyte-based potential biosensor

To establish the portable cardiomyocyte-based potential biosensors, cardiomyocytes and nano-platinum electroplated MEA are employed (Fig. 1(A)), and the portable wireless 8-channel MEA system is used for noninvasively monitoring electrical activity of cardiomyocytes. Fig. 1(B) illustrates the schematic of cardiomyocyte-based potential biosensor. Cardiomyocytes are cultured on the surface of MEA which is modified with self-assembled monolayer (SAM). When cell–electrode coupling forms, there still exists a nanoscale gap between them filled with trace amount of electrolyte. For electrogenic cells in excitation phase, transient transmembrane potential and ionic current generate and polarize the electrodes by reestablishing the charge distribution at electrode–electrolyte–cell interface. In that way, the electrodes record changed voltage as EFP. Generally, their components originate from the derivative of transmembrane potential and ionic flows including Na^+, Ca^{2+} and K^+ in different phases of action potential (Meyer et al., 2004).

EFP recording is performed by self-developed portable wireless 8-channel MEA system. The recording system is packaged into a 13 cm long, 10 cm wide, and 4 cm high plastic case which weighs about 400 g with battery packs installed (Fig. 1(D)). The recording system consists of three main parts: an 8-channel signal conditioning circuit that amplifiers and filters EFP signals from MEA chip, an embedded Wi-Fi module which integrates a Cortex-M3 microcontroller STM32F205 and a user interface to handle the data captured by STM32F205. Wi-Fi module acts as an access point, and PC or iPad device can wirelessly connect to the module

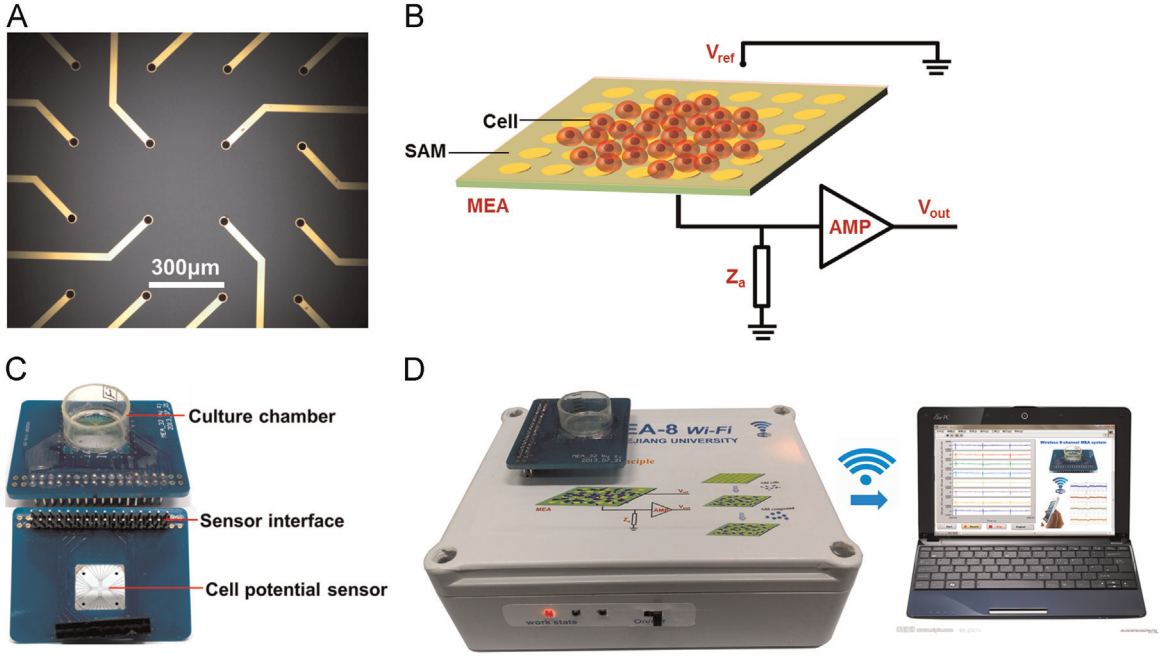

Fig. 1. Construction of the cardiomyocyte-based potential biosensor. (A) The MEA pattern with 32 channels. Electrode diameter is 30 μm and inter electrode distance is 300 μm. (B) The schematic of the cardiomyocyte-based potential biosensor. (C) The MEA sensor chip with a single culture chamber (inner diameter of 19 mm). (D) The wearable wireless 8-channel MEA system.

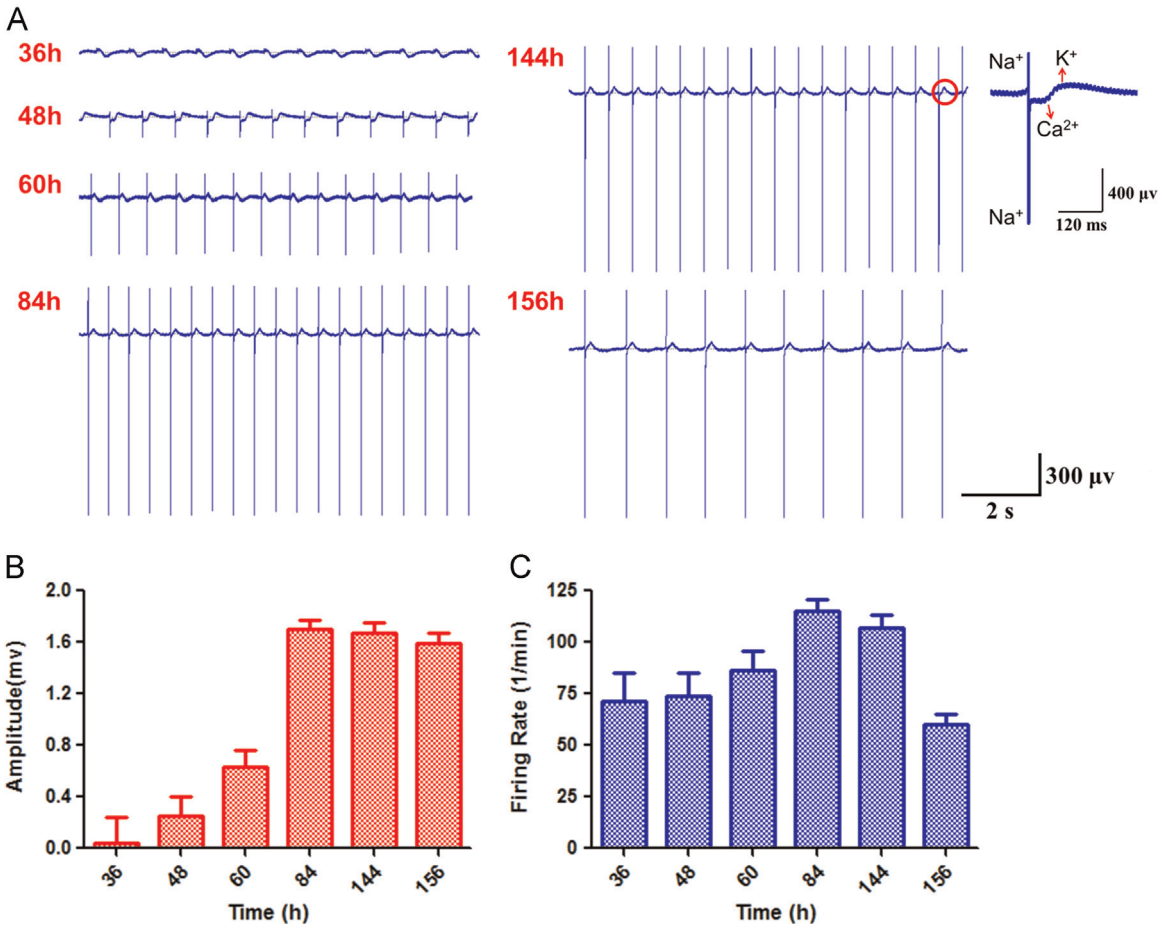

Fig. 2. Dynamic monitoring of spontaneous electrical activity of cardiomyocyte network using the cardiomyocyte-based potential biosensor. (A) Typical changes in EFP pattern over time at six selected time points. Each date segment is 10 s. (B) Spike amplitude of EFP signals at six selected time points. (C) Firing rate of EFP signals at six selected time points. Data represent mean ± STD of six independent experiments from three different isolations.

directly. The A/D conversion for each channel is set to 15 kHz sampling rate, and the up to 54 Mbits per second (Mbps) wireless link easily supports transmission 8 channels of captured cardiomyocyte EFP data synchronously. The sensor chip (Fig. 1(C)), mounted onto sensor interface, is connected to the signal conditioning circuit through a zero-insertion-force socket. Cardiomyocyte EFP signals are bandpass filtered from 0.5 Hz to 4 kHz and are amplified with a total voltage gain of 60 dB. The input-referred noise level of the system is maintained within 20 μV, which can easily afford the measurement of EFP from cardiomyocyte network. Since the packets are transmitted over the wireless local area network, the detection system provides a large transmission distance up to 50 m, which enables experimenters to be able to operate the device within almost any laboratory or open field context.

2.2. Chemical reagents

Saxitoxin (STX) and brevetoxin (PbTX-2) pure standards are obtained from Calbiochem (San Diego, CA) and Sigma-Aldrich (St. Louis, MO), respectively. STX stock solution (100 μM) is prepared in 0.05 mM acetic acid. PbTx-2 stock solution (100 μM) contains 1% ethanol. The effects of acetic acid and ethanol on the cardiomyocyte-based potential biosensor are not detectable at the concentrations used in this work. Working toxin solutions are freshly prepared minutes before use. Plating medium is used as diluent.

2.3. Cardiomyocyte culture

The potential sensor chips are coated with 0.1% gelatin overnight in 4 °C refrigerator. Ventricular tissue from neonatal Sprague-Dawley rats is received from hearts in ice-cold Dulbecco's minimum essential medium (DMEM, Invitrogen, Carlsbad, CA). The tissue is moved into a bottle with 2 ml hanks balanced salt solution (HBSS, Invitrogen, Carlsbad, CA) and shredded into fragments (1 mm^3). Tissue fragments are dissociated enzymatically by stepwise with trypsin/collagenase II (Invitrogen, Carlsbad, CA) mixture followed by mechanical trituration. Cell suspension is centrifuged with 800 rpm for 5 min. Supernatant is removed and cells are resuspended in 4 ml DMEM supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). Cells are plated on the previously prepared sensor ships at a density of 3.0×10^5 cells/ml on the sensor chips. A complete media exchange occurred every day to maintain cardiomyocyte viability.

2.4. Real-time monitoring of cardiomyocytes response to marine toxins

Toxin experiments are performed using the cardiomyocyte-based potential biosensor. Initially, basic electrical activity of cardiomyocyte networks is checked every 12 h to evaluate the performance of cardiomyocyte-based potential biosensors. Once the potential biosensors display the mature EFP signals (amplitude > 1.5 mV), they are ready for toxin treatment. Before the toxin administration, culture medium is removed and replaced

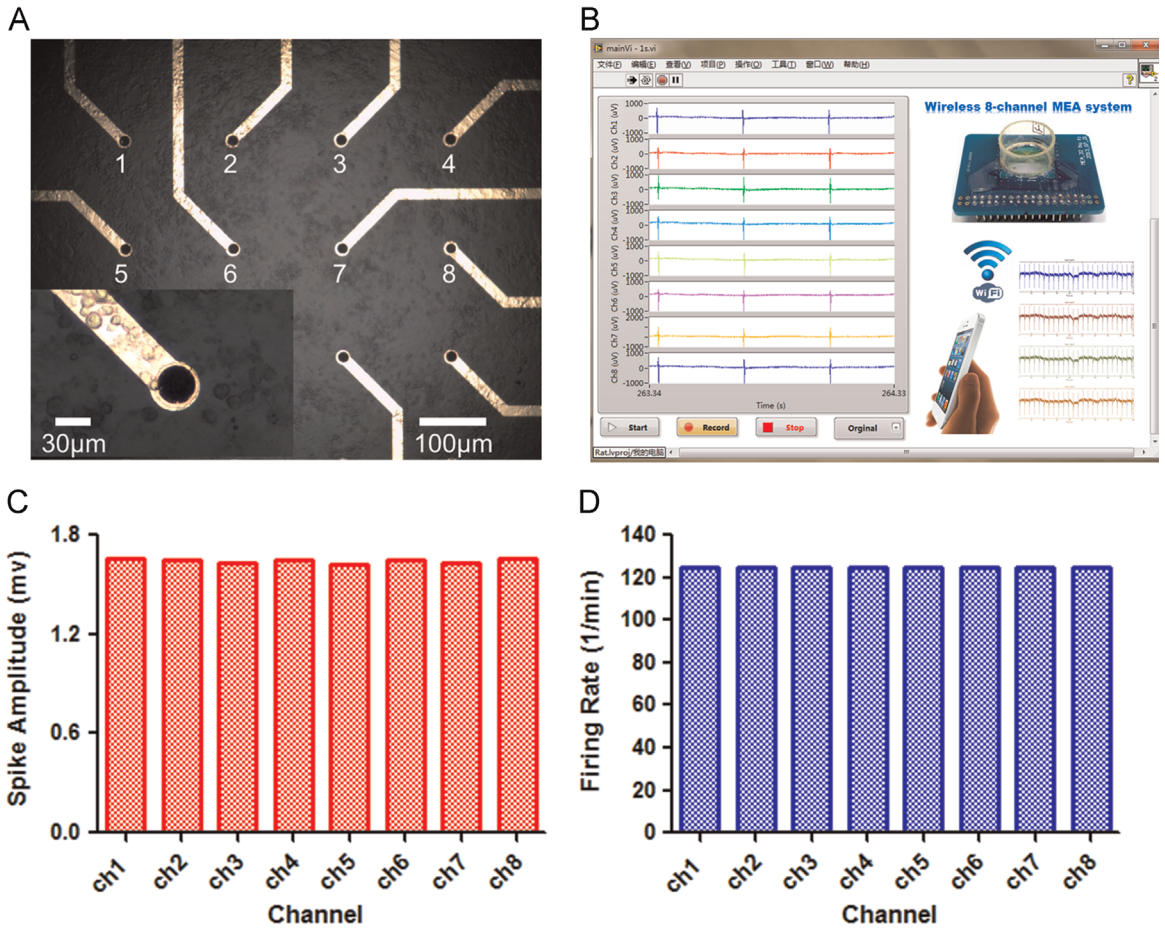

Fig. 3. Typical mature electrical activity of cardiomyocyte network at 84 h. (A) Typical cardiomyocyte network on MEA sensor chip. (B) Corresponding mature EFP signals from eight of the electrodes (numerical symbols). (C) Spike amplitude of typical mature EFP signals from 8 channels. (D) Firing rate of typical mature EFP signals from 8 channels.

with 990 μl fresh essential DMEM with 10% fetal bovine serum. After about 3 h, EFP recording is continued for 30 min in order to determine stable baselines of electrophysiological parameters of cardiomyocyte networks, and then 10 μl increasing concentrations of STX or PbTX-2 are added into the culture chambers to continuously record EFP signals for 30 min.

3. Results and discussion

3.1. Basic EFP pattern analysis of cardiomyocyte-based potential biosensors

To evaluate the performance of cardiomyocyte-based potential biosensors. The basic EFP pattern of the potential biosensor is assessed during a long monitoring term (about 7 days). The changes of EFP pattern of the potential biosensor over time is visualized in Fig. 2(A). Fig. 2(A) shows 10 s of typical EFP signals in one channel at six selected time points (36, 48, 60, 84, 144, and 156 h). Initially, no EFP signals are recorded from cardiomyocyte-based potential biosensor at the early time point (24 h) (data is not showed). Cardiomyocyte-based potential biosensor presents regular EFP signals with low spike amplitude (no more than 50 μV) at about 36 h. After approximately 48 h, more regular EFP signals appear but the spike amplitude of signals is still low. In the following time, the electrical activity increases rapidly over time until the potential biosensor displays a typical mature EFP pattern with high spike amplitude and firing rate at about 84 h (Fig. 3(B)), and the mature EFP pattern can continue for about 60 h.

Fig. 2(B) and (C) presents the statistical results of spike amplitude and firing rate of cardiomyocyte-based potential biosensor at different time points. As shown in Fig. 2(B), the spike amplitude of cardiomyocyte-based potential biosensor progressively increases, reaches plateau phase at about 84 h and maintains a stable level (spike amplitude of 1.6–1.8 mV). Likewise, the firing rate of cardiomyocyte-based potential biosensor gradually increases, reaches plateau phase at about 84 h and maintains a stable level (firing rate of 100–120 beats/min) for about 60 h (in Fig. 2(C)), while dramatically decreased after about 156 h. From the results, it can be seen that the high-consistent mature EFP signals appeared after about 84 h with high spike amplitude and firing rate, which can maintain for about 60 h. During this period, cardiomyocytes form a synchronously beating, confluent and uniform contracting cardiomyocyte network which couples high-closely to the sensor chips (Fig. 3(A)) and displays better consistency and more stable EFP signals (B). As shown in Fig. 3(B), different channels of cardiomyocyte-based potential biosensor exhibit high-synchronous and high-consistent EFP pattern at 84 h. The standard deviation (STD) of electrophysiological parameters of EFP signals (e.g., spike amplitude and firing rate) is less than 0.5% between each channel (Fig. 3(C) and (D)).

All the results reveal that the mature EFP signals (spike amplitude of 1.6–1.8 mV, firing rate of 100–120 beats/min) of cardiomyocytes-based potential biosensors are stable for about 60 h. Therefore, during this stable period, cardiomyocyte-based potential biosensor can be used for marine toxins experiments.

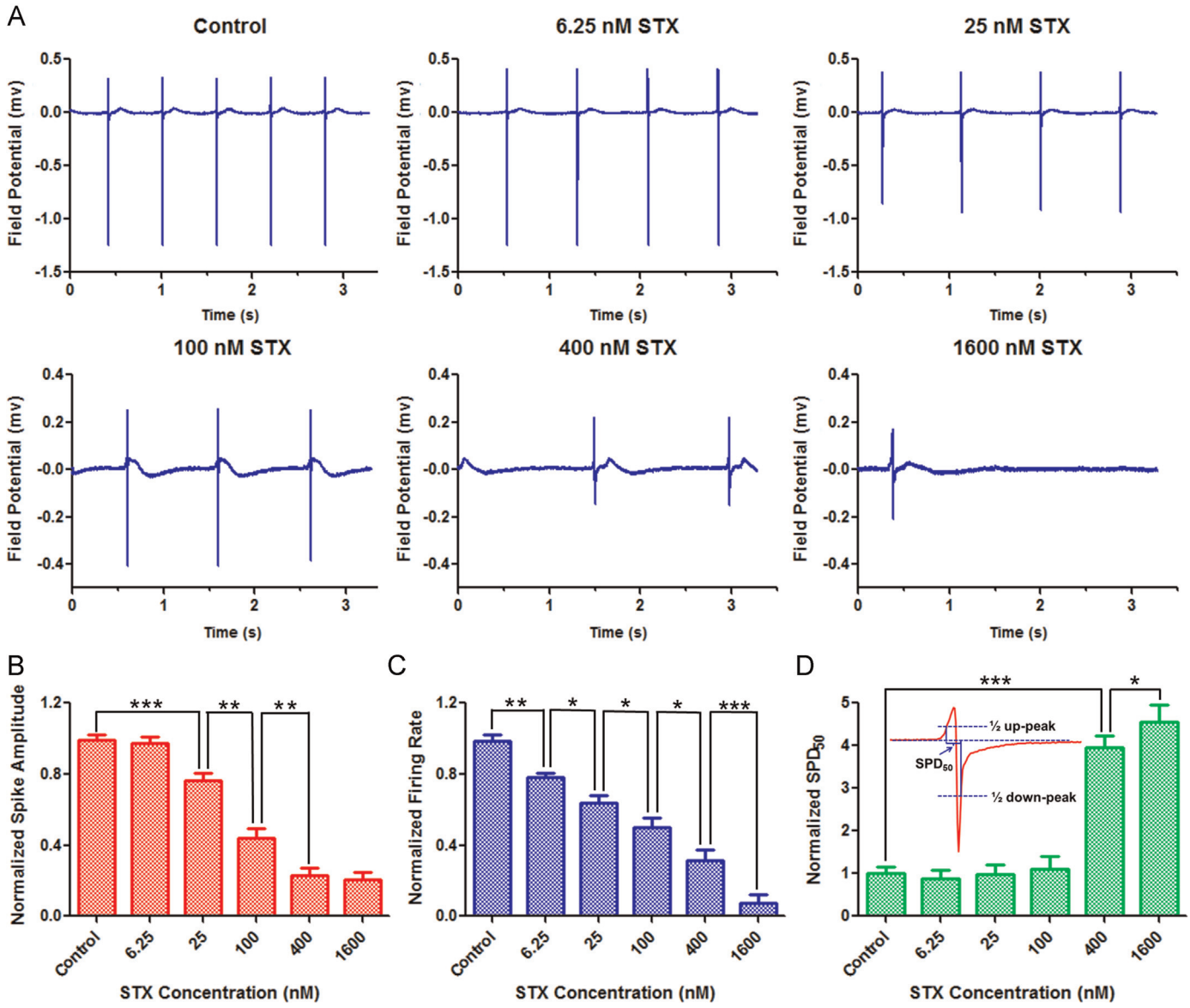

Fig. 4. Concentration-dependent effect on cardiomyocyte-based potential biosensor after exposure to the sodium channel inhibitor, STX after 5 min. (A) Changes in EFP signals before and after treatment with increasing concentrations of STX. (B)–(D) Normalized spike amplitude, normalized firing rate and normalized SPD_{50} of EFP signals before and after introduction of STX. Data represent mean ± S.E.M. of six independent experiments from three different isolations. Levels of significance: $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3.2. Assessment of STX and PbTX-2 by the cardiomyocyte-based potential biosensor

Cardiomyocytes are cultured for about 84 h on sensor chips, treated with increasing concentrations of STX or PbTX-2, and monitored for 30 min using the cardiomyocyte-based potential biosensor. The changes of EFP are analyzed to evaluate toxicity on the potential biosensor. Many different parameters can be derived from EFP recordings. Here, three of them are selected for the assessment of the changes induced by STX and PbTX-2. The analyzed results are normalized and then averaged with six independent experiments from three different isolations.

STX effect on the cardiomyocyte-based potential biosensors is shown in Fig. 4. Typical EFP signals which are recorded from a MEA channel are shown in Fig. 4(A) before and after exposure to different concentrations (6.25, 25, 100, 400, 1600 nM) of STX for 5 min. Obviously, administration of STX leads to a significant decrease in both spike amplitude and firing rate (see also Fig. 4 (B) and (C)). The effect of STX on cardiomyocyte network is consistent with the action of another sodium channel inhibitor, tetrodotoxin (Borkholder et al., 1998; Guo et al., 2011).

Three parameters (spike amplitude, firing rate and SPD_{50}) of the cardiomyocyte-based potential biosensor are derived from the original EFP signals. Fig. 4(B)–(D) illustrated the STX effects on the three parameters of the potential biosensor, respectively. As demonstrated in Fig. 4(B) and (C), STX shows a concentration-dependent inhibition effect on spike amplitude and firing rate. And in Fig. 4(D), STX shows no effect on SPD_{50} of the potential biosensor at lower concentrations (6.25–100 nM), and dramatically increases SPD_{50} at higher levels (400–1600 nM). Although STX causes concentration-dependent effects on all of the parameters, different parameters present different sensitivity to STX. From the statistical results in Fig. 4(B) and (D), it can be concluded that STX can significantly reduce the spike amplitude ($P < 0.001$, $n = 6$) at the concentration 25 nM, and STX can significantly increase the SPD_{50} ($P < 0.001$, $n = 6$) at the concentration 400 nM. While Fig. 4 (C) shows that the effect from the exposure to the lower concentration (6.25 nM) causes a statistically significant decrease

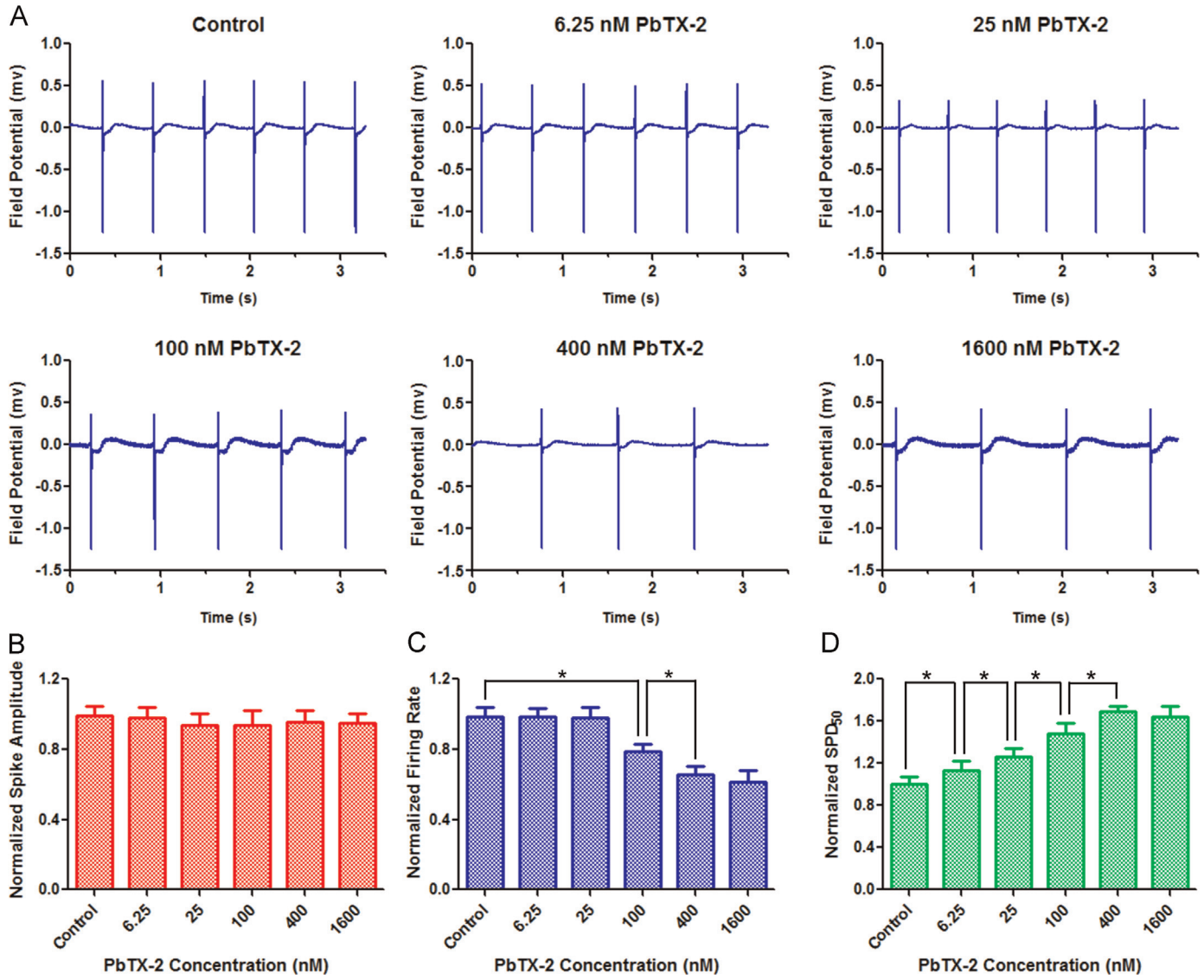

Fig. 5. Concentration-dependent effect on cardiomyocyte-based potential biosensor after exposure to PbTX-2 after 5 min. (A) Changes in EFP signals before and after treatment with increasing concentrations of PbTX-2. (B)–(D) Normalized spike amplitude, normalized firing rate and normalized SPD_{50} of EFP signals before and after introduction of PbTX-2. Data represent mean ± STD of six independent experiments from three different isolations. Levels of significance: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$.

($P<0.01$, $n=6$) in firing rate, indicating that firing rate of this potential biosensors is more sensitive to STX.

With the similar experiment protocol, cardiomyocyte-based potential biosensor is applied to study the effect of the sodium channel activator, PbTX-2. Exposure to PbTx-2 also induces profound changes on EFP signals of the cardiomyocyte-based potential biosensor. Fig. 5 shows the effect of PbTX-2 on the potential biosensors. Representative date segment of EFP signals recorded on a single sensor channel are displayed in Fig. 5(A) before and after PbTX-2 treatment for 5 min. PbTX-2 markedly reduces the firing rate of the potential biosensor (see also Fig. 5(C)), while PbTX-2 has no obvious effect on spike amplitude (also in Fig. 5B).

Three parameters (spike amplitude, firing rate, and SPD_{50}) of the cardiomyocyte-based potential biosensor are derived from the original EFP signals. Concentration-dependent effects of PbTX-2 on spike amplitude, firing rate, and SPD_{50} of this potential are displayed in Fig. 5(B), (C) and (D), respectively. No obvious effect is observed on spike amplitude after exposure to PbTX-2 (Fig. 5(B)). A concentration-dependent inhibition effect is observed with respect to firing rate after exposure to PbTx-2 (Fig. 5(C)). PbTX-2 has no effect on beating rate of cardiomyocyte-based biosensor at lower concentrations (6.25–25 nM), and shows an inhibition at higher levels (100–1600 nM). Also, PbTx-2 induces a concentration-dependent increase in SPD_{50} (Fig. 5(D)). From the statistical results in Fig. 5(B) and (C), it can be concluded that PbTx-2 could significantly reduce the beating rate ($P<0.05$, $n=6$) under the concentration 25 nM, while PbTx-2 has no significant difference ($P>0.05$, $n=6$) in spike amplitude in the tested concentration range (6.25–1600 nM). Fig. 5D shows that the effect from the exposure to the lowest tested concentration (6.25 nM) causes a statistically significant increase ($P<0.05$, $n=6$) in SPD_{50}, indicating that SPD_{50} of this potential biosensors is more sensitive to PbTx-2.

In this work, three representative electrophysiologic parameters (spike amplitude, firing rate and SPD_{50}) of EFP signals have been analyzed for the potential biosensor on the toxin concentrations. The marine toxin-induced changes in the parameters are obvious within 5 min of exposure and revealed sensitivity under nanomolar concentration. Based on the different action mechanism, the potential biosensor presented different responses

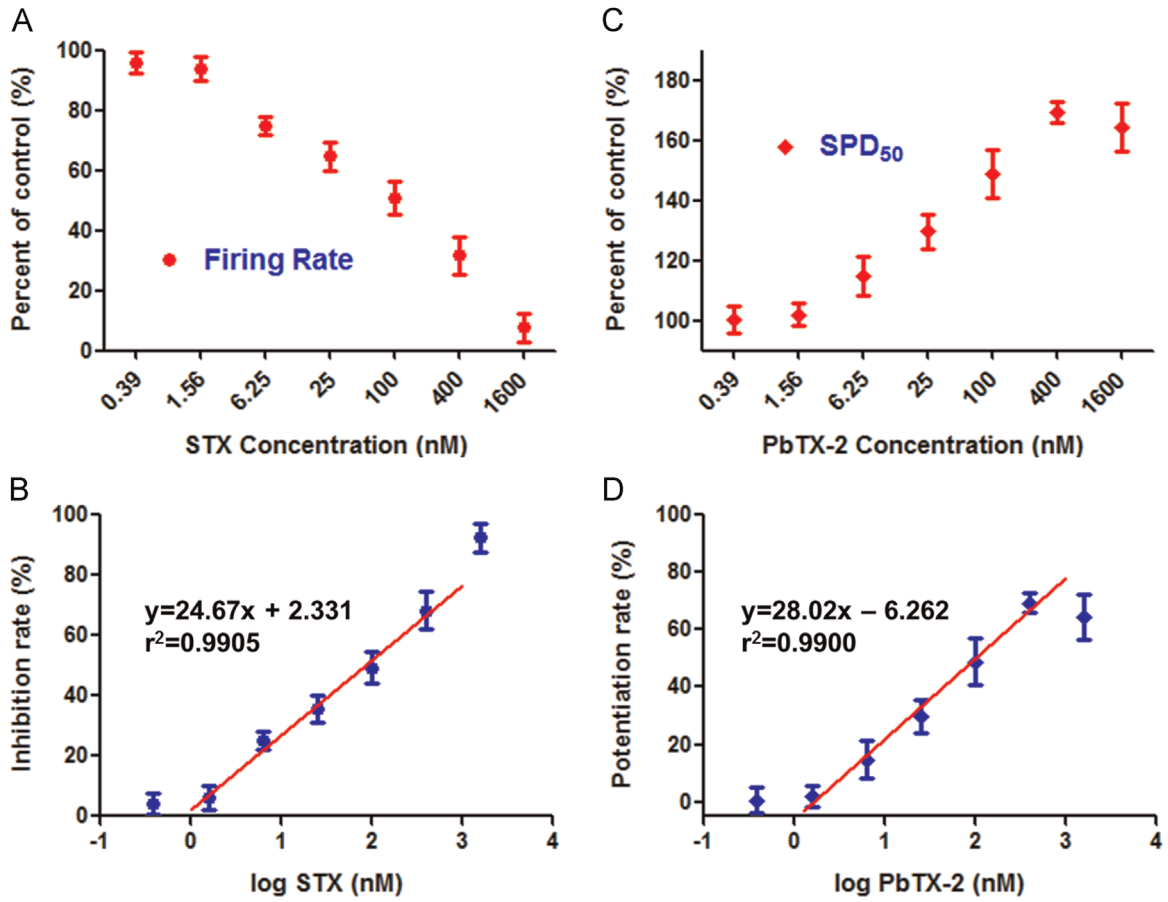

Fig. 6. The linear dependence of firing rate and SPD_{50} on the toxins concentration. (A) Concentration-dependent inhibition effect of STX on firing rate in the concentration range (0.39–1600 nM). (B) The linear dependence of firing rate response to STX. (C) Concentration-dependent potentiation effect of PbTX-2 on SPD_{50} in the concentration range (0.39–1600 nM). (D) The linear dependence of SPD_{50} response to PbTX-2. Data represent mean ± STD of six independent experiments from three different isolations.

to the two toxins on the three parameters. The results show that the most sensitive parameter to STX and PbTX-2 is firing rate and SPD_{50}, respectively. Consequently, the linear dependence of biosensors is analyzed on firing rate and SPD_{50} for STX and PbTX-2, respectively. Fig. 6(B) shows the corresponding results of inhibition rate of firing rate after treated with different concentrations of STX. The linear range of firing rate responding to STX is 0.19–2.60 log STX (nM); the sensitivity is 24.67%/log STX (nM). Fig. 6 (D) shows the corresponding results of potentiation rate of SPD_{50} after treated with different concentrations of PbTX-2. The linear range of SPD_{50} responding to PbTX-2 is 0.19–2.60 log PbTX-2 (nM), the sensitivity is 28.02%/log PbTX-2 (nM). The results imply that the cardiomyocyte-based potential biosensor may be exploited in the future for quantitative identification of these two toxins relying on their different sensitive parameters.

The limit of detection (LOD) of the biosensor is 1.17 nM (0.35 ppb) for STX, which is 2286 times below the regulatory limit (0.8 ppm) and 571 times below the mouse bioassay detection limit (0.2 ppm). And the LOD of the biosensor is 1.73 nM (1.55 ppb) for PbTX-2, which is approximately 516 times below the regulatory limit (0.8 ppm) set by the FDA. The LOD of STX is about 5 times lower than PbTX-2, indicating that the biosensor is more sensitive for STX. The repeatability of the cardiomyocyte-based potential biosensor response to STX and PbTX-2 is under 6% and 8%, respectively, indicating good consistency.

Up to date, a number of previous functional methods are applied for marine toxins detection. The traditional cell-based assay which rely on fluorescent dye (MTT or CCK8) is relatively time consuming and could not dynamically monitor the effects of STX and PbTX-2 (Cañete and Diogène, 2008; Caillaud et al., 2009), and the electrophysiological assays which rely on patch clamp limited the application of this assay outside the laboratory and need a skilled professional (Velez et al., 2001). Also, previous cell-based impedance biosensors established by our group are time-consuming due to their slow response to the toxins (Wang et al., 2015; Zou et al., 2015). The present study develops a new portable cardiomyocyte-based potential biosensor that improves these existing functional assays for STX and PbTX-2. The comparison of the previously developed system performance with the newly developed system is listed in Table S1 in supplementary materials. It has been demonstrated that the potential biosensor can be utilized to rapidly (within 5 min) detect these two toxins based on several important advantages. The portable wireless 8-channel MEA system provides a real-time monitoring and analysis of diverse and robust parameters in a label-free format for a given toxin with a single method. In addition, EFP directly reflects the changes of electrophysiological function which is induced by ion channel drugs or toxins, making the potential biosensor present flexibility to rapidly detect the ion channel toxins with high sensitivity. Furthermore, the portable potential biosensor exhibits robustness and high consistency with a long-term operational lifespan. It is anticipated that these unique attributes of the portable cardiomyocyte-based potential biosensor will lead to new applications in the marine toxin detection.

4. Conclusions

In this study, an improved and sensitive functional method is developed for rapid detection of STX and PbTX-2 using the

portable cardiomyocyte-based potential biosensor. This biosensor provides a label-free and convenient way to monitor the spontaneous electrical activity of cardiomyocyte network in real time. The results show that high-consistent mature EFP signals from the potential biosensor can be continuously recorded for approximately 60 h under basal conditions. The differences in toxin effect on three selected parameters of the potential biosensor provide useful information to discriminate between STX and PbTX-2. Compared with previous cell-based impedance biosensor established by our group, this portable biosensor greatly shortens the detection time of marine toxins. Therefore, the cardiomyocyte-based potential biosensor has the potential to be a novel platform for rapid detection of marine toxins in the future.

Acknowledgments

This work was supported by Major International Cooperation Project of Natural Science Foundation of China (No. 61320106002), Marine Public Welfare Project of China (No. 201305010), National Natural Science Foundation of China (No. 31228008), High-level Personnel Training Project of Cooperation Improvement of America and Oceania Region (No. 20142029), and China Postdoctoral Science Foundation (No. 2014M561764).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2015.04.028.

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