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Effects of perfluorooctane sulfonate on ion channels and glutamate-activated current in cultured rat hippocampal neurons

Chun-yang Liao[a,b], Lin Cui[b], Qun-fang Zhou[b], Shu-min Duan[c], Gui-bin Jiang[b,*]

[a] *Yantai Institute of Coastal Zone Research for Sustainable Development, Chinese Academy of Sciences, Yantai, Shandong 264003, China*
[b] *State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, P.O. Box 2871, Beijing 100085, China*
[c] *Institute of Neuroscience and State Key Laboratory of Neurobiology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China*

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ABSTRACT

Concern on an emerging persistent contaminant, perfluorooctane sulfonate (PFOS), is increasingly growing. Although the fate, transport, distribution and bioaccumulation of PFOS have been documented, its toxicological effects especially neurotoxicity remain largely unknown. In this study, the effects of PFOS on ion channels including potassium and sodium channels and exogenous glutamate-activated current in cultured rat hippocampal neurons were examined, based on whole-cell patch-clamp recording. PFOS markedly increased two subtypes of potassium currents, including transient outward current and delayed rectifier current, at doses over 10 μM. PFOS did not affect the amplitude of sodium current at all administrated doses (1, 10 or 100 μM) but clearly shifted the activation current–voltage curve toward negatively potential. Further, PFOS significantly altered the glutamate-activated current at all doses. Taken together these findings indicated that PFOS disturbs the neuronal physiological processes, which revealed the damage of this pollutant to nerve system and will be helpful for further exploration to its underlying mechanism.

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1. Introduction

Perfluorinated compounds (PFCs) are a class of emerging persistent contaminants. These chemicals are fully fluorinated man-made chemicals with unique properties that make them useful in wide array of industrial and household applications such as refrigerants, surfactants and polymers, and as components of pharmaceuticals, fire retardants, lubricants, adhesives, cosmetics, paper coatings, and insecticides (Renner, 2001; Giesy and Kannan, 2001, 2002; Houde et al., 2006a; Lau et al., 2007). Perfluorooctane sulfonate (PFOS) is the terminal degradation end product of many commercially used perfluorinated organic products and is the predominant PFC detected in multiple environmental matrices including water, air, sediment and biota (Kannan et al., 2001, 2002; Martin et al., 2002; Taniyasu et al., 2005; Houde et al., 2006b; Yeung et al., 2006). The widespread application, environmental persistence and bioaccumulative potential of PFOS increase governmental pressure and arise intense scientific and regulatory interest (Lau et al., 2004, 2007; Kennedy et al., 2004; Andersen et al., 2006; Houde et al., 2006a; Butenhoff et al., 2006).

The exceptional stability of PFOS also makes it potentially hazardous to wildlife and human. Human may be exposed to PFOS through air, water, food and household dust due to the global occurrence of PFOS in environment (Renner, 2001; Lau et al., 2007). PFOS is readily absorbed by organisms through oral uptake but difficulty eliminated out due to several years of half-life (Burris et al., 2002). Increasing evidences show that PFOS exposure can induce various toxic effects in animals such as hepatotoxicity, developmental, reproductive and systemic toxicity, interferences in thyroid hormone level, mitochondrial bioenergetics and cell–cell communication, neuroendocrine dysfunction and carcinogenicity (Hu et al., 2002, 2003; Austin et al., 2003; Lau et al., 2004; Kennedy et al., 2004). Using isolated guinea-pig ventricular myocyte and cultured rat cerebellar Purkinje cell as *in vitro* models, Harada et al. (2005, 2006) demonstrated the effects of PFOS on the calcium, potassium and sodium channels and action potential, based on whole-cell patch-clamp recording. Although there have been growing studies on fate, transport, distribution and bioaccumulation of PFOS, its toxicological effects especially neurotoxicity is still poorly understood. In this work, the primary cultured rat hippocampal neuron was used as an *in vitro* model to explore the effects of PFOS on

* Corresponding author. Tel.: +86 10 6284 9129; fax: +86 10 6284 9937.
E-mail address: gbjiang@rcees.ac.cn (G.-b. Jiang).

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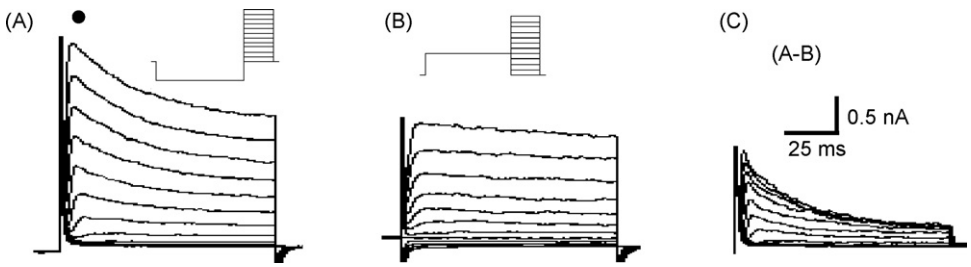

Fig. 1. Isolation of outward potassium currents in cultured rat hippocampal neurons. (A) Outward potassium currents were recorded by step voltages (100 ms, +10 mV increment) from −70 to +40 mV with a prepulse of −110 mV for 300 ms. The black circle indicates the position of peak currents as measured in Fig. 2. (B) Separate delayed rectifier current (I_K) was obtained by alteration of prepulse from −110 to −30 mV. (C) Isolation of transient outward current (I_A) was reached by subtraction of (B) from (A). Insets refer to protocols used. Scales, 0.5 nA, 25 ms. For this and subsequent figures, the pipette potential was held at −70 mV.

potassium, sodium and glutamate-activated currents and the underlying mechanism.

2. Materials and methods

2.1. Drugs and application

Heptadecafluorooctane sulfonic acid potassium salt [$CF_3(CF_2)_7SO_3K$, MW 538.22, 98%] was used as a standard for PFOS and purchased from Fluka (Buchs, Switzerland). Appropriate amount of PFOS was dissolved in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) giving a 100 mM stock solution. The working solutions were freshly prepared by dilution of the stock solution with the extracellular solution (ECS) just before use. Other reagents were from Sigma–Aldrich (St. Louis, MO) unless otherwise stated. For recording the potassium and sodium currents, drugs were applied to neurons by continuing perfusion using a HL-2 constant flow pump (Shanghai Huxi Analytical Instrument Co., China). For recording the glutamate-activated current, a miniature home-made Y-tube manipulated near the cell body (within 100 μm) was used to ensure rapid (within 100 ms) application of PFOS-containing saline to the neuron with minimal dilution or mixing during electrophysiological recording. The solutions were held in reservoirs higher than the recording bath and drawn through the arms of the Y-tube, which are of larger diameter tubing (PE 50) than the stem (PE 10).

2.2. Primary cell culture

The use and care of animals follow the guideline of the Shanghai Institutes for Biological Sciences Animal Research Advisory Committee. Primary hippocampal

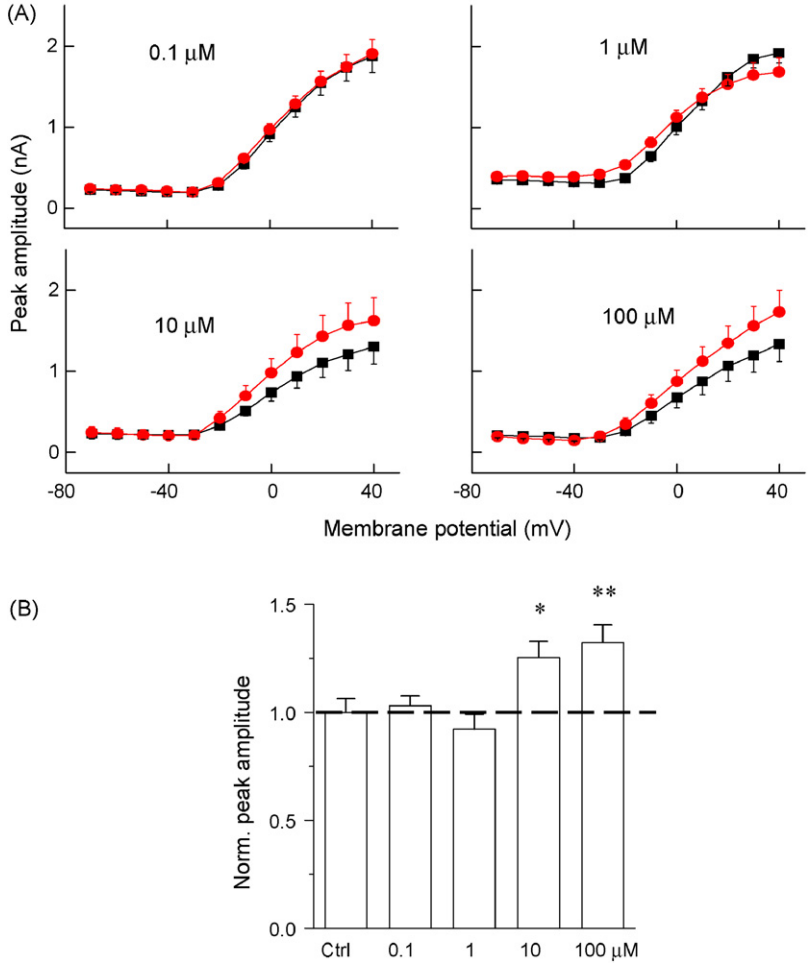

Fig. 2. PFOS increased the transient outward potassium current. (A) *I–V* relationships of the transient outward potassium current (I_A) were obtained by varying doses of PFOS. (B) Normalized amplitudes of I_A at +40 mV indicated effects of PFOS became evident at 10 μM. $n = 6–11$, $^*P < 0.05$, $^{**}P < 0.01$ vs. control, paired *t*-test. *Note*: in Figs. 2–4, black squares and red circles show the control and drug applications, respectively. Error bars show S.E.M. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

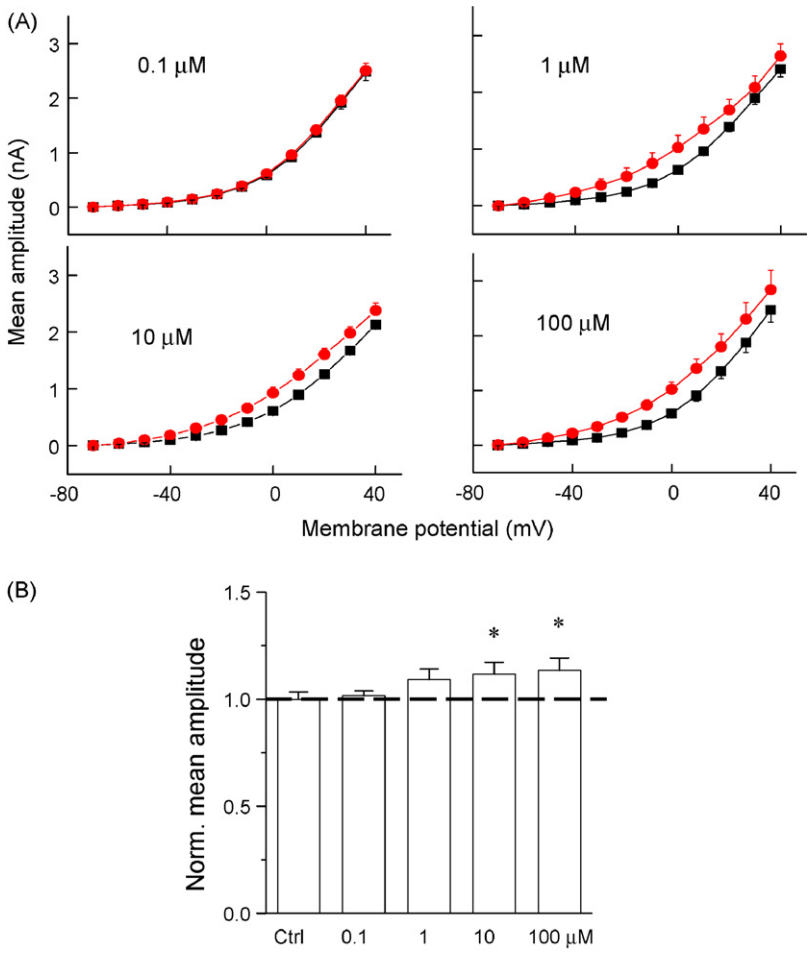

Fig. 3. PFOS increased the delayed rectifier potassium current. (A) *I–V* curves of the delayed rectifier potassium current (I_K) were obtained by applications of PFOS at various doses. (B) Summarized data regarding to amplitudes of I_K at +40 mV illustrated I_K was significantly increased by PFOS over 10 μM, in a similar way like I_A. $n = 6–12$, $^*P < 0.05$ vs. control, paired *t*-test. Error bars indicate S.E.M.

neuron was cultured as reported previously (Shen et al., 2006). Briefly, hippocampi were dissected from embryonic day 18 SD rats and digested in 0.25% trypsin (Sigma) for 12–15 min at 37 °C, followed by gentle trituration. Dissociated cells were plated at a density of 25,000–60,000 cells/mL on poly-D-lysine-coated 35 mm dishes (Costar, Cambridge, MA) in Dubecco's Modified Eagle Medium (DMEM; GIBCO, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO) and 10% Ham's F-12 (GIBCO). After 24 h *in vitro*, half of the medium was changed to serum-free neurobasal (NB) medium with 2% B27 supplement (GIBCO) and 0.25% glutamine (Sigma). Thereafter, half of the medium was replaced twice a week with NB medium containing 2% B27 supplement and 0.25% glutamine. After 5 days *in vitro* (DIV 5), cytosine arabinoside was added at 2–4 μM to inhibited glial cell proliferation.

2.3. Whole-cell recording

Voltage-clamp recordings were conducted pre- and post-treatment of PFOS at DIV 8–15 neurons from at least three separate cultures according to the whole-cell patch-clamp technique. Currents were recorded with a Multiclamp 700A or 700B amplifier, low-pass filtered at 1 kHz using the amplifier circuitry, sampled at 10 kHz and stored in a computer using a Digidata 1320A interface and Clampex 8.2 software (all from Axon Instrument, Foster City, CA). The patch pipettes were pulled by a P-97 micropipette puller from borosilicate glass capillaries (Sutter Instruments, Novato, CA). The pipette resistance was 2–6 MΩ when filled with intracellular solution (ICS). Neurons tested were held at −70 mV during electrophysiological recordings. For recording the potassium current, the ICS contained (in mM) 136.5 K-gluconate, 17.5 KCl, 9 NaCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, and 2 Na-ATP (pH 7.2). The ECS contained (in mM) 145 NaCl, 3 KCl, 3 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, 8 D-glucose, 0.0005 tetrodotoxin (TTX), and 0.1 $CdCl_2$ (pH 7.3). For recording the sodium current, the ICS contained (in mM) 135 CsCl, 10 TEA-Cl, 3 $MgCl_2$, 10 HEPES, 10 EGTA, 3 Na-ATP, 0.3 Na-GTP (pH 7.2). The ECS contained (in mM) 60 CsCl, 75 NaCl, 20 TEA-Cl, 1 $BaCl_2$, 2 4-AP, 2 $MgCl_2$, 10 HEPES, 10 D-glucose, and 0.1 $CdCl_2$ (pH 7.3). For recording the glutamate-activated current, the ICS was same as that of recording the potassium current and the ECS was Mg^{2+}-free ECS containing (in mM) 145 NaCl, 3 KCl, 3 $CaCl_2$, 10 HEPES, 8 glucose, 0.001 glycine, 0.0005 TTX (pH 7.3). All experiments were performed at room temperature and there is no discernable mortality of cells throughout each electrophysiological recording.

2.4. Data analysis

Data were analyzed with Clampfit 9.0 (Axon Instrument) and presented as mean ± S.E.M. Statistical comparisons were made using analysis of variance (ANOVA) or *t*-test where appropriate and $P < 0.05$ was considered significant.

3. Results

3.1. Effect of PFOS on voltage-dependent potassium channel currents

Outward potassium currents of neurons were examined with a bath saline containing both TTX and Cd^{2+} to eliminate possible competing inward Na^+ and Ca^{2+} currents (Meyers et al., 1992; Richmond et al., 1995). Two subtypes of K^+ currents, transient outward current (I_A) and delayed rectifier current (I_K), were recorded with a series graded concentrations of PFOS applications. Fig. 1 showed the example recordings of both subtypes of K^+ currents and their isolation. Outward K^+ currents, containing both I_A and I_K, were evoked by step voltages (100 ms, 10 mV) from −70 to +40 mV with a prepulse of −110 mV for 300 ms. Independent I_K was in turn recorded with the same protocol by altering prepulse from −110 to −30 mV. Isolation of I_A was reached by subtraction of I_K from total outward currents recorded (Duan and Cooke, 1999). The current densities of I_A and I_K were shown in Figs. 2A and 3A. The

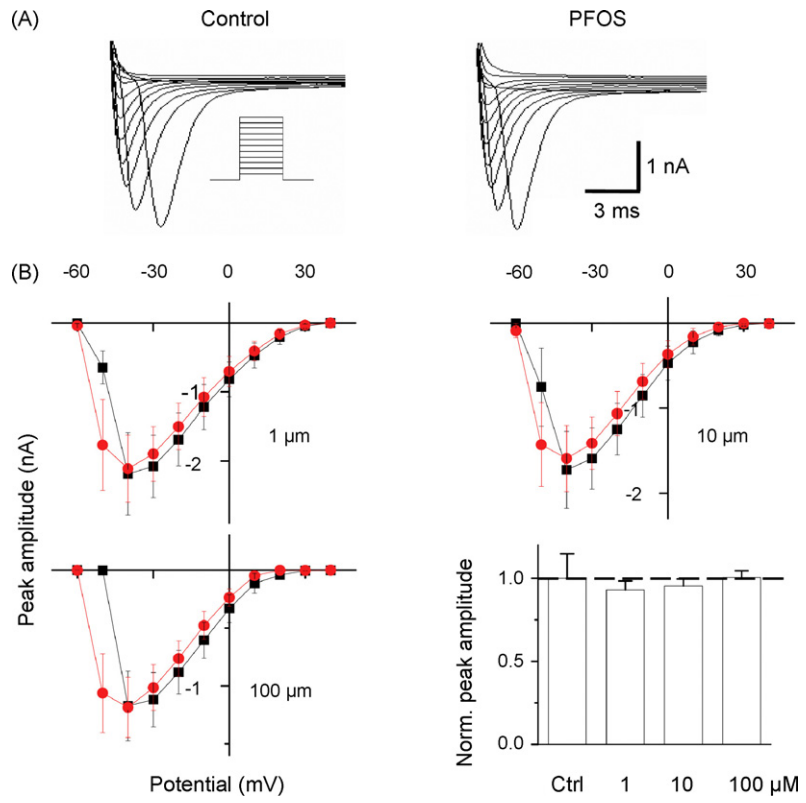

Fig. 4. PFOS left-ward shifted the voltage-dependent kinetics of sodium current. (A) Inward sodium current (I_{Na}) recordings were performed by 50 ms depolarizing voltage steps from −60 to +40 mV with +10 mV increment in absence (left panel) or presence (right panel) of PFOS. Inset shows protocol used. Scales, 1 nA, 3 ms. (B) *I–V* curves of I_{Na} with applications of PFOS at various doses and summarized maximum current amplitudes at −40 mV (right lower panel) suggested that PFOS left-ward shifted the *I–V* curves but not increased peak amplitudes. $n = 8–10$, $P > 0.05$ vs. control, paired *t*-test. Error bars represent S.E.M.

effects of PFOS became evident at 10 μM and the total magnifications were approximately 25 and 30% for I_A and I_K, respectively ($P < 0.05$, *t*-test). The maximum amplitudes of I_A and I_K at +40 mV in each series of recordings were compared subsequently in Figs. 2B and 3B. The current increase was common to two subtypes of K^+ channels tested and the dose–response relationships were observed, which demonstrated the actions of PFOS on potassium channels.

3.2. Effect of PFOS on voltage-dependent sodium channel current

The effect of PFOS on the voltage-dependent sodium current (I_{Na}) was examined by means of a Cd^{2+}-containing ECS and a Cs^+-rich ICS. The I_{Na} was recorded by 50 ms depolarizing voltage steps from −60 to +40 mV with +10 mV increment in the absence or presence of various doses of PFOS (1, 10 and 100 μM). As shown in Fig. 4B, PFOS negatively shifted the *I–V* curve of I_{Na} but not increased peak amplitudes at all administrated doses. Similarly the maximum amplitudes of I_{Na} at −40 mV in each series of recordings were averaged and the comparison results also demonstrated that no significant effects were found in amplitudes by PFOS (Fig. 4B, right lower panel).

3.3. Effect of PFOS on glutamate-activated current

The exogenous glutamate-activated inward currents were recorded by gravity ejection of glutamate (100 μM) in absence or presence of PFOS at various concentrations through a pair of

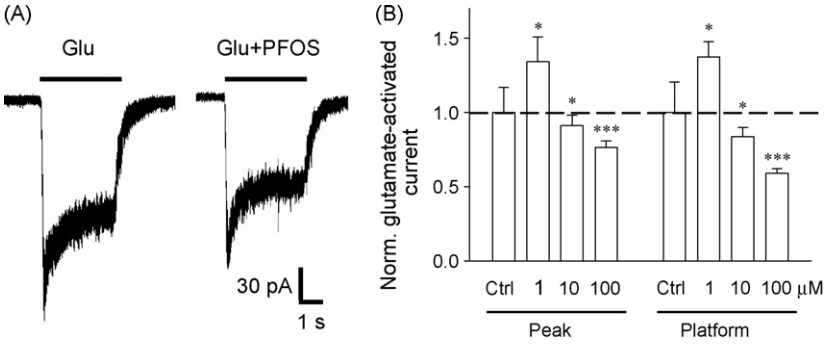

Fig. 5. PFOS changed the glutamate-activated current depending on doses. (A) The exogenous glutamate-activated inward currents were recorded in absence (left) and presence (right) of PFOS in Mg^{2+}-free ECS with co-application of glutamate (both 100 μM). Note: glutamate administration was continued for the duration of the overlying black bar. Scales, 30 pA, 1 s. (B) The relative current amplitudes normalized to the control showed that both peak and platform components were increased in a similar way by approximate 36% with application of PFOS at 1 μM and dose dependently reduced by about 13 and 32% with applications of PFOS at 10 and 100 μM, respectively. $n = 8–13$, $^*P < 0.05$, $^{***}P < 0.001$ vs. control, paired *t*-test. Error bars show S.E.M.

parallel polyethylene flow pipes. Glutamate induced currents in all neurons tested in Mg^{2+}-free ECS with co-applications of 1 μM glycine for magnification of the current and 0.5 μM TTX for inhibition of action potential. Fig. 5A showed the current response of a neuron induced by exogenous glutamate before and after 100 μM PFOS administration. The current reached a peak quickly (within 100 ms), then decayed to a steady-state level (platform) during glutamate application, and finally terminated with washout of ECS. It is known that the glutamate-activated current is induced by action of specific ligand, for example PFOS in this study, with α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate and *N*-methyl-D-aspartate (NMDA) receptors located on cellular membrane surface. The peak and platform reflect the AMPA/kainate and NMDA components of glutamate-activated current, respectively. Investigation of the glutamate-activated current with PFOS might preliminarily explore the effects of PFOS on AMPA/kainate and NMDA receptors. To examine whether the glutamate-activated current runs down over time, the currents were repeatedly recorded by ejection of glutamate at intervals of 5 min or more with Mg^{2+}-free ECS washout. We found that the currents did not decay over time under this experimental condition. So each peak or platform value of glutamate-activated currents was averaged by at least two repeated recordings with intervals of 5 min or more. After normalization to the control, the relative current amplitudes were displayed in Fig. 5B, indicating both peak and platform components in a similar way were elevated by about 36% over the control with application of PFOS at 1 μM and dose dependently suppressed by around 13 and 32% with applications of PFOS at 10 and 100 μM, respectively ($P < 0.05$, paired *t*-test).

4. Discussion

The present study showed evidences that PFOS disturbed the normal physiological processes of primary cultured rat hippocampal neurons, such as ion channels and glutamate-activated currents. Biological membranes are essential in maintaining cell integrity and function. Neurons are surrounded by a plasma membrane and the inside of a neuron contains a large amount of potassium ions (K^+). Even though they contain a lot of positively charged potassium ions, neurons contain a large number of negatively charged proteins to balance this out (to some extent). The outside of a neuron is generally a neutral environment containing large concentrations of sodium (Na^+) and chlorine (Cl^-), and smaller amounts of potassium (Koester and Siegelbaum, 2000). There are many kinds of ion channels in neuronal membranes, such as potassium channels, calcium channels and sodium channels, etc. Ion channels are targets for many toxins and chemicals. The possible effects of charged surfactants including PFOS on the modulation of ion channel function have been widely investigated. Our results obtained from cultured neurons confirm and further extend the studies by other researchers with other cell models (Ji et al., 1993; Xu and Rozanski, 1998; Harada et al., 2006). Given the amphiphilic structure, PFOS may exert its effect primarily on cell membrane. Using cell bioassay procedures, Hu et al. (2003) demonstrated that PFOS can increase the membrane fluidity and permeability through decrease in the cholesterol content of the membranes. Our findings illustrated that PFOS elevated the K^+ current (Figs. 2 and 3), left-ward shifted the voltage-dependent kinetics of Na^+ current (Fig. 4), and altered the glutamate-activated current (Fig. 5). This phenomenon is probably associated with alteration of membrane cholesterol content by PFOS and with possible actions on AMPA/kainate and NMDA receptors located on cellular membrane surface.

Ion channels play a critical role in various pathophysiological processes. For instance, K^+ channels may be an important determinant of cellular activities such as neural signal transduction, pacemaking and secretion to the regulation of cell volume and cell proliferation (Pongs, 1999). Na^+ channels produce the inward membrane current necessary for regenerative action potential production within the mammalian nervous system and are associated with the pathophysiology of pain (Waxman et al., 1999). K^+ ions tend to be in higher concentration inside (135 mM) the neuron than outside (3 mM). Also, Na^+ ions tend to be in higher concentration outside (145 mM) the neuron than inside (18 mM). When a neuron is at rest status, the plasma membrane is far more permeable to K^+ ions than to other ions present, such as Na^+ and Cl^- (Koester and Siegelbaum, 2000). Consequently K^+ ions diffuse out of the neuron along their concentration gradient a lot more quickly than Na^+ ions can move in. By using isolated cardiac ventricular myocyte as cell models, Huang et al. (1992) investigated the alterations of long-chain fatty acids in calcium channels and Ji et al. (1993) examined the modulation of voltage-dependent K^+ and Na^+ currents by charged amphiphiles containing negatively charged sodium dodecylsulfate (SDS) and positively charged dodecyltrimethylammonium (DDTMA). Recently Harada et al. (2005, 2006) reported that PFOS markedly increased Ca^{2+} current and altered the steady-state activation and inactivation of Ca^{2+}, K^+ and Na^+ currents toward the hyperpolarized direction. We further found that PFOS slightly elevated the amplitudes of K^+ currents (both I_A and I_K) in a dose-dependent fashion. K^+ channels control action potential duration and repolarization, release of neurotransmitters and hormones, and Ca^{2+}-dependent synaptic plasticity (Müller and Bittner, 2002). I_K and I_A contribute to action potential repolarization. Since I_A is transient, repolarization is mainly related to I_K. As in other neurons, I_A in hippocampal neurons was thought to modulate the timing of repetitive action potential generation and the time required to reach the threshold to fire an action potential (Gao and Ziskind-Conhaim, 1998). Enhancement of outward potassium currents leads to a reduction in cytoplasmic K^+ concentration, which is involved in the pathogenesis of neuronal death (Bortner et al., 1997). Thus it can be seen that PFOS might disturb the neuronal physiological processes through interference with the concentration of K^+ inside/outside (cytoplasm/surface of) the neurons. Nevertheless, PFOS did not appear to affect the amplitudes of Na^+ currents in cultured neurons, suggesting that PFOS might specifically act on ion channels. Additionally we also found PFOS shifted the activation current–voltage curve of Na^+ current toward negatively potential, which is mainly due to changes in the surface potential of the cell membrane.

Three families of ionotropic glutamate receptors, discriminated pharmacologically, are present in the central nervous system (CNS). These three families are AMPA, kainate and NMDA receptors, which all belong to a broad family of ligand-gated channels (Mayer, 2005). Ionotropic glutamate receptors mediate basic information processing in the brain and excitatory responses at the majority of CNS synapses (Wollmuth and Sobolevsky, 2004), such as those that are thought to be necessary for learning and memory, developing and maintaining cellular connections, and pain perception (Bliss and Collingridge, 1993; Woolf and Salter, 2000). AMPA/kainate receptors show fast gating kinetics, desensitize strongly, are permeable to Na^+ and K^+, and typically poorly permeable to Ca^{2+} (Hollmann and Heinemann, 1994). In contrast, NMDA receptors gate much more slowly, desensitize only weakly, are highly Ca^{2+}-permeable and are blocked by extracellular Mg^{2+} in a strongly voltage-dependent manner (Wollmuth and Sobolevsky, 2004). Our previous work exhibited that PFOS not only increased the amplitude but also left-ward shifted the *I–V* curves of Ca^{2+} currents in cultured hippocampal neurons (Liao et al., 2008). The present

work shows that PFOS slightly elevate the K^+ currents and alter the steady-state activation of Na^+ currents toward the hyperpolarized direction. These interferences might be association with the PFOS-induced alteration of glutamate-activated currents, including both AMPA/kainate and NMDA components. As shown in Fig. 5, it is possible that the PFOS effects in the membrane have multiple actions on the ionotropic glutamate receptors. The glutamate-activated currents were increased by PFOS at low concentration and suppressed at high concentrations, showing a non-monotone dose-dependent relationship. Since these glutamate receptors are particularly relevant to possible neurotoxicity, much more experiments need to be performed to elucidate the multiple actions of PFOS on glutamate-activated currents.

Recent studies have proved various toxicities of PFOS in animals (Lau et al., 2004, 2007; Kennedy et al., 2004; Beach et al., 2006). In the previous work (Liao et al., 2008), we revealed that acute perfusion of PFOS (10 μM and above) rapidly enhanced synaptic activity in both cultured hippocampal neurons and hippocampal brain slices, while chronic treatment with PFOS (50 μM) moderately inhibited neurite outgrowth and dramatically suppressed synaptogenesis in cultured neurons. We further showed that PFOS (10 μM and above) enhanced inward Ca^{2+} currents and increased intracellular Ca^{2+} in neurons through enhancement of L-type Ca^{2+} channels, a mechanism that may underlie PFOS' acute effects on synaptic transmission and chronic action on neuronal development. Previous studies reported that the tissue concentrations of PFOS detected in some animals can reach 1–10 mg/kg (approximately 2–20 μM) (Giesy and Kannan, 2001; Kannan et al., 2001). A study by Maestri et al. (2006) suggested that the concentrations of PFOS in non-occupationally exposed human liver and brain collected from Italy were 13.6 ng/g (~0.025 μM) and 1.3 ng/g (~0.002 μM), respectively. Recent evidences showed that the serum level of PFOS in U.S. general population has being gradually decreased after the 2000–2002 phase-out of PFOS-related products by the primary global manufacturer, 3 M Company (Calafat et al., 2007a,b). A pilot study by Olsen et al. (2007) showed that the plasma level of PFOS in American Red Cross blood donor samples in 2005 is approximate 0.03 μM. Nevertheless, the serum level of PFOS is up to much higher levels, approximate 1.82 μM, in occupational workers (Olsen et al., 2003). Although the level of PFOS in the tissues (such as liver and brain) and blood of general population is slightly lower than the lowest dose in our experiment, the intracellular Ca^{2+} might still be interfered by PFOS accumulated in the body over a long time. PFOS is very stable, not readily degraded and bioaccumulative, its toxicity thereby should raise serious concerns. Recent study shows that PFOS presents moderate acute toxicity by the oral route with a rat LD_{50} of 251 mg/kg (UNEP, 2002). Comparably the highest dose of PFOS (100 μM) used in our experiments is environmentally relevant, where the dose is comparable to the LD_{50} of 251 mg/kg. Further, the alteration in glutamate-activated current was evident even with 1 μM of PFOS application, which actually confirmed the potential damaging effects of PFOS to neurons.

In conclusion, our present results provided evidences that PFOS increased the amplitudes of two subtypes of K^+ currents including the transient outward current and delayed rectifier current in a similar way, negatively shifted the activation current–voltage curve of Na^+ current, and affect the exogenous glutamate-activated current in a dose-dependent manner. Many processes for actions of PFOS on neurons remain elusive and the underlying mechanisms still need further studies.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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