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# Cellular mechanism for spontaneous calcium oscillations in astrocytes[1]

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## Key words

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## Abstract

**Aim:** To determine the $Ca^{2+}$ source and cellular mechanisms of spontaneous $Ca^{2+}$ oscillations in hippocampal astrocytes. **Methods:** The cultured cells were loaded with Fluo-4 AM, the indicator of intracellular $Ca^{2+}$, and the dynamic $Ca^{2+}$ transients were visualized with confocal laser-scanning microscopy. **Results:** The spontaneous $Ca^{2+}$ oscillations in astrocytes were observed first in co-cultured hippocampal neurons and astrocytes. These oscillations were not affected by tetrodotoxin (TTX) treatment and kept up in purity cultured astrocytes. The spontaneous $Ca^{2+}$ oscillations were not impacted after blocking the voltage-gated $Ca^{2+}$ channels or ethylenediamine tetraacetic acid (EDTA) bathing, indicating that intracellular $Ca^{2+}$ elevation was not the result of extracellular $Ca^{2+}$ influx. Furthermore, the correlation between the spontaneous $Ca^{2+}$ oscillations and the $Ca^{2+}$ store in endoplasmic reticulum (ER) were investigated with pharmacological experiments. The oscillations were: 1) enhanced when cells were exposed to both low $Na^+$ (70 mmol/L) and high $Ca^{2+}$ (5 mmol/L) solution, and eliminated completely by 2 µmol/L thapsigargin, a blocker of sarcoplasmic reticulum $Ca^{2+}$-ATPase; and 2) still robust after the application with either 50 µmol/L ryanodine or 400 µmol/L tetracaine, two specific antagonists of ryanodine receptors, but depressed in a dose-dependent manner by 2-APB, an $InsP_3$ receptors ($InsP_3R$) blocker. **Conclusion:** $InsP_3R$-induced ER $Ca^{2+}$ release is an important cellular mechanism for the initiation of spontaneous $Ca^{2+}$ oscillation in hippocampal astrocytes.

## Introduction

Astrocytes were traditionally regarded as a passive glue that connects and supports neurons in the central nerve system (CNS). It builds the micro-environment in which neurons fulfill their tasks and recover from injury. However, growing evidence indicates that the role of astrocytes in the CNS may be underestimated, as bidirectional communication between neurons and astrocytes at the site of synapse has been found in types of astroglia from different tissues, leading to the concept of "tripartite synapse"[1–3]. With glutamate or other factors diffusing out of the synapse, the activation of neurons is able to affect astrocytes[4,5]. However, the activation of astrocytes can also affect neurons[6], via releasing varieties of neurotransmitters, including glutamate and ATP[7,8]. Although the mechanism of neurotransmitter secreting is unclear, this process is believed to couple with intracellular $Ca^{2+}$ elevation[9,10].

$Ca^{2+}$ is one of the most important second messengers and is thought to mediate communication between neurons and astrocytes[3,11]. Astrocytes are described as non-excitable cells, for their lack of voltage-gated sodium channels; however, they exhibit complicated intracellular $Ca^{2+}$ activity[12]. It has been reported that astrocytes express voltage-gated $Ca^{2+}$ channels[13] and the receptors of neurotransmitters[11]. Intracellular $Ca^{2+}$ oscillation in astrocyte *in situ*, coupled with neurotransmitter release, can drive NMDA receptor mediated neuronal excitation[14]. Many studies have shown that the spontaneous $Ca^{2+}$ oscillations existed in astrocytes and suggested that such $Ca^{2+}$ events are essential for communication between neurons and astrocytes[14–16]. These findings imply that astrocytes may act as command genera-

tors in neural regulation. Understanding the initiation of spontaneous $Ca^{2+}$ oscillations in astrocytes is thus substantial for better evaluation of the contribution of astrocytes to the whole neural system.

The increase in plasmic $Ca^{2+}$ concentrations during spontaneous $Ca^{2+}$ oscillations is either caused by the influx of extracellular $Ca^{2+}$ or the release from endoplasmic reticulum (ER) $Ca^{2+}$ store. In the present study, we sought to determine the $Ca^{2+}$ source in hippocampal astrocytes. With cultured hippocampal astrocytes from the neonatal rat, we investigated the correlation between the spontaneous $Ca^{2+}$ oscillations and ER $Ca^{2+}$ store with some pharmacological experiments using a confocal microscope. We found that the content of ER $Ca^{2+}$ store was necessary for the spontaneous $Ca^{2+}$ oscillations, and the activation of $InsP_3$ receptor ($InsP_3R$) played a key role in the process of the oscillations. Our finding suggests that $InsP_3R$-induced ER $Ca^{2+}$ release is an important cellular mechanism for the spontaneous $Ca^{2+}$ oscillation in hippocampal astrocytes.

## Materials and methods

**Cell cultures** After the brain of neonatal rats (Sprague-Dawley rats, purchased from Vital River Lab Animal Technology, China) were removed and placed into dissection solution (NaCl 137 mmol/L, KCl 5.4 mmol/L, $Na_2HPO_4 \cdot 12H_2O$ 0.67 mmol/L, $KH_2PO_4$ 0.22 mmol/L, HEPES 10 mmol/L, glucose 8.3 mmol/L and sucrose 11 mmol/L; pH 7.35), hippocampus were dissected and treated with 4 mL of 0.5% trypsin (Invitrogen, USA) at 37 °C for 30 min. Digestion was stopped by fetal bovine serum (FBS; HyClone, USA). Culture medium consisted of minimum essential medium (Invitrogen), containing 26 mmol/L $NaHCO_3$, 40 mmol/L glucose, 1 mmol/L pyruvate, $1\times10^5$ U/L penicillin, and 100 mg/L streptomycin, supplemented with 10% FBS and 2 mmol/L glutamine immediately before use. Cells were plated into 35-mm culture dishes for co-culture of neurons and astrocytes or 25 cm² culture flasks for purification later at a density of approximately $5\times10^8$ cells/L. Cells in flasks were grown to confluence at 37 °C in a humidified 5% $CO_2$/95% air. In order to get the purity culture of astrocytes, the flasks were shaken on a horizontal orbital shaker at 250 rpm for 18 h after 5–7 d. The remaining adherent cells were enzymatically detached with trypsin (0.5%) plus EDTA (0.06%), resuspended in culture medium, and plated onto poly-*D*-lysine-coated (12.5 mg/L) glass coverslips. Cells were fed every 3–4 d by replacing the medium with fresh medium. The cells were used in experiments after 1–4 d, by which time they had grown to confluence.

**$Ca^{2+}$ imaging** The bathing solution consisted of NaCl 141 mmol/L, KCl 2.5 mmol/L, $MgCl_2$ 1.3 mmol/L, $CaCl_2$ 2.4 mmol/L, $NaH_2PO_4$ 1.25 mmol/L, glucose 11 mmol/L, HEPES 10 mmol/L, pH 7.35. Cells were loaded with the $Ca^{2+}$ indicator, Fluo-4-AM (Invitrogen), at a concentration of 1.82 μmol/L in bathing solution for 5 min at room temperature. Confocal series-scan imaging was performed by using a Zeiss LSM 510 confocal microscope equipped with an argon laser (488 nm) and 40×, 1.3 NA oil immersion objectives. Series-image scanning was used to record the $Ca^{2+}$ oscillation in cells. The sampling rate was 1 Hz, and the optical slice was approximately 3 μm.

## Results

### Spontaneous $Ca^{2+}$ oscillations in hippocampal astrocytes

To evaluate the effects of neurons on spontaneous $Ca^{2+}$ oscillations of astrocytes, the co-cultured hippocampal neurons and astrocytes were loaded with Fluo-4 AM first, and the intracellular $Ca^{2+}$ oscillations in astrocytes were investigated with confocal-laser-scanning microscope. The cells were then exposed to 1 μmol/L TTX (Figure 1B), a selective antagonist of voltage-gated $Na^+$ channels, which can effectively block the action potential of neurons, in order to examine whether this activity comes from neurons or originates from astrocytes themselves. We found that the robust activity of astrocytes was not impacted (Figure 1C; *n*=30). These results indicated that the process of the oscillations was neuronal action potential-independent.

Considering the possibility that the treatment of TTX may not completely occlude neurons' effects, eg, via spontaneous transmitter release, purified astrocytes (Figure 1A) from the co-cultured system were employed in all other experiments. To determine the purity of astrocytes, we labeled astrocytes with anti-GFAP antibody and cell nuclei with Hoechst 33258, after the culture was fixed by 4% paraformaldehyde in PBS. Immunofluorescence analysis showed that approximately 97% (132 in 136) cells were GFAP marked astrocytes (Figure 1E–1G). We observed the $Ca^{2+}$ activity in purity cultured astrocytes as above, and there was no significant change in the activity when compared with the cells in co-culture (Figure 1D). This result implied that the spontaneous $Ca^{2+}$ oscillations in astrocytes did not require the participation of neurons.

### Spontaneous $Ca^{2+}$ oscillation does not depend on extracellular $Ca^{2+}$
The results mentioned above suggested an astrocytic-originated $Ca^{2+}$ signal in the network, we then sought the cellular mechanism of such spontaneous action. The elevation of intracellular $Ca^{2+}$ may result either from the $Ca^{2+}$ influx of the extracellular environment, or from the $Ca^{2+}$ release of intracellular $Ca^{2+}$ stores. We first tested the former,
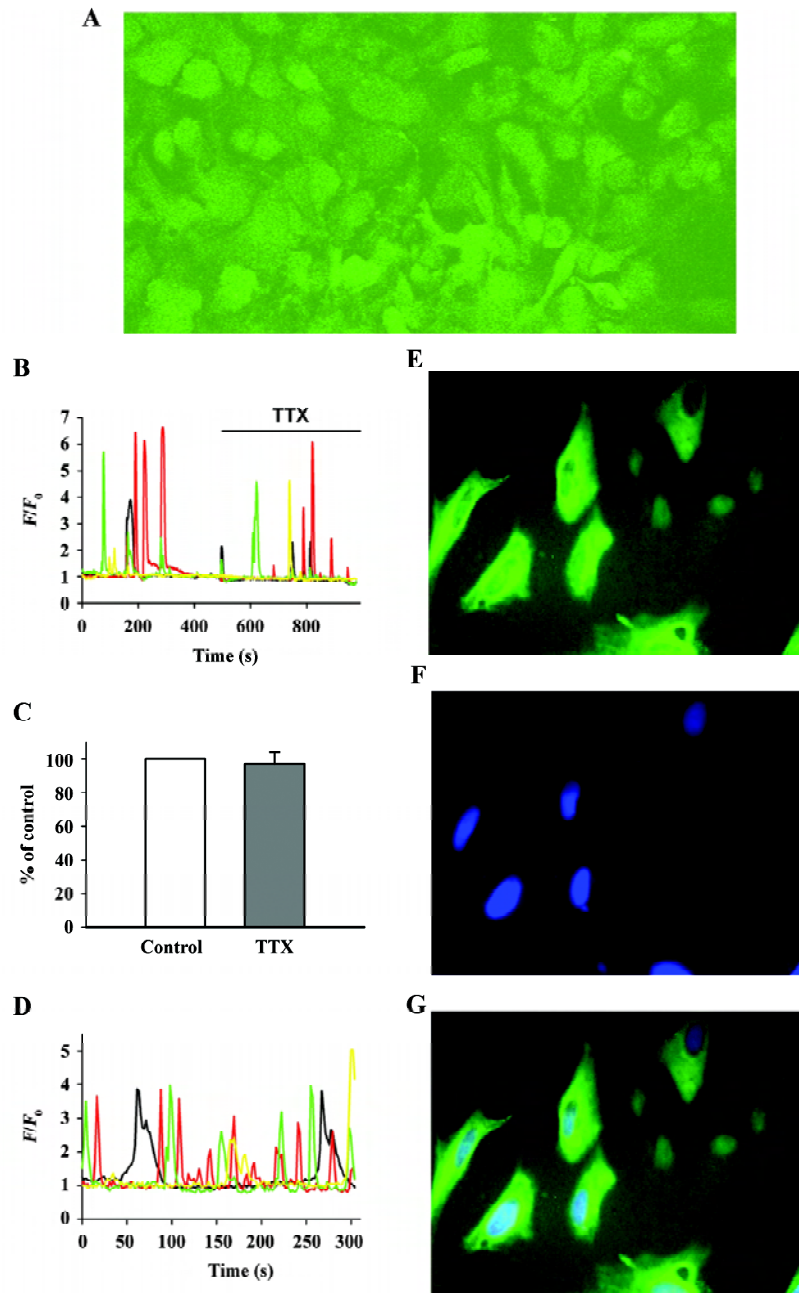
**Figure 1.** The neuronal-independent activity in astrocytes. (A) A sample image of purity culture of astrocytes that loaded with Fluo-4 AM (40× oil immersion objectives). (B) The intracellular $Ca^{2+}$ oscillations in astrocytes from the co-cultured astrocytes network were insensitive to the treatment of TTX (1 μmol/L). The different colors indicated the recording from different cells respectively. (C) Data analysis from (B), the numbers of action for 5 min before and after TTX application ($n$=30). (D) The spontaneous $Ca^{2+}$ oscillations in astrocytes from purity cultured astrocytes network. (E,F) Sample images of purified astrocytes (40× optical objectives) labeled with anti-GFAP antibody (E) and Hoechst 33258 (F). (G) Overlay of (E) and (F).

and found that the spontaneous $Ca^{2+}$ oscillations in astrocytes were insensitive to the treatment of nifedipine, which can selectively block the L-type $Ca^{2+}$ channels. Neither the frequency nor the amplitude of the intracellular $Ca^{2+}$ oscilla-

tion was altered (Figure 2A, 2B). This data indicated that the elevation of intracellular $Ca^{2+}$ was not resulted from $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels.

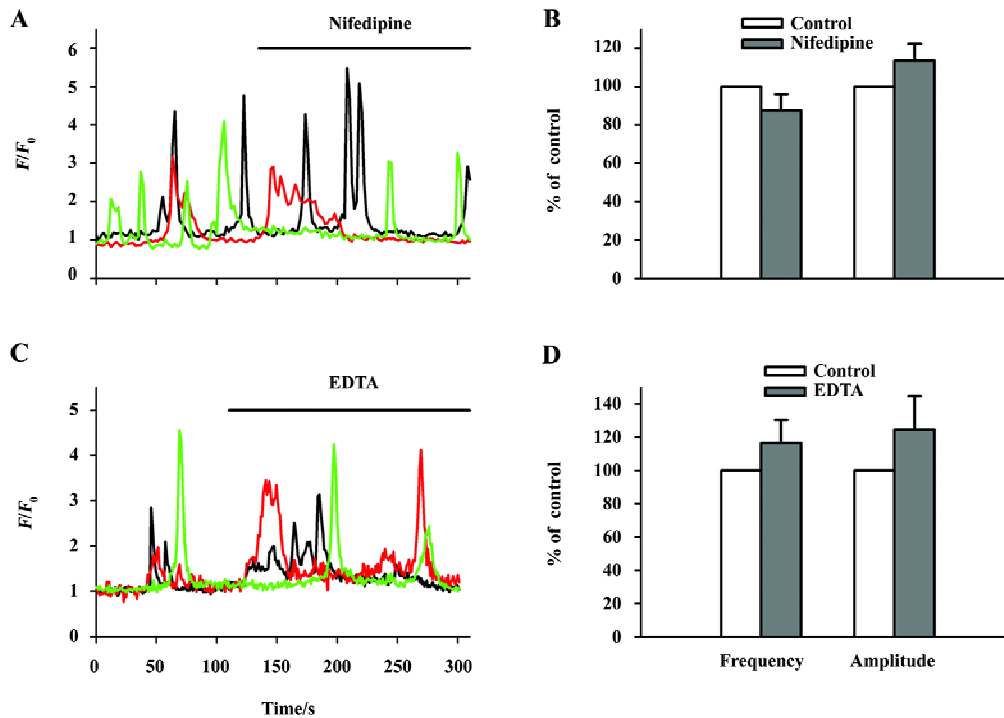Apart from L-type $Ca^{2+}$ channels, there might be other

**Figure 2.** The extracellular-independence of the spontaneous $Ca^{2+}$ oscillations in astrocytes. (A) The intracellular $Ca^{2+}$ oscillations in purity culture of astrocytes were insensitive to the treatment of nifedipine (10 µmol/L). (B) Data analysis from (A), no significant difference was observed in either frequency or amplitude between control cells and nifedipine treated cells. (C) The intracellular $Ca^{2+}$ oscillations in purity culture of astrocytes were insensitive to the treatment of EDTA (20 mmol/L). (D) Data analysis from (C). The value of each group in (B) and (D) was from three separated experiments with over 20 cells in each experiment.

$Ca^{2+}$-permeable channels on plasma membrane. So we bathed the cells in 20 mmol/L EDTA, which eliminated the source of extracellular $Ca^{2+}$. Even under this condition, the spontaneous $Ca^{2+}$ oscillations in astrocytes were not impacted (Figure 2C, 2D). This evidence does not support the extracellular origination of the spontaneous $Ca^{2+}$ oscillations.

**ER $Ca^{2+}$ store is necessary in spontaneous $Ca^{2+}$ oscillations** The results above implied that an intracellular mechanism must be responsible for spontaneous $Ca^{2+}$ oscillations in astrocytes. In order to realize the role of ER $Ca^{2+}$ store in the process, the cells were exposed to low $Na^+$ (70 mmol/L) solution (Figure 3A), which could increase the content of ER $Ca^{2+}$ store without significant change of intracellular $Ca^{2+}$ concentration[17]. The frequency of spontaneous $Ca^{2+}$ oscillations was enhanced to 237%±17% of control by low $Na^+$ solution treatment (Figure 3B; $P<0.01$, $n=51$ and $n=82$ in control and low $Na^+$ group respectively). High $Ca^{2+}$ solution (5 mmol/L) was also applied (Figure 3C), and a similar result was observed (Figure 3D; enhanced to 172%±25% of control; $P<0.05$, $n=30$ in both the control and high $Ca^{2+}$ group). We next blocked the sarcoendoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) on ER with its specific antagonist, thapsigargin,

and found that 2 µmol/L thapsigargin completely eliminated the spontaneous $Ca^{2+}$ oscillations in astrocytes (Figure 3E). The frequency of oscillations was 1.06±0.12 time/min ($n=35$) before and no event after thapsigargin treatment. These results suggest that the content of ER $Ca^{2+}$ store is necessary for generating spontaneous $Ca^{2+}$ oscillations.

**Essential role of InsP$_3$R in the spontaneous $Ca^{2+}$ oscillations** The ER $Ca^{2+}$ store may generate intracellular $Ca^{2+}$ signal through two types of $Ca^{2+}$ release channels, the InsP$_3$Rs and ryanodine receptors (RyRs). To test the role of RyRs in spontaneous $Ca^{2+}$ oscillations, the cells were treated with 50 µmol/L ryanodine or 400 µmol/L tetracaine, two specific antagonists of RyRs. The spontaneous $Ca^{2+}$ oscillations were still robust after blockers application (Figure 4A, 4B). However, treating the cells with 100 µmol/L 2-APB (Figure 4C), the blocker of InsP$_3$Rs, depressed the spontaneous $Ca^{2+}$ oscillations by approximately 90% (Figure 4D, $n=115$). Furthermore, the inhibition of 2-APB behaved in a dose-dependent manner (Figure 4E). In the presence of tetracaine, the effect of 2-APB was more potent than in the absence of tetracaine (Figure 4E), suggesting a potential interaction between InsP$_3$Rs and RyRs. The above evidence suggests
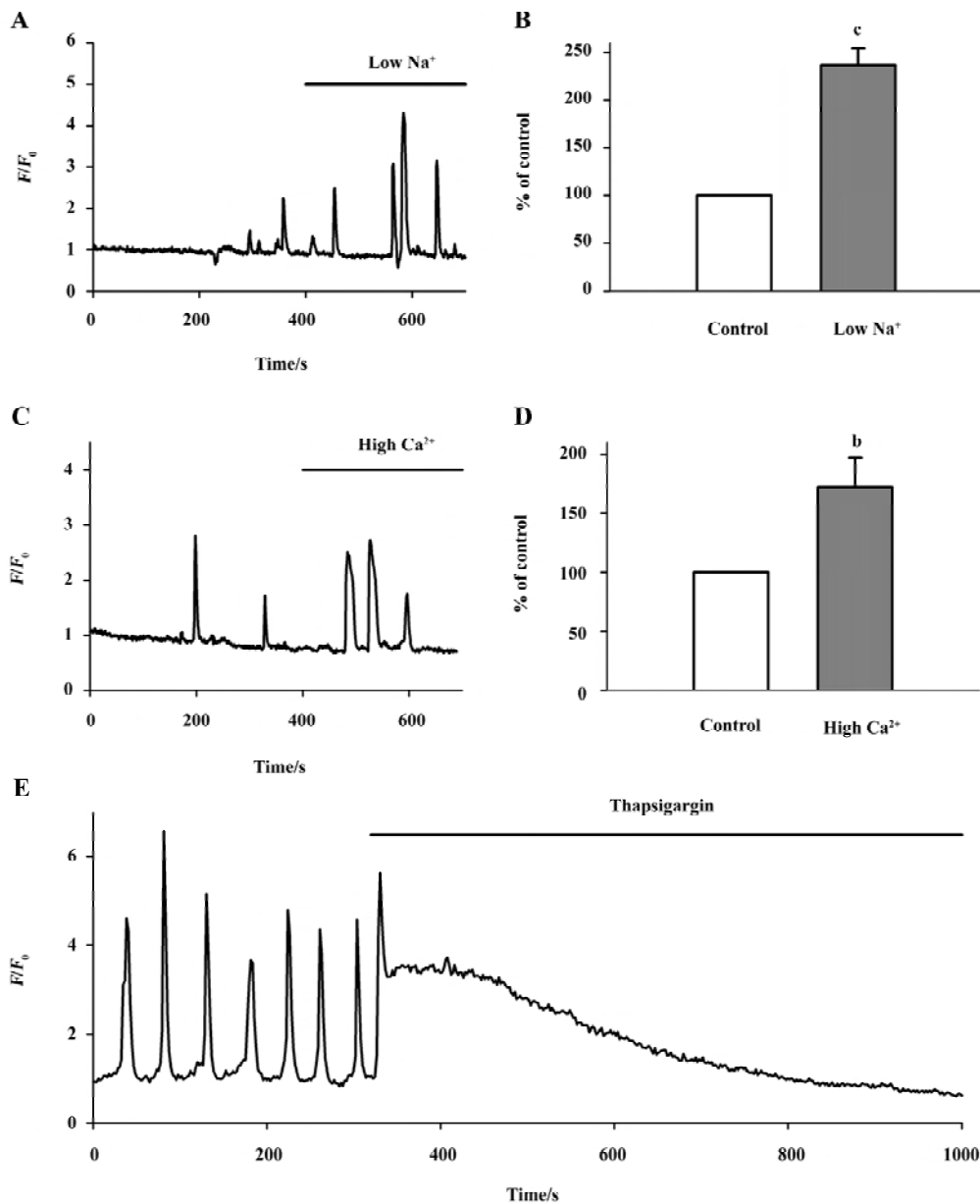
**Figure 3.** The content of ER $Ca^{2+}$ store is a necessary factor for the spontaneous $Ca^{2+}$ oscillations. (A) Loading the ER $Ca^{2+}$ store with low $Na^+$ (70 mmol/L, 50% alternative with Li) bath solution enhanced the spontaneous $Ca^{2+}$ oscillations. (B) Data analysis from (A), the frequency of spontaneous $Ca^{2+}$ oscillations was significantly increased by low $Na^+$ ($n=51$ and $n=82$ in control and low $Na^+$ group, respectively). [c]$P<0.01$ *vs* control group (Student's *t*-test). (C) Loading the ER $Ca^{2+}$ store with high $Ca^{2+}$ (5 mmol/L) bath solution enhanced the spontaneous $Ca^{2+}$ oscillations. (D) Data analysis from (C), the frequency of spontaneous $Ca^{2+}$ oscillations was significant increased by high $Ca^{2+}$ ($n=30$ in both control and high $Ca^{2+}$ group). [b]$P<0.05$ *vs* control group (Student's *t*-test). (E) Depleting the ER $Ca^{2+}$ store with thapsigargin (2 μmol/L), which completely blocks the SERCA, abolished the spontaneous $Ca^{2+}$ oscillations.

that, in the process of spontaneous $Ca^{2+}$ oscillations, InsP₃Rs plays an essential role while RyRs may play an assistant role.

## Discussion

Astrocytes might have a much more essential role than has been revealed in CNS. Thereby understanding the initiation of spontaneous $Ca^{2+}$ oscillations in astrocytes becomes very important. Using confocal laser-scanning microscopy we found that: 1) the content of ER $Ca^{2+}$ store was necessary for the spontaneous $Ca^{2+}$ oscillations; and 2) the activation of InsP₃R played a key role in the process of spon-
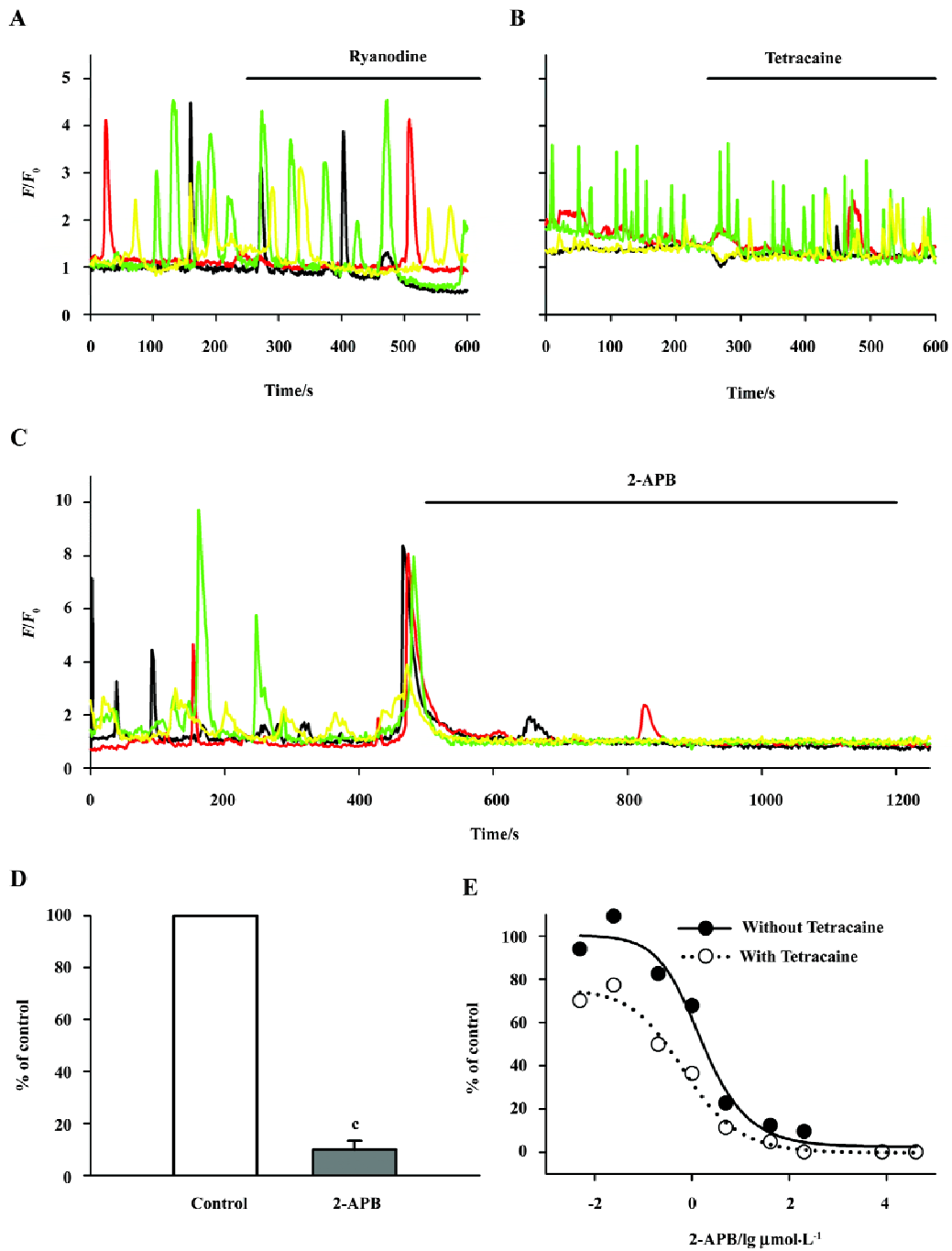
**Figure 4.** InsP$_3$R plays an essential role in the spontaneous Ca$^{2+}$ oscillations in astrocytes. Both ryanodine (50 µmol/L; A) and tetracaine (400 µmol/L; B) treatment did not affect the spontaneous Ca$^{2+}$ oscillation in astrocytes. (C) Incubating the cells in 2-APB (100 µmol/L), which blocked InsP$_3$R, depressed the cell activity. (D) Data analysis from (C), 2-APB significantly reduced the frequency of spontaneous Ca$^{2+}$ oscillations in astrocytes (*n*=115). $^c$*P*<0.01 *vs* control group (Student's *t*-test). (E) The dose-response curve of 2-APB in normal bath solution (—•—) or tetracaine (400 µmol/L) containing bath solution (···o···). The inhibition of 2-APB was increased in the presence of tetracaine, revealing that RyRs may have an indirect effect on the oscillations of astrocytes.

taneous Ca$^{2+}$ oscillation. Our results suggest that InsP$_3$R-induced ER Ca$^{2+}$ release is an important cellular mechanism for the spontaneous Ca$^{2+}$ oscillation in hippocampal astrocytes.

Although there is still an argument that the intracellular $Ca^{2+}$ oscillations in astrocytes comes from neurons[18], most believe the existence of spontaneous $Ca^{2+}$ oscillations in astrocytes. Many investigators have been trying to probe the mechanism for the initiation of spontaneous $Ca^{2+}$ oscillations, but the results have been rather inconsistent. Some reports showed that the spontaneous $Ca^{2+}$ oscillations in astrocytes required extracellular $Ca^{2+[14,16]}$, while others supported the contribution of intracellular $Ca^{2+[19]}$. This disagreement may be the result of different subtypes of astrocytes and different preparations conditions. Our results support the view that the spontaneous $Ca^{2+}$ oscillations in astrocytes originate via intracellular mechanism, and the ER $Ca^{2+}$ store is necessary for the process.

Most of studies supported that the InsP$_3$Rs played an essential role in the process of spontaneous $Ca^{2+}$ oscillations in astrocytes[19–21], and our results is consistent with these reports. However, RyRs are also richly expressed on the ER of astrocytes[12], and a functional $Ca^{2+}$ sensitive store has been reported[22]. All these findings lead to an open question of what the role is of RyRs in spontaneous $Ca^{2+}$ of astrocytes. In the present study, we compared the property of the spontaneous $Ca^{2+}$ oscillations in astrocytes before and after blocking RyRs. Although RyRs blockers-perfusion could not block the spontaneous $Ca^{2+}$ oscillations, we found that tetracaine had some depressing effect when coapplied with 2-APB. Therefore, there may be some interaction between RyRs and InsP$_3$Rs. The crosstalk between RyRs and InsP$_3$Rs has been reported[20], but its physiological significance needs to be investigated further.

Recently studies have revealed that $Ca^{2+}$ signaling in astrocytes-mediated control of cerebral blood flow, is a mechanism of neurovascular coupling[23,24]. It has been shown also that the activity of astrocytes may lead to synchronized $Ca^{2+}$ oscillation in neurons[6]. We believe that the spontaneous $Ca^{2+}$ oscillations in astrocytes play a substantial role in the process of information transferring from astrocytes to neurons. In summary, we demonstrated that the spontaneous $Ca^{2+}$ oscillations in astrocytes were dependent on release from ER $Ca^{2+}$ stores through InsP$_3$Rs, and there was interaction between RyRs and InsP$_3$Rs in the process of spontaneous $Ca^{2+}$ oscillation. Our findings present a new aspect for understanding the $Ca^{2+}$ signal in astrocytes and the essential role of astrocytes in CNS.

## Acknowledgement

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