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Full-length article

Cellular mechanism for spontaneous calcium oscillations in astrocytes[1]

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Key words

hippocampal astrocytes; spontaneous Ca^{2+} oscillations; endoplasmic reticulum Ca^{2+} store; $InsP_3$ receptors; confocal laser scanning microscope

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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 ºC 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, 1×10^5 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 cm^2 culture flasks for purification later at a density of approximately 5×10^8 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,

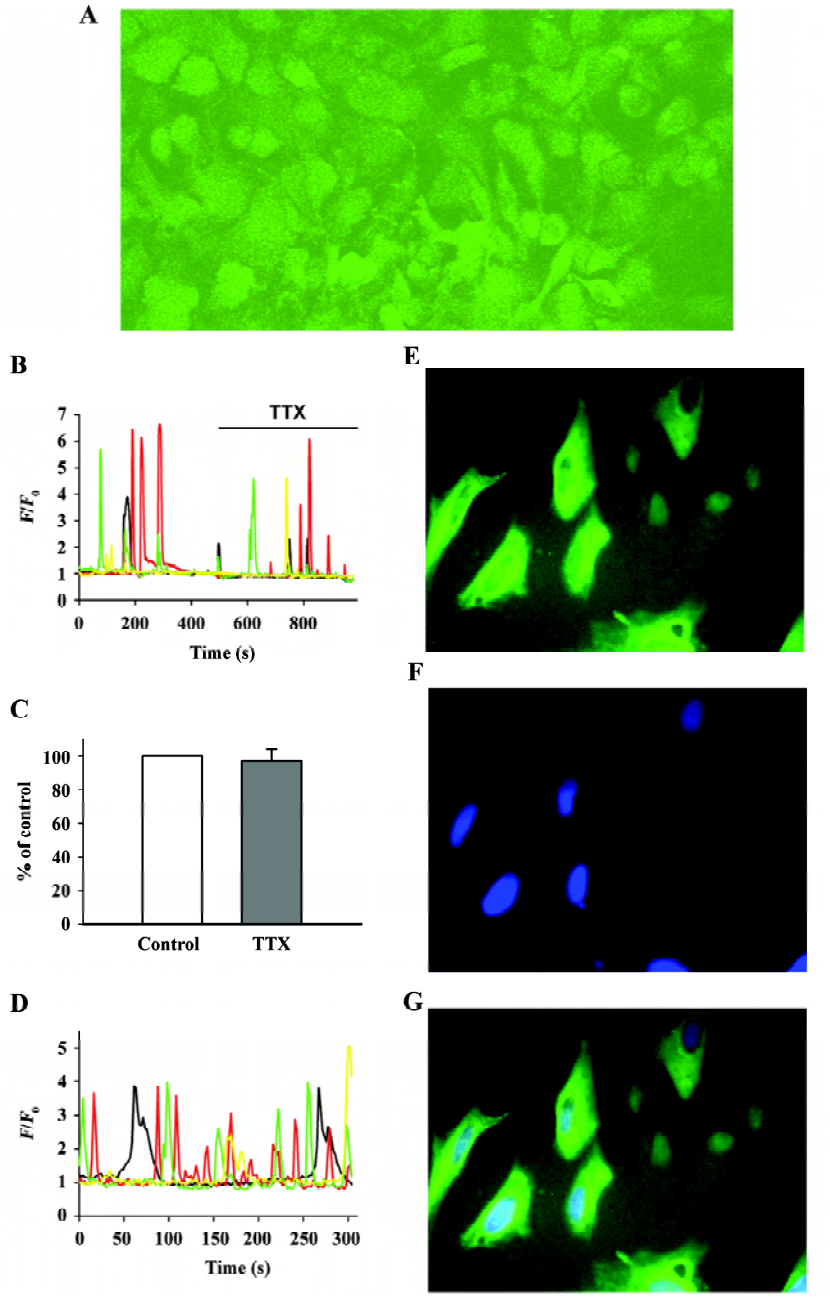

Figure 1. The neuronal-independent activity in astrocytes. (A) A sample image of purity culture of astrocytes that loaded with Fluo-4 AM (40× oil immersion objectives). (B) The intracellular Ca^{2+} oscillations in astrocytes from the co-cultured astrocytes network were insensitive to the treatment of 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+} oscillation 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

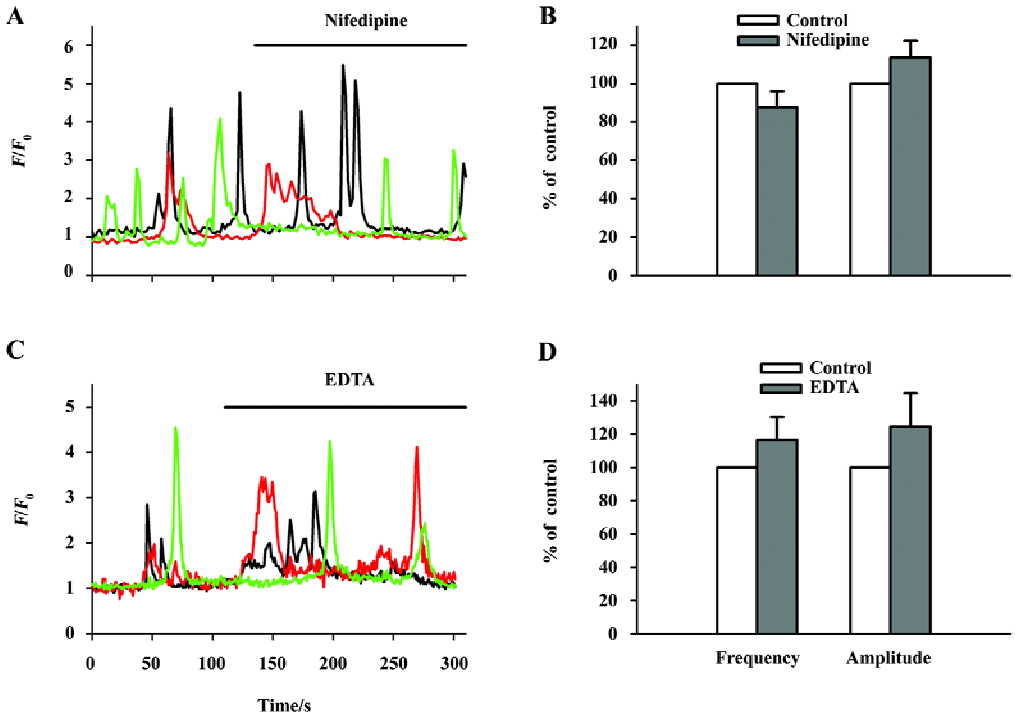

Figure 2. The extracellular-independence of the spontaneous Ca^{2+} oscillations in astrocytes. (A) The intracellular Ca^{2+} oscillations in purity culture of astrocytes were insensitive to the treatment of nifedipine (10 μmol/L). (B) Data analysis from (A), no significant difference was observed in 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 $InsP_3R$ 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 $InsP_3Rs$ 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 $InsP_3Rs$, 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 $InsP_3Rs$ and RyRs. The above evidence suggests

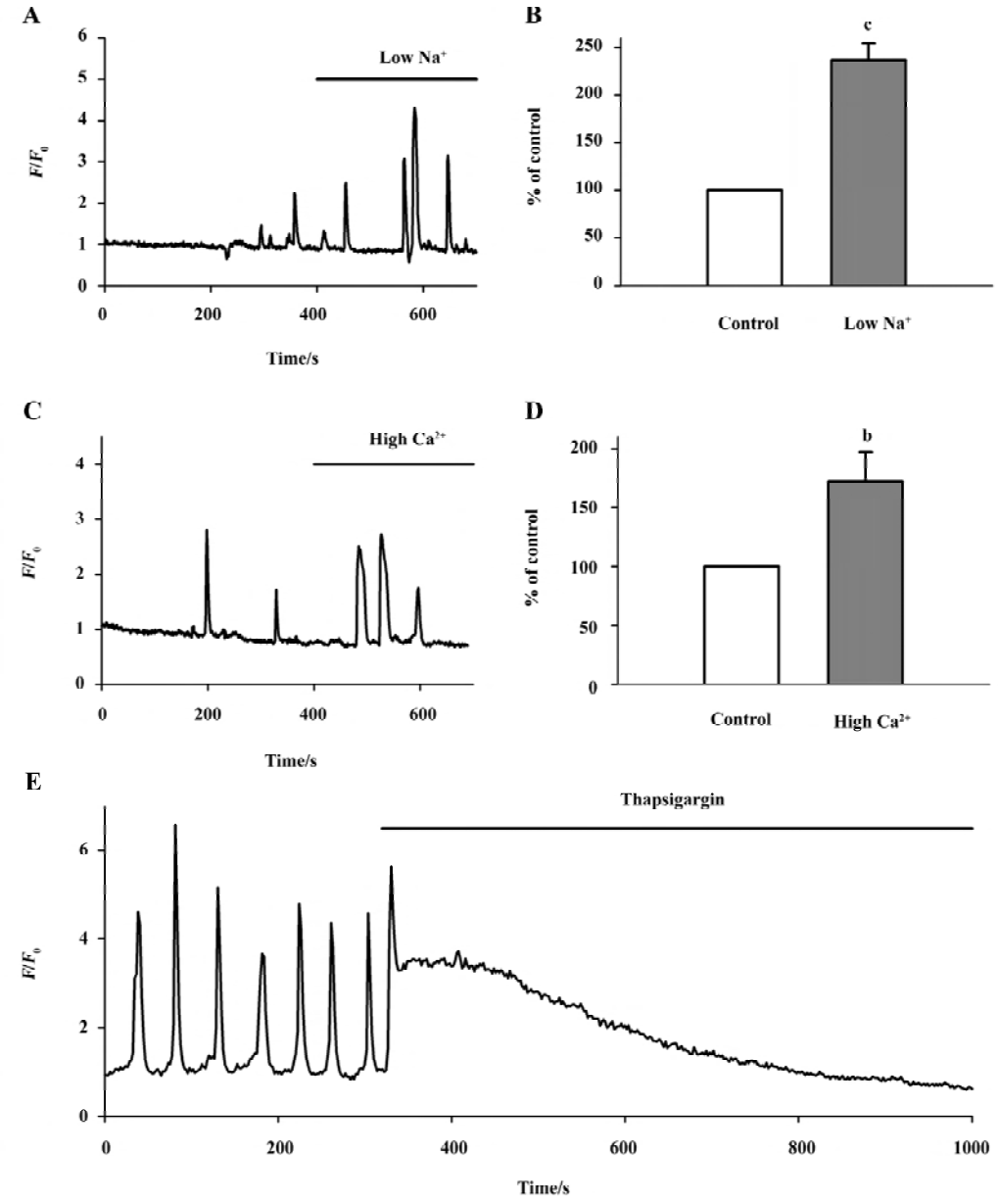

Figure 3. The content of ER Ca^{2+} store is a necessary factor for the spontaneous Ca^{2+} oscillations. (A) Loading the ER Ca^{2+} store with low Na^+ (70 mmol/L, 50% alternative with Li) bath solution enhanced the spontaneous Ca^{2+} oscillations. (B) Data analysis from (A), the frequency of spontaneous Ca^{2+} oscillations was significantly increased by low Na^+ (n=51 and n=82 in control and low Na^+ group, respectively). ^cP<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). ^bP<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, $InsP_3Rs$ 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 $InsP_3R$ played a key role in the process of spon-

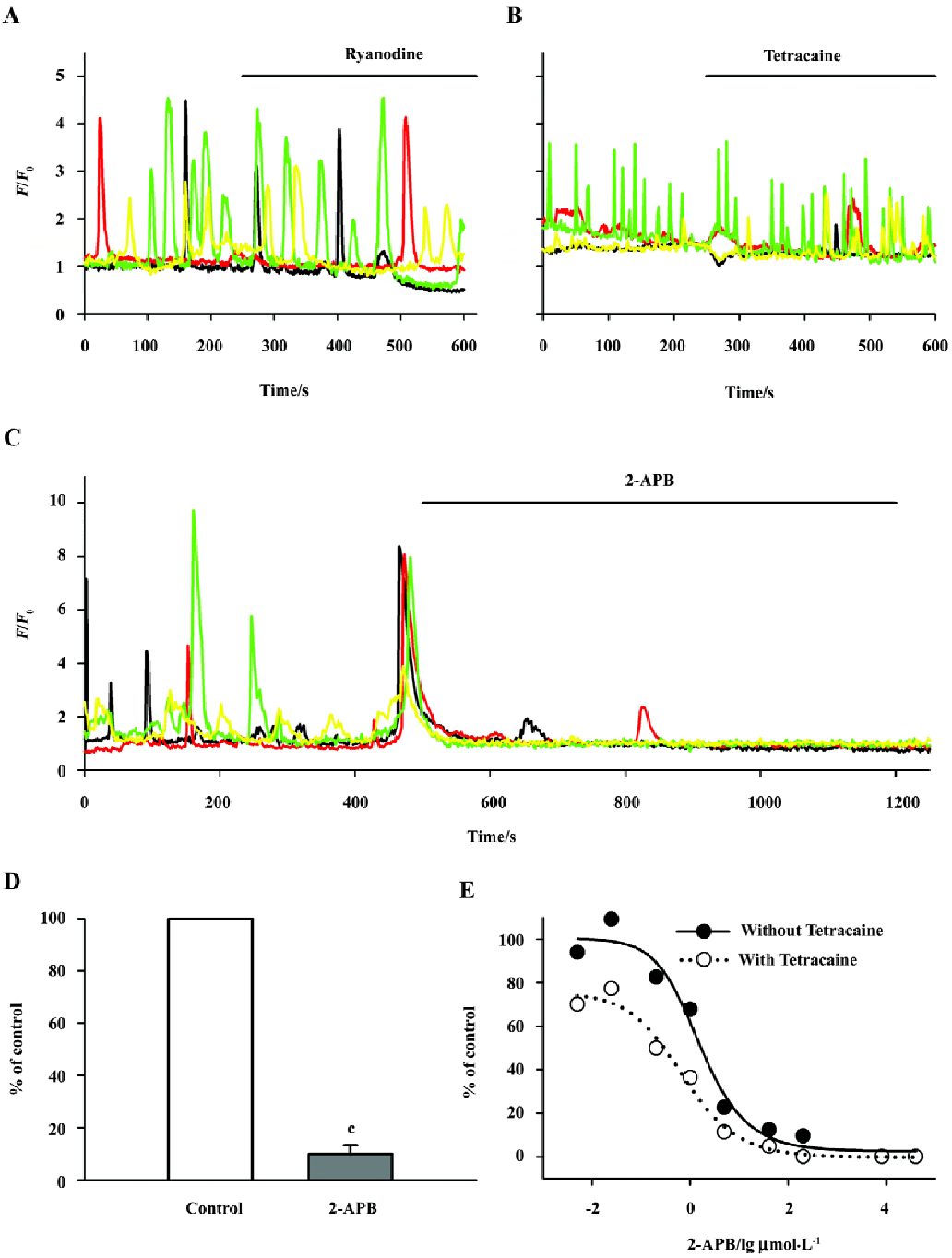

Figure 4. $InsP_3R$ plays an essential role in the spontaneous Ca^{2+} oscillations in astrocytes. Both ryanodine (50 μmol/L; A) and tetracaine (400 μmol/L; B) treatment did not affect the spontaneous Ca^{2+} oscillation in astrocytes. (C) Incubating the cells in 2-APB (100 μmol/L), which blocked $InsP_3R$, depressed the cell activity. (D) Data analysis from (C), 2-APB significantly reduced the frequency of spontaneous Ca^{2+} oscillations in astrocytes (n=115). ^cP<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 $InsP_3R$-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 $InsP_3Rs$ 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 $InsP_3Rs$. The crosstalk between RyRs and $InsP_3Rs$ 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 $InsP_3Rs$, and there was interaction between RyRs and $InsP_3Rs$ 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.

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Most cited papers published in 2003 and 2004

The following are the most cited papers published in ***Acta Pharmacologica Sinica*** during 2003 and 2004, ranked by total citations up to date according to the ISI Web of Science. We are very grateful to all authors' long-term support and excellent contributions to our journal.

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A 3D model of SARS_CoV 3CL proteinase and its inhibitors design by virtual screening. (2003; 24: 497–504)
Times Cited: 42

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Putative hAPN receptor binding sites in SARS_CoV spike protein. (2003; 24: 481–8)
Times Cited: 15

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Small envelope protein E of SARS: cloning, expression, purification, CD determination, and bioinformatics analysis. (2003; 24: 505–11)
Times Cited: 14

4 **Cao LZ, Lin ZB.**
Regulatory effect of *Ganoderma lucidum* polysaccharides on cytotoxic T-lymphocytes induced by dendritic cells *in vitro*. (2003; 24: 321–6)
Times Cited: 12

5 **Zhang Y, Wu LJ, Tashiro S, Onodera S, Ikejima T.**
Evodiamine induces tumor cell death through different pathways: apoptosis and necrosis. (2004; 25: 83–9)
Times Cited: 11

6 **Fan CL, Li Y, Gao PJ, Liu JJ, Zhang XJ, Zhu DL.**
Differentiation of endothelial progenitor cells from human umbilical cord blood CD 34(+) cells *in vitro*. (2003; 24: 212–8)
Times Cited: 11

7 **Zhang M, Zhang X, Bai CX, Chen J, Wei MQ.**
Inhibition of epidermal growth factor receptor expression by RNA interference in A549 cells. (2004; 25: 61–7)
Times Cited: 10

8 **He SH, Chen P, Chen HQ.**
Modulation of enzymatic activity of human mast cell tryptase and chymase by protease inhibitors. (2003; 24: 923–9)
Times Cited: 10

9 **Chang L, Du JB, Gao LR, Pang YZ, Tang CS.**
Effect of ghrelin on septic shock in rats. (2003; 24: 45–9)
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Association of vitamin D receptor and estrogen receptor-α gene polymorphism with peak bone mass and bone size in Chinese women. (2004; 25: 462–8)
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Inhibition of cytochrome P450 by furanocoumarins in grapefruit juice and herbal medicines. (2004;25: 129–36)
Times Cited: 9

12 **Iskit AB, Guc O.**
Effects of endothelin and nitric oxide on organ injury, mesenteric ischemia, and survival in experimental models of septic shock. (2003; 24 : 953–7)
Times Cited: 9

13 **Li XH, Li JJ, Zhang HW, Sun P, Zhang YL, Cai SH, *et al*.**
Nimesulide inhibits tumor growth in mice implanted hepatoma: overexpression of Bax over Bcl-2. (2003; 24: 1045–50)
Times Cited: 9

14 **Wise H.**
Multiple signalling options for prostacyclin. (2003; 24: 625–30)
Times Cited: 9

15 **Liu BC, Chen Q, Luo DD, Sun J, Phillips AO, Ruan XZ, *et al*.**
Mechanisms of irbesartan in prevention of renal lesion in streptozotocin-induced diabetic rats. (2003; 24: 67–73)
Times Cited: 9