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Original Research

Calcium oscillations in freshly isolated neonatal rat cortical neurons[1]

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KEY WORDS calcium; neurons; cerebral cortex; potassium channels; Sprague-Dawley rats

ABSTRACT

AIM: To investigate the mechanisms of intracellular calcium concentration ($[Ca^{2+}]_i$) oscillations in freshly isolated neonatal rat cortical neurons. **METHODS**: Cortical neurons were isolated from neonatal rats 6 - 7 d after birth by enzymatic digestion. $[Ca^{2+}]_i$ changes were measured in a microscopic calcium measurement system with Fura-2 as indicator. **RESULTS**: In a total of 82 neurons recorded, 47 showed spontaneous $[Ca^{2+}]_i$ oscillations. The spontaneous $[Ca^{2+}]_i$ oscillations were dependent on $[Ca^{2+}]_o$. Removal of $[Ca^{2+}]_o$ completely abolished spontaneous oscillations. Tetraethylammonium 1 mmol/L increased both the amplitude and frequency of calcium oscillations, whereas the frequency was increased by Cs^+ 1 mmol/L. Ba^{2+} 1 mmol/L, in contrast, induced $[Ca^{2+}]_i$ oscillations superimposed on a sustained phasic increase. **CONCLUSION**: Spontaneous $[Ca^{2+}]_i$ oscillation is an intrinsic property of neonatal rat cortical neurons. Potassium channels play an important role in the control of both the amplitude and frequency of $[Ca^{2+}]_i$ oscillations in cortical neurons.

INTRODUCTION

Intracellular calcium ($[Ca^{2+}]_i$) is a ubiquitous intracellular signal which by way of calmodulin, Ca^{2+}/calmodulin-dependent protein kinases, and corresponding substrates, modulates multiple cellular functions in different types of cell. At the single cell level, $[Ca^{2+}]_i$ increases are by the form of oscillations, being subject to both amplitude and frequency modulation (AM and FM). AM and FM modulation confers complex encoding of Ca^{2+} signaling, to ensure specificity of modulation. The mechanism of Ca^{2+} oscillation occupies a pivotal position in cell physiology.

It has been shown that phospholipase C signaling pathway plays a critical role in the generation of Ca^{2+} oscillation in non-excitable cells such as the pancreatic acinar cells[1]. But in electrically excitable cells, voltage-dependent ionic channels are very important, eg, the cyclic opening of the ATP-sensitive potassium channel K_{ATP} is critical for Ca^{2+} oscillations in pancreatic β cells[2]. In cultured neuronal cells, spontaneous Ca^{2+} oscillations are frequently observed. In these cases, neurotransmitter release and synaptic connection was found to play an important role in the generation of spontaneous Ca^{2+} oscillation[3]. But whether spontaneous Ca^{2+} oscillation occurs in synaptically disconnected neurons is not known.

In this work, we found that freshly isolated (with no synaptic connection) and perfused neonatal rat cortical neurons showed spontaneous Ca^{2+} oscillations, indicating that Ca^{2+} oscillation may be an intrinsic property of neurons. It was also found that potassium channels played an important role in the encoding of calcium oscillatory signal.

MATERIALS AND METHODS

New born Sprague-Dawley rats of 6 - 7 d old were killed by cervical dislocation. Brain slices (400 μm) were cut in artificial cerebrospinal fluid solution (ACFS) at 4 ℃ on a vibroslicer (ZQP-86, China). The slices were held at 22 - 25 ℃ in oxygenated ACFS for 51 - 60

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min before and 40 min after digestion in Pronase E (0.04 %, 20 min). A piece of brain tissue was mechanically dissociated by gentle trituration with fire polished pipettes with decreasing diameters (ϕ 700 – 100 μm) in Sykus-Moore chamber with the bottom of coverslip treated with Cell-Tak (Becton Dickinson, Bedford, MA, USA). Dissociated neurons were allowed to attach for 15 min before perfusion with oxygenated perfusion buffer.

ACFS used in this work had the following composition (mmol/L): NaCl 126, KCl 5, NaH_2PO_4 1.25, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, and glucose 10. Perfusion buffer had the following composition (mmol/L): NaCl 138, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, glucose 20, and *N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid] (HEPES) 10, pH 7.4. Freshly isolated cortical neurons were incubated in perfusion buffer containing Fura-2 AM 3 μmol/L for 40 min at room temperature with gentle shaking by the hand every 10 min. After loading, neurons were perfused with buffer for 20 min to remove unloaded Fura-2 AM. Sykus-Moore chamber containing Fura-2 loaded cells was positioned on the microscope stage of an inverted fluorescence microscope (Olympus IX70) coupled to a microfluorometric calcium measurement system (M40, PTI, USA). Fura-2 was excited at 340 nm/380 nm and fluorescence recorded at 510 nm ($1 \times 10^4 - 1 \times 10^6$ counts per second), $[Ca^{2+}]_i$ changes being recorded as changes in fluorescence ratio F_{340}/F_{380}. Neurons were continuously perfused (1 mL/min), and stimulating chemical was introduced to the neurons by a change of perfusing buffer.

Fura-2 AM and Cell-Tak were purchased from Molecular Probes (Eugene, Oregan, USA) and from Becton Dickinson (Bedford, MA, USA) respectively. Pronase E, tetraethylammonium (TEA), $BaCl_2$, and CsCl were from Sigma (St Louis, MO, USA). All other chemicals were of analytical grade.

RESULTS

The dissociated cortical neurons (>90 % viable by Trypan Blue exclusion) readily attached to Cell-Tak treated cover-slips, and maintained at least one neurite. After Fura-2 loading, the neurons remained alive for >2 h.

In a total of 82 untreated neurons, 47 showed spontaneous $[Ca^{2+}]_i$ oscillations (57.3 %). These oscillations were rather sporadic, the frequency and amplitude showed some variation (Fig 1, $n = 47$).

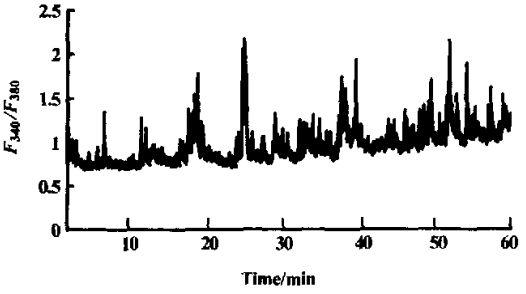

Fig 1. Spontaneous $[Ca^{2+}]_i$ oscillations in freshly isolated rat cortical neurons. Similar oscillations were observed in 46 other cells ($n = 47$).

In the oscillating cells, it was found that extracellular calcium was important for the maintenance of oscillations. Removal of $[Ca^{2+}]_o$ (omission of Ca^{2+} from the medium) completely abolished these $[Ca^{2+}]_i$ oscillations (Fig 2, $n = 8$).

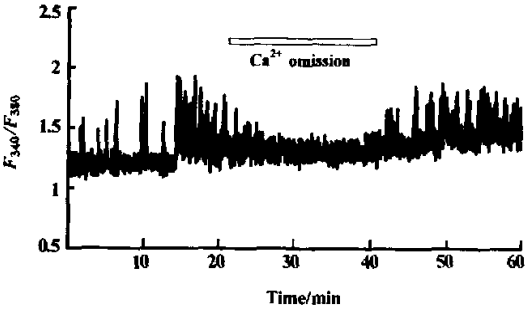

Fig 2. Removal of extracellular calcium abolished spontaneous Ca^{2+} oscillations. $[Ca^{2+}]_o$ was removed during the period indicated by the horizontal bar. Similar effects were observed in 7 other cells ($n = 8$).

To determine the effects of potassium channels in the generation of calcium oscillations, a number of potassium channel inhibitors were used. These include inhibitors for the outward current (TEA), the inward rectifiers (Cs^+), and both the outward current and inward rectifier (Ba^{2+}). When TEA 1 mmol/L was added to the perfusion medium, the amplitude of oscillations increased markedly, the frequency also showed significant increase. The TEA effect was reversible (Fig 3, $n = 6$). In non-oscillating cells, TEA could also induce oscillations (not shown). It is interesting to note that in comparison with Cs^+ and Ba^{2+}, the onset of TEA effect was significantly

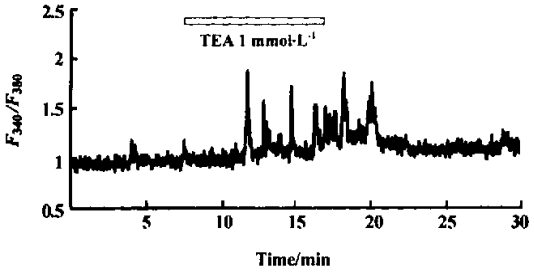

Fig 3. Effect of TEA on spontaneous $[Ca^{2+}]_i$ oscillations in freshly isolated rat cortical neurons. TEA was added during the period indicated by the horizontal bar. Similar effects were observed in 5 other cells ($n=6$).

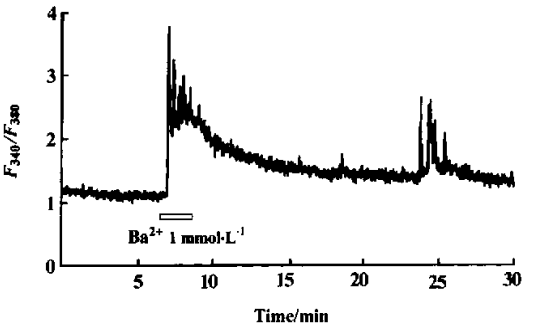

Fig 5. Effect of Ba^{2+} on spontaneous $[Ca^{2+}]_i$ oscillations in freshly isolated rat cortical neurons. Ba^{2+} was added during the period indicated by the horizontal bar. Similar effects were observed in 6 other cells ($n=7$).

delayed (5 min).

In comparison, when Cs^+ 1 mmol/L was added to the perfusion medium, the frequency of oscillations increased (Fig 4, $n=6$). The effect was reversible upon wash-out of Cs^+. Ba^{2+} 1 mmol/L, a dual inhibitor for both outward current and inward rectifier current, induced bursting $[Ca^{2+}]_i$ oscillations superimposed on a long-lasting phasic increase in $[Ca^{2+}]_i$ (Fig 5, $n=7$). This latter increase was long-lasting or quite permanent, in this particular cell, a secondary burst also occurred at a later time.

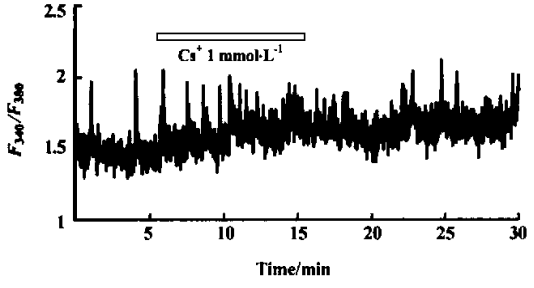

Fig 4. Effect of Cs^+ on spontaneous $[Ca^{2+}]_i$ oscillations in freshly isolated rat cortical neurons. Cs^+ was added during the period indicated by the horizontal bar. Similar effects were observed in 5 other cells ($n=6$).

DISCUSSION

In this work, it was found that spontaneous calcium oscillations occurred in freshly isolated neonatal rat cortical neurons (Fig 1). The percentage of oscillating cells was 57 % (47 out of 82), significantly higher than primary culture of the same neurons. Shimizu *et al* (1992), using primary cultured embryonic rat cortical neurons, found that about 30 % of the cultured neurons showed spontaneous calcium oscillations. It was also determined that synaptic connections and NMDA receptors were important in the generation of these spontaneous calcium oscillations[3].

In a more recent report, it was found that in primary cultured embryonic rat cortical neurons, both removal of Mg^{2+} from extracellular medium and addition of 4-aminopyridine (4-AP) induced synchronized calcium oscillations. These synchronized oscillations were triggered by release of glutamate, and subsequent NMDA (Mg^{2+} induction) and AMPA/KA (4-AP induction) receptor activation. Synapse formation was essential in the generation of synchronized oscillations, both in the initiation and propagation phases. These authors speculated that spontaneous or constitutive synaptic vesicle fusion and glutamate release were particularly important in the initiation of this process[4]. The freshly isolated neurons do not form synaptic connections, therefore these oscillations must be of a different mechanism.

Spontaneous electrical activities are a common feature for many types of excitable cells. Spontaneous action potentials are routinely observed in gonadotropin-releasing hormone (GnRH) neurons[5,6], smooth muscle cells[7,8], cerebellar Purkinje cells[9], and anterior and intermediate pituitary cells[10,11]. Spontaneous EPSP are routinely observed in all cortical neurons[12], and neurons in the cortex are known to show intrinsic electrical oscillations[12,13]. Membrane depolarization leads to the opening of voltage-dependent calcium channels (L, N,

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T, and P/Q) which have been shown to exist in isolated rat cortical neurons[4], and this leads to subsequent calcium influx. Spontaneous action potential is a direct cause for increases in $[Ca^{2+}]_i$. The fact that omission of calcium from the extracellular solution abolished $[Ca^{2+}]_i$ oscillation (Fig 2) confirmed that calcium influx was important for the maintenance of spontaneous $[Ca^{2+}]_i$ oscillations. Therefore bursts of action potentials will account for the generation of spontaneous $[Ca^{2+}]_i$ oscillations in the freshly isolated neonatal rat cortical neurons.

Potassium channel inhibitors modified both the frequency and amplitude of calcium oscillations (Fig 3 – 5). The outward potassium current had been identified before in isolated rat cortical neurons[14]. TEA induced increase in the amplitude of $[Ca^{2+}]_i$ oscillations was due to its effect to prolong the duration of action potential and subsequently increased action potential-carried calcium influx. But it also increased the frequency of calcium oscillations, indicating that increased calcium entry may have some positive effect on the neuron itself for subsequent ability to fire action potentials in oscillating neurons (which may also make non-oscillating neurons tend to oscillate). The inward K^+ rectifier current is known to oppose depolarizing influences[15], to dampen neuronal excitability[16], and to slow heart rate or be involved in GABA-mediated inhibition in CNS neurons[17,18]. Inhibition of the inward K^+ current increases the likelihood of action potential bursts. Therefore inhibition of the inward K^+ current by Cs^+ increased the frequency of calcium oscillations. Similar effects of potassium channel inhibitors on spontaneous calcium oscillations have been observed in the anterior pituitary lactotrophes also[19]. Ba^{2+} inhibited both inward and outward K^+ currents and subsequently induced a phasic increase in $[Ca^{2+}]_i$ upon which bursting oscillations occurred. The sustained elevation in $[Ca^{2+}]_i$ also led to secondary burst of calcium oscillations. Ba^{2+} induced phasic or sustained increase in F_{340}/F_{380} was probably due to a lack of efficiency of the calcium pump to handle Ba^{2+}.

In contrast to the regular, synchronized $[Ca^{2+}]_i$ oscillation observed in cultured neurons, the spontaneous calcium oscillations in the freshly isolated cortical neurons were rather sporadic or stochastic, with much less consistency both in frequency and amplitude. Some excitable cells are known to generate spontaneous electrical impulses at random[20]. The opening and closure of individual ion channels occur randomly[21]. Single channel currents are known to trigger action potentials[22]. Action potentials drive calcium influx, sporadic or stochastic bursts of action potentials thus lead to sporadic or stochastic $[Ca^{2+}]_i$ oscillations. The identities of the oscillating and non-oscillating neurons remain to be clarified. These spontaneous activities may, however, play a role in CNS development and maturation. The potassium channels seemed very important in damping or clamping the occurence of $[Ca^{2+}]_i$ oscillations. In organized neuron network, synaptic connection leads to a lower percentage of spontaneously oscillating cells.

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新鲜分离的新生大鼠皮层神经元的钙振荡[1]

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关键词 钙；神经元；大脑皮质；钾通道；Sprague-Dawley 大鼠

目的：研究新鲜分离的新生大鼠皮层神经元胞内钙离子浓度($[Ca^{2+}]_i$)发生振荡的机制. **方法**：采用酶解结合机械分离法从 6 – 7 日龄大鼠分离皮层神经元. 用 M40 钙离子测量系统(PTI)测量细胞内钙离子浓度的变化. 用 Fura-2 作为钙离子指示剂. **结果**：在观察到的 82 个神经元细胞中，47 个产生了自发钙振荡. 自发钙振荡依赖于胞外钙离子浓度. 去除外钙后自发钙振荡立即停止. 四乙铵 1 mmol/L 引起钙振荡振幅增大，频率变快. CsCl 1 mmol/L 主要引起频率增加. $BaCl_2$ 1 mmol/L 可使振幅、频率增高，并有明显的高台样基线增加. **结论**：皮层神经元在无突触联系的情况下具有产生自发$[Ca^{2+}]_i$振荡的特性，K^+通道在决定钙振荡的幅值和频率方面起重要作用.

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