Mechanisms underlying low $[Ca^{2+}]_o$ -induced increased excitability of hippocampal neurons

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Abstract: Objective Concentration of extracellular calcium ($[Ca^{2+}]_o$) in the central nervous system decreases substantially in different conditions. It results in facilitating neuronal excitability. The goal of this study is to examine the mechanisms of enhanced neuronal excitation in low $[Ca^{2+}]_o$ in order to provide new clues to treat the hyperexcitability diseases in clinic. **Methods** Whole-cell patch-clamp technique and neuron culture were used in the study. **Results** The firing threshold of cultured hippocampal neurons decreased markedly in low $[Ca^{2+}]_o$ saline. Unexpectedly, apamine and isoprenaline, antagonists of medium afterhyperpolarization (mAHP) and slow AHP (sAHP) respectively, had no statistic significant effect on excitability of neurons. TTX at a low concentration was sufficient to inhibit I_{NaP} , which blocked the increase of firing frequency in low $[Ca^{2+}]_o$. It also reduced the number of spikes in normal $[Ca^{2+}]_o$. **Conclusion** These results suggest that in cultured hippocampal neurons, modulation of spiking threshold but not AHP may cause the increased excitability in low $[Ca^{2+}]_o$.

Keywords: small-conductance calcium-activated potassium channels (SK_{Ca}); hippocampus; calcium

1 Introduction

Concentrations of extracellular calcium ($[Ca^{2+}]_o$) in the central nervous system decrease substantially in different conditions. Decreases in $[Ca^{2+}]_o$ can be seen during brain ischemia^[1], or by experimentally induced seizure activity^[2,3]. It has been reported that decreases in $[Ca^{2+}]_o$ facilitated neuronal excitability^[2,4]. Infusion of low-calcium solutions into the brains of cats and other species has been demonstrated to induce seizure-like activity. Thus $[Ca^{2+}]_o$ reduction *in vivo* could trigger a seizure, inasmuch as the intense neuronal activity associated with seizures could further decrease $[Ca^{2+}]_o$ in a feed-forward manner^[5]. It is important to find out the detailed mechanisms underlying enhanced neuronal excitation in low $[Ca^{2+}]_o$ in order to explore new method to treat the

hyperexcitability diseases in clinic.

Afterhyperpolarizations (AHPs) which follow action potentials are important factors for controlling neuronal excitability. In hippocampal neurons, there are three distinct components that can form AHP: fast, medium and slow AHP (fAHP, mAHP, sAHP). The fAHP, mediated by both Ca^{2+} - and voltage-sensitive channel, lasts a few milliseconds and contributes to spike repolarization^[6]. The mAHP, which is mediated by voltage-independent, small conductance Ca^{2+} -activated K^+ channels (SK_{Ca}), can be found after single action potential, but is more pronounced after spike trains. The mAHP is characterized by a time constant of decay of 100 ms about and by its sensitivity to the bee venom toxin, apamin, and to the scorpion toxins, scyllatoxin and tamapin. It regulates firing frequency and may contribute to spike frequency adaptation. sAHP has a slower time course (in the range of seconds), and is insensitive to apamin, but can be modulated by several neurotransmitters^[7,8], which may underlie spike frequency adaptation. Garduño *et al.* reported that 1-Ethyl-2-benzimidazolinone (EBIO), a drug that enhances the I_{AHPs} , suppressed epileptiform activity in hippocampus slice^[9].

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Also, the excitability of neurons is modulated by persistent sodium current (I_{NaP}). Compared with the transient voltage-dependent sodium current, which has rapid kinetics and contributes to the generation of action potential, the I_{NaP} sustains after the rapid decay of the transient sodium current. I_{NaP} ordinarily accounts for about 1% of the peak inward sodium current^[10]. Despite its small amplitude compared with peak sodium current, I_{NaP} can alter firing behavior profoundly, especially in the subthreshold voltage range. It has been shown that I_{NaP} is involved in generation of the afterdepolarization (ADP) and the role of Na^+ channels in ADP is enhanced in low $[Ca^{2+}]_o$ solution. I_{NaP} plays a key role in spontaneous activity and burst generation in hippocampal neurons in zero-calcium solutions^[3,5].

Besides, cell properties such as resting membrane potential, threshold, are also participated in regulating the excitation of neurons. In this study, we examined three hypotheses concerning the underlying mechanism of hippocampal neurons in low $[Ca^{2+}]_o$ solution: (1) decrease of spike threshold; (2) suppression of Ca^{2+} -activated K^+ currents; and (3) upmodulation of persistent Na^+ current (I_{NaP}). Our data are consistent with the first hypothesis.

2 Materials and methods

2.1 Culture of hippocampal neurons The method used for culture of embryonic rat hippocampal neurons was described in previous study^[11]. Carefully scooped brain out and separated the hippocampi from embryonic day 17-18 (E17-18) rats. Added trypsin to digest for 15 min at 37 °C, washed, and then dispersed cells by gentle trituration. Planted the cells on poly L-lysine precoated glass coverslips. The cells were incubated with the medium composed of DMEM (BioWhittaker), 10% bovine calf serum (HyClone), 10% Ham's F-12 with glutamine (Bio Whittaker), 50 units/mL penicillin-streptomycin (Sigma), and 1- B-27 supplement (Invitrogen-Gibco). One third of the culture medium was replaced with the same medium supplemented with 20 mmol/L KCl 24 h later. Cytosine arabinoside (Sigma) was used to prevent overgrowth of glial cells around 7-10 d. The neurons were used in patch-clamp experiment on the day 14-21.

2.2 Patch-clamp recording Whole-cell perforated patch-clamp technique was obtained from cultured hippocampal neurons at room temperature, using amphotericin B (Sigma) for perforation. The composition of the pipette solution was (in

mmol/L): potassium gluconate 136.5, KCl 17.5, NaCl 9, $MgCl_2$ 1, Hepes 10, EGTA 0.2, and 200 μ g/mL amphotericin B, pH 7.2-7.4. The external bath solution contained (in mmol/L) NaCl 150, KCl 3, $CaCl_2$ 3, $MgCl_2$ 2, Hepes 10, and glucose 5, pH 7.2-7.4.

2.3 Statistical analysis The cells were perfused with fresh bath solution at a slow rate throughout the experiment. In current-clamp recordings, voltage responses were recorded with an Axocalp 700A amplifier (Axon instruments, USA). pCLAMP software (Axon Instruments, USA) was used to perform signal acquisition and data analysis. Results were presented at means $\pm$ SD. Statistical parameters were assessed with Student's *t*-test. $P < 0.05$ was considered statistically significant.

3 Results

3.1 Frequency of spikes vs $[Ca^{2+}]_o$ To identify whether the GABAergic neurons had the same responses as glutamatergic neurons in low $[Ca^{2+}]_o$, neuron classification was achieved by recording pairs of neurons that had formed functional synaptic connections simultaneously. In voltage-clamp mode, stimulate one neuron with 1 ms depolarization from -70 mV to +30 mV and record the responses from the other neuron. In our recording conditions, both excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) were inward currents, but the IPSCs had longer decay time and more negative reversal potentials than EPSCs. The details were described in the previous study^[12].

After confirming cell type, 10 μ mol/L 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), 10 μ mol/L bicuculline methiodide (BMI) and 20 μ mol/L 2-amino-5-phosphonopentanoic acid (AP5) were added to the bath as AMPA receptor blocker, GABA_A receptor blocker, and NMDA receptor blocker respectively to break off the neuronal network. In current-clamp mode, suprathreshold current injections (300 ms) elicited repetitive firing of hippocampal neurons.

Spike frequency increased clearly after 4-5 min superfusion with low $[Ca^{2+}]_o$ 1.5 mmol/L and free- Ca^{2+} solution, and recovered when standard external solution was reapplied. GABAergic ($n = 4$) and glutamatergic ($n = 16$) neurons increased their firing frequency (62.2 $\pm$ 30.3)%, (61.7 $\pm$ 37.0)% respectively when the $[Ca^{2+}]_o$ was lowered from 3 to 1.5 mmol/L, and increased firing frequency (126.4 $\pm$ 49.3)%, (125.7 $\pm$ 59.7)% respectively when changed from 3 mmol/L to zero- Ca^{2+} solution. There is no significant difference of spike frequency

between GABAergic and glutamatergic neurons ($P > 0.05$). Figure 1A shows a sample of increased spike rate of neuron when it was perfused with 1.5 mmol/L $[Ca^{2+}]_o$ and free $[Ca^{2+}]_o$ solution.

3.2 Membrane properties vs $[Ca^{2+}]_o$ We compared membrane properties in control $[Ca^{2+}]_o$ (3 mmol/L), low $[Ca^{2+}]_o$ (1.5 mmol/L) and zero- $[Ca^{2+}]_o$ solutions. Resting membrane potential, spike amplitude, depolarization, threshold, and AHP amplitude were observed in hippocampal neurons (Table 1). The resting membrane potential did not change significantly, but the threshold decreased and the amplitude of action potential increased markedly in low $[Ca^{2+}]_o$ and free $[Ca^{2+}]_o$ solution ($P < 0.05$, $n = 5$) (Fig. 1B). The AHP amplitude decreased in low $[Ca^{2+}]_o$ and free $[Ca^{2+}]_o$ ($P < 0.05$) compared with the control $[Ca^{2+}]_o$, but there was no significant difference between low $[Ca^{2+}]_o$ and free $[Ca^{2+}]_o$ ($P > 0.05$). The decrease of threshold and AHP may be the key reasons for

the increased firing rate of hippocampal neuron in low $[Ca^{2+}]_o$.

3.3 Number of spikes vs AHP AHP is an important factor in modulating neuronal firing rate in many kinds of neurons. It can be predicted that the inhibition of AHP would increase firing rate of neurons. We therefore examined how pharmacological antagonisms of the AHP affected spike frequency in hippocampal neurons. The sAHP can be blocked by 8 μ mol/L isoprenaline (Iso) and mAHP can be blocked by 100 nmol/L apamin^[8]. The firing rates were increased ($66.1 \pm 34.3\%$) in 10 of 24 neurons while decreased ($40.9 \pm 28.9\%$) with apamin treatment in 14 neurons. In 6 of 14 neurons, the firing rates were increased ($59.7 \pm 46.0\%$) and in 8 neurons decreased ($44.7 \pm 28.2\%$) with Iso treatment. Neither block of SK_{Ca} channels by apamin ($n = 24$, $P > 0.05$) nor block of sAHP by Iso ($n = 14$, $P > 0.05$) had a statistically significant effect on spike frequency. Figure 2 showed 4 sample neurons which reflected the diverse effects of apamin and Iso on firing rate of neurons.

Table 1 A comparison of the physiological properties of hippocampal neurons in different $[Ca^{2+}]_o$ ($n = 5$).

	3 mmol/L $[Ca^{2+}]_o$	1.5 mmol/L $[Ca^{2+}]_o$	0 mmol/L $[Ca^{2+}]_o$
Resting potential (mV)	-61.6 ± 5.8	-60.9 ± 5.0	-61.3 ± 5.2
Firing threshold (mV)	-23.3 ± 8.7	$-34.2 \pm 9.1^*$	$-42.36 \pm 9.3^*$
AP amplitude (mV)	92.0 ± 14.9	$101.6 \pm 15.2^*$	$115.4 \pm 15.3^*$
AHP (mV)	-65.1 ± 4.7	$-63.2 \pm 4.1^*$	$-63.3 \pm 4.4^*$

Values are presented as means $\pm$ SD. * Significantly different from control ($P < 0.05$)

Fig. 1 Effects of $[Ca^{2+}]_o$ on firing frequency and single action potential of hippocampal neurons. A: Neurons stimulated with depolarizing current injections (300 ms, 160 pA) elicited trains of action potentials. Low $[Ca^{2+}]_o$ and zero- $[Ca^{2+}]_o$ increased firing rate. B: The changes of single action potential of neurons in low $[Ca^{2+}]_o$ and zero- $[Ca^{2+}]_o$. Threshold and AHP decreased markedly and amplitude of action potential increased. Short line represented the stimulating time, and long line, the recording time.

Fig. 2 The diverse effects of mAHP antagonist apamin and sAHP antagonist Iso on firing rate of hippocampal neurons. A: Apamin increased but Iso decreased firing rate. B: Apamin decreased but Iso enhanced firing rate. C: Both apamin and Iso increased firing rate; D: Neither apamin nor Iso increased firing rate.

To confirm the results, we also permuted and combined different experiments conditions on one cell as follows: 3 mmol/L $[Ca^{2+}]_o$; apamine + 3 mmol/L $[Ca^{2+}]_o$; Iso + 3 mmol/L $[Ca^{2+}]_o$; apamin + Iso + 3 mmol/L $[Ca^{2+}]_o$; 1.5 mmol/L $[Ca^{2+}]_o$; 1.5 mmol/L $[Ca^{2+}]_o$ + apamin + Iso; free $[Ca^{2+}]_o$. Every condition was treated after the neurons recovered completely in normal bath. In this process, we found that even the apamin and Iso decreased the spike frequency, the low $[Ca^{2+}]_o$ saline could still increase the firing rate on the same cell (Fig. 3). Also, spike frequency in low $[Ca^{2+}]_o$ + apamine + Iso solution is much slower than that in low $[Ca^{2+}]_o$ alone in some cells (data not shown). We can conclude that mAHP and sAHP did not play an important role in enhanced excitability of cultured hippocampal neurons in low $[Ca^{2+}]_o$. The mechanisms of bidirectional effects of AHP antagonist are not understood and may be caused by variation of the density of SK_{Ca} channels in different cells. Another possibility is that the preponderant SK_{Ca} channels are apamin-insensitive and Iso-insensitive in cultured hippocampal neurons.

3.4 I_{NaP} vs $[Ca^{2+}]_o$ It has been reported that lowering $[Ca^{2+}]_o$

Fig. 3 Neither apamin nor Iso enhanced firing rate of hippocampal neurons, while the neuron fired faster in low (1.5 mmol/L Ca^{2+}) and free $[Ca^{2+}]_o$ (0 mmol/L Ca^{2+}).

Fig. 4 TTX (0.01 μ mol/L), antagonist of I_{NaP} , blocked the increased spike rate of hippocampal neuron in zero- Ca^{2+} . It also blocked the repetitive firing in control bath solution.

augmented the size of the ADP in a concentration-dependent manner in hippocampal pyramidal neurons^[31]. The enlargement of the spike ADP was associated with the induction of intrinsic bursting by low $[Ca^{2+}]_o$. The phenomenon was suppressed by inhibiting I_{NaP} . It was hypothesized that I_{NaP} mediated the increased spike rate in low $[Ca^{2+}]_o$. TTX (0.01 μ mol/L), antagonist of I_{NaP} , blocked the increased firing frequency in low $[Ca^{2+}]_o$. As a result, we speculated that I_{NaP} contributed to the enhanced excitation in low $[Ca^{2+}]_o$. But TTX also blocked the following spikes in normal $[Ca^{2+}]_o$ (Fig. 4). Thus, it is difficult to conclude whether I_{NaP} play an impor-

tant role in the enhanced excitability of hippocampal neurons in low $[Ca^{2+}]_o$.

4 Discussion

Excitatory activity causes a transient decrease in the extracellular concentration of Ca^{2+} and these changes are most pronounced in the pyramidal cell layers of the hippocampus^[13]. The changes in Ca^{2+} are further enhanced during seizure activity. Although synaptic transmission plays a key role in the epileptic seizures in brain, it also can be induced when chemical synaptic transmissions are blocked by low-calcium artificial cerebrospinal fluid solution in hippocampal slices. Excessive excitation is thought to be one of the important keys for the development of the low- Ca^{2+} induced epileptic activity. The mechanisms of enhanced excitation of neurons in low $[Ca^{2+}]_o$ are still puzzled.

There are several mechanisms underlying the enhanced neuronal excitability such as reducing the surface charge screening, blocking calcium-activated hyperpolarizing currents (I_h) and suppressing afterhyperpolarizations, as well as mereaing field effects and gap junction^[14,15]. In recent decades, AHP has been identified to play critical roles in regulating neuronal excitability. The main finding in this study is that suppressed AHP is not the major modulator for excitability of cultured hippocampal neurons in low $[Ca^{2+}]_o$. Even though we blocked AHP, the excitability of hippocampal neurons enhanced greatly. The obviously decreased threshold of neurons in low $[Ca^{2+}]_o$ may be one of the important mechanisms underlying enhanced excitation in low $[Ca^{2+}]_o$ saline.

4.1 Low $[Ca^{2+}]_o$ and threshold In our study, the threshold of neurons decreased substantially in low- and zero- $[Ca^{2+}]_o$. The reason is maybe that lowering $[Ca^{2+}]_o$ reduces charge-shielding effects on groups located at the membrane surface, and thus influences the activation of voltage-dependent currents^[16]. As early as 1957, Frankenhaeuser and Hodgkin found that Ca^{2+} could adsorb to negative surface charges present at the outer edge of the membrane. As a consequence of the adsorption of Ca^{2+} to these charges, the gating machinery would sense a steeper electric field, and the gating curve would shift in the depolarizing direction on the voltage axis. Thus, sodium channels need more depolarization to be activated. Lowing Ca^{2+} , the adsorption of Ca^{2+} to surface charge was depressed, thus, less depolarization could activate the sodium channels^[17].

Besides the effects of Ca^{2+} on Na^+ channels gating, external Ca^{2+} also blocks the current that flows through Na^+ channels in a voltage-dependent manner. The Ca^{2+} -induced gating shifts observed in negatively charged membranes reflect the sum of the effects due to Ca^{2+} interacting directly with the Na^+ channel protein and to Ca^{2+} interacting with the negatively charged membrane lipids^[17].

4.2 AHP and excitability There are three different afterhyperpotentials: fAHP, mAHP, and sAHP following action potentials in hippocampal pyramidal neurons^[7]. Several types of K^+ currents underlie the AHP. A Ca^{2+} - and voltage-dependent, TEA-sensitive potassium current (I_c) contributes to the fast and medium AHP^[18]. At least two other potassium conductances contribute to the mAHP: I_{AHP} , due to the activation of SK_{Ca} of the apamin-sensitive type^[19], and I_M , a voltage-gated K^+ current suppressed by muscarinic agonists^[20]. In all hippocampal regions, muscarine mostly excited interneurons^[21]. The slow AHP is instead mediated by I_{sAHP} , and apamin-insensitive Ca^{2+} -activated K^+ current lasting more than 1 s and sensitive to modulation by many neurotransmitters.

Although Ca^{2+} -dependent SK channels underlie the mAHP, there was no precise match between the τ decay of I_{mAHP} and τ decay of the bulk $[Ca^{2+}]_i$ in soma or dendrite of pyramid cells. Neither matches between I_{sAHP} and somatic $[Ca^{2+}]_i$ ^[22]. In our experiment, AHP amplitude of neurons had no obvious difference between low $[Ca^{2+}]_o$ and zero $[Ca^{2+}]_o$.

It has been reported that mAHP and sAHP play a critical role in regulating neuronal excitability and the spread of epileptiform activity^[9]. The enhancer of AHP could provide new therapeutic targets for the management of epileptic patients. In our research, apamin reduced mAHP and increased the firing rate in control solution in some pyramidal neurons. However, in some other neurons, apamin did not increase, but decreased the firing frequency in control saline. The effect of apamin on firing rate is not significant. It may be due to the various density of small Ca^{2+} -activated K^+ channels or due to the occurrence of the apamine-insensitive mAHP and Iso-insensitive sAHP in cultured hippocampal neurons. In those hippocampal cells which apamin did not change their spike frequency, the spike rates also increased in low and free- Ca^{2+} solutions. So, we can conclude that the apamin-sensitive mAHP and Iso-sensitive sAHP, although present in hippocampal neurons, do not appear to play a role in controlling spike frequency in low Ca^{2+} and free Ca^{2+} saline. This

result is consistent with the research in suprachiasmatic nucleus^[6]. In suprachiasmatic nucleus brain slice, neither block of SK_{Ca} channels by apamin nor block BK_{Ca} channels by iberiotoxin had a statistically significant effect on spike frequency. In lateral amygdala (LA) principal neurons, apamin, which clearly blocked I_{AHP} , had no effect on the number of spikes fired^[23]. The exact physiological role of SK channels in the neurons remains elusive.

4.3 I_{NaP} and excitability The existence of I_{NaP} was first discovered in hippocampus and cerebellum. It is a noninactivating component of the total sodium current which modulates the neuronal excitability. It adds to synaptic current to boost membrane potential toward threshold for spike generation and control repetitive firing behavior. Some sodium channel mutations associated with human epilepsy syndromes exhibit increased I_{NaP} ^[24].

It has been reported that lowering $[Ca^{2+}]_o$ augmented the size of the ADP in hippocampal slice and supraoptic hypothalamic neurons which was associated with the induction of intrinsic bursting by low $[Ca^{2+}]_o$ ^[3]. The phenomenon was suppressed by inhibiting I_{NaP} . In our experiment, TTX, antagonist of I_{NaP} , blocked increasing firing rate in both low $[Ca^{2+}]_o$ and control $[Ca^{2+}]_o$. So, whether or not I_{NaP} played an important role in the enhanced excitability of cultured hippocampal neurons in low $[Ca^{2+}]_o$ was elusive.

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细胞外钙离子浓度对海马神经元兴奋性的影响

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摘要: **目的** 在不同的生理、病理情况下, 中枢神经系统细胞外钙离子浓度 ($[Ca^{2+}]_o$) 下降, 导致神经元兴奋性增高。本研究的主要目的是探讨低钙条件下神经元兴奋性增高的机制, 从而为临床治疗神经元过度兴奋性疾病探索新的治疗途径。 **方法** 应用穿孔膜片钳及细胞培养技术, 记录不同细胞外钙离子浓度对海马神经元兴奋性的影响。 **结果** 低钙环境使神经元兴奋性显著增高, 阈电位水平显著降低, 动作电位幅度显著增高。并且出乎意料的是, mAHP 拮抗剂 apamin 及 sAHP 拮抗剂 Iso 对海马神经元兴奋性的影响没有统计学的意义。作为 I_{NaP} 拮抗剂, 低浓度的河豚毒 (TTX) 虽然阻断了低钙环境中神经元兴奋性的增加, 但同时也阻断了正常钙离子浓度下的动作电位的发放。 **结论** 低钙环境中海马神经元阈电位的显著下降可能是导致神经元兴奋性增高的主要原因。

关键词: 小电导 Ca^{2+} -激活的钾通道 (SK_{Ca}); 海马; 钙