Ca²⁺ channel currents of cortical neurons from pure and mixed cultures

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Abstract Voltage-gated Ca²⁺ channels (VGCCs) are key regulators of many neuronal functions, and involved in multiple central nervous system diseases. In the last 30 years, a large number of injury and disease models have been established based on cultured neurons. Culture with serum develops a mixture of neurons and glial cells, while culture without serum develops pure neurons. Both of these neuronal-culture methods are widely used. However, the properties of Ca²⁺ currents in neurons from these two cultures have not been compared. In this study, we cultured rat cortical neurons in serum-containing or -free medium and then recorded the Ca²⁺ channel currents using patch-clamp technique. Our results showed that there were significant differences in the amplitude and activation properties of whole-cell Ca²⁺ channel currents, and of non-L-type Ca²⁺ channel currents between the neurons from these two culture systems. Our data suggested that the difference

of whole-cell Ca²⁺ currents may result from the differences in non-L-type currents. Understanding of these properties will considerably advance studies of VGCCs in neurons from pure or mixed culture.

Keywords Pure cultured neurons · Mixed cultured neurons · Ca²⁺ currents · Patch-clamp recording

Introduction

Ca²⁺ is an important second messenger and involved in many cell functions, such as development, apoptosis, gene expression, and cell migration. The voltage-gated Ca²⁺ channels (VGCCs) are critical components of the Ca²⁺ signal and widely expressed in the central nervous system (CNS). In neurons, Ca²⁺ influx through VGCCs regulates a number of neuronal functions including excitability and synaptic plasticity. For example, N- or P/Q-type VGCCs are mainly distributed in presynaptic terminals and control the neurotransmitters release (Qian and Noebels 2001; Spafford and Zamponi 2003). L-type Ca²⁺ channels located on the cell body are related to more functions, such as the modulation of intracellular Ca²⁺ homeostasis (Davare and Hell 2003) and the migration of newborn neurons in the postnatal olfactory bulb (Darcy and Isaacson 2009). Many studies have reported the roles of VGCCs in CNS injuries and diseases. For instance, N-type VGCC is a key component in controlling the transmission of nociceptive signals

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(Altier et al. 2006), and the inhibition of P/Q-type VGCCs mediates the suppression of spontaneous synaptic activity by amyloid β oligomers (Nimmrich et al. 2008). In addition, the entry of L-type VGCCs has been associated with neuronal death in Alzheimer's disease (Yagami et al. 2004), ischemia (Li et al. 2007), and glutamate toxicity (Sribnick et al. 2009).

The cultured neurons were often used in the studies of CNS injury and disease. Fetal bovine serum (FBS) was added into culture medium in earlier studies, however unknown and inconstant FBS compositions introduced unavoidable variables into the experimental system. In 1980s, a serum-free medium was devised to obtain a pure culture of neurons to allow the expression of characteristic neuron properties to form mature synapses (Ahmed et al. 1983; Weiss et al. 1986). The most common serum-free medium is Neurobasal with B-27 supplement. Electrical properties of neurons in serum-free culture have been investigated (Evans et al. 1998).

We used a whole-cell patch-clamp recording technique to measure and compare Ca^{2+} channel currents of neurons from serum-containing and -free cultures. Our results brought a new understanding for the VGCCs research in cultured neurons.

Materials and methods

Mixed culture

A primary culture of neurons was prepared from 18- to 19-day-old fetal rats (Sprague–Dawley rats) that were purchased from Beijing Vital River Experimental Animal Technical Co. Ltd, Beijing, China. All procedures were measured according to the national standard. Cerebral cortices without hippocampus and dura mater were treated with 4 mL of 0.25% trypsin for 3–5 min at room temperature (RT). Cells were collected by centrifugation for 10 min at 900g. The pellet was re-suspended in MEM (pH 7.4 with KOH) with 0.7 mM sodium pyruvate, 14 mM KCl, 7 mM NaHCO_3 , 7 g/L glucose, and 1% penicillin/streptomycin. Approximately 0.5×10^6 cells were placed on a 22×22 mm glass cover slip pretreated with 12.5 $\mu\text{g/mL}$ Poly-D-Lysine, and incubated in a 35 mm tissue culture dish. Glutamine (2 mM), 10% fetal bovine serum (FBS; HyClone, USA) and 1% B-27 supplement were added to the culture medium

immediately before use. Dishes were incubated at 37 °C, 5% CO_2 /95% air and half of the culture medium was changed every 3 days. All recordings were performed on day 8 to day 12 in vitro.

To examine the proportion of neurons, the cell nuclei were labeled with Hoechst 33258 and the neurons were stained with anti-NeuN (a neuronal specific nuclear protein) antibody after the cells were fixed in 4% paraformaldehyde. The results (from two independent experiments with four parallels) showed that the proportion of neurons was about 30%.

Pure culture

The cells were cultured serum-free in Neurobasal-A Medium with 2% B-27, 2 mM glutamine and 0.5% penicillin/streptomycin. Approximately 0.8×10^6 cells were placed on a 22×22 mm glass cover slip pretreated with 50 $\mu\text{g/mL}$ Poly-D-Lysine. To eliminate proliferative cells, arabinosylcytosine C (a selective inhibitor of DNA synthesis; 10 μM) was added to the medium at day 2 for 24 h. Half of the culture medium was changed every 3 days.

The neuron purity was determined with the same method as described above. The proportion of neurons was more than 95%.

Whole-cell recording

The size of neurons in mixed culture seemed more uniform. In our experiments, the recording was performed on bipolar neurons with the same size (Fig. 1a). The similarity of the neurons from two cultures was supported by their similar membrane capacitances. There was no difference in membrane capacitances between mixed (11.1 ± 0.6 pF; $n = 35$) and pure (10.7 ± 0.6 pF; $n = 26$) cultures (Fig. 1b). All recordings were performed on day 8 to day 12 in vitro.

For whole-cell recordings of voltage-gated Ca^{2+} channel activity, the bath solution had the following composition (in mM): 135 TEA-Cl, 10 BaCl_2 , 10 HEPES and 10 glucose, pH 7.4 with tetraethylammonium hydroxide (TEA-OH); the pipette solution contained (in mM): 130 CsCl, 1 MgCl_2 , 8 EGTA, 4 TEA-Cl, 10 HEPES, 4 ATP-Mg, 10 phosphocreatine and 0.1 GTP- Li_4 , pH 7.2 with TEA-OH. Patch pipettes were pulled to a tip resistance of 5–10 M Ω from borosilicate glass capillary tube on the P-97 micropipette puller (Sutter Instrument, USA) and fire-polished (Narishige,

Fig. 1 The morphological and membrane capacitance of neurons in pure and mixed culture. Typical bipolar neurons (indicated by arrow) from pure (left) and mixed (right) culture

Japan) before use. The voltage-clamp recording was obtained using an EPC-7 amplifier (HEKA, Germany). Data were digitized at 2.5 kHz. The neurons were held at a potential of -100 mV and depolarized to -80 mV for 10 ms to measure their capacitance. For recording of the whole-cell Ca^{2+} currents, neurons were held at -80 mV and depolarized to potentials ranging from -80 to $+80$ mV for 140 ms with a frequency of 0.5 Hz. The recordings leak was subtracted using off-line software. The whole-cell Ca^{2+} current densities were defined as peak current amplitude divided by cell capacitance. The liquid junction potential between the pipette and bath solutions was zeroed prior to seal formation.

Nifedipine (Sigma, USA) was used to determine the proportion of L-type Ca^{2+} channel current in the whole-cell current, which was added to the 4 mL bath solution with a final concentration of $10 \mu\text{M}$ following the recording. The non-L-type Ca^{2+} channel current was recorded 2 min after the application of nifedipine.

Data were analyzed using pClamp 6.0 or 8.0 (Axon, USA) and SPSS 11.5 (SPSS Inc., USA). A two-tailed Student's *t* test was used for comparisons, with $P < 0.05$ indicating statistical difference. All data were expressed as mean $\pm$ SE.

Results

Amplitude of whole-cell Ca^{2+} channel currents

In our experiments, Ba^{2+} was used instead of Ca^{2+} in the bath solution to reduce the influence of rundown (Eckert and Tillotson 1981). Whole-cell Ba^{2+} current evoked at potentials from -80 to $+80$ mV was recorded

were selected (arrows; a). The membrane capacitances in pure ($n = 17$) and mixed ($n = 35$) neurons were not different (b)

(Fig. 2a). The current density in the neurons from pure culture ($n = 17$) was significantly greater than those from mixed culture ($n = 35$; Fig. 2b; $P < 0.01$). The peak current occurred at -10 mV in the pure cultured neurons, while at 0 mV in the mixed cultured neurons. The maximal current densities of the neurons from pure and mixed-cultures were -31.6 ± 4.0 pA/pF and -18.1 ± 2.0 pA/pF, respectively.

Activation properties

The activation properties of the Ca^{2+} channel currents were analyzed further. Normalized conductance of Ca^{2+} channels at each voltage was calculated and fitted by the Boltzmann equation. The voltage-dependant activation curve of Ca^{2+} currents from the pure cultured neurons was shifted leftwards (Fig. 3a). Half-activation voltages and slope factor, two parameters reflected activation curves, were significantly different between neurons from pure and mixed cultures. Half-activation voltages were -21.9 ± 1.5 mV ($n = 17$) and -15.1 ± 1.0 mV ($n = 35$; Fig. 3b; $P < 0.01$), and the slope factors were 4.9 ± 0.6 and 6.7 ± 0.3 (Fig. 3c; $P < 0.01$) in groups of neurons from pure and mixed cultures, respectively. These data indicated that there were significant differences in the activation properties of Ca^{2+} currents between both groups.

Amplitude of L- and non-L-type Ca^{2+} channels currents

In cortical neurons, a variety of Ca^{2+} channels are expressed, including L-, N-, R-, and P/Q-type, where L-type Ca^{2+} channels (LCCs) were thought to play

Fig. 2 The whole-cell Ca^{2+} currents in pure and mixed cultures. The neurons were held at -80 mV and then depolarized to elicit the whole cell Ca^{2+} channel currents (a) in the pure (upon) and mixed (below) cultured neurons. There were significant differences in current densities at voltage ranges from -30 to $+10$ mV in pure (circle; $n = 17$) and mixed (triangle; $n = 35$) cultured neurons (b). * $P < 0.05$ and ** $P < 0.01$ as compared with pure cultured neurons (students t test)

key roles in many pathogenic conditions. We treated the neurons with $10 \mu\text{M}$ nifedipine, a selective LCC antagonist, and obtained the L-type Ca^{2+} currents by subtracting the whole-cell Ca^{2+} currents after nifedipine treatment from the one before it (Fig. 4a). The percentage of L-type currents in whole-cell currents from pure cultured neurons ($n = 6$) and mixed cultured neurons ($n = 12$) were $41.8 \pm 8.5\%$ and $62.4 \pm 4.4\%$ respectively (Fig. 4b; $P < 0.05$). There was no significant difference in the current density of LCC between these two groups (Fig. 4c). However, the peak current of LCC occurred at the same voltage

Fig. 3 Activation properties of VGCCs in pure and mixed cultured neurons. The voltage-dependent activation curves were fitted by Boltzman equation (a) and showed significantly different half-activation potential (b) and slope factor (c) between pure (circle; $n = 17$) and mixed (triangle; $n = 35$) cultured neurons. ** $P < 0.01$ as compared with pure cultured neurons (students t test)

as the whole-cell Ca^{2+} currents (Fig. 2b). On the other hand, nifedipine-insensitive currents (non-L-type currents) in neurons from these two cultures were different (Fig. 4d; $P < 0.05$ and $P < 0.01$).

Activation properties of LCC and non-L-channels

Figure 5a, b showed the activation curves of L-type current ($n = 6$) and non-L-type currents ($n = 12$) from the pure and mixed cultured neurons. The half-activation voltages of L-type current were $-20.1 \pm$

Fig. 4 LCC current and non-L-currents in pure and mixed cultured neurons. The LCC currents were dissociated by nifedipine (10 μ M) from whole cell Ca^{2+} currents (a). The percentage of LCC currents in whole cell currents from mixed cultured neurons (triangle; $n = 12$) was higher than from pure (circle; $n = 6$) cultured neurons (b). The current densities were not different for L-type current (c), but were significantly different for non-L-type currents (d) between pure and mixed cultured neurons. * $P < 0.05$ and ** $P < 0.01$ as compared with pure cultured neurons (students t test)

1.1 mV and -16.4 ± 1.4 mV, respectively (Fig. 5c left; $P = 0.07$). There was a remarkable difference of the non-L-type currents between two cultures (Fig. 5c right; -19.5 ± 2.2 mV, $n = 6$; -8.7 ± 1.2 mV, $n = 10$; $P < 0.01$). The slope factor was similar for the L-type current (4.7 ± 0.6 vs. 5.4 ± 0.4 ; Fig. 5d left; $P = 0.32$), but significantly different for non-L-type currents (3.0 ± 0.4 in pure cultured neurons and 4.7 ± 0.4 in mixed cultured one; Fig. 5d right; $P < 0.05$). These data suggested that the difference in amplitude and activation properties of whole-cell Ca^{2+} currents resulted from the difference of non-L-type currents between both cultures.

Discussion

Our data showed that both amplitude and activation properties of whole-cell Ca^{2+} channel currents were significantly different between cortical neurons from pure and mixed cultures. The larger peak current density in neurons from pure culture may result from more functional Ca^{2+} channel expressed on the plasma membrane. Our data also showed that there was no remarkable difference in LCC current between the neurons from both cultures, but the non-L-type

currents from pure culture were significantly higher. These results implied that the non-L-type currents contributed mainly to larger whole-cell Ca^{2+} currents in neurons from pure culture.

The peaks of whole-cell Ca^{2+} channel currents were observed at 0 and -10 mV in neurons cultured with and without serum, respectively. The significant differences between these two groups in both half-activation voltage and slope factor were also detected. The voltage of peak currents in pure cultured neurons was more hyperpolarized. This difference may result partially from the increase of the proportion of N-type currents (as the percentage of L-type current was significantly lower in pure cultured neurons; Fig. 4b). The N-type channel current, another important population of Ca^{2+} currents in neurons, reached the peak at -20 mV in pure cultured neurons (Zhou et al. 2006). Compared with the neurons from the mixed culture, the amplitude of non-L-type currents was lower, and the activation properties were different. These findings suggested that the major reason for the differences in whole cell Ca^{2+} currents between the neurons from both cultures was due to the differences in the non-L-type currents.

In addition, the mixed culture contained glial cells while the pure culture did not. Over the past decade, many studies have shown that glial cells play a key

Fig. 5 Activation properties of L-type currents and non-L-type currents in pure and mixed cultured neurons. The voltage-dependent activation curves of L-type current (a) and non-L-currents (b) were fitted by Boltzman equation and showed that both of half-activation potential (c) and slope factor (d) were significantly different between pure (circle; $n = 6$) and mixed (triangle; $n = 10$) cultured neurons in the non-L-type currents. * $P < 0.05$ and ** $P < 0.01$ as compared with pure cultured neurons (students t test)

role in the regulation of synaptic transmission (Pfrieger and Barres 1997; Ullian et al. 2004; Steinmetz et al. 2006). Glial cells do not just regulate the postsynaptic receptor, but also affect the presynaptic elements, e.g. upregulating the expression of LCCs (Wang et al. 2008) and enhancing the N-type currents (Mazzanti and Haydon 2003). But these findings could not explain the larger Ca^{2+} currents in pure cultured neurons from our experiments. It is possible that the upregulation of Ca^{2+} channels by the condition of pure culture (e.g. B27) was stronger than the function of glial cells in mixed culture.

In conclusion, Ca^{2+} currents in neurons could be influenced by culture conditions. Therefore the culture condition should be considered as an important part when designing new experiments.

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