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Research Paper

Whole-cell recordings of voltage-gated Calcium, Potassium and Sodium currents in acutely isolated hippocampal pyramidal neurons ☆

Shuyun Huang[a], Qing Cai[a*], Weitian Liu[b], Xiaoling Wang[a], Tao Wang[a]

[a]*Department of physiology, Tianjin University of Traditional Medicine, Tianjin 300193,China*

[b]*Department of cerebral surgery, People Hospital of FengRun, Tangshan 064000, China*

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Abstract

Objective: To record Calcium, Potassium and Sodium currents in acutely isolated hippocampal pyramidal neurons. **Methods:**Hippocampal CA3 neurons were freshly isolated by 1 mg protease /3 ml SES and mechanical trituration with polished pipettes of progressively smaller tip diameters. Patch clamp technique in whole-cell mode was employed to record voltage-gated channel currents. **Results:**The procedure dissociated hippocampal neurons, preserving apical dendrites and several basal dendrites, without impairing the electrical characteristics of the neurons. Whole-cell patch clamp configuration was successfully used to record voltage-gated Ca^{2+} currents, delayed rectifier K^+ current and voltage-gated Na^+ currents. **Conclusion:**Protease combined with mechanical trituration may be used for the dissociation of neurons from rat hippocampus. Voltage-gated channels currents could be recorded using a patch clamp technique.

Keywords: patch clamp; hippocampus; voltage-gated channels; whole-cell

INTRODUCTION

Ca^{2+}, K^+ and Na^+ channels are important for the regulation of neuronal excitability because they depolarize and repolarize neurons in response to depolarizing events, respectively. Voltage-gated K^+ channels have been implicated in governing cell excitability[1] setting the resting potential[2] and cell apoptosis[3]. Calcium entry mediated by voltage-gated calcium channels triggers a wide array of cellular responses that range from muscle contraction and activation of calcium-depend enzymes, to calcium-triggered exocytosis and synaptic activity[4-6]. Voltage-gated sodium channels mediate the depolarization phase of the action potential and neuronal excitability[7-10]. Patch clamp technique is a powerful tool for studying the electrophysiological properties of biological membranes. The hippocampus is an important region for learning, memory and stress[11-12]. The purpose of the present study is to establish the methods of wholecell recordings of calcium, potassium and sodium currents in acutely isolated hippocampal pyramidal neurons.

MATERIALS AND METHODS

Reagents

The ionic composition of source-artificial cerebrospinal fluid(CSF), pH 7.4, included(in mmol/L):248 sucrose, 1.25 NaH_2PO_4, 2 KCl, 1 $CaCl_2$, 2 $MgSO_4$, 10 glucose, 1 $MgCl_2$, 26 $NaHCO_3$. Source-CSF instead of artificial cerebrospinal fluid and the standard external solution could reduce excessive Na^+ influx when 400 μm slices were prepared. The standard external solution(SES) contained(in mmol/L): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$,10 N'-A-hydroxythylpiperazine-N'-ethanesulfanic acid(HEPES), 10 glucose, and the pH was adjusted to 7.4 with Tris-base. Both external and internal solutions were made up using salts of analytical grade in twice-distilled deionized water. Tetrodotoxin(TTX), Ethylene glycol-bis-(2-aminoethyl) tetraacetic acid(EGTA), MgATP, Na_2GTP, HEPES, CsCl, TEACl, Tris-base, and CsOH were obtained from

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*Corresponding author

*E-mail address:*qingcai@tjutcm.edu.cn

Sigma Chemical Co., USA.

Preparation of hippocampal slices and acute dissociation of neurons

Animals were provided by the experimental animal center of Tianjin University of Traditional Medicine, and were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Hippocampal pyramidal neurons were prepared as described previously[13-14] with minor modifications. Sprague-Dawley rats(15~20 days old) were anesthetized with ether and then decapitated. The brains were quickly excised and placed into ice-cold oxygenated source-artificial cerebrospinal fluid for 2 min. The brains were glued to the chilled stage of a vibratome(MA752, Campden, UK) and sliced to a horizontal thickness of 400 μ m. Slices were incubated in an incubation chamber filled with oxygenated SES at 18~22℃ for at least 1 h. Then they were incubated for 30 min at 32℃ in 6 ml oxygenated SES with 2 mg protease(protease Type XIV, Sigma). After that, tissue slices were rinsed three times in oxygenated SES and incubated in an incubation chamber for containing oxygenated SES at 18~22℃ for 1.5 h. The CA3 region was punched out with a fire-polished injection needle and was transferred to a conical polystyrene test tube that contained 2 ml SES. The tissue punch was triturated by heat polished different diameters pipettes. Two minutes later, the cell suspension was transferred to a clean cover slip, and after 15 min the cover slip was washed twice with extracellular recording medium.

Electrophysiological recordings

The isolated CA3 neurons were transferred to a recording chamber mounted on an inverted microscope (DMIRB, Leica, Germany). Only neurons that exhibited the stereotypical morphology of pyramidal neurons and no visible evidence of injury were selected for recording. Electrophysiological recordings were made with standard whole-cell voltage-clamp technique[15]. Recording pipettes were made from borosilicate glass capillaries using a puller (model p-97, Sutter Instrument, Novato, CA) and heat-polished to easily allow gigaohm seal formation. The resistance of the recording pipettes filled with pipette solution was 3~6 MΩ in the bath solution. The junction potential between the patch pipettes and bath solution was adjusted to zero before gigaohm seal formation. Having compensated pipette and cell transient capacitance with the appropriate controls on the amplifier, the membrane was ruptured with gentle suction to obtain the whole cell voltage-clamp configuration. Series resistance was routinely compensated by 60~70% and monitored periodically. Leak current was subtracted using a P/4 protocol. Whole-cell recordings of high-voltage-activated(HVA) Ca^{2+} currents were obtained under conditions optimized to ensure their complete isolation from other voltage-gated currents. TTX(0.5 μmol/L) was used to block sodium channels. Potassium currents were blocked by replacing internal K^+ with Cs^+ and tetraethylammonium (TEA^+) as a dominant cation in the external solution and 4-aminopyridine(4-AP) externally. For recording potassium currents, SES was used as the bath solution, and 0.5 μmol/L TTX and 0.2 mmol/L $CdCl_2$ were used to block sodium channels and voltage dependent Ca^{2+} channels respectively. For recording Na^+ currents, K^+ currents were blocked by replacing internal K^+ with Cs^+ and TEA^+, 4-AP in the extracellular solution. The pH of the extracellular solution and intracellular solution was adjusted to 7.4 and 7.2 with Tris-base and CsOH, respectively(***Table 1***). The internal recording solutions was filtered(using a Millipore 0.2 μm syringe filter) before use and frozen until the day of the experiment. Electrophysiological recordings were performed at room temperature(22~25℃).

Table 1 **The ionic composition of external and internal solutions**

	external solutions(mmol/L)	internal solutions(mmol/L)
Ca^{2+} current	140 TEACl, 10 $CaCl_2$, 5 4-AP, 10 HEPES, 10 glucose, and 0.5 μmol/L TTX.	130 CsCl, 10 EGTA, 3 MgATP, 0.3 Na_2GTP, 10 HEPES, 10 glucose.
K^+ current	150 NaCl, 5 KCl, 1 $MgCl_2$, 2 CaCl2, 10 HEPES, 10 glucose, 0.2 $CdCl_2$ and 0.5 μmol/L TTX.	140 KCl, 10 EGTA, 3 MgATP, 10 HEPES, 10 glucose.
Na^+ current	50 NaCl, 100 TEACl, 1 $MgCl_2$, 1 CaCl2, 5 HEPES, 5 D-glucose, 5 4-AP, 0.2 $CdCl_2$	140 CsCl, 2 $MgCl_2$, 10 HEPES, 5 EGTA, and 3 Na_2ATP.

Statistical analysis

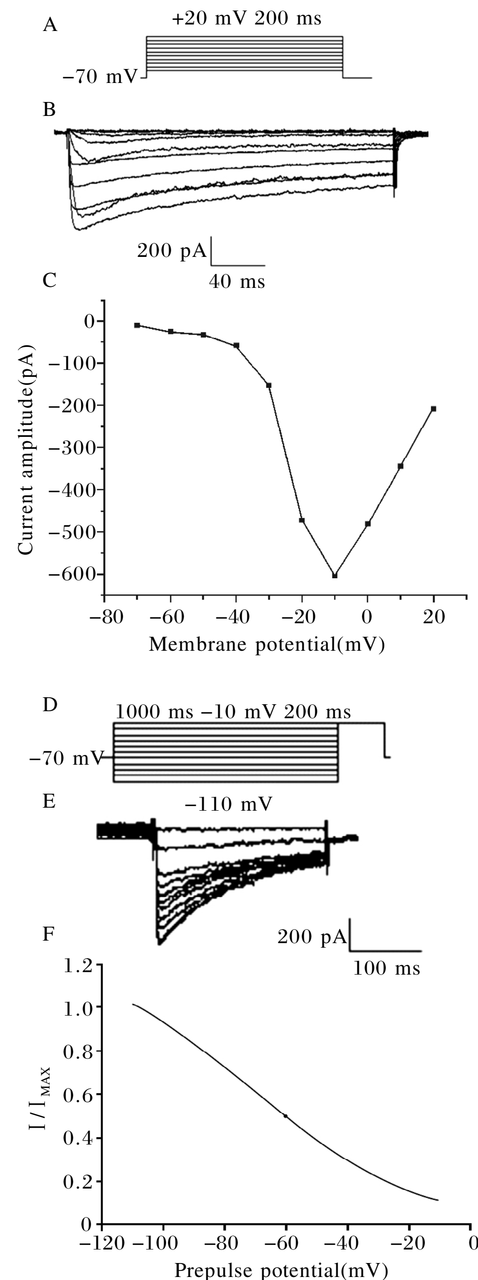
All data were analyzed by using pCLAMP CLAMPFIT procedures(Axon Instrument, USA). Current-voltage relations of voltage-gated channels were analyzed by Origin 6.0 software(Microcal Software, USA).

RESULTS

Voltage-gated Ca^{2+} currents

Voltage-gated Ca^{2+} currents were recorded as previously described[16-17]. Voltage-gated Ca^{2+} currents were elicited by 200 ms depolarizing potentials from-70 mV to +20 mV with an increment of 10 mV and the holding potential was −70 mV(***Fig. 1A***). The example of raw Ca^{2+} current traces for CA3 neuron is shown in ***Fig. 1B***. No significant inward current was observed until

the depolarizing step reached –40 mV. Currents reached maximum amplitude at approximately –10 mV in each case, and became small with stronger depolarization. The current amplitude dependence on the potential is shown as an I-V curve(***Fig. 1C***). The maximal current peak amplitude of Ca^{2+} channel in ***Fig. 1B*** was –616.52 pA. The voltage dependence of steady-state inactivation was studied using a double pulse protocol. Initially, the membrane potential was kept at a predetermined value using a 1s step prepulse. The value of the first prepulse was –110 mV. Voltage increment was 10 mV and the last prepulse reached a value of –10mV. This conditioning pulse was followed by a second fixed step depolarization to –10 mV lasting 200 ms, testing the fraction of the maximum Ca^{2+} current of each pre-pulse (***Fig. 1D***). The original current recordings in response to the test voltage step are demonstrated in ***Fig. 1E***. The evoked peak current amplitudes were normalized with respect to the maximal peak current amplitude evoked and then plotted against the prepulse. The data fitted well with the Boltzmann equation(***Fig. 1F***).



A:The pulse protocols for recording voltage-gated Ca^{2+} current; B:Raw traces of calcium current elicited in freshly isolated CA3 neuron; C:Current-voltage relation of calcium current of freshly isolated CA3 neuron; D:The pulse protocols for recording inactivation kinetics of voltage-gated Ca^{2+} currents. E:Raw traces of whole-cell calcium currents elicited in freshly isolated CA3 neurons. F:Steady-state inactivation curve of Ca^{2+} currents in CA3 neurons. The curve was fitted by the Boltzmann equation.

Fig. 1 Ca^{2+} currents in freshly isolated hippocampal CA3 pyramidal neuron

Delayed rectifier potassium current

Delayed rectifier potassium currents(I_{KD}) were recorded as previously described[13, 18]. I_{KD} was elicited by a protocol consisting of multiple depolarizing 200 ms pulses(–60 mV to +50 mV), preceded by a single prepulse step to –50 mV, with inactivated transient outward potassium currents from a holding potential of –80 mV(***Fig. 2A***). ***Fig. 2B*** shows a series of evoked raw current traces. The outward currents showed a slow activation and inactivation during depolarizing 200 ms pulses, and increased with the test potentials. The currents were sensitive to TEA, a potassium currents blocker. The currents recorded under these conditions were outward delayed rectifier potassium currents. The current-voltage(I-V) curve of I_{KD} was obtained by plotting the evoked currents against the test pulse(***Fig.2C***). For activation, the current at each test potential was converted to conductance(G) using the following equation:$G = I/(V-V_{rev})$, where V is membrane potential and V_{rev} is reverse membrane potential. The peak conductance value for each membrane potential was normalized to G_{max}. The data were fitted using the Boltzmann equation:$G/G_{max} =1/\{1+\exp[-(V-V_{1/2})/k]$, $V_{1/2}$ is the potential at G at 0.5 G_{max} and k is the slope factor. ***Fig. 2D*** shows the conductance-voltage curve of I_{KD}. The normalized conductance was fitted with the Boltzmann equation.

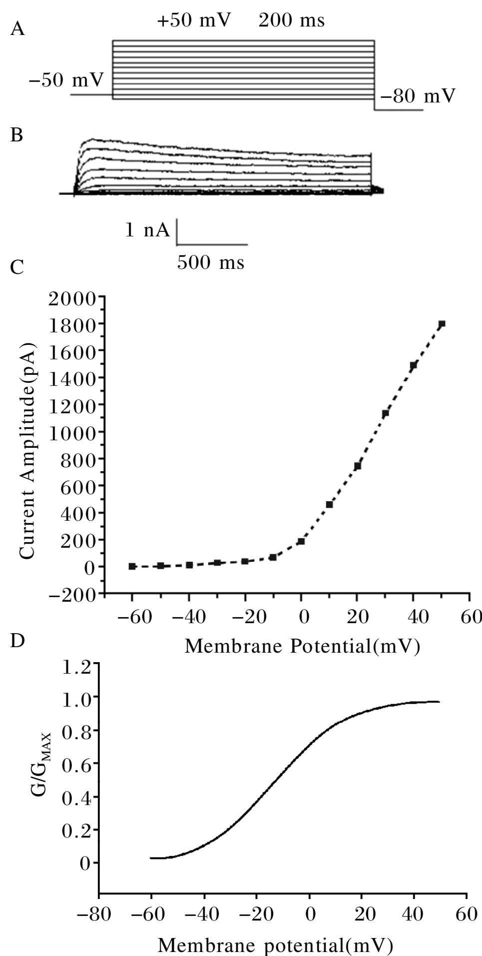
Voltage-gated Na^+ currents

Voltage-gated Na^+ currents were recorded as previously described[19-20] with minor modifications. Neuronal cells were held at holding potential(HP) of -90 mV, and 12 ms depolarizing potentials from a HP of -90 to +30 mV at 10-mV steps activated inward currents (***Fig. 3A***). The example of raw Na^+ current traces for CA3 neuron is shown in ***Fig. 3B***. They were completely and reversibly blocked by 0.5 μmol/L TTX, so these inward currents were attributed to sodium currents(I_{Na}). The current amplitude dependence of the

potential is shown as an I - V curve(***Fig. 3C***). Na^+ currents of steady-state inactivation were elicited with a 12 ms –20 mV test pulse preceded by 500 ms prepulses to potentials between –120 and –20 mV. For inactivation, the current value at the corresponding prepulse potential was normalized to I_{max}, and was fitted using Boltzmann equation:$I/I_{max}=1/[1+exp(V-V_{1/2})/k]$, where $V_{1/2}$ is the membrane potential at which 50% inactivation of the current is observed, V is the prepulse membrane, and k is the slope factor. The example of raw Na^+ currents of steady-state inactivation for CA3 neuron is shown in ***Fig. 3D*** and ***Fig. 3E.*** Steady-state inactivation curve of Na^+ current is shown ***Fig. 3F***.

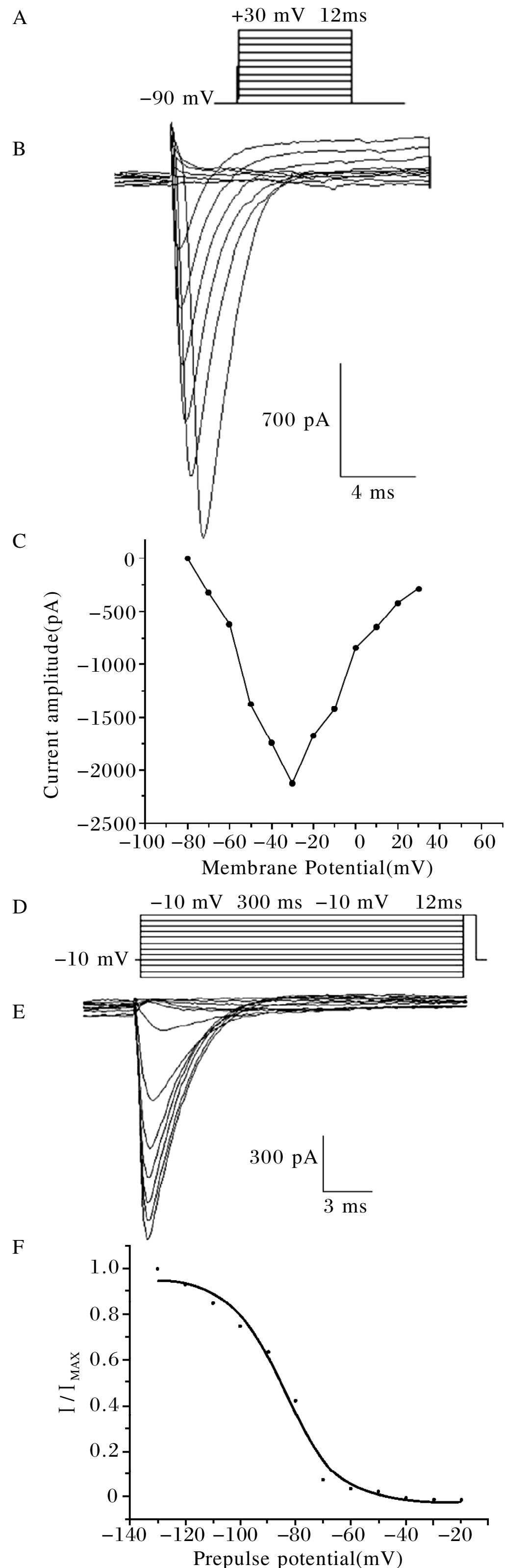
DISCUSSION

During our isolation, there were many differences from previous methods. First, source-artificial cerebrospinal fluid was used when the brain slices were made. This reduced sodium influx when an eqimolar concentration of sucrose replaced NaCl during



A:The pulse protocols for recording I_{KD}; B:Raw traces of I_{KD} elicited in freshly isolated CA3 neuron; C:Current-voltage relation (I-V curve) of I_{KD} obtained from CA3 neuron. D:The conductance-voltage curve of I_{KD}.

Fig. 2 Outward delayed-rectifier K^+ currents in freshly isolated hippocampal CA3 pyramidal neuron



A:Na^+ currents are evoked by 12 ms depolarizing potentials from a holding potential of -90 to +30 mV at 10-mV steps; B Raw traces of I_{Na} elicited in freshly isolated CA3 neuron; C:Current-voltage relation(I - V curve) of I_{Na} obtained from CA3 neuron. D,E:Na^+ currents of steady-state inactivation were elicited with a 12 ms -20 mV test pulse preceded by 500 ms prepulses to potentials between -120 and -20 mV. F:Steady-state inactivation curve of Na^+ current.

Fig. 3 Na^+ currents in freshly isolated hippocampal CA3 pyramidal neuron

decapitation, dissection and slicing. Second, only one kind of protease was used in our study, instead of multiple enzymes. Finally, the standard external solution was used to incubate the slices twice. The pH of the solution was more stable compared to artificial cerebrospinal fluid. It provided a stable extracellular environment for the dissecting procedure. Therefore, the quality and the quantity of the cells could be ensured so that the patch clamp experiment could be performed successfully. Theoretically, isolation with enzyme will irreversibly digest the protein, including ion channels in the cell membrane. In the present study, we successfully recorded voltage-gated Ca^{2+} currents, delayed rectifier K^+ current and voltage-gated Na^+ currents in acutely isolated hippocampal pyramidal neurons. Our successful experience mostly lies in the following:①Great care was taken in handling the brain slices to avoid applying pressure to the slices and using a polished thin glass wand to transfer slices. ② The activity and dose of enzyme can influence the neurons' quality and quantity. Excessive enzyme will destroy the normal structure of neurons and cause neuronal swelling. The membranes of the remaining living neurons could be easy to break before making a GΩ seal, which is key for successful patch clamp experiments. On the other hand, insufficient enzyme is not enough to disrupt the connections among neurons. ③Incubating twice is beneficial to the recovery of the slices, and the time course should not be short. ④Careful and slow trituration of tissue punches through heat-polished Pasteur pipettes of progressively smaller tip diameter is necessary, and air bubbles should be avoided to prevent cell damage.

In the present study, voltage-gated Ca^{2+}, K^+ and Na^+ channels currents were successfully recorded in acutely isolated hippocampal pyramidal neurons. This method is ideal for the dissociation of neurons from rat hippocampus, and offers a powerful tool for functional analysis at the level of the individual cell.

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