ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Feb；21 (2)：156－160
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Corticosterone impairs cultured hippocampal neurons and facilitates Ca^{2+} influx through voltage-dependent Ca^{2+} channel[1]

ZHOU Jian-Zheng[2], ZHENG Jian-Quan, ZHANG Yong-Xiang, ZHOU Jin-Huang
(*Beijing Institute of Pharmacology and Toxicology, Beijing 100850, China*)

KEY WORDS corticosterone; hippocampus; patch-clamp techniques; calcium channels; neurons; cultured cells; cell survival; inhibitory concentration 50

ABSTRACT

AIM: To investigate the effect of corticosterone (Cor) on the viability of cultured hippocampal neurons as well as voltage-dependent Ca^{2+} channel (VDCC) on the membrane of the hippocampal neurons. METHODS: The primary cultured hippocampal neurons were prepared and the viability of hippocampal neurons was determined by MTT assays. Inward Ca^{2+} currents of VDCC on the membrane of the hippocampal neurons were measured with the whole-cell patch-clamp technique. RESULTS: Treatment with Cor concentration-dependently reduced the survival of hippocampal neurons. The IC_{50} of Cor was 3.2 $\mu mol \cdot L^{-1}$. Neurons from cerebral cortex were affected only by high concentrations of Cor (10 $\mu mol \cdot L^{-1}$ and 0.1 $mmol \cdot L^{-1}$) with the IC_{50}, 85 $\mu mol \cdot L^{-1}$, 20 times larger than the former. Whole-cell patch-clamp experiment showed that Cor (1 $\mu mol \cdot L^{-1}$－0.1 $mmol \cdot L^{-1}$) sprayed to the surface of the hippocampal neurons instantly facilitated Ca^{2+} influx through VDCC with the maximal elevation of 53 %, 191 %, and 84 % above the baseline respectively and this effect was shown to be concentration-independent. In addition, changing the membrane potentials from －40 mV to －10 mV did not affect the facilitating effect of Cor on the Ca^{2+} influx, indicating that Cor-induced Ca^{2+} influx was membrane potential-independent. CONCLUSION: Cor markedly facilitated Ca^{2+} influx into the hippocampal neurons, which may be one of the important mechanisms underlying the neurotoxicity of Cor to hippocampus.

INTRODUCTION

Corticosterone (Cor) was an important steroid hormone in regulating metabolism of fat, protein and glucose in various peripheral tissues. Recent studies[1,2] indicated that high concentrations of glucocorticoids showed adverse effects on the central nervous system (CNS), especially on the hippocampus. The *in vitro* toxic effects of some excitotoxins were shown to be exacerbated by the exposure to Cor[3] and administration of Cor to rats for three months led to significant impairment of learning and memory[2]. However, the direct effect of Cor on the neurons was not completely understood yet. It was reported recently that adrenalectomy in rats significantly reduced the amplitude of Ca^{2+}-dependent-K^+-mediated afterhyperpolarization (AHP) and supplemented with Cor to adrenalectomized rats restored the amplitude of AHP[4,5]. Because the AHP amplitude directly reflects the cytosolic free Ca^{2+} level at the end of action potential, it seemed that Cor could affect the homeostasis of cytosolic free Ca^{2+} level in the hippocampal neurons. Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCC) was one of the main pathways for elevation of cytosoilc free Ca^{2+} level. Some VDCC blockers, such as nimodipine, prevented the Ca^{2+} overload-induced neurons death by blocking Ca^{2+} influx through VDCC[6]. In the present paper, in order to investigate the neurotoxic effect of Cor and its possible mechanism, effects of Cor on the viability of primary cultured neurons and Ca^{2+} influx through VDCC were investigated.

MATERIALS AND METHODS

Primary hippocampal and cerebral cortex culture

The whole brains were taken from day 18 embryo Sprague-Dawley rats (Experimental Animal Center of Beijing Medical University, Grade Ⅱ, Certificate № 01-3055, the Administrative Commission of Beijing) and the hippocampi and cerebral cortex were dissected. Pri-

[1] Project supported by the National Natural Science Foundation of China, № 39570851 and the 95' Climbing Program — the National Key Projects for Fundamental Research.
[2] Correspondence to Dr ZHOU Jian-Zheng. Phn 86-10-6693-1627. Fax 86-10-6821-1656. E-mail zhoujz@nic.bmi.ac.cn
Received 1998-11-16 Accepted 1999-06-25

mary neuronal cultures of the hippocampus and cerebral cortex were prepared as Banker and Cowan[7]. The hippocampi and cerebral cortex were pooled and enzymatically dispersed respectively with 0.125 % trypsin and 0.01 % DNase I (Sigma) at 37 ℃ for 20 min. The cells were washed and counted in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing glutamine 2, glucose 5.0 mmol·L^{-1}, 10 % fetal calf serum (FCS) and 10 % horse serum, and then plated into 96-well plates which had been coated with 0.1 mL of poly-*D*-lysine (10 mg·L^{-1}) (Sigma) at a density of 5.0×10^4 cells/well in 100 μL. The cultures were incubated at 37 ℃ in 5 % CO_2 + 95 % air. The medium was changed to serum-free DMEM medium containing insulin 10 mg·L^{-1}, transferrin 10 mg·L^{-1} and sodium selenite 10 mg·L^{-1} (Sigma) 24 h after the primary incubation. The cells were treated with Cor (Sigma) in different concentrations (10 nmol·L^{-1} - 0.1 mmol·L^{-1}) for 24 h 3 d after medium change. The cell viability was measured using the MTT tetrazolium (Aldrich) cytotoxic assays 24 h after Cor treatment.

Quantitation of cell viability Quantitation of cell viability was conducted using MTT tetrazolium cytotoxic assays[8]. Briefly, MTT 10 μL in phosphate-buffer saline (5.0 g·L^{-1}) was added to the hippocampal culture and further incubated for two and a half hour at 37 ℃. Then, 10 % SDS in HCl 0.01 mol·L^{-1} was added. After dissolution of the dark blue formazan crystals, the absorbance were examined on a microplate spectrometer (Mltiscan MCC 340, Titertek Co. USA) at test wavelength, 570 nm with reference wavelength, 630 nm.

Ca^{2+} currents recording Hippocampal neurons in 7 - 10 d were used and recordings of evoked Ca^{2+} currents were carried out at 20 - 25 ℃ using the whole-cell patch-clamp technique as previously described[9]. The patch-pipette was filled with solution containing CsCl 140, HEPES 10, egtazic acid 10 and ATP 2 mmol·L^{-1} with the electric resistance of 1 - 5 MΩ. The extracellular solution contained NaCl 140, KCl 5, $CaCl_2$ 3, $MgCl_2$ 1, HEPES 10, glucose 10, tetraethylammonium chloride 140, tetrodotoxin 0.001 mmol·L^{-1}. Following seal formation and prior to entering the whole-cell mode, electrode capacitance was neutralized by using the capacitance compensation circuitry of Axopatch 1D (Axon Instruments, Foster City CA, USA). In the whole-cell mode, the Axopatch was further adjusted to correct for 80 % - 85 % of the series resistance. Command potential sequences were delivered to the patch-clamp amplifier and the data were simultaneously collected with a computer. Evoked currents were filtered at 10 kHz (- 3 dB, 8-pole low-pass Bessel filter, Frequency Devices, Haverhill, MA), digitally sampled at 500 μs per point and stored on magnetic media in digital form for later analysis. Capacitative and leakage currents digitally subtracted from all records, which was carried out on line by using pCLAMP 5.5.1 (Axon Instruments).

Applications of drugs Cor was applied by a puff pipette connected to a pressure injector (BH-2, Medical System Co Ltd). The puff pipette consisted of 3 microtubes with a diameter of 5 - 10 μm. The distance between the pipette and the recorded neuron was 20 - 30 μm, and 50 - 60 kPa of N_2 pressure was delivered.

Statistics The amplitudes of Cor-induced Ca^{2+} currents (I_{Ca}) were measured with the program of pCLAMP 5.5.1. Mean values were given with means ± standard deviation ($\bar{x} \pm s$). The program of Statistics Analysis System (SAS) was used for ANOVA analysis followed by Duncan test.

RESULTS

Neurotoxic effect of Cor on primary cultured hippocampal and cerebral cortex neurons Cor showed concentration-dependent ($P<0.05$ or $P<0.01$) toxic effect on both hippocampal and cerebral cortex neurons. The hippocampal neurons swelled with disappeared dendrites and became round-shaped cells, indicating the death of the neurons after Cor treatment at the highest concentration of Cor 0.1 mmol·L^{-1}. The IC_{50} of Cor to hippocampal cells was about 3.2 μmol·L^{-1}, while that to cerebral cortex was 85 μmol·L^{-1}, 20 times larger than the former (Tab 1).

Effect of Cor on the Ca^{2+} currents of VDCC After spraying Cor (1 μmol·L^{-1} - 0.1 mmol·L^{-1}) to the membrane of hippocampal neurons, Ca^{2+} currents were instantly facilitated. However, Ca^{2+} currents induced by Cor 10 μmol·L^{-1} was the largest with the maximal elevated amplitude of 191 % above the baseline while those induced by Cor 1 μmol·L^{-1} and 0.1 mmol·L^{-1} were relatively smaller with the maximal elevation of 53 % and 84 % respectively. (Fig 1).

Effect of Cor on the Ca^{2+} current-membrane potential (I-V) relationship In our present experiment, the Ca^{2+} currents were recorded by 300 ms depolarization steps from - 85 mV to + 5 mV at a holding potential

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Feb；21 (2)
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Tab 1. Toxic effect of Cor on primary cultured hippocampal and cortical neurons. $n=7-8$. $\bar{x}\pm s$.
$^bP<0.05$, $^cP<0.01$ *vs* control.

Cor/ μmol·L^{-1}	Survival/% Hippocampus	Cerebral cortex
0	100	100
0.01	81 ± 20^b	100 ± 7
0.1	63 ± 13^c	93 ± 10
1	68 ± 14^c	88 ± 17
10	15 ± 7^c	77 ± 10^c
100	11 ± 4^c	43 ± 8^c

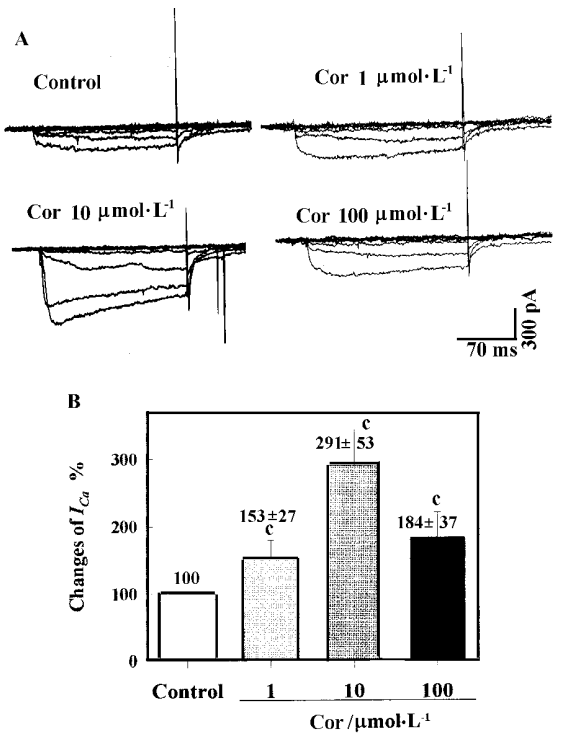

Fig 1. Changes of Ca^{2+} currents of VDCC after spraying Cor (1.0 μmol·L^{-1} − 0.1 mmol·L^{-1}) on the membrane of hippocampal neurons. Ca^{2+} currents were elicited by 300 ms depolarization steps from −85 mV to +5 mV at a holding potential of −100 mV. A) four evoked Ca^{2+} currents graphs demonstrating Cor-induced changes of Ca^{2+} influx through VDCC; B) a figure showing Cor-facilitated Ca^{2+} influx into the hippocampal neurons when the membrane potential was depolarized at −10 mV. $n=6-8$. $\bar{x}\pm s$. $^cP<0.01$ *vs* control.

of −100 mV. The relationship between Ca^{2+} currents and membrane potentials showed that the VDCC began to open when the membrane potential was depolarized at −60 mV. After that, the Ca^{2+} currents were gradually increased with the elevation of the membrane potentials. When the membrane potential was depolarized at −10 mV, the inward Ca^{2+} influx reached its peak. Cor (1 μmol·L^{-1} − 0.1 mmol·L^{-1}) sprayed on the surface of hippocampal neurons shifted the I-V curve down. When the membrane potentials were depolarized at −40 mV, −25 mV, −10 mV, or even 5 mV, the corresponding Ca^{2+} currents were all significantly ($P<0.05$, or $P<0.01$, Fig 2) larger that those in control.

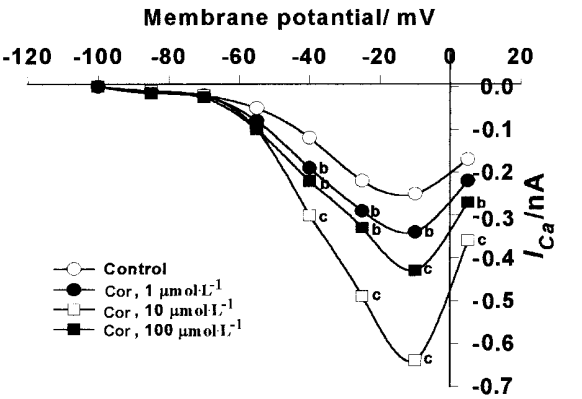

Fig 2. Effect of Cor (1.0 μmol·L^{-1} − 0.1 mmol·L^{-1}) on the relationship of Ca^{2+} currents (nA) of VDCC and membrane potentials (mV) on the hippocampal neurons. Ca^{2+} currents were elicited by 300 ms depolarization steps from −85 mV to +5 mV at a holding potential of −100 mV. $n=6-8$. $\bar{x}\pm s$.
$^bP<0.05$, $^cP<0.01$ *vs* control.

Cor-induced facilitation of Ca^{2+} influx was voltage-independent Ca^{2+} influx through VDCC at various membrane potentials were all greatly increased by Cor at the concentration of 10 μmol·L^{-1} (Tab 2). However, the elevated amplitudes among the three groups were similar and no statistical significance was observed ($P>0.05$).

Tab 2. Cor-facilitated Ca^{2+} influx through VDCC on the membrane of neurons is potential-independent.
$n=6-8$. $\bar{x}\pm s$. $^aP>0.05$, $^cP<0.01$ *vs* control.

Potentials/ mV	Ca^{2+} currents/pA Control	Cor 10 μmol·L^{-1}	Elevation/ %
−10	250 ± 83	643 ± 252^c	257 ± 38^a
−25	225 ± 56	494 ± 126^c	220 ± 26^a
−40	123 ± 24	302 ± 55^c	246 ± 29^a

DISCUSSION

In our previous studies[10], it was suggested that elevated Cor level in plasma was responsible for the ATP and ADP deficiency and glutamate accumulation in hippocampus in senescence-accelerated mouse (SAM). However, the direct effect of Cor on the hippocampus was controversial. Packan and Sapolsky[11] demonstrated that administration of Cor exacerbated many insults such as hypoxia and glutamate without direct neurotoxicity on primary hippocampal culture. In the present study, Cor application to serum-free medium concentration-dependently reduced the survival of the primary cultured hippocampal neurons. In addition, the neurotoxicity of Cor to cortical culture was also found at the concentrations of 10 $\mu mol \cdot L^{-1}$ and 0.1 $mmol \cdot L^{-1}$. The IC_{50} of Cor to hippocampal cells and cortical cells was 3.2 $\mu mol \cdot L^{-1}$ and 85 $\mu mol \cdot L^{-1}$ respectively, suggesting that Cor impaired both hippocampal and cortical neurons directly. The IC_{50} of Cor to hippocampus was smaller than that to cerebral cortex, suggesting that hippocampus was more sensitive to the toxic effect of Cor.

Ca^{2+} is one of the most important second messenger in mediating the cell functions. However, overload of cytosolic Ca^{2+} has been considered to be a common pathway for cell death[6]. Our results in the whole-cell recording experiment showed that Cor sprayed to the surface of hippocampal neurons significantly augmented the Ca^{2+} currents, demonstrating that Cor accelerated Ca^{2+} influx into hippocampal neurons. The I-V relationship curve showed that the VDCC began to open when the membrane potential was depolarized at -60 mV, indicating that the Ca^{2+} currents recorded in the present experiment were low voltage-activated currents. In addition, application of Cor shifted the I-V relationship curve down and the Ca^{2+} currents were greatly enhanced by Cor when the membrane potentials were depolarized from -85 mV to $+5$ mV. The Cor-induced facilitation of Ca^{2+} influx may be one of the important mechanisms underlying its toxic effect on hippocampal neurons. Although Cor at three doses all facilitated the Ca^{2+} influx, the maximal elevations did not show positively proportional to Cor concentrations. In our study, Cor at concentration of 10 $\mu mol \cdot L^{-1}$ induced the largest Ca^{2+} currents with the maximal elevation of 191 % on the baseline while Cor, 10 $\mu mol \cdot L^{-1}$ and 0.1 $mmol \cdot L^{-1}$, promoted Ca^{2+} influx with the maximal amplitude of 53 % and 84 % respectively, indicating that Cor-induced facilitation of Ca^{2+} influx was concentration-independent. Thus, there is discrepancy between Cor-induced neurotoxicity and Cor-induced facilitation Ca^{2+} influx, suggesting that there may be other mechanisms mediating the Cor-induced neurotoxicity on hippocampal neurons besides Ca^{2+} influx through VDCC. In the present experiment, depolarizing voltage steps from -40 mV to -10 mV had no effect on the Cor-induced facilitation of Ca^{2+} influx, suggesting that this effect was potential-independent. Our previous studies demonstrated that pretreatment with NMDA-receptor antagonist, MK-801, antagonized the Cor-induced neurotoxicity to hippocampal neurons[12] and incubation of Cor with hippocampal slice significantly accelerated the glutamate release[13]. Other studies indicated that Cor promoted stress-induced glutamate release in hippocampus[14] and Cor administration inhibited the uptake of glutamate in the hippocampal cells[15]. These results suggested that Cor application might lead to accumulation of glutamate in hippocampal synapse. Increasing evidence indicated that glutamate released from presynapse during synaptic activation bound the NMDA-receptor in post-synaptic membrane and then led to activation of the ligand-gated Ca^{2+} channels (LGCC), which usually depolarized the postsynaptic membrane and subsequently opened the VDCC[16]. For example, during formation of LTP in hippocampal CA1 neurons, activation of NMDA-receptor facilitated the Ca^{2+} influx mainly through VDCC, not through LGCC itself[16]. With these findings, it is reasonable to postulate that the potential-independent facilitation of Ca^{2+} influx induced by Cor may also be related to accumulation of glutamate.

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ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2000 Feb；21 (2)
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皮质酮损伤培养的海马神经细胞并促进其电压依赖性钙内流[1]

周建政[2]，郑健全，张永祥，周金黄
(北京毒理药理研究所，北京 100850，中国)

关键词 皮质酮；海马；膜片箝技术；钙通道；神经元；培养的细胞；细胞存活；半数抑制浓度

目的：研究皮质酮(Cor)对原代培养海马神经细胞存活和海马神经细胞电压依赖性钙通道(VDCC)的影响. **方法**：原代海马神经细胞存活率测定用 MTT 比色法. 海马神经细胞上 VDCC 内向 Ca^{2+} 电流检测采用全细胞膜片箝技术. **结果**：Cor 可浓度依赖地损伤原代海马神经细胞和皮层神经细胞，IC_{50} 分别为 3.2 $\mu mol \cdot L^{-1}$ 和 85 $\mu mol \cdot L^{-1}$. Cor (1 $\mu mol \cdot L^{-1}$－0.1 $mmol \cdot L^{-1}$)喷射于海马神经细胞表面即刻显著促进电压依赖性 Ca^{2+} 内流，其最大升幅分别是 53 %，191 % 和 84 %，而且 Cor 诱导的钙内流增加是非浓度依赖和非电压依赖的. **结论**：Cor 可显著促进海马神经细胞电压依赖性钙通道开放，该作用可能是 Cor 海马神经毒性作用的机制之一.

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