Nikethamide increases sodium current of inspiratory neuron via PKC pathway in mNRF of neonatal rats in vitro

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Abstract: To elucidate the roles nikethamide on sodium currents of inspiratory neurons in the medial area of nucleus retrofacialis (mNRF) and whether PKC take part in those roles, whole-cell patch were performed to record sodium current of inspiratory neuron(I neuron) and ELISA double-antibody sandwich method were used to measured concentration of PKC in mNRF. Nikethamide increases persistent and transient sodium current of I neuron. It makes steady activation curves shifted to more negative potential and steady inactivation curves shifted to more positive potential of sodium channel. Determination the concentration of PKC of neurons in mNRF by ELISA , nikethamide increases concentration of PKC of neurons in mNRF. Nikethamide makes sodium chaanel open at a lower membrane potential and close at a higher membrane potential, it increases open lasting time and open probability of sodium channel via PKC pathway.

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1. Introduction

Our researches have demonstrated that the medial area of nucleus retrofacialis (mNRF) is the site of respiratory rhythmogenesis[1,2]. Smith stated that the pre-B Ö tzinger complex (PBC) was the site of respiratory rhythmogenesis in 1991. Both mNRF and PBC are located in rostral ventrolateral medulla, although the anatomical position are different, but they are overlapped partly. Nikethamide (N, N-diethylnicotinamide) has been used widely in clinic as : first, used as a respiratory central stimulator, it can excite respiratory center selectively[3]; second, nikethamide decreases the aminopherase and jaundice levels of infant[4]; thirdly, niketnamide comprise with the polarity part and the nonpolarity part, it can be used as chaotropic agent to dissolve drugs which is difficult to dissolve in water[5]. Nikethamide increase basic rhythmic resoiratory discharge activities of medullary slice and action potential of inspiratory neuron(I neuron) of mNRF. The present study was designed and performed to to know how nikethamide increases excitability of inspiratory neuron and whether sodium current and PKC take part in those roles.

2. Materials and methods

2.1 Materials

Neonatal Sprague–Dawley rats, both males and females, 0-3 days, n=7, were supplied by Experimental Animal Center of Xinxiang Medical University. Nikethamide were bought from Sigma, others for artificial cerebral spinal fluid (ACSF) are Of analytical grade.

2.2 Medullary slice preparation.

All experiments used the transverse, rhythmic medullary slice, which can generate respiratory-related motor output. The Office for the Protection of Research Subjects, Xinxiang medical university Research Committee approved all protocols. Neonatal Sprague–Dawley rats were deeply anesthetized with ether (delivered by inhalation) and quickly decapitated at the C3-C4 spinal level. The brainstem was dissected in ice-cold ACSF containing in mM: 124 NaCl, 5 KCl, 2.4 $CaCl_2$, 1.3 $MgSO_4$, 26 $NaHCO_3$, 1.2 KH_2PO_4, and 30 D-glucose, equilibrated with carbogen (95% O_2 and 5% CO_2), pH 7.4. The brainstem was serially sectioned in the transverse plane until the nucleus ambiguus and inferior olive were visible. Then a rhythmic 750μm-thick slice containing the mNRF was obtained by slicing the medulla using a Vibratome (VT1000S, Leica, Germany). Slice was quickly transferred to a recording chamber and continuously perfused with oxygen-saturated ACSF at a rate of 8–10 ml/min at 27–29℃ in a 3ml recording chamber.

2.3 Electrophysiological recordings

The discharge of hypoglossal nerve (XII nerve) rootlets were recorded serving as a marker of fictive inspiration by using suction electrodes(inner diameter 180μm). Signals were amplified and band-pass filtered (100 Hz–3.3 kHz), data were sampled (5 kHz) and stored in the computer via BL-420F biological signal processing system. Whole-cell patch-clamp recordings were performed using an MultiClamp 700B amplifier (Molecular Devices) in current-clamp and voltage-clamp mode. I neuron were visualized using

infrared-enhanced differential interference contrast (IR-DIC) video microscopy(NIKON). Electrodes were pulled from borosilicate glass (outer diameter, 1.5mm;inner diameter, 0.86mm) by a horizontal puller (model P-97; Sutter Instruments, Novato, CA). The electrodes used for voltage-clamp recordings to isolate sodium currents were filled with a solution containing: 110 mM CsCl, 30 mM TEA-Cl, 1 mM CaCl2, 10 mM EGTA, 2 mM MgCl2, 4 mM Na2ATP, and 10 mM HEPES, adjusted pH to 7.2 by CsOH. The result of this pipette solution was small LJP (3mV), which was not corrected in this study.

Only I neuron active in phase with population activity were considered in this study[2,6]. The discharge pattern of each cell type was first identified in the cell-attached mode. After recording, CNQX, CPP, strychnine, and bicuculline were bath applied to isolate chemical synaptic input of neuron, the neuron which stop to burst in the absence of inspiratory population bursts is I neuron, show in Fig. 1.

2.4 Determination the concentration of PKC of neurons in mNRF by ELISA double-antibody sandwich method.

Prepare the "mNRF island" from medullary slices according Johnson SM[7], Sixteen islands were divided into two equal groups randomly: control group and nikethamide group. The control islands were incubated for 30 minutes in ACSF, islands of nikethamide group were incubated for 30 minutes in ACSF in which dissolved 0.02mol / L nikethamide. After incubated, determination the concentration of PKC by the P-PKC kit of ELISA double-antibody sandwich method(repeated three times)[8].

2.5 Statistical analysis

All values were expressed as means±SEM and statistical comparisons were evaluated using repeated measures analysis followed by Student's t-test where appropriate. A probability value less than 0.05 ($P < 0.05$) was considered as statistically significant.

3. Results

3.1 Nikethamide increased transient sodium current and persistent sodium current of I neurons.

Experiments were performed in the whole-cell patch-clamp mode with the neurons recorded in current clamp at the zero potential. Establishing the whole-cell patch-clamp configuration did not alter the firing pattern of recorded neurons. In voltage-clamp configuration, voltage steps from -80 to +20 mV and slow voltage ramps from -80 to +20 mV (90 mV/ sec) were applied to elicit transient and persistent sodium currents, respectively[9,10]. Both the persistent and transient inward currents recorded in I neurons were sensitive to TTX. The persistent sodium currents and the transient currents of I neurons were changed from 1628.33pA±276.94 pA to 1961.33±252.86 pA, 295±58 pA to 320±71 pA before and after nikethamide were purfused, show in Fig. 2($n=7$, $P < 0.05$).

3.2 Effects of nikethamide on steady-state activation curves and steady-state inactivation curves of sodium channel in I neurons.

To establish steady-state activation curves, cells were held at −70 mV, and a series of 20 ms pulse from −70 to 0 mV in 10 mV increments were followed by a 500 ms test pulse of −130 mV[9,10]. The peak currents of membrane potential were converted into conductance calculated from the equation: $G= I/(Vm -Vrev)$, where Vm is the membrane potential and Vrev is the reversal potential. The normalized conductance was fitted by a Boltzmann equation: $I/Imax=I/ \{ 1+\exp[(V-V1/2)/\kappa] \}$ where V1/2 is the membrane potential at half-activation and κ is the slope factor. The steady-state activation curves of sodium current before and after exposure to nikethamide are shown in Fig. 3A. The curve was shifted to more negative potential after application of nikethamide. The value of $V_{1/2}$ for INa activation changed from −34.49 ± 0.73 mV in the absence of nikethamide to −40.45± 2.00mV in the presence of nikethamide, with a corresponding change in slope factor κ from 5.67 ± 0.47 to 4.54± 0.32, show in Fig. 3 ($n=7$, $P < 0.05$).

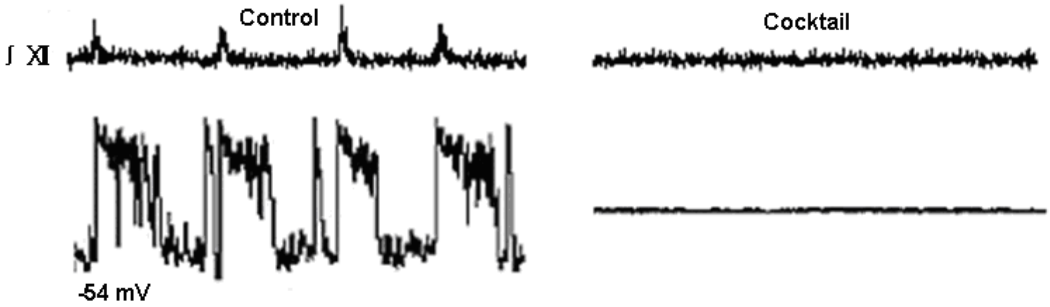

Fig. 1 Identification of inspiratory neuron

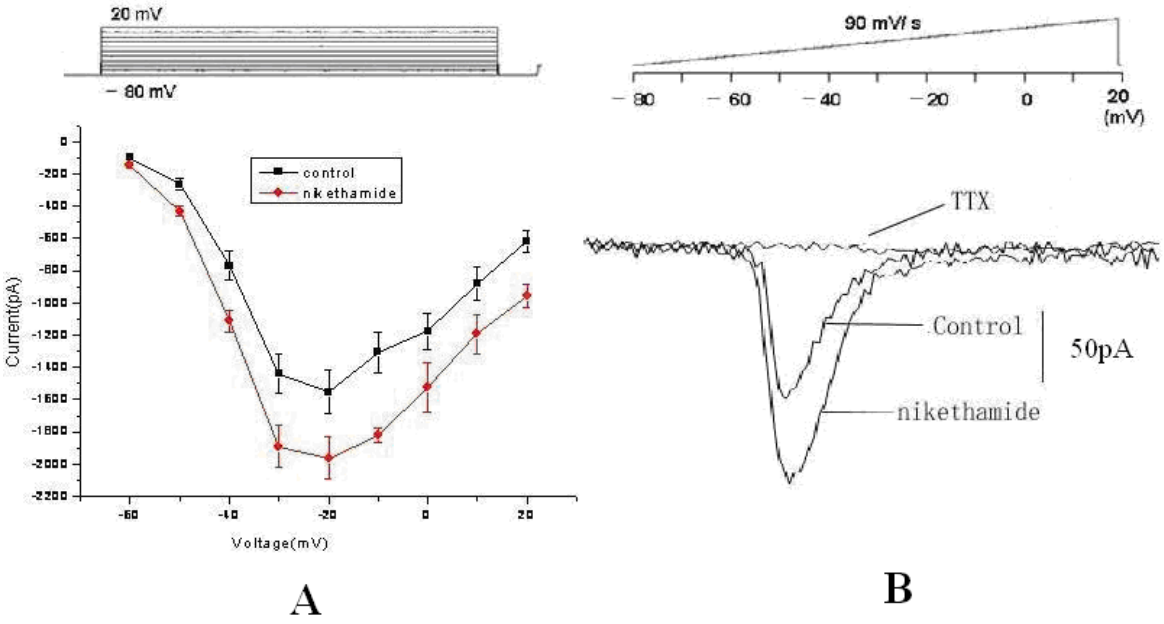

Fig. 2 Nikethamide increases transient sodium current(I-V curve, A) and persistent sodium current(B) of I neurons

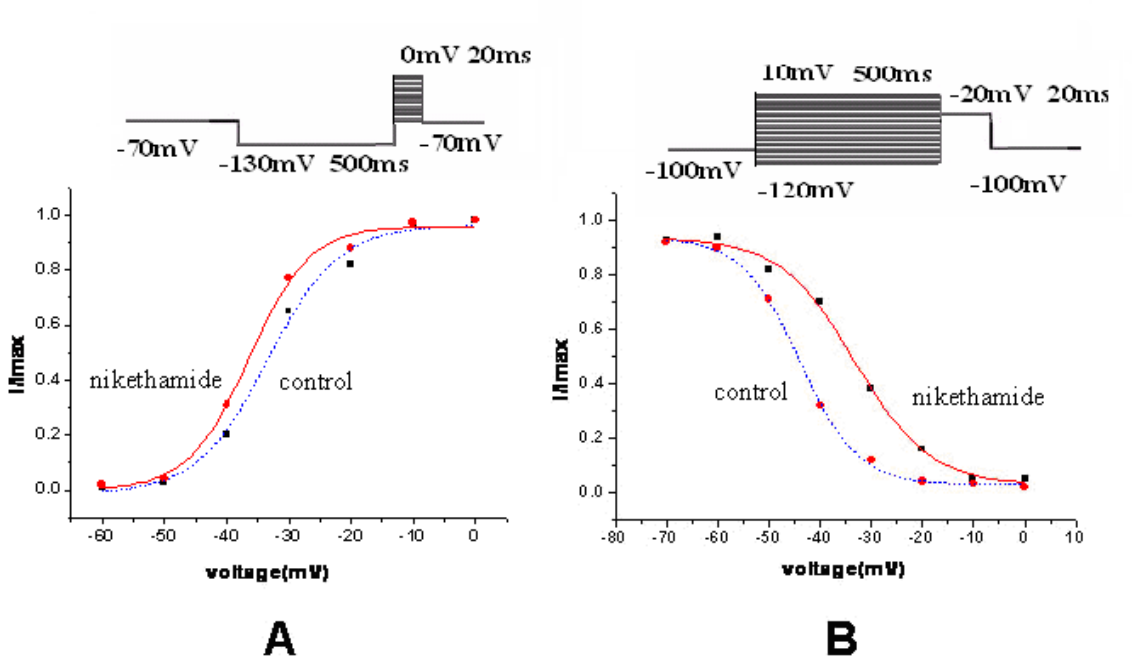

Fig. 3 Roles of nikethamide on steady-state activation curves (A) and steady-state inactivation curves (B)of sodium channel I neurons

To investigate the effects of nikethamide on steady-state inactivation properties of INa. Cells were held at −100 mV, and a series of 500 ms prepulse from −120 to 10 mV in 10 mV increments were followed by a 20 ms test pulse of −20 mV[9,10]. The inactivation curves were fitted by a Boltzmann equation:$I/I_{max}=I/\{1+\exp[(V-V_{1/2})/\kappa]\}$, where $V_{1/2}$ is the membrane potential at half-activation and κ is the slope factor. The curve was shifted to more positive potential after application of nikethamide, Fig. 3B. The value of $V_{1/2}$ was −46.85 ± 1.59 mV without nikethamide, but was changed to −35.02±1.55 mV in

the presence of nikethamide, with κ varying from 5.34 ± 0.33 to 6.67± 0.30, show in Fig. 2 (n = 7, P < 0.05).

3.3 The concentration of PKC of neurons in mNRF was 29.24% higher than control group.

After incubation with nikethamide the concentration of PKC of neurons in mNRF was 29.24% higher than control group. The concentration of PKC of niketahmide is 0.31±0.014, and the control group is 0.24±0.010.

4. Discussions

This study was performed in in-vitro brainstem slices containing the neurons critical for integration of respiratory drive. The respiratory frequency of this preparation was markedly slower than that in vivo due to the isolation of nervous system from mechanosensory afferent inputs and the removal of vagal mechanosensory afferent inputs[11]. However, the discharge patterns of respiratory motor neurons in vitro were similar to that in the intact mammal but different from gasping[12,13]. The I neurons, which appear to be fundamental components of the inspiratory pattern generation, have been proposed to be responsible for respiratory rhythm.

In this study, nikethamide has a exciting roles on sodium current, it increases transient and persistent sodium current of I neurons. Nikethamide makes sodium channel steady activation curves change towards hyperpolarization, and makes steady inactivation curves change towards depolarization. Nikethamide makes sodium chaanel open at a lower membrane potential and close at a higher membrane potential, meaning nikethamide increase open lasting time and open probability of sodium channel, increase exciting of I neuron.

After enter neuron, nikethamide transform into nicotinic amide through deacetylation, nicotinic amide transform into N-Methylnicotinamide through methylation. Nikethamide excreted through kidney in N-Methylnicotinamide. Nicotinic amide is a material for synthesizing $NADP^+$[14]. Nikethamide increase concentration of $NADP^+$, $NADP^+ \rightarrow$ superoxide $\rightarrow$ superoxide dismutase $\rightarrow$ H_2O_2, increase reactive oxygen species (ROS), and ROS activate Protein kinase C[15,16]. In our study, after being incubated with nikethamide, the concentration of PKC was higher than control. PKC increase open probability of sodium channel through phosphorylation and increase concentration of reactive oxygen species[17,18]. Reactive oxygen species can increase open probability of sodium channel too[19]. The increasing open probability of sodium channel increasing the excitability of I neurons and respiratory center.

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