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Nitric oxide modulation of voltage-gated calcium current by *S*-nitrosylation and cGMP pathway in cultured rat hippocampal neurons

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Abstract

Nitric oxide (NO) plays an important role in many physiological and pathophysiological processes in the brain. In this study, we examined the mechanistic effects of an NO donor, diethylenetriamine/nitric oxide adduct (DETA/NO) on the voltage-gated calcium currents in cultured rat hippocampal neurons. DETA/NO stimulated the calcium currents and slightly increased the channel sensitivity to depolarizing voltages. The effect of DETA/NO on the calcium current was blocked by either depleting the NO in DETA/NO or by pretreating the neurons with NEM, a thiol-specific alkylating agent, suggesting an involvement of *S*-nitrosylation in the current response to NO. In addition, activation of the cGMP pathway by 8-Br-cGMP inhibited the calcium current in the neurons. Also, inhibition of guanylyl cyclase by 1H-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one (ODQ) increased the current response to DETA/NO. Taken together, our results demonstrate that both *S*-nitrosylation and cGMP pathway are involved in the NO modulation of the hippocampal calcium current.

Keywords: Nitric oxide; *S*-Nitrosylation; cGMP pathway; Voltage-gated calcium currents; Hippocampus

Nitric oxide (NO) and its signaling mediators have been shown to be involved in many physiological and pathophysiological processes in the brain. In hippocampus, all NO synthase (NOS) isoforms have been found to be expressed in physiological conditions and pathogenic processes such as ischemic injury [1,2]. Two major signaling mechanisms, namely cGMP pathway and *S*-nitrosylation, mediate the cellular effects of NO. The cGMP pathway has been extensively documented for the effect on proteins by phosphorylation, whereas *S*-nitrosylation of proteins by chemical reactions with NO can modify protein functions. Recently, more attention has been focused on *S*-nitrosylation showing the post-translational modification of the protein at the thiol side-chains of cysteine residues [3–6]. Over 100 proteins have been identified as the targets for *S*-nitrosylation and it can be at extracellular or intracellular part of the protein [7].

Calcium influx regulated by voltage-gated calcium channels plays an important physiological role in the calcium signaling [8–10] and in pathophysiological events such as ischemia or hypoxia [1,11]. Calcium-dependent NOS activity can be activated by the calcium influx for the NO production during membrane depolarization with the opening of the calcium channels [1,3]. In the hippocampal pyramidal neurons, voltage-gated calcium channels are prevalent and the L-type channel contributes up to about 50% of the total calcium currents [12]. Therefore, modulation of the voltage-gated calcium channel activity may have dramatic effects on the calcium influx and signaling in the hippocampal neuron. NO has been extensively reported to be able to regulate many kinds of ion channels [3,5,13,14]. Until now, it is still unclear the mechanistic effects of NO on the voltage-gated calcium current in the hippocampal

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neuron. We have reported that activation of NOS can slowly depolarize the cultured hippocampal neuron [15]. Thus, the aim of this study was to examine the hypothesis that NO modulation of the voltage-gated calcium current might be mediated by *S*-nitrosylation and cGMP pathway in cultured rat hippocampal neurons. Here we reported that NO donor diethylenetriamine/nitric oxide adduct (DETA/NO) increased the calcium current by *S*-nitrosylation but the current was inhibited by the cGMP pathway, suggesting a feedback control of the calcium influx by NO modulation of the voltage-gated calcium channels.

Materials and methods

Primary hippocampal neuronal cultures were prepared from 1-d-old (postnatal day 1) Sprague–Dawley rats. Hippocampi were dissociated and digested by 0.125% Trypsin. Cells were plated at a density of 4×10^5 cells/cm^2 on poly-L-lysine-coated culture surface. Culture medium consisted of 90% DMEM supplemented with 10% fetal bovine serum. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 and 95% air. Glial cell growth was controlled by 10^{-5} M cytosine arabinoside for 48 h at 2 days in vitro. A one-half volume medium replacement was conducted every 3 days. All electrophysiological experiments were performed on cells cultured for 10–16 days.

Whole-cell calcium currents were recorded by using patch clamp techniques as described previously [16]. The bath solution contained (in mM): 75 choline chloride, 50 TEA-Cl, 20 $BaCl_2$, 5 CsCl, 2 $MgCl_2$, 10 glucose, 10 Hepes and 0.001 TTX, pH 7.3 with TEA-OH. The pipette solution contained (in mM): 145 methanesulfonic acid, 11 EGTA, 10 TEA-Cl, 5 $MgCl_2$, 1 $CaCl_2$, 10 Hepes, 5 Mg-ATP, 0.5 Na-GTP and 0.1 leupeptin, pH 7.3 with CsOH. Pipettes with resistance of 3–4 MΩ were pulled from borosilicate glass using a micropipette puller (model P-97, Sutter Instruments, CA). Currents were recorded at room temperature using an Axopatch 200B amplifier (Axon Instruments, CA). Signals were low-pass filtered at 1–2 kHz and digitized at 5 kHz with Digidata 1320 interface and pCLAMP 8.0 software (Axon Instruments). Currents were leak subtracted and elicited by depolarizing pulses to 0 mV for 100 ms from a holding potential of −50 mV at intervals of 10 s and denoted as I_{Ba}. Current–voltage (*I–V*) relations were elicited from a holding potential of −50 mV using 100 ms steps (10 s between steps) to test potentials over a range of −20 to +60 mV in 10 mV increments. Ramp currents were elicited from a holding potential of −50 mV using 180 ms ramp to +70 mV.

All chemicals were obtained from Sigma except for tetrodotoxin (Fisheries Research Institute of Hebei, China). Stocks of *N*-ethylmaleimide (NEM) and 1H-[1,2,4] oxadiazolo [4,3-*a*] quinoxalin-1-one (ODQ) were stored at −20 °C. Stocks of 8-Br-cGMP was dissolved in distilled water and used freshly. Stock of diethylenetriamine/nitric oxide adduct (DETA/NO) was stored at 4 °C and used within 2 days.

Pooled data are presented as mean ± SEM. Statistical significance was determined by a paired *t*-test, an independent-sample *t*-test or a one-way ANOVA where appropriate. p values <0.05 were considered significant.

Results

A sustained inward current (I_{Ba}) was activated by depolarizing voltage steps from a holding potential of −50 mV with 20 mM Ba^{2+} as the charge carrier. Nifedipine (NFDP, 20 μM), a dihydropyridine L-type voltage sensitive calcium-channel blocker, suppressed the current by

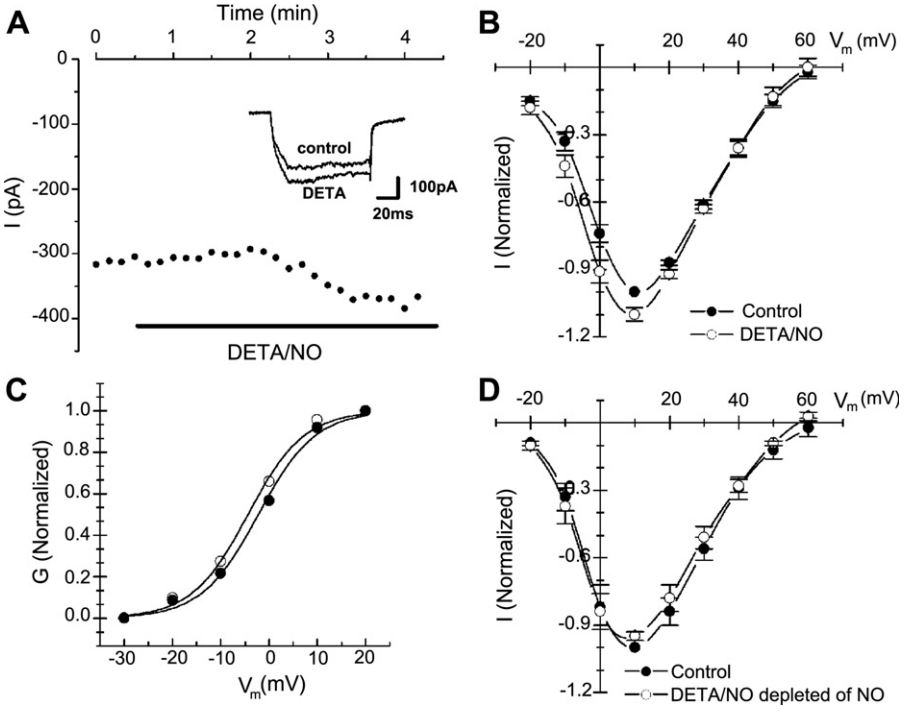

Fig. 1. Stimulating effects of DETA/NO on the calcium current in cultured hippocampal neurons. (A) An example of the time course of DETA/NO on the current. DETA/NO (0.5 mM) started to increase the current about 1.5 min after administration and reached to the maximumal level after 1 min. (B) Current–voltage relationship before and after exposure to DETA/NO showing a voltage dependent modulation of the current. DETA/NO increased the current within the range between −20 and 10 mV of the *I–V* relationship. The current was increased about 23% by steps at 0 mV ($n = 22$, $p < 0.01$). (C) Activation curve of the calcium current obtained from the data shown in (B). The continuous curve is the Boltzmann fit. In control, the midpoint is at -2.2 ± 0.9 mV with a slope of 5.9 ± 0.5. Following DETA/NO treatment, there is a slight leftward shift of the midpoint at -4.4 ± 0.5 mV with slope of 5.8 ± 0.5. (D) DETA/NO with NO depletion had no effect on the current.