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MECHANISM OF MOTILIN-MEDIATED INHIBITION ON VOLTAGE-DEPENDENT POTASSIUM CURRENTS IN HIPPOCAMPAL NEURONS

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Abstract—*Objective:* The effects of motilin on voltage-dependent K^+ currents in hippocampal neurons with the addition of L-arginine (L-AA), D-arginine (D-AA) and N-nitro-L-arginine methyl ester (L-NAME) were investigated in this study.

***Methods:* Mice (1–3 days old) were randomly assigned to different groups according to the addition of motilin, L-AA, D-AA, and L-NAME. The K^+ current signals were detected by the whole-cell patch-clamp technique.**

***Results:* Compared with the control group, the transient outward voltage-dependent K^+ current was significantly inhibited by motilin added with L-AA. In contrast, the addition of motilin and L-NAME significantly increased the K^+ current, while no significant change was detected by the addition of motilin accompanied with D-AA.**

***Conclusion:* The inhibiting effects of motilin on the voltage-dependent K^+ current in hippocampal neurons indicate that motilin acts as a regulatory factor for the nitric oxide pathway.**

Key words: motilin, hippocampal neurons, whole-cell patch clamp, nitric oxide.

INTRODUCTION

Motilin, a 22-amino-acid peptide (Brown et al., 1971), is known to stimulate gastrointestinal motility (Brown et al., 1973) and mainly involved in the regulation of the migrating motor complex in the fasting state (Smet et al., 2009). Motilin mRNA and motilin receptors have been detected in the gastric body and the dodecadactylon of humans (Miller et al., 2000), as well as in the mammalian central nervous system (CNS), including the cortex, cerebellum, hippocampus, amygdala and hypothalamus (Depoortere et al., 1997a,b; Lange et al., 1986). The highest expression level of motilin receptors is found in the hippocampus (Lin and Dong, 2005). In recent years, motilin has been considered as a new treatment modality (Chapman et al., 2013), and it is reported that the motilin receptor agonist erythromycin can improve gastric emptying half-time in adult cystic fibrosis patients with gastroparesis and induce the intestinal function recovery of the anastomosis for small intestinal atresia (Tonelli et al., 2009; Lu et al., 2010a). However, the mechanism underlying the regulatory effect of motilin on gastrointestinal motility has not been clearly demonstrated so far. Additionally, the stimulatory role of motilin on the gastrointestinal motility in the hippocampus remains to be elucidated.

While previous study proved that L-arginine (L-AA) can increase the excited function of gastric distension neurons in the hippocampus in rats (Lu et al., 2007), nitric oxide synthase (NOS) is newly found in the hippocampus (Lu et al., 2010b), leading to the question whether nitric oxide (NO) is involved in the regulatory effect of motilin on gastrointestinal motility. In order to determine the role of NO in motilin-mediated effects on hippocampal neurons, the interaction between motilin and NO in the hippocampus using the whole-cell patch-clamp was investigated in the present study.

EXPERIMENTAL PROCEDURES

Animals and grouping

Mice of 1–3 days old (12 ± 2.3 g) were provided by Experimental Animal Center of the Heze Medical College. Motilin, L-AA, D-arginine (D-AA), and N-nitro-L-arginine methyl ester (L-NAME) were all purchased from Sigma, USA. Mice were randomized into the eight groups: M (n = 6, treated with 10^{-6} M motilin only); L-AA (n = 6, treated with 10^{-6} M L-AA only), D-AA (n = 6, treated with 10^{-6} M D-AA only), L-NAME (n = 6,

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Abbreviations: ACSF, Artificial Cerebrospinal Fluid; D-AA, D-arginine; EGTA, ethylene glycol tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; L-AA, L-arginine; L-NAME, N-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; SD, standard deviation.

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treated with 10^{-6} M L-NAME only), ML ($n = 6$, treated with 10^{-6} M L-AA then followed by 10^{-6} M motilin), MD ($n = 6$, treated with 10^{-6} M D-AA then followed by 10^{-6} M motilin), MN ($n = 6$, treated with 10^{-6} M L-NAME then followed by 10^{-6} M motilin), and the control ($n = 6$, without drug treatment). All animal care and procedures were approved by the Institutional Animal Care and Use Committee of the Medical College of Qingdao University. And mice were used in accordance with the ethical guidelines of the Medical College of Qingdao University.

Dissociation of hippocampal neurons cells

The mice hippocampus CA1 was dissociated in the Artificial Cerebrospinal Fluid (ACSF) (NaCl 126 mM, KCl 5.0 mM, $CaCl_2$ 2 mM, $MgSO_4$ 2 mM, $NaHCO_3$ 25 mM, NaH_2PO_4 1.5 mM, and Glucose 10 mM, pH 7.4) at 0–4 °C for 1 min and sliced into sections with the thickness of 400–500 μm. Sections were incubated at 32 °C with 95% of O_2 and 5% of CO_2, then digested with 0.05 mg/ml of trypsin (Solarbio, China) for 30 min. Sections were perfused with ACSF for 2–3 times after the digestion, and further digested with 0.05 mg/ml of pronase E (Solarbio, China) for 30 min. After washing with ACSF for three times, slices were transferred to a centrifuge tube containing oxygen-saturated standard extracellular solution, and dispersed by a pipette to prepare a single-cell suspension. The upper layer of the cell suspension was transferred to a culture dish containing oxygen-saturated standard extracellular solution after 5 min, and the cells were attached to the wall after a further 20-min culture and subsequently used for whole-cell patch-clamp recording.

Whole-cell patch-clamp recording

Motilin, L-AA, D-AA, and L-NAME were added directly to the incubation dish before and after the current signals were recorded.

A glass capillary was pulled to form the recording electrode using a PP-830-type microelectrode puller (Narishige, Japan). The resistance at the tip was 2–4 MΩ when filled with the electrode solution (KCl 140 mM, EGTA 10 mM, HEPES 10 mM, $MaCl_2$ 1 mM, and Na_2ATP 4 mM, pH 7.5). The current signals were saved on the computer (Lenovel, China) hard drive via a 2-kHz Digidatal 200B A/D and D/A converter. The sampling frequency was 1 kHz. During the experiment, the setting of the holding and storage was maintained with a HEKA Pulsefit 8.5 (HECK EPC-9, Germany).

Statistical analysis

All data were analyzed using the HEKA Pulsefit 8.5 and Origin 6.0 software, and the results were represented as mean ± standard deviation (SD). The difference caused by the drug administration was analyzed using the analysis of variance (ANOVA) and multiple comparison test. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Effects of arginine and motilin on the peak of outward K^+ currents

In order to understand the effect of motilin and NO precursor L-AA on the transient outward K^+ currents, whole-cell patch clamp was performed. The membrane potential was clamped at −80 mV, and the outward currents were elicited by a step voltage command pulse from −80 mV to +30 mV for 400 ms with step of 10 mV at intervals of 30 s. In group M, motilin at the concentration of 10^{-6} M was added after recording the normal currents of the control group. Compared with the control group, motilin significantly inhibited the currents in group M (Fig. 1A, C). In group L-AA, with the addition of 10^{-6} M L-AA to the incubation dish after recording the normal currents of the control group for 10 min, a significant inhibition was also observed (Fig. 1B, C). With the further addition of 10^{-6} M motilin (group ML), the currents were sharply decreased (Fig. 1B, C). Compared with group M, the inhibition effect of group ML on the K^+ currents was significantly more effective (Fig. 1C and Table 1).

When adding D-AA (isolog of L-AA) at the concentration of 10^{-6} M after washing out the cells (group D-AA), outward K^+ currents did not show any significant change compared to the control group (Fig. 2). In contrast, the further addition of 10^{-6} M motilin (group MD) after 5 min resulted in the significant decrease in outward K^+ currents (Fig. 2), but there was no significant difference between the group M and group MD (Fig. 2 and Table 2). The above results suggest that L-AA enhances the inhibitory effect of motilin on the transient outward K^+ currents, whereas no significant changes are observed by the addition of D-AA.

Effects of arginine and motilin on the current–voltage (*I–V*) curve of outward K^+ currents

To further determine the interaction between motilin and L-AA, the effects of arginine and motilin on the *I–V* curve of outward potassium currents were observed. With the holding potential set at −80 mV, after a series of outward currents were elicited by stimulating the hippocampal neurons with depolarizing pulses from −80 mV to +30 mV in 10 mV steps, the *I–V* curves were generated at every level of membrane potential by plotting the peak values of the K^+ currents. The outward K^+ currents were gradually increased with the rise of voltage (Fig. 3A), and evident changes in the *I–V* curve were observed after the addition of motilin at the concentration of 10^{-6} M (Fig. 3E). Notably, although the overall shape of the *I–V* curves was not changed, the motilin shifted the *I–V* curve downward, suggesting a decrease in the outward K^+ currents and an increase in the voltage as compared to the control group (Fig. 3B). When 10^{-6} M L-AA was added after 5 min, the currents were decreased significantly (Fig. 3C), and the *I–V* curve was also shifted downward (Fig. 3E). After motilin was added (group ML), the currents showed an obvious decrease compared with the control group and group M