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Acute Ethanol Reduces Calcium Signaling Elicited by K^+ Depolarization in Cultured Cerebellar Granule Neurons

Jong-Nam Kim*

Center for Neurodynamics. Korea University, Seoul 136-701, Korea
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ABSTRACTS *: The effects of acute ethanol on the high K^+ induced Ca^{2+} signals were examined from primary cultures of cerebellar granule neurons. Ca^{2+} signals were measured with Calcium Green-1 based microscopic video imaging. Because Ca^{2+} signal was low in most of granule neurons without stimuli, high KCl was used for depolarization. In most case, acute exposure to ethanol reduced the peak amplitude of the Ca^{2+} signals, induced by high K^+, even though low concentration of ethanol (2~10 mM) was used and the effects lasted more than 30 min. It was also possible to see differences of ethanol inhibition, i.e. the temporal pattern of Ca^{2+} signal reductions and the strength of inhibition of Ca^{2+} signals among neurons. These results indicate that low concentration of ethanol has diverse actions on the Ca^{2+} signals in cerebellar granule neurons.*

Key Words *: Alcohol, Cerebellar Granule neurons, Intracellular Ca^{2+}, Calcium Green-1*

I. INTRODUCTION

Consumption of ethanol during pregnancy at levels considered to be in the moderate range (less than 20 mM) can generate fetal alcohol effects (behavioral, cognitive anomalies) in the offspring (Eckardt *et al.*, 1998). The cerebellar and hippocampal regions of the CNS might be sensitive ones to acute alcohol exposure as well as chronic exposure. In these CNS regions, alterations in neuronal firing properties, the response to synaptic input and the sensitivity to transmitter chemicals have been described following alcohol application, i.e. acute administration of ethanol partially inhibits various aspects of glutamate receptor activity, in particular NMDA receptor-mediated functional responses such as Ca^{2+} influx, transmitter release and neurotoxicity (Bloom and Siggins, 1987; Franklin and Gruol, 1987; Dildy and Leslie, 1989; Hoffman *et al.*, 1989; Urrutia and Gruol, 1992; Randall *et al.*, 1995; Gruol and Curry, 1995; Hou *et al.*, 1996; Gruol *et al.*, 1997). However, the concentrations of ethanol used during above studies were higher than 20 mM or chronic ethanol applications were adopted. In the present experiment, the effects of low dose (2~10 mM) of acutely applied ethanol were tested in the cultured cerebellar granule neurons. Ca^{2+} signals were used as indices of ethanol effects.

*To whom correspondence should be addressed
ABBREVIATIONS
ACSF, artificial cerebrospinal fluid, NMDA receptor, N-methyl-D-aspartate receptor

II. MATERALS AND METHODS

1. Cell culture

Primary neuronal cultures were prepared using cerebellar tissue from 6- to 8-day-old Sprague-Dawley rats. After removal of the pial membrane, the tissue was cut into pieces with scalpels and treated with 0.25% trypsin solution (Sigma, USA) at 37°C for 10 min. The action of trypsin was halted in a HBSS solution containing 0.01% soybean trypsin inhibitor (Sigma, USA) and 0.05% DNase (Sigma, USA). The cells were then mechanically dissociated by gentle trituration with a fire-polished, siliconized Pasteur pipette. The suspension was centrifuged (5 min, 1000 rpm) and the pellets were resuspended in tissue culture medium consisting of Dulbecco's modified Eagle's medium (DMEM, GIBCO), 10% fetal bovine serum (GIBCO), 25 mM KCl, and 1% penicillin-streptomycin (GIBCO). Aggregates were removed by filtration through

a 80-μm stainless mesh. Tissue culture dishes (Falcon, Beckton Dickinson) were coated with 100 ug/ml of poly-L-lysine for 2 h, empted, and air dried. Dissociated cells were plated (at a density of 10^5 cells/2 ml/dish) onto the tissue culture dishes, and maintained in culture at 37°C in an atmosphere of 5% CO_2/95% and used at on days 4 or 5 *in vitro*. The culture medium was changed every 3-4 days.

2. Dye loading and image collection

The culture medium was replaced with artificial cerebrospinal fluid (ACSF) containing 1~2 μM Calcium Green-1/AM (Molecular Probes, USA) in a CO_6 incubator (Vision Scientific Co., Korea). The ACSF consisted of 124 mM NaCl, 3 mM KCl, 26 mM NH_2CO_4, 1.25 mM Na_2HPO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 1.3 mM glucose. After 2~6 h of the loading procedure, the solution was replaced with a fresh ACSF for an additional 1 h to allow for cleavage of the acetoxymethyl (AM) ester. The culture dish was mounted on the stage of an inverted phase-contrast microscope (Olympus IX70, Japan) and perfused constantly with oxygenated ACSF by a peristaltic pump (ISM1-B, Cole Parmer Instrument Company, Switzerland) at a flow rate of 4 ml/min. A heating unit was attached to the infusion tube to maintain the recording medium at the desired temperature (35~37°C). Fields of neurons selected for study under phase contrast or bright field optics and photographed with a cooled CCD camera (MicroMax. Princeton Inst. Inc., USA) attached to the microscope and granules cells were identified by morphological criteria (Gruol and Crimi, 1988). Calcium Green-1 was excited at 488 nm and its fluorescence emission collected at 530 nm. Live video images of selected microscopic fields were recorded with a cooled CCD and digitized by computer. Real time digitized display, image acquisition, and Ca^{2+} measurements were maid with WinView imaging program (Princeton Inst.. Inc., USA). For each microscopic field, data were collected at 1.5 sec interval during recording.

3. Drug application

30 mM KCl was dissolved in oxygenated ACSF (KCl+ACSF) and ethanol (2 or 10 mM) was dissolved in KCl+ACSF (Alcohol+KCl+ACSF). The normal ACSF perfusion was switched to KCl+ACSF or Alcohol+KCl+ACSF not more than 2 min (usually 90 sec) and induced changes of Ca^{2+} signals were recorded more than 3 min. The bath was exchanged after each drug application. All three solutions were bubbled constantly with 95% O_2 and 5% CO_2.

4. Data analysis

From the saved images on the hard disk drive, Ca^{2+} signals were quantified by measurement of the fluorescence intensities in the somatic region. Data from neurons, examined in the same experiment, were pooled together but error bars were not shown for figure clarity. In general, error bar is bigger when the fluorescence intensity was higher.

III. RESULTS AND DISCUSSION

1. Cell identification

The granule neurons were distinguished from other cells by morphological features (round cell bodies, less than 10 μm in diameter). In a field of the 40× objective, several granule neurons were recorded simultaneously (Fig. 1A). Granule neurons, in the same filed of Fig. 1A, were preferentially loaded with the calcium-sensitive dye, Calcium Green-1. Fluorescence intensities were increased when the granule neurons were stimulated with KCl (Fig. 1B). Some cells did not show any fluorescence but the reason for preferential loading of granule neurons is not known. The cerebellar cultures prepared from the cortical region of fetal rat cerebellum contained all the neuronal types located in the cortical region of the cerebellum in vivo (Groul and Crimi, 1988).

2. Effects of acute ethanol on the Ca^{2+} signals induced by high K^+

Even thought high doses (higher than 10 mM) of ethanol produce inhibition of Ca^{2+} flux via NMDA receptor in the brain (Barrios and Liljequist, 1997), it is necessary to find the effect of low dose of alcohol. In the beginning of experiments. 10 mM ethanol was used but the reduction of Ca^{2+} signals was not recov-

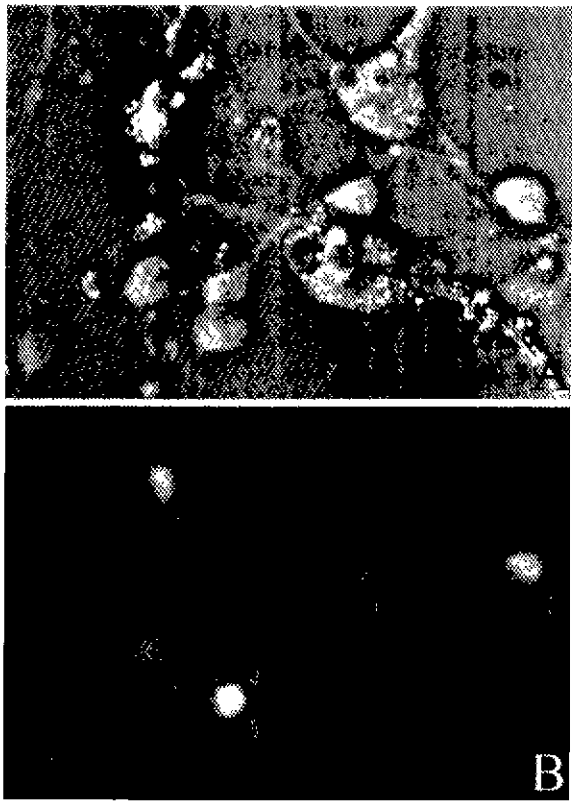

Fig. 1. The light field (A) and the corresponding fluorescent image (B) of cerebellar granule neurons. The fluorescence image (B) of the same field as in A was obtained by loading with calcium Green-1. The culture dish was incubated in ACSF containing 1.5 μM Calcium Green for 2 h (see Material and Methods). Bright spots show the stimulated granule cells by KCl.

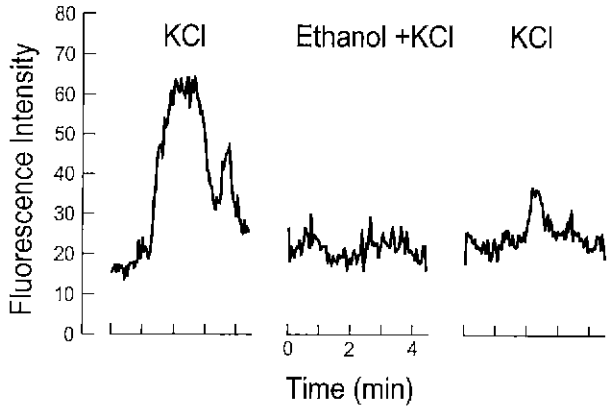

Fig. 2. Effect of ethanol exposure on KCl induced increase of Ca^{2+} in the primary cultures of cerebellar granule neurons. (responses of cerebellar granule cell to KCl (left). Ethanol+KCl (middle) and KCl (right)). Typical Ca^{2+} increase in 7 granule neurons (shown on Fig. 1) in responses to 30 mM KCl with or without 2 mM Ethanol. Measurements were made in the somatic region of the neuron. The y axis represents the relative fluorescence intensity of Calcium Green. Note that Ca^{2+} increase induced by KCl (left) was abolished by ethanol (middle) and recovered partially after 30 min (right). The main effect of ethanol was to reduce the peak amplitude of the Ca^{2+} signal.

ered well within 60 min. Therefore, for later experiments, 2 mM ethanol was used instead. Examples of granule neurons (cell number=7) responding to KCl with a rise in Ca^{2+} are shown in Fig. 2 (left). Ethanol reduced the increase induced by KCl (middle) and recovered partially after 30 min.

Higher doses of ethanol were not tested here. However, several other studies have shown that high doses of ethanol depress granule neuron's activity *in vivo* and *in vitro* (Sorenson *et al.*, 1981). On the other hand, Seil *et al.* (1977) reported that low doses of acute ethanol increased the firing rate and the regularity of cerebellar neurons, whereas high doses depressed activity. From the current results, the degree of reduction was variable but no excitation was found. The granules cells provide excitatory synaptic input to the putkinje neurons and other cerebellar interneurons. These reductions shown on granule neurons by ethanol might affect other cells as well. The underestimation of ethanol volatility is a potential source of experimental error especially in studies of cumulative of chronic effects of ethanol exposure. Indeed, when the cells are exposed to ethanol in an open system at 37°C, more than 70% of the ethanol is lost within 72 h (Borgs *et al.*, 1993). But, acute ethanol application does not show such problem.

The most interesting observations in the present study are, first, that the enhancement of Ca^{2+} by high K^+ is inhibited by very low dose ethanol (2 mM), and second, that each neurons show differing patterns of Ca^{2-} to ethanol at specific time points after acute exposure which indicates different mechanisms are exist.

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