Modification of Nifedipine Inhibitory Effect on Calcium Spike and L-Type Calcium Current by Ethanol in F1 Neuron of *Helix aspersa*

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ABSTRACT

There is strong evidence demonstrating that nifedipine dissolved in ethanol selectively inhibits only L-type Ca^{2+} current. In addition, acute ethanol exposure reduces voltage-dependent calcium currents. In the present study, we investigated the antagonistic effect of fixed concentration of nifedipine dissolved in different concentration of ethanol on L-type Ca^{2+} current. In a Na^{+}-K^{+} free solution, nifedipine dissolved in 60 and 120 mM ethanol decreased resting membrane potential of Ca^{2+} spikes and caused a significant reduction in amplitude, duration and an increase in threshold of Ca^{2+} spikes. Furthermore, Ca^{2+} current was inhibited by ethanol in a concentration-dependent manner, so that the reduction of L-type Ca^{2+} current by nifedipine/60 and 120 mM ethanol was statistically significant. Meanwhile, ethanol concentration-dependent response of Ca^{2+} currents was observed at its late component in more positive potentials. These results may be consistent with ethanol-dependent inhibition of L-type Ca^{2+} currents and ethanol-dependent enhancment of a Ca^{2+}-activated potassium current. *Iran. Biomed. J. 7 (3): 99-105, 2003*

Keywords: Ethanol, Nifedipine, Ca^{2+} spikes, L-type Ca^{2+} current, F1 neuron

INTRODUCTION

Nifedipine, a classical dihydropyridine calcium antagonist has been used to purify and characterize selectively the long-lasting (L-type) calcium channels [1, 2]. As a poorly water- soluble compound, nifedipine is usually dissolved in organic solvents such as acetone [3], dimethylsulfoxide (DMSO) and ethanol [4]. The inhibitory effect of nifedipine is mainly depends on its solvent. In neuroblastoma cells, nifedipine dissolved in ethanol blocked L-type calcium channels but had no effect on T channels [5]. On the other hand, there are many studies demonstrating that several ionic channels are sensitive to pharmacological concentrations of ethanol. The acute ethanol exposure reduces voltage-dependent calcium currents in a variety of systems including dorsal root ganglion cells in rat [6], nerve cells of aplysia [7], rat neurohypophysial terminals [8], PC12 cells [9] and rat pinealocytes [10]. It also inhibits calcium uptake into mouse synaptosomes [11]. The expression of calcium channels in hepatic stellate cells of rat is upregulated by chronic treatment of ethanol [12] and ethanol reduces the duration of single evoked spikes through specific inhibition of voltage-activated calcium currents in acutely dissociated supraoptic neurons in rat [13]. Furthermore, ethanol enhances a calcium-activated potassium current in F1 neuron of *Helix aspersa* [14], isolated neurohypophysial terminals [15], planar lipid bilayer [16] and clonal pituitary (GH3) cells [17]. Since

both nifedipine and ethanol individually block calcium currents, it is possible that nifedipine dissolved in ethanol exerts an additive inhibitory effect on L-type calcium channels. The present study is designed to examine the effect of different concentrations of ethanol on antagonistic effect of nifedipine on L-type calcium channels in F1 neuron of *Helix aspersa* using voltage- and current-clamp techniques.

MATERIALS AND METHODS

Animals and dissection. Experiments were performed on the somata of isolated F1 neurons of the sub-oesophageal ganglia of *Helix aspersa* (Iranian garden snail). Specimens were collected locally with average in weight of 4 to 7 g. For dissection, the animal was pinned onto a corkboard in an extended position. The ganglionic mass with its main peripheral nerves and aorta was dissected out and then pinned by the nerves and the edges of the connective tissue into a sylgard-grounded recording chamber (Dowcorning, Midland, MI). The overlying layers of connective tissue covering the ganglia were gently torn using two pairs of fine forceps without any pretreatment with proteolytic enzymes. F1 neurons were visually identified by their size and colour within the right parietal ganglion [18]. F1 neurons were equilibrated for two hours in normal snail Ringer at room temperature (20-23°C) before recording.

Solutions and drugs. The normal snail Ringer contained (in mM): NaCl, 80; $CaCl_2$, 10; KCl, 4; $MgCl_2$, 5; glucose, 10; 4-(2-hydroxyethyl)-1- piperazine-N-ethanesulfonic acid (HEPES), 5; as described by Taylor [19]. The solution used for detection of calcium current contained (in mM): TEA-Cl, 84; $CaCl_2$, 10; KCl, 4; $MgCl_2$, 5; glucose, 10; HEPES, 5.

During recording of calcium currents, 4-aminopyridine (5 mM) and nifedipine were dissolved in different concentrations of absolute ethanol (10, 30, 60 and 120 mM) and were applied to the isolated F1 neurons by bath superfusion. The final concentration of nifedipine in the bath was 1µM in different concentrations of ethanol. The potential-recording and current-passing electrodes were filled with 3 M KCl (pH 7.4). The pH of the solutions was adjusted to 7.8 with either Trizma hydrochloride or Trizma base. All drugs were purchased from Sigma (Sigma, St. Louis, MO, USA). Osmolarity of the solutions was from 212 to 216 mOsm/L of H_2O and it was routinely checked using a Gonotec Osmometer (Osmo Mat 030, UK).

Recording techniques and equipments. A conventional two-microelectrode voltage and current clamp method was applied using Axoclamp-2B amplifier (Axon Instruments, CA, USA). The reference electrode in all experiments was a silver-silver chloride wire within an agar bridge (4% agar in snail Ringer). Microelectrodes were freshly pulled from borosilicate glass capillaries with external diameter of 1 mm and internal diameter of 0.58 mm (Clark Electromedical Instruments, Pangbourne, UK) using a horizontal puller (Stoelting, USA) and then were coated with parafilm. Voltage steps were elicited for 390 ms from holding potentials of -90mV and -40mV to various potentials (-90to +90or -40to +90mV) in 5 mV increments. Command potentials were generated using IBM-compatible computer with Matlab software program. Signals were digitized on-line and stored on the computer for offline analysis. All current traces obtained from voltage clamp experiments were corrected for linear leak and capacity currents. Spontaneous action potential of F1 neuron was recorded in standard Ringer and Na^+ and K^+ free solution before and after adding nifedipine dissolved in differerent concentrations of ethanol. The action potential duration was measured at half peak amplitude.

Data analysis. Data were filtered at 30 KHz. Current and voltage records were sampled at 20 KHz and were digitized online using a 16-bit A/D converter and stored for further analysis. At the end of each experiment, the tip potential was measured and if it was greater than ±5 mV, the related data were discarded. Furthermore, to minimize the errors due to tip potentials, microelectrodes with a low resistance of 6 ± 0.63 MΩ were used. Leak currents were obtained by blocking all known ionic currents with this assumption that the remaining current is leak current. Leak currents were digitally subtracted from the presented data. All values were given as means ± S.E.M. Statistical significance was indicated by $P<0.05$ which was obtained from Student's paired *t*-test and one-way analysis of variance (ANOVA).

RESULTS

The presented data were collected from 42 F 1 neurons of parietal ganglion of *Helix aspersa*.

Calcium spikes of F1 neurons. In standard Ringer, F1 neuron exhibited spontaneous firing activity with a

direct repolarizing phase of action potential (Fig. 1A). To characterize calcium spikes and their underlying calcium inward currents in somata of F1 neuron, recording was performed through blocking voltage-activated K^+ outward currents (Tetraethyammonium, TEA and 4-Aminopyridine, 4-AP added and/or K^+ omitted from bath) as well as blocking voltage- activated Na^+ inward currents (Na^+ omitted from bath). Under these conditions, a type of calcium spike with plateau was spontaneously elicited from a holding potential of -35.3 ± 1.40 mV. These spikes showed large amplitude with a plateau phase compared to Na^+ action potential in standard Ringer (85.5 ±3.6 versus 61.53 ± 2.3 mV; Fig. 1B), but like fast action potential were evoked when threshold was exceeded. Since their threshold was near -32.14 ± 1.28 mV, they could be referred to as high-threshold calcium spike (HTS) [20]. Duration of calcium spikes at half spike amplitude was longer than that of Na^+ spikes (229.66 ± 13.33 vs. 6.07 ± 0.89 ms).

The effect of different concentrations of ethanol on antagonistic action of nifedipine on calcium spi hyperpolarization. The slow calcium spikes were not observed unless Na^+ and K^+ currents were blocked. Ni organic blocker of L-type calcium channels dissolved in30 60and 120 mM ethanol caused a significant rec (35-40%), duration (17-21%) and an increase in threshold (29-41%) and threshold latency (more than 100% and eliminated plateau potential, but did not fully abolish the HTS (Figs. 1C, D and E; Table 1). In standard action potentials of F1 neuron were followed by a shallow after spike hyperpolarization that its amplitude and ± 1.30 mV and 3.78 ± 0.50 ms, respectively (Fig. 1A). In a Na^+-K^+ free solution, after spike hyperpolarizati but after application of nifedipine dissolved in 60 and, in particular 120 mM ethanol, a prominent after spik was appeared following calcium spike (Figs. 1D and E). Amplitude and duration of this after spike hyperpol 2.09 mV and 54.12 ± 1.07 ms, respectively.

The effect of different concentrations of ethanol on antagonistic action of nifedipine on calcium currents. We have focused on L-type calcium channels in F1 neuron because of its sensitivity to ethanol. In addition, this channel can be easily isolated from other calcium channels using depolarized holding potential of -40 mV.

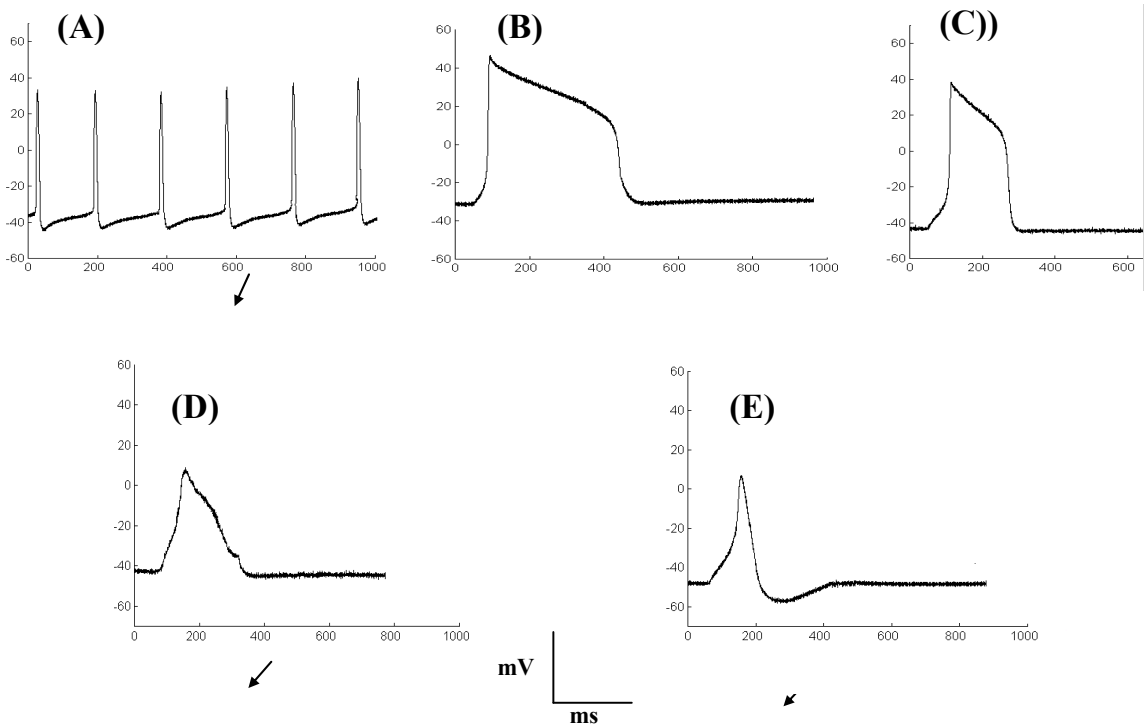

Fig. 1. Intracellular records showing typical spontaneous firing activity in F1 neuron of *Helix aspersa* in standard Ringer **(A)**. Ir spontaneous high-threshold Ca^{2+} spikes were recorded with reduced K^+ currents and blocked Na^+ currents before **(B)** and after adding

30 **(C)**, 60 **(D)** and 120 **(E)** mM ethanol. The arrows represent after spike hyperpolarization.

Table 1. The effect of different concentrations of ethanol on Ca^{2+} spike characteristics in F1 neuron. The final concentration of nifedipine in the bath was fixed at 1 μM for each concentration of ethanol.

Membrane electrical properties	Standard Ringer (n = 20)	Na^+-K^+ free Solution (pre- exposure) (n = 14)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (10 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (30 mM) (n = 11)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (60 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (120 mM) (n = 10)
Resting membrane potential (mV)	-37.10 ± 1.1	-31.2 ± 1.60	-36.3 ± 2.30	-42.70 ± 3.70	-42.1 ± 1.1	-47.7 ± 4.90
Action potential amplitude (mV)	61.50 ± 2.3	85.5 ± 3.60	70.3 ± 5.30	67.10 ± 1.10	56.1 ± 2.2*	51.3 ± 2.04*
Action potential duration (ms)	6.07 ± 0.8	229.6 ± 13.30	201.3 ± 10.50	199.01 ± 12.10	191.5 ± 5.4	183.8 ± 4.40*
Action potential threshold (mV)	-33.80 ± 2.1	-32.1 ± 1.28	-30.1 ± 2.18	-29.20 ± 1.29	-22.9 ± 2.3	-19.0 ± 1.60
Threshold latency (ms)	22.70 ± 5.4	53.2 ± 1.20	61.3 ± 2.03	90.50 ± 1.20	92.3 ± 5.2*	107.5 ± 5.89*

*P<0.05 [compared with characteristics of Ca^{2+} spike in Na^+-K^+ free solution before exposing to nifedipine/ethanol]

Different concentrations of ethanol influences the antagonstic action of nifedipine on L-type calci

elimination of K^+ and Na^+ currents, we determined the sensitivity of calcium current carried through L-ty concentration of nifedipine (1 μM) dissolved in
10-120 mM ethanol using two-electrode voltage clamp. The membrane potential was stepped to +90 mV for potentials of -90 and
-40 mV in sequential 5 mV increments. The ethanol concentration-dependent response of calcium current is 2A shows maximum calcium current elicited from a holding potential of
-40 mV to +90 mV before and after exposing individual neurons to nifidepine dissolved in different concentra min and Figures 2B and 2C plot their peak current as a function of voltage (I-V) at holding potentials respectively. According to I-V relations, in a Na^+-K^+ free solution, peak calcium current amplitude evoked f was -5.2 ± 0.18 and -4.6 ± 0.33 nA, respectively (Table 2). The I-V relations also showed a characteristic sha current components at a holding potential of -90in contrast to -40mV . The peak current evoked from a hol mV increased gradually with increaseing potential up to about -55 mV, when a plateau was reached. Above again increased with increasing potential to reach a maximum at steps up to near -5 mV (Fig. 2B). The I-V rela evoked from a holding potential of -40mV showed a single peak near 10 mV (Fig. 2C). Calcium currents reversible manner by nifedipine dissolved in ethanol (Figs.
2A and B), but this inhibition was not to the same extent in all concentrations of ethanol. Meanwhile, as relations, the inhibitory effect of nifedipine dissolved in ethanol has exerted on late component of calcium cu holding potential of -90mV and single peak evoked from a holding potential of -40 mV in more positive pot C). Peak calcium currents evoked from both holding potentials in neurons exposed to nifedipine/60 mM ethanc mM ethanol were inhibited 60% and 85% respectively (Fig. 2 and Table 2). In all conditions, calcium currents inward to outward below about +30 mV, but the reversal potentials shifted to more negative potentials in an e dependent manner.

After application of nifedipine/10 mM ethanol, at both holding potentials, the peak current potential shift potentials, so that peak current potential evoked from -90mV before and after application of nifedipine /10 m 0 and -10 mV and from -40mV was 15 and

Table 2. The effect of different concentrations of ethanol on peak Ca^{2+} currents at different holding potentials. The concentration of nifedipine in the bath was fixed at 1μM for each concentration of ethanol.

Ca^{2+} current properties	Na^+-K^+ free Solution (pre-exposure) (n = 14)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (10 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (30mM) (n = 11)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (60 mM) (n = 12)	Na^+-K^+ free Solution + Nifedipine/ Ethanol (120 mM) (n = 10)
Peak Ca^{2+}currents amplitude (nA) -40 mV	-4.6 ± 0.33	-3.7 ± 0.23	-2.9 ± 0.15	-1.9 ± 0.5*	-0.7 ± 0.34*
	-5.2 ± 0.18	-3.7 ± 0.23	-2.9 ± 0.15	-2.1 ± 0.2*	-0.98 ± 0.44*

Peak Ca^{2+}currents amplitude (nA) - 90 mV

*P<0.05 [compared with peak Ca^{2+} currents in Na^{+}-K^{+} free solution before exposing to nifedipine/ethanol]

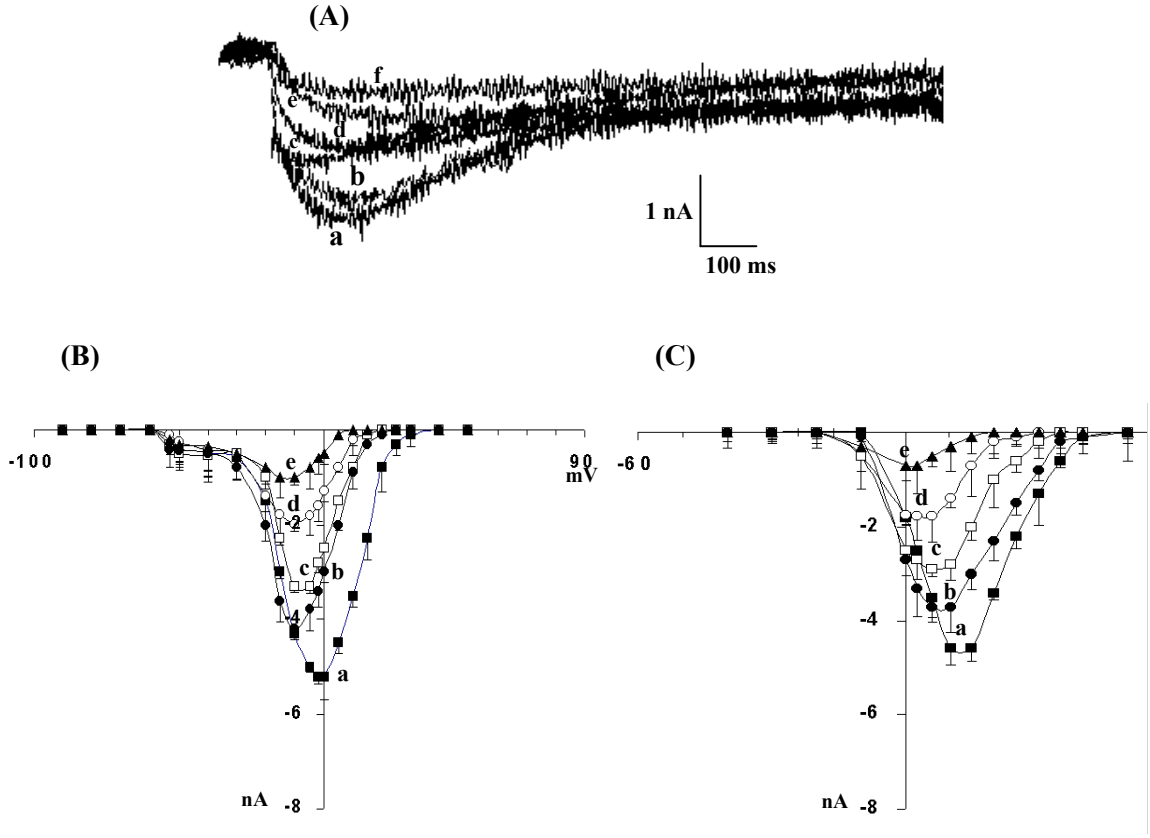

Fig. 2. Ca^{2+} currents in a Na^{+}-K^{+} free solution. **(A)**, Peak current responses in 390 ms voltage steps from a holding potential of -40 **f)** and after (wash; **b**) a 5-min. exposure to 1 µM nifedipine dissolved in 10 **(c)**, 30 **(d)**, 60 **(e)** and 120 **(f)** mM ethanol. **(B)**, I-V rela evoked from a holding potential of -90 mV before **(a)** and after **(b-e)** a 5-min. exposure to 1 µM nifedipine dissolved in 10 **(b)**, 30 **(c** ethanol. **(C)**, I-V relation for the peak currents evoked from a holding potential of -40mV before **(a)** and after **(b-e)** a 5-min. expo dissolved in 10 **(b)**, 30 **(c)**, 60 **(d)** and 120 **(e)** mM ethanol.

5 mV respectively, but there was no considerable change in peak current voltages with increasing of ethanol co

DISCUSSION

The present study has emphasized on changes in activity of neuronal L-type calcium channels that could concentration of nifedipine dissolved in different concentrations of ethanol (10-120 mM). In normal Ringer are characterized by spontaneous action potentials associated with short spike duration and after spike hyperpo K^{+} free solution, F1 neurons display high-threshold calcium spikes that are not detected unless Na^{+}- K^{+} curr neurons have at least two types of calcium currents: nifedipine-sensitive and nifedipine-insensitive calcium c dependency of the recorded membrane calcium currents indicated two kinds of currents: low-threshold cu activated near -55mV and high -threshold current that is mainly activated near -35mV . That these two curr through different Ca^{2+} channels is supported by the difference in voltage dependency of activation as well as d to nifedipine.

As mentioned previously, nifedipine is a relatively selective blocker of L-type calcium channel but what is u the inhibitory effect of nifedipine is dependent on concentrations of ethanol. The finding of the present stu increasing concentration of nifedipine dissolved in fixed concentration of ethanol had no effect on low-thresh [21], acute exposure of F1 neuron to fixed concentration of nifedipine dissolved in different concentration mM) has also no effect on low-threshold calcium channels and specifically blocks only L-type calcium cha

same extent, so that in the presence of ethanol concentrations of 60-120 mM, nifedipine inhibited L-ty significantly.

Several possible processes could explain ethanol concentration-dependent effect on L-type calcium cha concentration-dependent inhibitory effect could be due to its direct effect on L-type calcium channels t effective number of channels or affecting channels gating properties, as many previous studies have also der ethanol exposure induce reduction of L-type calcium current [9]. On the other hand, the result of previous stuc neuron have revealed that in normal physiological solution, ethanol (5-50mM) hyperpolarized resting mer depressed both spontaneous action potential duration and its firing rate by enhancement of the repo hyperpolarization phases respectively [14].

These observations indicate that the major effect of ethanol in concentrations less than 100 mM on activity mediated by potassium currents. Similar findings have been reported elsewhere in tissue slice preparations neurons it was concluded that 5-20mM ethanol increased a calcium - activated current [22]. It has been sug potassium conductance may be increased by all central nervous system depressant drugs [23], and in particu calcium may be involved in this enhancement [24]. Therefore, it is possible that in addition to direct effect calcium channels, there is an ethanol concentration-dependent enhancing effect on a calcium-activated pot explanation is strengthened by the fact that after exposure of F1 neuron to nifedipine dissolved in a high conce prominent after spike hyperpolarization was appeared and resting membrane potential decreased. Be pharmacodynamic changes in neuronal ionic channel activity observed when ethanol is chosen as nifedipine important application of appropriate concentration of ethanol for an optimal antagonistic effect of nifedipin channels. Further studies are warranted to investigate the possible mechanisms that are involved in modifying nifedipine by its solvent, ethanol.

In conclusion, nifedipine is used extensively in the treatment of cardiovascular and neurological disorders been usually dissolved in organic solvents including ethanol. Since ethanol has also direct inhibitory and p membrane ionic currents, for optimal efficacy of nifedipine on L-type calcium channels, it is important concentration of ethanol is used as nifedipine solvent. The present data suggest that increasing of ethanol con the properties of high-threshold calcium spikes and consequently the neuronal excitability through an et dependent inhibitory effect on L-type calcium channels and/or ethanol concentration-dependent potentiatin potassium current.

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