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Full-length article

Action of aluminum on high voltage-dependent calcium current and its modulation by ginkgolide B

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Key words

hippocampus; aluminum; voltage-dependent calcium channels; ginkgolide B; patch-clamp technique

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Abstract

Aim: To investigate the effect of aluminum (Al) on high voltage-dependent calcium current (I_{HVA}) and its modulation by ginkgolide B (Gin B). **Methods:** The whole-cell, patch-clamp technique was used to record I_{HVA} from acutely isolated hippocampal CA1 pyramydal neurons in rats. **Results:** Al 0.1 mmol/L (low concentration) reduced I_{HVA}; Al 0.75 and 1.0 mmol/L (high concentrations) increased I_{HVA}, and Al decreased and increased I_{HVA} at intermediate concentrations of 0.25 and 0.5 mmol/L. The increase of I_{HVA} by Al 1.0 mmol/L was enhanced by the adenylyl cyclase (AC) agonist forskolin and was partly abolished by the cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) antagonist H-89, whereas the decrease observed with Al 0.1 mmol/L was neither reversed by forskolin nor affected by H-89. Gin B had no effect on I_{HVA} in normal neurons, but canceled the increase in I_{HVA} by 1.0 mmol/L Al. **Conclusion:** The results indicate that the mechanism of Al affecting I_{HVA} differs at different concentrations, and this may be attributed to its complex actions. Gin B could prevent neurons from injury by inhibiting calcium influx.

Introduction

The accumulation of aluminum (Al) within the body can result in many mental diseases. For example, Al is concentrated in the neurofibrillary tangles and senile plaques of patients with Alzheimer disease (AD)[1]. Al can be attributed to several neurological disorders, such as dialysis syndrome and Guamanian amylotrophic lateral sclerosis-Parkinson's dementia[2]. A number of studies have implicated that Al has no effect on long-term potentiation (LTP)[3]. However, more and more studies have shown that Al can impair LTP[4] and evoke potential in the hippocampus[5]. Studies have also shown that Al affects amino acid neurotransmitters in the hippocampus and enhances glutamate-mediated excitotoxicity, which may be one of the causes of its toxicity[6]. Reports about its mechanism involving ion channels are few and controversial[7–9].

Extracts from leaves of *Ginkgo biloba* (*EG*b) and one of its constituents ginkgolide B have been demonstrated to protect cardiomyocytes and cultured neurons from the injury induced by hypoxia, ischemia, and the neurotoxicity induced by Aβ[10–12]. However, it is not known whether the mechanism of this protection of neurons involves ion channels, such as voltage-dependent calcium channels (VDCC).

The present study investigated the actions of Al on I_{HVA} and its modulation by Gin B to examine the neurotoxic mechanisms of Al and the neuroprotective mechanisms of Gin B.

Materials and methods

Reagents Pronase E, forskolin, TEA-Cl, H-89, and HEPES were purchased from Sigma Chemical Company (St Louis, MO, USA). H-89 was dissolved in pipette solution and stored at -20°C. After the whole-cell configuration was constructed, H-89 was dialyzed into the cell through the pipette. Ginkgolide B (BN52021, purity 98.2%) was from the Wuhan Institute of Botany, Chinese Academy of Sciences (Wuhan, China). $AlCl_3$ was from Jinghua Chemical Company (Beijing, China). The remaining chemicals, unless otherwise

stated, were all purchased from the Shanghai Chemical Reagent Plant (Shanghai, China).

Cell isolation Animals were provided by the experimental animal center of Tongji Medical College (Grade II, Certificate No 19-050). Hippocampal CA1 neurons were acutely isolated by enzymatic digestion and mechanical dispersion from 7 to 10-d-old Wistar rats as described in a previous study[13], with a few modifications. After the animals were killed, the hippocampi were removed and coronary slices were cut at a thickness of approximately 500 μm in ice-cold oxygenated incubation solution within 30 s. The slices were incubated in an external solution saturated with pure O_2 at 32 °C for 1 h, treated with Pronase E 6.0–7.0 kU/L for 25 min in the oxygenated external solution at 32 °C. After digestion the slices were washed six times with external solution and incubated in the same solution saturated with pure O_2 at room temperature. CA1 regions were dissected out and transferred into centrifuge tubes. Hippocampal neurons were dispersed by gentle pipetting using fine glass tubes. After 5 min, the cell suspension was transferred into the recording chamber with a glass coverslip filled with external solution. The cells were left for approximately 30 min before beginning the experiments.

Electrophysiology The cells were placed in a recording chamber mounted on the stage of an inverted microscope (Carl Zeiss, Germany) and superfused with extra cellular solution at room temperature (21–22 °C). Extracellular solution for recording I_{HVA} was composed of (mmol/L): NaCl 150, KCl 5, $MgCl_2$ 1.1, $CaCl_2$ 2.5, HEPES 10, glucose 10, TTX 0.001, and the pH was adjusted to 7.4 with NaOH. Extracellular application of drugs was carried out by perfusing cells with extracellular solution containing the drugs.

Whole-cell patch experiments were carried out using an Axopatch 200A amplifier (Axon Instruments, Foster City, CA, USA) driven by ISO2 software (MFK, Frankfurt, Germany). In the voltage-clamp experiments, the cells were stepped from -80 mV (50 ms) to -40 mV (200 ms), and then depolarized to 0 mV (200 ms) after briefly hyperpolarizing the membrane potential for 10 ms to -45 mV. The I_{HVA} was activated by the second depolarization. The protocol was applied every 5 s. For analysis of the current-voltage (*I–V*) relationship, voltage steps (200 ms) were used to depolarize from -40 mV to +40 mV in 10 mV increments. Glass pipettes were used with a resistance of about 3–5 MΩ when filled with a pipette solution composed of (mmol/L): CsCl 140, $MgCl_2$ 2, Mg-ATP 4, TEA-Cl 2, HEPES 10, egtazic acid 11, and the pH was adjusted to 7.2 with CsOH.

Data were acquired at a sampling rate of 10 kHz, filtered at 2 kHz, stored on hard disk and analyzed off-line using the ISO2 analysis software package (MFK, Frankfurt, Germany).

Data analysis The amplitude of I_{HVA} was calculated as the difference between the instantaneous current at the beginning of the experiment and the maximum activating current. Currents were normalized to membrane capacitance to calculate current densities ($pA \cdot pF^{-1}$). Cell membrane capacitance (Cm) was determined online using the ISO2 software program. The activation rate constant and inactivation rate constant were obtained using the ISO2 analysis software. Graphical and statistical data analyses were carried out using Sigmaplot 2001 (SPSS, Chicago, IL, USA) and Origin 6.0 (Microcal Software, Inc, Northampton, MA01060, USA). Data were presented as mean±SEM where appropriate. Statistical analysis were carried out using Student's paired and unpaired *t*-tests and values of $P<0.05$ were considered statistically significant.

Results

Action of Al on I_{HVA} Bath application of $AlCl_3$ 0.01 mmol/L had no effect on I_{HVA}. The current densities before and after $AlCl_3$ application were 18.5±2.4 $pA \cdot pF^{-1}$ and 18.5±2.2 $pA \cdot pF^{-1}$, respectively (n=11, $P>0.05$) (Figure 1A).

Bath application of $AlCl_3$ 0.1 mmol/L caused a reduction in I_{HVA} from 17.7±1.6 $pA \cdot pF^{-1}$ to 12.7±1.4 $pA \cdot pF^{-1}$ (n=27, $P<0.01$), that is, a reduction of 30.5%± 4.1%. The reduction of I_{HVA} by $AlCl_3$ did not recover after the $AlCl_3$ was washed out (Figure 1B).

$AlCl_3$ 0.25 mmol/L caused a reduction in I_{HVA} in 80% (8/15) of the neurons, and an increase in 20% (4/15) of the neurons. $AlCl_3$ 0.50 mmol/L caused a reduction in I_{HVA} in 50% (7/14) of the neurons, and an increase in I_{HVA} in 50% (7/14) of the neurons. In contrast, $AlCl_3$ 0.75 mmol/L increased I_{HVA} by 30.8%±5.2% (n=15, $P<0.01$) in all neurons tested (from 17.8± 1.8 $pA \cdot pF^{-1}$ to 23.0±2.5 $pA \cdot pF^{-1}$). $AlCl_3$ 1.0 mmol/L increased I_{HVA} by 37.3%±7.8% (from 19.6±3.1 $pA \cdot pF^{-1}$ to 26.2±4.3 $pA \cdot pF^{-1}$) (n=21, $P<0.01$). I_{HVA} increased by $AlCl_3$ was irreversible after $AlCl_3$ was washed out (Figure 1C).

At both low and high concentrations, $AlCl_3$ inhibited or increased the maximum amplitude of I_{HVA}, but had no effect on the activation threshold potential of I_{HVA} in the *I–V* relationship (Figure 2A, B). The *G-V* curve was unaffected by $AlCl_3$ 0.1 mmol/L (n=5, $P>0.05$) or $AlCl_3$ 1.0 mmol/L (n=5, $P>0.05$) (control: $V_{0.5}$=-12.8 mV±4.4 mV, k=5.5±3.8; $AlCl_3$ 0.1 mmol/L: $V_{0.5}$=-12.0 mV±4.5 mV, k=5.0±4.0; $AlCl_3$ 1.0 mmol/L: $V_{0.5}$=-13.6 mV±5.3 mV, k=5.2±4.4) (Figure 2C). In addition, AlCl3 had no effect on the activation rate constants at concentrations of 0.1 mmol/L (n=8, $P>0.05$) or 1.0 mmol/L (n=9, $P>0.05$).

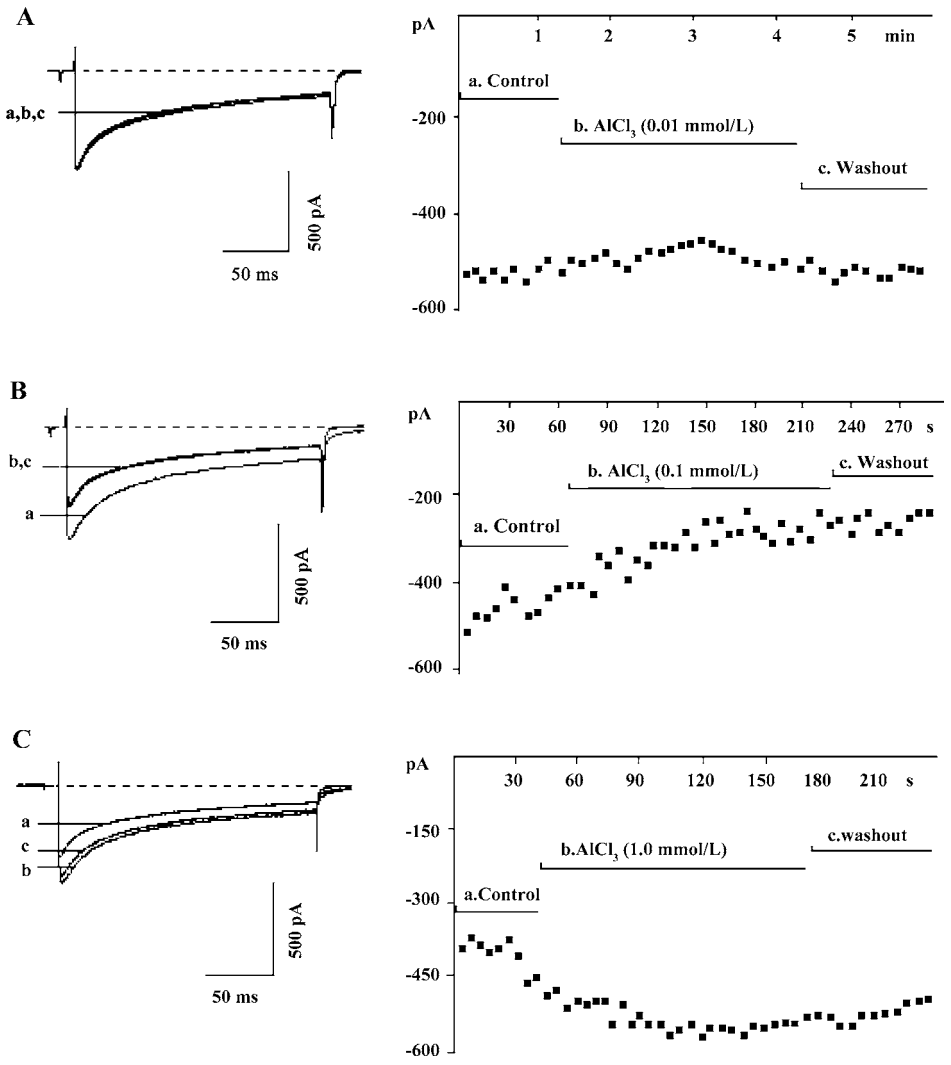

Figure 1. Effect of $AlCl_3$ on I_{HVA} at concentrations of 0.01, 0.1 and 1.0 mmol/L (A, B, C, respectively). I_{HVA} current was recorded at different concentrations of $AlCl_3$ (left). (a) Before the application of $AlCl_3$; (b) Application of $AlCl_3$; (c) washout. The time course of the experiment corresponding to left-hand panels (right).

To gain a better understanding of the action of Al on I_{HVA}, we explored its action on the steady-state inactivation curve of I_{HVA}. $AlCl_3$ shifted the curve to a depolarizing voltage at 1.0 mmol/L (n=5, P<0.05), whereas it shifted the inactivation curve to a hyperpolarizing voltage at 0.1 mmol/L (n=5, P< 0.05). (Control: $V_{0.5}$=-35.4±3.3 mV, k=-14.3±2.5; 0.1 mmol/L $AlCl_3$: $V_{0.5}$=-41.1±2.7 mV, k=-9.2±2.0; 1.0 mmol/L $AlCl_3$: $V_{0.5}$= -29.8±6.9 mV, k=-10.8±2.4). $AlCl_3$ 0.1 mmol/L decreased the inactivation rate constant by 27.3%±6.3% (n=5, P<0.01), whereas 1.0 mmol/L $AlCl_3$ increased the inactivation rate constant by 44.7%±3.4% (n=7, P<0.01) (Figure 3).

Effect of Gin B on I_{HVA} in hippocampal neurons Gin B at doses of 0.01–20 μmol/L had no effect on I_{HVA} in normal hippocampal neurons (P>0.05) (Table 1). Gin B inhibited the increase of I_{HVA} by $AlCl_3$ 1.0 mmol/L. After a steady increase in the action of $AlCl_3$, Gin B at concentrations of 0.01 μmol/L,

Table 1. Effect of Gin B at different concentrations (0.01, 0.1, 1.0, 10, and 20 μmol/L) on the amplitude of I_{HVA}. n=6. Mean±SD.

Gin B/μmol·L^{-1}	I_{HVA}/pA·pF^{-1}	
	Control	Gin B
0.01	19.6±2.9	19.1±3.9
0.1	19.1±3.2	19.6±2.2
1.0	19.4±1.9	18.8±1.6
10	20.0±1.2	18.7±0.8
20	17.2±0.9	17.2±1.3

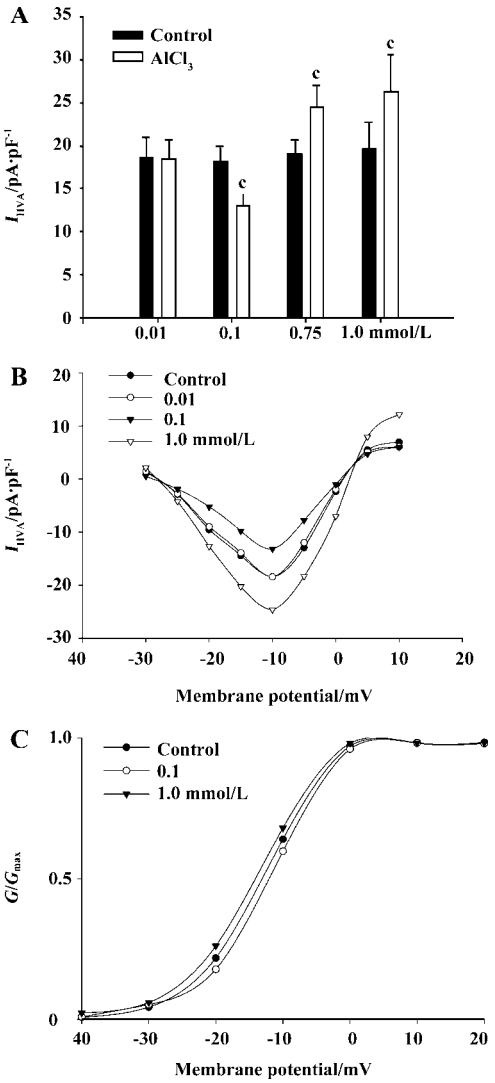

Figure 2. (A) Effect of Al at different concentrations on the amplitude of I_{HVA}. $^cP<0.01$ *vs* control. (B) Effect of Al at different concentrations on the current-voltage (*I–V*) relationship of HVA. (C) Effect of Al at different concentrations on the steady-state conductance (G) and voltage (V) curve. Data were transformed from the *I–V* data shown in B. *G–V* parameters were fitted to the Boltzman equation: $G/G_{max}=1/[1+\exp(V_m-V_{1/2})/k]$, where G_{max} is the maximum conductance, $V_{1/2}$ is the membrane potential at which 50% of activation was observed, and *k* is the slop of the function.

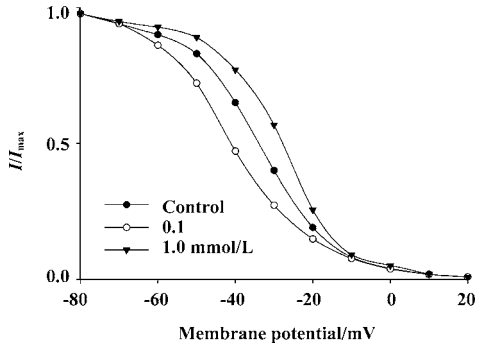

Figure 3. Effects of Al at different concentrations on the steady-state inactivation curve. I_{HVA} was measured using a 200 ms test pulse to 10 mV by 3 s conditioning prepulse ranging from -80 mV to +20 mV, with 10 mV increments. Data were fitted to the Boltzman equation: $I/I_{max}=1/[1+\exp(V_{1/2}-V_m)/k]$, where $V_{1/2}$ is the membrane potential at which 50% of activation was observed, and *k* is the slop of the function.

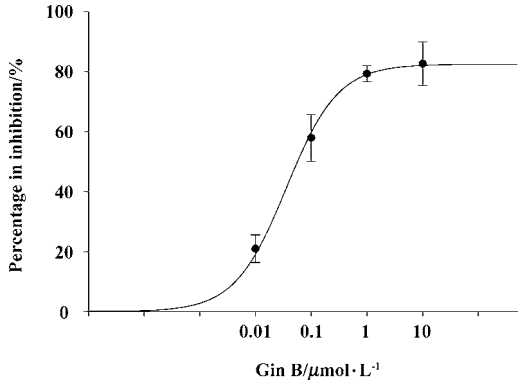

Figure 4. Concentration-response relationship for the inhibition of Gin B on the action of $AlCl_3$ (1.0 mmol/L) in hippocampal neurons. In the concentration-response curve for Gin B each point represents the mean±SEM of the percentage inhibition of Gin B from six to seven cells. The curve shown is the fit of the data to the logistic equation $Y=Y_{max}/[1+(IC_{50}/C)^n]$, where *C* is the concentration of Gin B, *Y* is the fraction of the maximal inhibition response value, *n* is the Hill coefficient, and IC_{50} is the concentration of Gin B producing 50% inhibition on the increase in I_{HVA} by 1.0 mmol/L Al.

0.1μmol/L, 1.0 μmol/L, and 10 μmol/L reduced I_{HVA} by 21.0%±4.6% (*n*=7, *P*<0.05), 57.9%±7.8% (*n*=6, *P*<0.01), 79.3%±2.7% (*n*=6, *P*<0.01), and 82.4%±7.3% (*n*=6, *P*<0.05), respectively. The concentration producing 50% inhibition by Gin B of Al 1.0 mmol/L is 0.0359 μmol/L±0.0038 μmol/L (Figure 4).

Co-superfusion $AlCl_3$ 0.1 mmol/L plus Gin B 10 μmol/L was applied in the same way as $AlCl_3$ 1.0 mmol/L. For all tested neurons (*n*=15), there was no change in I_{HVA} in 53.3% of the neurons and a slight increase in I_{HVA} in the remaining neurons (*P*>0.05). This result indicated that Gin B had no effect on the action of Al 0.1 mmol/L .

Mechanism of action of high concentrations of Al on I_{HVA} Application of forskolin 10 μmol/L (an agonist of adenylyl cyclase) increased I_{HVA} by 30.8%±7.5% (*n*=14, *P*< 0.05). Bath application of forskolin 10 μmol/L in combination with Al 1.0 mmol/L increased I_{HVA} by 68.3%±8.7% (*n*=31, *P*< 0.05) (Figure 5A).

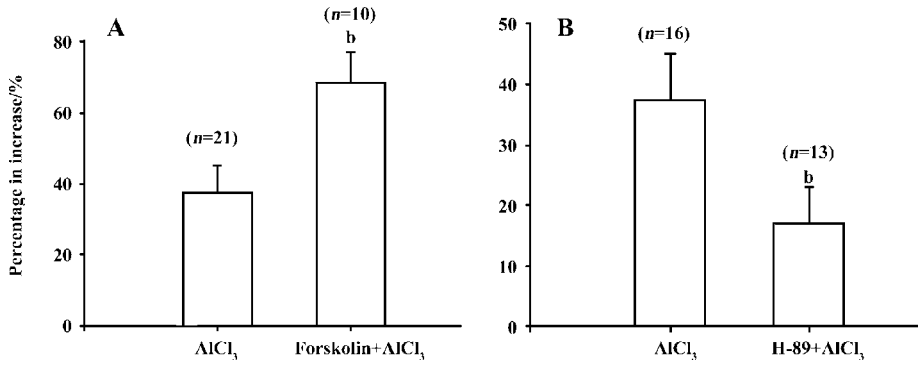

Figure 5. (A) Percentage of increased action by $AlCl_3$ 1.0 mmol/L and $AlCl_3$ co-superfusion with forskolin. (B) Percentage of increased action by $AlCl_3$ 1.0 mmol/L in the presence and the absence of H-89. $^bP<0.05$ *vs* $AlCl_3$ 1.0 mmol/L.

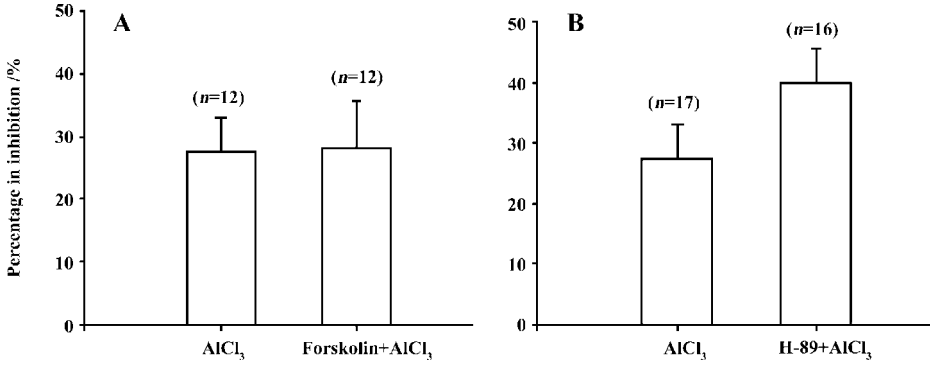

Figure 6. (A) Percentage of inhibitory action by $AlCl_3$ 0.1 mmol/L (27.5%±5.6%) and $AlCl_3$ co-superfusion with forskolin (28.2%± 7.3%). (B) Percentage of inhibitory action by $AlCl_3$ 0.1 mmol/L in the presence (39.9%±5.7%) and absence of H-89 (27.5%±5.6%).

H-89 is a selective antagonist of PKA. In this study, adding H-89 in the pipette solution reduced the amplitude of I_{HVA} by 42.0%±4.1% (from 20.2±3.3 pA·pF^{-1} to 13.9±3.1 pA·pF^{-1}, n=6, P<0.01) within approximately 80–100 s. $AlCl_3$ 1.0 mmol/L was bath applied in the presence of H-89 (10 μmol/L) in the pipette solution. Aluminum increased I_{HVA} by 17.2%±5.8% (n=10, P<0.05). Compared with the effect of Al 1.0 mmol/L on I_{HVA} without H-89 (n=29, P<0.05), the reduction in I_{HVA} in the presence of H-89 was significant, indicating that H-89 could, in part, abolish the increase in I_{HVA} by Al at high concentrations (Figure 5B).

Mechanism of action of low concentrations of Al on I_{HVA} To investigate the mechanism by which Al inhibited I_{HVA} at low concentrations, $AlCl_3$ 0.1 mmol/L was applied first and I_{HVA} was reduced to 12.9±1.1 pA·pF^{-1} from 18.5±1.7 pA·pF^{-1} (n=12, P<0.01). After the current was stable, forskolin 10 μmol/L and $AlCl_3$ 0.1 mmol/L were co-applied. Forskolin did not cancel the inhibition of I_{HVA} by 0.1 mmol/L $AlCl_3$. The I_{HVA} after forskolin application was 12.5±0.9 pA·pF^{-1} (n=12, P>0.05) (Figure 6A).

In the presence of H-89 (10 μmol/L), I_{HVA} was reduced to 13.1±2.5 pA·pF^{-1} from 20.1±4.2 pA·pF^{-1} (n=8, P<0.01). After the current was stable, $AlCl_3$ 0.1 mmol/L was bath applied, and I_{HVA} was reduced to 12.5±2.5 pA·pF^{-1}. There was no difference in the percentage inhibition with and without H-89 application (n=33, P>0.05) (Figure 6B).

Discussion

VDCC in hippocampal neurons are divided into high voltage-dependent channels (HVA) and low voltage-dependent channels (LVA) according to the difference in activation threshold. In the present study we demonstrated that the effect of Al on I_{HVA} differed at different concentrations. Al reduced the amplitude of I_{HVA} irreversibly at low concentrations (0.1 mmol/L). This result supports a previous report on dorsal root ganglion (DRG) neurons[7]. However, Al inhibited and enhanced I_{HVA} as Al concentrations increased

(between 0.25 mmol/L and 0.50 mmol/L), and the percentage of enhanced I_{HVA} by Al in the neurons examined increased with increased Al concentrations. When 0.75 mmol/L and 1.0 mmol/L Al were bath applied, the amplitude of I_{HVA} in all neurons tested increased.

The toxic effect of Al in humans is chronic and accumulative and leads to degradation and apoptosis of cells[14]. Acute application of Al inhibits LTP on hippocampal slices of rats as well as *in vivo* by intracerebroventricular injection. Studies have shown that a series of molecular mechanisms involved in synaptic plasticity, including protein phosphorylation, gene expression, and neurotransmitter release, were regulated by VDCC[15]. LTP induced in different areas of the hippocampus has an intimate relationship with VDCC[16]. The inhibition of I_{HVA} by Al at low concentrations could lead to a reduction in calcium influx, resulting in the reduced release of some neurotransmitters, which might explain impaired LTP in this concentration range. Aluminum increased the amplitude of I_{HVA} at high concentrations and, thus, led to increased calcium influx, resulting in a series of pathological changes, which could cause impairment of LTP and neuronal damage.

In our study, the actions of Al on I_{HVA} differed at different concentrations; thus, it is possible that the mechanism of action is different at different Al concentrations. Protein phosphorylation modulates the function of VDCC and the AC-cAMP-PKA system plays a key role[17]. Thus, forskolin and H-89 were used to investigate whether the action of Al on I_{HVA} is involved in this mechanism. H-89 markedly abolished the increase of I_{HVA} by Al 1.0 mmol/L. Co-superfusion with forskolin plus Al at high concentrations caused more Ca^{2+} influx. Together these results indicate that an Al-induced increase in I_{HVA} possibly results from activating cAMP-PKA. However, H-89 did not reverse the action of $AlCl_3$ totally, suggesting that other mechanisms must contribute to its action on I_{HVA} at high concentrations.

Platt[7] reported that the interactions of aluminum with two different binding sites (within and outside) of calcium channels might contribute to the reduction of VDCCs on DRG neurons. Al has been reported to inhibit Mg-dependent enzymes and to interact with phosphorylation sites[18]. In the present study, the co-application of forskolin and Al did not cancel the reduction and the action of 0.1 mmol/L Al was not affected by H-89, indicating that the mechanism by which Al reduces I_{HVA} at low concentrations might not be involved in the cAMP-PKA system. In addition, Gin B effectively canceled the increase of I_{HVA} by Al at high concentrations, but had almost no effect on the reduction of I_{HVA} by Al at low concentrations, further suggesting that the action of Al at low concentrations on I_{HVA} occurs via a different mechanism. The mechanism by which Al reduced I_{HVA} requires further examination. At intermediate concentration ranges, Al both reduced and enhanced I_{HVA}. The mechanism is not known, but may result from a difference in neurons or from the concentration of Al itself, which indicated that this concentration might be the point at which I_{HVA} moves from being inhibited to enhanced and this might be the reason for its complexity and diversity.

*EG*b is a complex mixture containing 24% flavonoid glycosides, 6% terpene lactones, such as ginkgolide A, B, C, J and bilobalide, a number of organic acids, and various other constituents. Studies have shown that Gin B has many pharmacological effects (ie preventing atherosclerosis, diminishing coagulation of platelets, ameliorating the circulation system) and has a distinctively protective effect on the central nerve and cardiovascular systems. Clinical studies have shown that oral administration of *EG*b in human patients with dementia is effective[19]. Gin B can protect cardiocmyocytes and cultured neurons from injury by hypoxia and ischemia through many pathways, for example, by acting as an anti-oxidant[20], acting as the antagonist of platelet-activating factor[21] and by inhibiting NO-stimulated PKC activity[22]. Furthermore, Gin B has been shown to prevent neurons from glutamate excitoxicity through a reduction in $[Ca^{2+}]_i$[23] and to have an effect on the glycine-gated chloride channel[24]. The present study provides the first evidence that Gin B can cancel the increase of I_{HVA} by Al, and that Gin B can protect neurons by inhibiting I_{HVA}, providing a possible mechanism for clinical treatment in a number of nervous system diseases. The detailed mechanism by which Gin B inhibits I_{HVA} remains to be investigated.

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