Interleukin-6 inhibits L-type calcium channel activity of cultured cerebellar granule neurons

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Abstract Our previous work has shown that interleukin-6 (IL-6) implements its neuroprotective effect by inhibiting the intracellular Ca²⁺ overload in neurons. Here, we examined whether regulation of L-type calcium channels (LCCs) activities is involved in the neuroprotective action of IL-6. In cultured cerebellar granule neurons (CGNs), patch-clamp recording showed that the whole-cell Ca²⁺ current and LCC current were significantly reduced by IL-6 pretreatment (120 ng/ml, for 24 h). Calcium imaging data indicated that IL-6 significantly suppressed high K⁺-induced intracellular Ca²⁺ overload and LCC Ca²⁺ influx. Moreover, expression of the LCC subunit, Caᵥ1.2, was remarkably downregulated by IL-6 in cultured CGNs. These findings suggest that IL-6 exerts a neurotrophic effect by preventing Ca²⁺ overload, at least partly through inhibition of LCC activity in cultured CGNs.

Keywords Interleukin-6 · L-type calcium channels · Whole-cell recording · Calcium imaging · Cerebellar granule neurons

Introduction

Interleukin-6 (IL-6), a member of pleiotropic cytokine family, has complex effects on the central nervous system (CNS) [1]. Under normal physiological conditions, the IL-6 level in the CNS is low. In neural functional disorders, such as brain diseases and injuries, IL-6 expression increases greatly [2–7]. The increased IL-6 may reflect a harmful process as an injurious mediator. For example, IL-6 is a detrimental player in the CNS, contributing to pathogenesis of neurodegenerative diseases, e.g., Alzheimer’s and Parkinson’s disease [8, 9]. However, the IL-6 increase may also represent a compensative mechanism for neural repair. For instance, IL-6 regulates neuronal function and development in the innate response of the CNS to injury and diseases [10, 11], and exerts neurotrophic and neuroprotective effects on glutamate- and N-methyl-D-aspartic acid (NMDA)-induced neuronal damage [12–15]. Hereby, further exploration is needed to understand the roles of IL-6 in brain physiology and pathology.

It is well known that Ca²⁺ is not only an important signaling molecule in neurons, but also a mediator leading to neuronal injury and death when it accumulates in the cytosol of cells, termed Ca²⁺ overload. Neuronal Ca²⁺ overload mainly involves three mechanisms: Ca²⁺ influx through ligand-gated channels, Ca²⁺ influx through voltage-gated Ca²⁺ channels (VGCCs) activated by membrane depolarization, and Ca²⁺ release from intracellular store induced by an increase in cytosolic Ca²⁺ [16]. By means of confocal laser scanning microscope (CLSM), we previously found that IL-6 suppressed neuronal intracellular Ca²⁺ overload induced by glutamate or NMDA, and exerted a neuroprotective effect [13, 15]. However, the mechanism underlying the IL-6 suppression of intracellular Ca²⁺ overload is not clear. We hypothesized that IL-6 exerts its neuroprotective effect by inhibiting the activity of LCCs.
function by suppressing the expression of VGCCs in cerebellar granule neurons (CGNs).

VGCCs are expressed in neurons and have multiple types, such as L-, N-, P/Q-, R-, and T-type Ca\(^{2+}\) channels [17–21]. Among these various types of VGCCs, L-type calcium channels (LCCs) are widely distributed on the cell body of neurons in mammalian CNS, including CGNs [22–24]. Calcium influx through LCCs in response to membrane depolarization serves essential functions in the regulation of intracellular Ca\(^{2+}\) homeostasis and neuronal excitability [25, 26]. Excessive Ca\(^{2+}\) influx through LCCs results in intracellular Ca\(^{2+}\) overload, which has been implicated in the pathogenesis of neurodegenerative disorders resulting from brain ischemia [16, 27, 28]. Therefore, in the present study, we firstly focused on LCCs to clarify the mechanism of the neuroprotective effect of IL-6 on LCCs by means of whole-cell patch clamp methods and calcium imaging.

**Materials and methods**

Isolation and culture of rat CGNs

Primary cultures of CGNs were obtained from neonatal Sprague-Dawley rats (The Center of Experimental Animals, Nantong University, China) at 8 days of age using previously described procedures [29]. Briefly, the cerebellum was removed from rats and minced with sterile surgical blades. The minced cerebellum was chemically dissociated in the presence of trypsin (Amresco, USA) and DNase I (Worthington, USA), and resuspended in the dissociated in the presence of trypsin (Amresco, USA) and surgical blades. The minced cerebellum was chemically dissociated in the presence of trypsin (Amresco, USA) and DNase I (Worthington, USA), and resuspended in the fol-

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Electrophysiological recording

Current through the Ca channel was isolated by blocking the Na channel with TTX and recorded using an Axopatch 200B patch-clamp amplifier (Axon, USA) at room temperature (20–22 °C). The bath solution was composed of TEA-Cl 144, BaCl\(_2\) 10, MgCl\(_2\) 2, CsCl 3, HEPES 10, glucose 10, 4-aminopyridine 2, and TTX 0.001 (all in mM), and adjusted to pH 7.4 with TEA-OH. Patch pipettes were pulled on a micropipette puller (pp830, Narishige, Japan) to a tip resistance of 3–5 MΩ when filled with internal solution. The pipette solution contained CsCl 140, HEPES 10, EGTA 10, TEA-Cl 5, and Na\(_2\)-ATP 2 (all in mM), and was adjusted to pH 7.2 with CsOH. Current responses were low-pass filtered at 1 kHz and analyzed with pClamp10.2 (Axon, USA). Linear components of capacitive and leak currents were subtracted using the P/4 protocol. \(I_{\text{Ca}}\), carried by Ba\(^{2+}\), was elicited by a series of command potentials from −60 to +40 mV for 250 ms in 10-mV steps from a holding potential of −80 mV. The whole-cell current densities were defined as peak current amplitude divided by cell capacitance. Nifedipine (Sigma), a blocker for LCCs, was used to determine the proportion of LCC current in the whole-cell current. It was added to 2 ml of bath solution with a final concentration of 10 μM, and 2-min later, the non-L-type channel current was recorded [30]. To determine the voltage-dependent activation property of LCCs, values of currents obtained were normalized to conductance with the form \(g = I/(V_m - V_{\text{rev}})\), and fitted to a single Boltzmann function of the form \(g/g_{\text{max}} = 1 - \{1 + \exp[(V_m - V_{1/2})/k]\}^{-1}\), where \(g\) is conductance, \(I\) is the amplitude of whole-cell LCC current, \(V_m\) is the membrane voltage, \(V_{\text{rev}}\) is the reversal potential, \(k\) is the slope factor, and \(g_{\text{max}}\) is the maximal conductance.

Measurement of intracellular Ca\(^{2+}\) fluorescence intensity

Intracellular Ca\(^{2+}\) level was quantified by single cell fluo-3 fluorescence intensity as described previously [29] with a small modification. Briefly, cultured CGNs were rinsed twice with balanced salt solution (BSS), then incubated at 37 °C for 45 min in the presence of 5 μM fluo-3/acetoxymethyl ester (Fluo-3/AM, Calbiochem), washed twice again with BSS, and incubated for an additional 20 min prior to imaging. The BSS was composed of (in mM): 145 NaCl, 5.6 KCl, 5 HEPES, 3.6 NaHCO\(_3\), 5.6 glucose, and 2.3 CaCl\(_2\). Calcium imaging was recorded by CLSM (Leica TCS SPE, Germany). Successive images were collected at 5-s intervals. Fluo-3 fluorescence was excited at 488 nm, and emitted light was measured at 530 nm. Quantification of the fluorescence intensity was performed using TCS-SPE software from Leica. To depolarize neurons and activate VGCCs, neurons were stimulated with high K\(^{+}\)-solution (150 mM KCl), whose composition was the same as that of BSS, but Na\(^{+}\) was replaced by K\(^{+}\). When
the high-K solution was applied to stimulate neurons, 100 μl of solution containing 150 mM KCl was added to 100 μl of BSS, and therefore the high K concentration was about 75 mM. Because the concentration of other constituents than K in the high-K solution was the same as that in BSS, the addition of the high-K solution to BSS did not alter the concentration of other constituents such as HEPES, NaHCO3, glucose, and CaCl2. Nifedipine (10 μM) was applied to neurons 25 min before high K stimulation. In one-scanned visual field, 30 neurons were randomly selected to obtain their dynamic intracellular Ca2+ levels. Neuronal basal Ca2+ fluorescence intensity before high K stimulation was firstly recorded for about 90 s, and then these neurons were stimulated by high K and scanned for 6 min. Neuronal maximal fluo-3 fluorescence intensity after high K stimulation was statistically analyzed. The same experiment was repeated four times.

Western blot assay

For measurement of expression of the LCC subunit, pore-forming α1c (also known as Ca1.2), the cultured CGNs were lysed by boiling sample buffer (125 mM Tris-HCl, pH 6.8, containing 4 % SDS, 12 % β-mercaptoethanol, and 20 % glycerol). The cell extracts were boiled for 5 min and loaded onto gels in each electrophoresis. After SDS-PAGE, the separated proteins in the gel were electrotransferred onto a PVDF membrane (Millipore) in tris-glycine-methanol buffer. The membrane was blocked in blocking solution (5 % non-fat dry milk in TBS), and then incubated with primary antibody in blocking solution (rabbit anti-α1c, 1:200; Alomone) overnight at 4°C. After washing with TBS/Tween-20, the membrane was incubated in secondary antibody (1:5,000 dilution) coupled to HRP, washed as above, and visualized by chemiluminescence using the ECL system.

Statistical analysis

Data were analyzed using pClamp 10.2 (Axon Instruments). One-way analysis or Student’s t test was used for comparisons, with p < 0.05 indicating statistical difference. All data were presented as mean ± standard deviation (M ± SD).

Results

Influence of IL-6 on whole-cell LCC current

Under the condition of Ba2+ instead of Ca2+ in the bath solution, which reduced the influence of Ca2+ current rundown [31], the whole-cell current through the Ca channel, evoked by depolarization from −60 to +40 mV at a holding potential of −80 mV, in neurons pretreated with IL-6 (120 ng/ml) was smaller than that in control neurons (Fig. 1a, b). Statistical analysis of current density displayed that the effect of IL-6 diminishing Ca-channel current was significant between −20 and +10 mV of depolarization (Fig. 1c).

The effect of IL-6 on LCC current was examined using the selective LCC antagonist, nifedipine. In control neurons, depolarization from a holding potential of −80 mV to a test potential of −10 mV evoked an inward Ca-channel current, and perfusion with nifedipine (10 μM) diminished the Ca-channel current (Fig. 2a). This demonstrated that opening of LCCs contributed to the inward current through the Ca channel. In IL-6-pretreated neurons, the depolarization from −80 to −10 mV also evoked an inward Ca-channel current, but the current was smaller than that in control neurons (Fig. 2a), demonstrating an inhibitory effect of IL-6 on Ca-channel current. The nifedipine perfusion also decreased the current through Ca-channel in IL-6-pretreated neurons (Fig. 2a). However, between IL-6-treated and control neurons, the nifedipine-insensitive
Ca-channel current was not significantly different (Fig. 2b), indicating that IL-6 did not alter the non-L-type Ca-channel current. On the other hand, the nifedipine-sensitive Ca-channel current was remarkably suppressed by IL-6 exposure (Fig. 2c). This revealed that the suppressive effect of IL-6 on the Ca-channel current was a result of its inhibition of LCCs. Moreover, to examine whether the voltage-dependent activation property of I_LCC was modified by IL-6 exposure, we calculated normalized conductance of LCCs using Boltzmann’s equation. The value of the reversal potential was close to 60 mV. The fitted values of $V_{1/2}$ were $-25.05 \pm 1.93$ and $-26.84 \pm 1.64$ mV, and the slope factors were $-5.84 \pm 1.81$ and $-4.75 \pm 1.30$ in control and IL-6-treated neurons, respectively. These data showed that neuronal voltage dependence on activation was not changed following incubation of the neurons with IL-6 (Fig. 2c3).

Effect of IL-6 on high K$^+$-evoked [Ca$^{2+}$]i increase

To further demonstrate the effect of IL-6 on LCCs, we measured dynamic changes of intracellular Ca$^{2+}$ fluorescence intensity in cultured CGNs by CLSM. In control neurons, depolarization stimulation by high K$^+$ evoked an acute elevation of intracellular Ca$^{2+}$ level (Fig. 3). In IL-6-pretreated neurons, high K$^+$ stimulation evoked significantly less elevation of the intracellular Ca$^{2+}$ level than in control neurons (Fig. 3), indicating that IL-6 suppressed

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**Fig. 2** Influence of IL-6 on whole-cell LCC current in cultured CGNs. Depolarization voltage was set to $-10$ mV from a holding potential of $-80$ mV, and whole-cell inward current through the Ca channel was recorded in control and IL-6-exposed neurons. Perfusion of control or IL-6-exposed neurons with 10 μM of nifedipine, a blocker for LCCs, reduced the inward current through the Ca channel (a). The inward current after nifedipine action was non-L-type Ca-channel current (b1), and it was not significantly different between IL-6-pretreated and control neurons (b2). The inward current blocked by nifedipine was the LCC current (c1). The LCC current density was evidently lower in IL-6-exposed neurons than in controls (c2). $**p < 0.01$, compared with controls ($n = 8$). Voltage-dependent activation curves were obtained by the Boltzmann equation, $g/g_{\text{max}} = 1 - \exp[(V_m - V_{1/2})/K]^{-1}$. The fitted values of $V_{1/2}$ were $-25.05 \pm 1.93$ and $-26.84 \pm 1.64$ mV, and the k (slope factor) was $-5.84 \pm 1.81$ and $-4.75 \pm 1.30$ in control and IL-6-treated neurons, respectively. No significant differences in the data were found between IL-6-treated and control neurons (c3, $n = 6$).
high K+-induced intracellular Ca$^{2+}$ overload. After exposure to nifedipine (10 μM), an LCC antagonist, for 25 min, high K$^+$ stimulation resulted in a reduction of intracellular Ca$^{2+}$ overload compared with control neurons lacking nifedipine exposure (Fig. 3). This suggests that the reduction of intracellular Ca$^{2+}$ was attributable to a reduction of Ca$^{2+}$ influx through LCCs. However, the inhibitory effect of nifedipine on high K$^+$-induced intracellular Ca$^{2+}$ overload did not have a notable difference in the presence and the absence of IL-6 (Fig. 3). This indicated that IL-6 did not significantly alter nifedipine-resistant Ca$^{2+}$ overload components and therefore suggested that IL-6 exerted its suppressive effect on high K$^+$-evoked intracellular Ca$^{2+}$ overload by attenuating nifedipine-dependent LCC Ca$^{2+}$ influx.

IL-6 downregulates protein expression of LCC subunit

Expression of the LCC subunit, pore-forming α_{1c} (also known as Ca_{1.2}), in cultured CGNs was measured in order to reveal the mechanism underlying IL-6 suppression of the LCC current and LCC Ca$^{2+}$ influx. The LCC subunit protein expression was remarkably downregulated by IL-6 pretreatment (Fig. 4). This showed that via the downregulation, IL-6 carried out its inhibitory effect on LCC function.

Discussion

In this study, IL-6 pretreatment of cultured CGNs significantly reduced the inward current through the Ca channel evoked by depolarization from −20 to +10 mV at a holding potential of −80 mV, suggesting that IL-6 inhibits VGCC opening. To examine the contribution of LCCs, a type of VGCCs, to the inward Ca-channel current, we used nifedipine to block LCCs and found that the inward Ca-channel current was diminished. This suggests that depolarizing stimulation causes opening of LCCs and consequent influx of Ca$^{2+}$ current in cultured CGNs. The report that extracellular Ca$^{2+}$ influx occurs not only directly through the glutamate-activated membrane channel, but also indirectly through activated VGCCs by membrane depolarization [32] supports our present results. Importantly, after neurons were pretreated with IL-6, the effect of the nifedipine-sensitive inward Ca-channel current was significantly suppressed. The result suggests that IL-6 inhibits LCC activity. Some other cytokines, such as interleukin-1β, tumor necrosis factor α, and ciliary neurotrophic factor, have been reported to modulate various types of VGCC currents in neurons [30, 33, 34]. Thus, our present data provide more evidence for IL-6 regulating the LCC current in cultured CGNs.

To further demonstrate the modulation of LCC activity by IL-6, we observed the influence of IL-6 on intracellular Ca$^{2+}$ overload evoked by high K$^+$-depolarization stimulation in cultured CGNs. The IL-6 pretreatment significantly reduced the high K$^+$-evoked intracellular Ca$^{2+}$ overload. The result is consistent with the data obtained from the patch-clamp experiments and demonstrates that IL-6 inhibits VGCC activity. In our previous work, we indicated that IL-6 suppresses glutamate- or NMDA-induced intracellular Ca$^{2+}$ overload and neuronal apoptosis in cultured CGNs, and therefore suggest that IL-6 has a neuroprotective effect [13, 15, 29]. Here we add evidence for the IL-6 neuroprotection at the profile of its suppression of VGCCs. Further, we hypothesized that the inhibitory effect of IL-6 on VGCC-dependent Ca$^{2+}$ influx is mediated by LCC-activity suppression. We observed that nifedipine attenuated intracellular Ca$^{2+}$ overload triggered by high K$^+$-depolarization stimulation, demonstrating that LCC opening is involved in the high K$^+$-induced intracellular Ca$^{2+}$ overload. The inhibitory effect of nifedipine on intracellular Ca$^{2+}$ overload occurred similarly in IL-6-exposed and control neurons. It indicates that IL-6 does not significantly alter the nifedipine-insensitive Ca$^{2+}$-influx component. Therefore, the suppression of intracellular Ca$^{2+}$ overload by IL-6 is attributed to its suppression of the nifedipine-sensitive Ca$^{2+}$-influx component. These findings are consistent with the conclusion from the whole-cell recording that IL-6 suppresses LCC activity. Thus, we suggest that IL-6 neuroprotection through suppression of intracellular Ca$^{2+}$ overload is implemented, at least partly, by the inhibition of the LCC current.

Since the voltage-dependent property of $I_{\text{LCC}}$ was not modified by IL-6 pretreatment in the current study, the mechanism underlying the IL-6 inhibition of LCC activity needs to be explained. We found that expression of the LCC pore-forming subunit Ca_{1.2} was significantly downregulated by IL-6 exposure in cultured CGNs. The downregulation reached 60 %, and it was quite consistent with the reduction in $I_{\text{LCC}}$ peak current density in IL-6-treated neurons. On the basis of these findings, we suggest that the suppression of LCC function by IL-6 is related to a decrease in LCC protein expression.

As we previously reported [13, 15, 29], the present study represents a neuroprotective role of IL-6. However, since IL-6 is a pleiotropic cytokine, it exerts neurotrophic and neuroprotective effects, and yet can also function as a mediator of inflammation, demyelination, and astrogliosis, depending on the cellular context [35]. Therefore, the dosage of IL-6, degree of neuronal damage, type and environment of neurons, and existence of soluble IL-6 receptors can influence IL-6 effects [36, 37]. For example, Nelson et al. [38] showed that a lower dose of IL-6 (5 ng/ml) exposure enhances the mean amplitude of the Ca$^{2+}$ signal
in response to glutamate receptor agonists in cultured cerebellar Purkinje neurons, whereas a higher concentration of IL-6 (10 ng/ml) has no effect on the Ca\(^{2+}\) signal in response to the same agonists. On the other hand, Vereyken et al. [39] report that transient high-K\(^+\) stimulation (0.5 s) enhances the Ca\(^{2+}\) signal, but longer high-K\(^+\) stimulation (>1 s) attenuates the Ca\(^{2+}\) signal in IL-6-treated neurons. In addition, NMDA infusion into rat striatum results in a decrease in striatal cholinergic and GABAergic neurons, and co-infusion of IL-6 and NMDA reduces the loss of cholinergic neurons, but fails to prevent the loss of GABAergic neurons [37]. These differences of response to IL-6 among different IL-6 dosages, neuron-damaged degrees, and neuronal types explain the distinct and complex effects of IL-6, neuroprotective, neuroinjured, or non-effective. Further exploration is needed to clarify the mechanisms underlying the different effects of IL-6.

In general, in the presence of IL-6 receptor, IL-6 acts on target cells and promotes dimerization of gp130, a signal-transducing subunit coupled with IL-6 receptor. CGNs have been reported to express IL-6 receptor and gp130 signal protein [40, 41]. In our previous work, anti-gp130 antibody blocked the inhibitory effect of IL-6 on glutamate-induced intracellular Ca\(^{2+}\) overload, indicating that the IL-6 receptor is involved in the neuroprotective effect of IL-6 [29]. On the basis of these findings, we suggest that the suppressed LCC activity caused by IL-6 is mediated by the IL-6 receptor.

Fig. 3 Role of LCCs in IL-6 suppressing high K\(^+\)-trigged intracellular Ca\(^{2+}\) overload. LCC blocker nifedipine (10 \(\mu\)M) treated neurons for 25 min before high K\(^+\)-stimulation. The neurons were incubated at 37 °C for 45 min in the presence of 5 \(\mu\)M of Fluo-3/AM, and then dynamic changes in intracellular Ca\(^{2+}\) levels were tested by CLSM during the whole 6-min high-K\(^+\) stimulation. In each treatment, 30 neurons were randomly selected to analyze dynamic intracellular Ca\(^{2+}\) levels (a). The compilation of data for the mean and SD of four separate experiments as in a is presented in b. The peak intracellular Ca\(^{2+}\) levels following high K\(^+\) stimulation were compared for statistical significance of the differences between the various treatments (c). The arrows denote the beginning time when KCl was applied. *\(p < 0.05\) and NS means no significant difference.

Fig. 4 IL-6 downregulates LCC subunit expression in cultured CGNs. The CGNs from 8-day-old rats were incubated for 7 days and then exposed to IL-6 (120 ng/ml) for 24 h. The protein expression of the LCC subunit, pore-forming \(\alpha_{1c}\) (also known as Ca\(_{\text{v}1.2}\), was significantly downregulated by IL-6 pretreatment (a). The data are from three separate experiments (b). **\(p < 0.01\), compared with control.
In conclusion, we revealed that IL-6 inhibits the activity of LCCs in cultured CGNs and this inhibition is associated with downregulation of LCC protein expression. These results imply that a neuroprotective role of IL-6 in the CNS is implemented, at least partially, by suppression of the neuronal LCC current and therefore a reduction in intracellular Ca\(^{2+}\) overload.

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