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L-type Ca^{2+} channel blocker attenuates impairment induced by acidosis in hippocampal neurons

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Accumulation of acid in the brain during ischemia may contribute to the activation of voltage-gated Ca^{2+} channels and subsequent neuronal injury. However, information regarding the role of L-type Ca^{2+} channel under acidosis remains unclear. In the present study, we examined the role of L-type Ca^{2+} channel in acidosis induced neuronal death and subsequent pathogenesis events responsible for neuron degeneration. Here we report that preincubation of cells with nifedipine (5 μM, 10 μM), an inhibitor of L-type Ca^{2+} channel markedly reduced neuronal death induced by moderate extracellular acidosis (pH 6.5) on cultured hippocampus neurons. Furthermore, nifedipine decreased the hippocampus neuronal swelling, as well as the accumulation of Ca^{2+} and collapse of mitochondrial membrane potential induced by acidosis. These findings demonstrate that pharmacological inhibition of L-type Ca^{2+} channel would attenuate neuronal degeneration caused by toxic low pH exposure in rat hippocampus neuron.

1. Introduction

Brain neurons are vulnerable to acidosis, which often occurs during stroke, inflammation, hypoxia and seizures (Deitmer et al. 1996; Li et al. 1997). In neurons, ischemia causes the extracellular pH fall, which activates Ca^{2+}-permeable acid-sensing ion channels (ASICs), resulting in Ca^{2+} overload (Xiong et al. 2004). The increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) leads to a cascade of events, which can precipitate necrosis and/or apoptosis of susceptible neurons (Randall et al. 1992). Activation of ASICs promotes Ca^{2+} influx by generating membrane depolarization, which may also facilitate opening of voltage-gated Ca^{2+} channels (VGCCs) (Gao et al. 2005). VGCCs are known to be very sensitive to pH in a variety of cell types including neurons (Dixon et al. 1993).

Several lines of evidence suggest that many membrane effects of H^+ ions arise from their action on VGCCs (Tombaugh et al. 1997). *In vivo* and *in vitro* experiments have also indicated that anoxic/cerebral ischemia induces L-type VGCC activation (Pisani et al. 1998). These observations led to the hypothesis that the inhibition of the activation of calcium channels might be useful in the treatment of acidosis. In the present study, to test this hypothetical proposal, we measured cell viability and cell swelling of hippocampal neurons exposed to acidosis in the presence of nifedipine, a L-type calcium channel blocker by comparing those of amiloride, which has been recognized as an ASICs channel blocker that protects the brain from ischemic injury (Xiong et al. 2004). We also examined whether nifedipine would have effects on changes of intracellular Ca^{2+} and mitochondrial membrane potential (MMP) induced by acidosis.

2. Investigations and results

2.1. Nifedipine reduces acidosis-induced neuronal death

Cell death was quantitatively analyzed by counting dead neurons under the fluorescence microscope and total neurons under the DIC microscope in three randomly chosen fields in each dish. Under the fluorescence microscope, PI stained the dead neurons red. Exposure to pH 6.5 aCSF resulted in neuronal death within 24 h (by approximately 47%, Fig. 1). Co-administration of 5 μM, 10 μM nifedipine or 0.1 mM amiloride showed protective effects against acidosis-induced neuronal

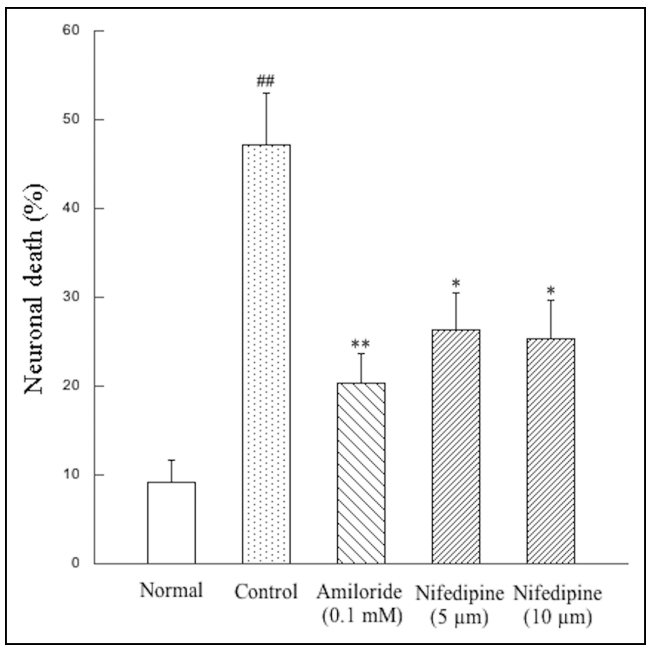

Fig. 1: Neuronal death of hippocampal neurons after acidosis treatment in presence and absence 0.1 mM amiloride, 5 μM nifedipine or 10 μM nifedipine. Normal, pH 7.4 aCSF and vehicle treatment; Control, pH 6.5 aCSF and vehicle treatment. The data represent mean ± SEM from 4 experiments. ##$P < 0.01$ vs. Normal; *$P < 0.05$, **$P < 0.01$ vs. Control.

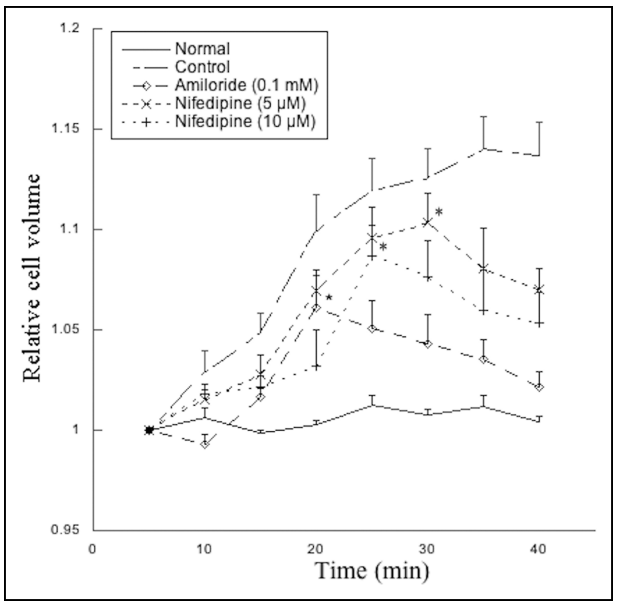

Fig. 2: The time course of relative cell volume of hippocampal neurons after acidosis treatment in presence and absence 0.1 mM amiloride, 5 μM nifedipine or 10 μM nifedipine. Normal, pH 7.4 aCSF and vehicle treatment; Control, pH 6.5 aCSF and vehicle treatment. The data represent mean ± SEM from 4 experiments. *$P < 0.05$ vs. Control at the same time point.

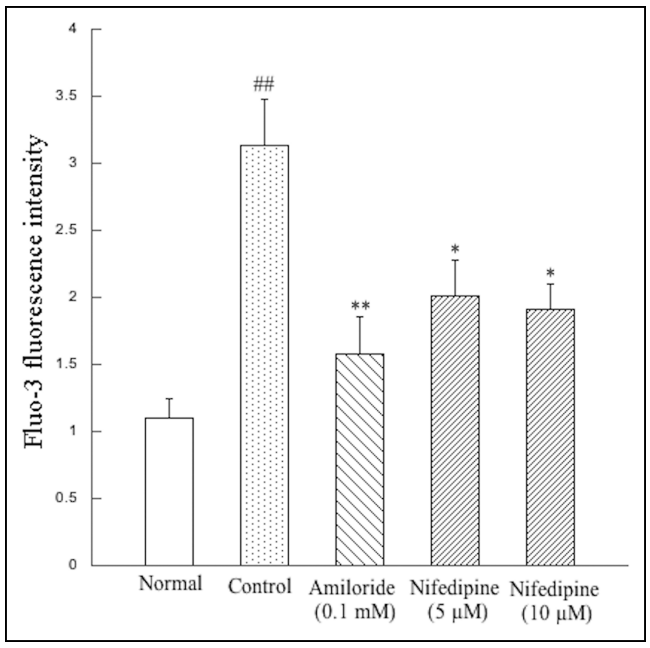

Fig. 3: Peak rise of fluo-3 fluorescence in hippocampal neurons after acidosis treatment in presence and absence 0.1 mM amiloride, 5 μM nifedipine or 10 μM nifedipine. Normal, pH 7.4 aCSF and vehicle treatment; Control, pH 6.5 aCSF and vehicle treatment. The data represent mean ± SEM from 4 experiments. ##$P < 0.01$ vs. Normal; *$P < 0.05$, **$P < 0.01$ vs. Control.

death. Figure 1 shows the protective effects of nifedipine and amiloride against acidosis-induced neuronal death (neuronal death [%]: pH 6.5 aCSF, 36.9 ± 4.9; with 5.0 and 10 μM nifedipine, 27.4 ± 5.7, and 25.3 ± 3.5, respectively; with 0.1 mM amiloride, 19.1 ± 3.1). Nifedipine showed significant protection against acidosis-induced neuronal death, and reduced the rate by approximately 20%. Amiloride, a ASICs channel antagonist, considerable inhibited neuronal death, indicating that the death process was acidotoxicity.

2.2. *Nifedipine inhibits acidosis-induced cellular swelling*

After aCSF (pH 6.5) exposure, cellular swelling developed over 30 min, and the area of the neuron gradually increased to 1.146 ± 0.025 of the baseline (Fig. 2). Amiloride and nifedipine (5 μM, 10 μM) significantly suppressed cellular swelling 1.061 ± 0.016 (20 min); 1.102 ± 0.018 (30 min); 1.087 ± 0.020 (25 min) of baseline. Neither amiloride nor nifedipine alone induced morphological changes. Cells treated with amiloride underwent a complete volume recovery after 35 min treatment.

2.3. *Nifedipine suppresses acidosis-induced increase in $[Ca^{2+}]_i$*

Neurons stained with fluo-3 were exposed to pH 6.5 aCSF by dropping aliquot, and observed every 60 s under a confocal microscope. Figure 3 shows the fold increase in fluorescence intensity. Acid aCSF (pH 6.5) induced a persistent rise of $[Ca^{2+}]_i$ in 30 min, with the fluorescence intensity being 3.2-fold stronger at the end of the observation. Nifedipine significantly suppressed the peaks of $[Ca^{2+}]_i$ at both concentrations (Fig. 3). This result indicated a comparable inhibitory effect of nifedipine and amiloride with respect to the persistent $[Ca^{2+}]_i$ increase after acidosis application during the observation period. Neither nifedipine nor amiloride alone affected $[Ca^{2+}]_i$ in the neurons.

2.4. *Nifedipine improves neuronal mitochondrial membrane potential during acidosis*

Acid aCSF (pH 6.5) gradually evoked a decrease in the fluorescence intensity of Rho123, which reached 57.6% of the basal value after 6 h acidosis (Fig. 4), indicating a decrease in mitochondrial membrane potential (MMP). Figure 4 shows that addition of nifedipine (5, 10 μM) significantly restored mitochondrial membrane potentials to around 80% of basal levels after acid aCSF exposure. Amiloride (0.1 mM) also significantly inhibited reduction of MMP (84.4% of the basal value). Neither

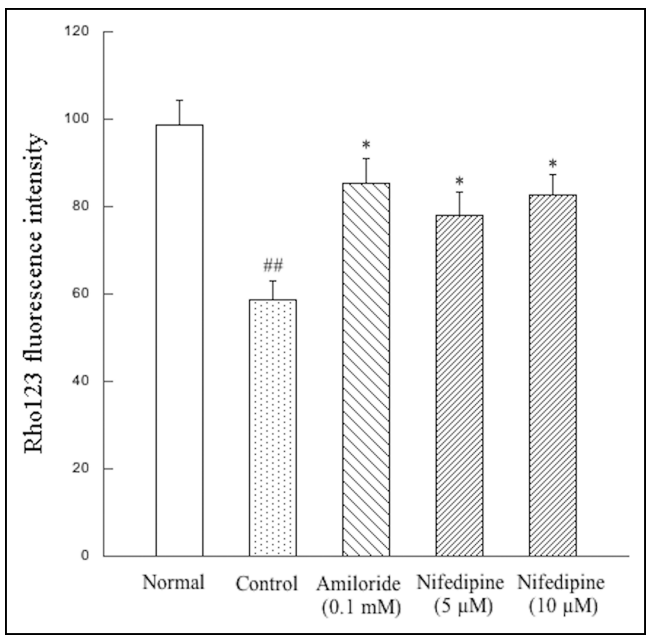

Fig. 4: Rho123 fluorescence intensity in hippocampal neurons after acidosis treatment in presence and absence 0.1 mM amiloride, 5 μM nifedipine or 10 μM nifedipine. Normal, pH 7.4 aCSF and vehicle treatment; Control, pH 6.5 aCSF and vehicle treatment. The data represent mean ± SEM from 4 experiments. ##$P < 0.01$ vs. Normal; *$P < 0.05$ vs. Control.

nifedipine nor amiloride alone affected fluorescence intensity of Rho123.

3. Discussion

In the present study we used a well-established cell model of acidosis impairment (changing extracellular pH) to verify whether L-VGCC blocker contribute to acidosis-induced neuronal death. The acid-sensing ion channels (ASICs) are voltage-insensitive cation channels activated by extracellular acidification (Waldmann et al. 1997) and moderate activation of ASICs is expected to induce membrane depolarization and facilitate neuronal damage (Xiong et al. 2004). Our data show that the L-VGCC inhibitor, nifedipine, could exert a neuroprotective effect against acidotoxicity in rat hippocampal neurons. In addition, attenuation of cell swelling and neuronal death appeared to be mediated by nifedipine's action on regulating intracellular calcium levels and MMP.

It is well established that intracellular Ca^{2+} overload is essential for neuronal dysfunction, which is associated with neuropathological syndromes due to the ensuing cascade of neurodestructive processes (Choi et al. 1988). Activation of ASICs causes dramatic depolarization of membrane potential, it is expected that VGCCs (e.g. L-type which is nifedipine sensitive) be activated following ASICs activation, and this secondary activation of voltage-gated calcium channel may contribute to acid-induced increase of intracellular calcium and neuronal injury (Chu et al. 2004). In this study, we demonstrated that nifedipine markedly suppressed the calcium overload. We found that both nifedipine and amiloride protect the neuron from acid-injury, possibly due to their abilities to inhibit Ca^{2+} influx. It may be suggested that the persistent increase in $[Ca^{2+}]_i$ induced by acidosis can be mediated not only by the ASICs but also by L-VGCC. In this regard, inhibition of L-VGCC channels may also play a role in preventing neurons from injury in acidosis conditions.

Numerous studies have reported that mitochondrial dysfunction contributes to neurodegenerative diseases through reducing ATP production, increasing ROS formation, and releasing death regulatory and signaling molecules from the intermembrane space (Knott et al. 2008). Mitochondrial disturbances are consistent with changes in MMP during neuronal impairment, and MMP has been shown to be involved in a variety of pathophysiological conditions, in particular for apoptosis (Fiskum et al. 2003). Here, we demonstrated that exposure to acidosis resulted in a marked dissipation of MMP in cultured neurons, and this dissipation was effectively prevented by L-VGCC inhibition, and this is consistent with previous results that nifedipine attenuated the AMPA induced mitochondrial dysfunction and degeneration of motor neurons (Joshi et al. 2011). These findings reveal that both L-VGCC and ASICs participate in acidosis-induced mitochondrial dysfunction. This suggests one common mechanism of action for both drugs to reduce cell impairment appeared through their ability to regulate MMP following acid injury. Further studies are imperative to elucidate the precise relation of varying causes of Ca^{2+} to MMP.

Brain edema is a key feature of acid injury, and it is defined as a cellular swelling with fluid accumulating within cells in the brain (Staub et al. 1993). Factors that induce neuron swelling are likely multifactorial, including increased levels of Ca^{2+}, elevated Na^{+}, acidosis, and breakdown of energy metabolism (Dreier et al. 2011), especially mitochondrial dysfunction (Macgregor et al. 2003). Our results showed that acid-induced cellular swelling was inhibited by nifedipine, providing the evidence for activation of the L-VGCC during the process. Since the activated L-VGCC induces Ca^{2+} influx, which must contribute to mitochondrial dysfunction as well as the cellular swelling, and the energy failure due to mitochondrial dysfunction induced by Ca^{2+} influx impairs cellular energy-consuming systems and leads to further cellular swelling. Therefore, it is inferred that nifedipine suppressed the cellular swelling through regulation of the main factors like calcium overload and mitochondrial dysfunction. Irrespective of the mechanism, the cellular swelling eventually leads to the rupture of the cell membrane and then neuronal death. Thus, suppression of the cellular swelling via L-VGCC inhibition can be neuroprotective.

L-VGCC blocker may have neuroprotective effects against acidosis *in vivo*. The L-type calcium channel blocker nimodipine significantly raised local cerebral pH on the rat model of regional cerebral acidosis (Vogel et al. 1988). It is also reported that preoperative nimodipine treatment reduced intraoperative CSF acidosis in elderly comorbid patients (Oehmke et al. 2003). The explanation may be that the inhibition of L-type calcium channel prevents a pathologically increased Ca^{2+} influx, and then accelerates the elimination proton load (Oehmke et al. 2003). Our results which show that L-VGCC blocker nifedipine reduced neuronal death induced by acidosis further support the viewpoint above. However, we found that the inhibition of other possible Ca^{2+} channels by CNQX and MK801 did not suppress acidosis-induced neuronal impairment (data not shown). Therefore there is still much work to do to explore whether there is a functional linkage between L-VGCC-mediated and ASICs mediated acid toxicity.

In summary, the results in the present study potentially indicate that the L-VGCC inhibitor is neuroprotective against acid toxicity through inhibition of both calcium overload and MMP dissipation in neurons. Thus, the blockade of L-type Ca^{2+} channel is likely to be of therapeutic benefit in the treatment of both neurological and psychological disorders associated with acidosis.

4. Experimental

4.1. Materials

Nifedipine, amiloride, dimethyl sulphoxide, cytosine β-D-arabinofuranoside and propidium iodide (PI) were obtained from Sigma Aldrich (St. Louis, MO, USA), fluo-3/acetoxymethylester (Fluo-3) and Rhodamine 123 from Molecular Probes. Chemicals were dissolved in dimethyl sulfoxide (DMSO) and diluted by PBS. All other chemicals were of analytic grade and were obtained from commercial sources.

4.2. Primary culture of hippocampal neurons

Primary cell cultures derived from rat hippocampi, containing mainly neurons over astrocytes, were established as described previously (Askwith et al. 2004). In brief, cerebral hippocampi were dissected from postnatal day 0–1 pups, freed from extraneous tissue, and cut into pieces. Dissected tissue was transferred into Leibovitz's L-15 medium and incubated for 15 min at 37 °C. After incubation, the dissected tissue was washed three times with mouse M5-5 medium and triturated. Dissociated cells were then centrifuged and medium was aspirated. Cells were resuspended in supplemented neurobasal-A medium (1% B27 supplement containing antioxidants, 0.5 mM L-glutamine, 0.5 mg/mL gentamycin, and 2.5 mg/L insulin, 16 nM selenite, and 1.4 mg/L transferrin). Cells were plated in 6-well plates containing 10 mm poly-D-lysine-coated glass coverslips at a density of 2×10^5 cells per well. After 48–72 h, 10 μM cytosine β-D-arabinofuranoside was added to inhibit glial proliferation. Neurons were maintained at 37 °C with 5% CO_2 for 14–21 d before experiments were performed.

4.3. Induction of acidosis toxicity and assessment of neuronal death

Acidosis toxicity was induced in primary cultures as described (Xiong et al. 2004). After washing the cultures with artificial cerebrospinal fluid (aCSF)—NaCl, 140 mM; KCl, 5 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2 mM; glucose, 10 mM; and HEPES, 10 mM (with a pH adjusted to 7.4 or 6.5), they were incubated at 37 °C for 24 h with aCSF. In some experiments, nifedipine (5 μM, 10 μM) or amiloride (0.1 mM) was added to the aCSF 10 min

before acidosis exposure. Appropriate stock solutions of drugs were prepared and the solvents (0.1% DMSO) had no effect on the cell viability in the concentrations used here. Control neurons received the acidic extracellular solution (aCSF, pH 6.5) containing 0.1% DMSO, and normal group only received the neutral extracellular solution (aCSF, pH 7.4) without any drug treatment. The pH was monitored by a laboratory pH meter (WTW inoLab, Germany). The mortality of the cultures was assayed by using PI, which does not penetrate viable cells but enters injured cells, yielding the red staining. At the end of acidosis exposure, the cultures were incubated with aCSF-containing PI (3 μM) at 37 °C for 10 min. To assess the neuronal death, we employed a differential interference contrast (DIC) microscope (Olympus IX 70, Japan) with a 20 × DIC objective lens and fluorescence optics (excitation at 515 nm, emission at 620 nm). The percentage of neuronal death was calculated by counting the number of all neurons (in the DIC mode) and that of the neurons stained with PI (in the fluorescence mode). Under the microscope, 3 fields (30 to 50 neurons per field) were chosen at random in each dish (3 dishes each group).

4.4. Cell volume measurements

All experiments were performed using aCSF (pH adjusted to 7.4 or 6.5) containing 5 μM, 10 μM nifedipine or 0.1 mM amiloride. Neurons were imaged using methods described previously (Churchwell et al. 1996). Briefly, neurons grown on cover slips were placed in 35 mm diameter tissue culture dishes. These dishes were prepared for use by drilling a 17 mm diameter hole in the bottom of the dish. A 25 mm diameter cover slip was cemented over the bottom of the hole, creating a shallow well. The culture dish was placed in the MIU-IBC-IF Chamber (Olympus, Japan) mounted on the stage of a motorized inverted research microscope (Olympus IX81, Japan). Images of neurons were recorded using a high-resolution microscope camera (QImaging MicroPublisher 5.0 RTV, Canada).

To estimate cell volume, the area of the cell was measured by tracing its outline of the image (Hosoi et al. 2004). The area of the traced regions was determined by image analysis software (Optimas, China). Each image was traced twice, and the values were averaged. The average value obtained from five images measured in the first 5 min was used as the baseline value (A_0). The relative volume of the neuron was expressed as $V/V_0 = (A/A_0)^{1.5}$, where V is the volume, A the area, and the subscript 0 indicates the baseline value. Thus, the values of relative cell volume (V/V_0) were normalized to the baseline value.

4.5. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

The intracellular Ca^{2+} concentration was measured by fluorescence imaging with Fluo-3, as described previously (Deng et al. 2005). Neurons were rinsed 3 times with aCSF and incubated at 37 °C with aCSF-containing Fluo-3 (5 μM) for 30 min. Fluorescence measurements of $[Ca^{2+}]_i$ was performed using a confocal laser scanning microscopy (Zeiss LSM510, Germany). After recording the fluorescence images of baseline, the neurons were exposed to acid aCSF containing nifedepine (5 μM, 10 μM), and the fluorescence images were taken for 30 min. Increases of $[Ca^{2+}]_i$ are expressed as the ratio of fluorescence intensity of Fluo-3 over baseline (F_X/F_0). This ratio method was used because it is independent of factors such as dye concentration, excitation intensity and detector efficiency. In some experiments, drugs or vehicles were added to the medium 10 minutes before acidosis application.

4.6. Mitochondrial transmembrane potential (MMP) analysis

Cells were cultivated in the absence and presence of 5 μM, 10 μM nifedipine or 0.1 mM amiloride exposed to acid aCSF for 6 h. Control neurons were incubated in acid aCSF for 6 h, and other acidosis procedure was prepared as described above. To quantify the changes of MMP (Lok et al. 2011), medium were aspirated and neurons were washed, trypsinized, re-suspended in PBS and stained with Rhodamine 123 (final concentration of 10 μM) for 30 min in the dark. Cells were washed again with PBS and ten thousand cells per sample were measured by flow cytometry (Becton-Dickinson, USA).

4.7. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Statistical analysis was performed by ANOVA with Tukey's HSD post-hoc test using SPSS11.0. The *P* values less than 0.05 were considered statistically significant.

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