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·Original Article·

Formaldehyde increases intracellular calcium concentration in primary cultured hippocampal neurons partly through NMDA receptors and T-type calcium channels

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Abstract: Objective Formaldehyde at high concentrations is a contributor to air pollution. It is also an endogenous metabolic product in cells, and when beyond physiological concentrations, has pathological effects on neurons. Formaldehyde induces mis-folding and aggregation of neuronal tau protein, hippocampal neuronal apoptosis, cognitive impairment and loss of memory functions, as well as excitation of peripheral nociceptive neurons in cancer pain models. Intracellular calcium ($[Ca^{2+}]_i$) is an important intracellular messenger, and plays a key role in many pathological processes. The present study aimed to investigate the effect of formaldehyde on $[Ca^{2+}]_i$ and the possible involvement of N-methyl-*D*-aspartate receptors (NMDARs) and T-type Ca^{2+} channels on the cell membrane. **Methods** Using primary cultured hippocampal neurons as a model, changes of $[Ca^{2+}]_i$ in the presence of formaldehyde at a low concentration were detected by confocal laser scanning microscopy. **Results** Formaldehyde at 1 mmol/L approximately doubled $[Ca^{2+}]_i$. (2*R*)-amino-5-phosphonopentanoate (AP5, 25 μmol/L, an NMDAR antagonist) and mibefradil (MIB, 1 μmol/L, a T-type Ca^{2+} channel blocker), given 5 min after formaldehyde perfusion, each partly inhibited the formaldehyde-induced increase of $[Ca^{2+}]_i$, and this inhibitory effect was reinforced by combined application of AP5 and MIB. When applied 3 min before formaldehyde perfusion, AP5 (even at 50 μmol/L) did not inhibit the formaldehyde-induced increase of $[Ca^{2+}]_i$, but MIB (1 μmol/L) significantly inhibited this increase by 70%. **Conclusion** These results suggest that formaldehyde at a low concentration increases $[Ca^{2+}]_i$ in cultured hippocampal neurons; NMDARs and T-type Ca^{2+} channels may be involved in this process.

Keywords: formaldehyde; intracellular calcium; neuronal activation; NMDA receptors; T-type calcium channels

1 Introduction

It is generally acknowledged that formaldehyde is a component of air pollution. Inhaled formaldehyde is toxic or lethal to human beings when its concentration reaches 0.08% (~26.7 mmol/L)[1]. High concentrations induce not only DNA damage, leukemia, and carcinoma, but also neuronal degeneration in Parkinson's disease[2,3]. In fact, formaldehyde is produced as a universal metabolic product by methylation and demethylation during biological processes within cells under physiological conditions[4]. The physiological concentration of endogenous formaldehyde in human blood was found at approximately 0.1 mmol/L[5]. In recent years, researchers have found that endogenous, metabolically-produced formaldehyde also has pathological effects when it exceeds physiological concentrations.

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We found that formaldehyde concentration is elevated in the spinal cord of rats with chronic pain[6]. Formaldehyde derived from local metastasized tumor tissues excites peripheral nociceptive neurons and thus can induce cancer pain[7]. More surprisingly, we and others found that formaldehyde at low pathological concentrations induces misfolding and aggregation of neuronal tau protein, leading to apoptosis of hippocampal neurons *in vitro*[8], and even cognitive impairment in human beings and loss of memory functions in animals[9,10].

Neuronal activation is a common event during these pathological processes. Increased intracellular calcium concentration ($[Ca^{2+}]_i$) is an important trigger for neuronal activation[11]. These findings raised the interesting question of whether formaldehyde increases $[Ca^{2+}]_i$ in neurons.

Voltage-dependent and ligand-gated Ca^{2+} channels in the cell membrane are the major pathways for Ca^{2+} entry into neurons. It is well known that N-methyl-*D*-aspartate receptors (NMDARs) are the dominant Ca^{2+} influx channels in the cell membrane. They are activated by their agonists and membrane depolarization. T-type voltage-gated Ca^{2+} channels in neurons are also important for Ca^{2+} entry. They are sensitive to membrane potential and have a low voltage threshold. For example, the threshold for T-type Ca^{2+} channel activation is around −60 mV in hippocampal neurons[12]. Activation of these channels mediates low-threshold Ca^{2+} spikes and after-depolarization potentials which are important for regulating neuronal excitability[13,14].

In the present study, using primary cultured hippocampal neurons as a model, we assessed the effect of formaldehyde at a low pathological concentration on $[Ca^{2+}]_i$. The possible involvement of NMDARs and T-type voltage-gated Ca^{2+} channels in this process was also investigated.

2 Materials and methods

2.1 Animals Pregnant female Sprague-Dawley rats were provided by the Department of Experimental Animal Sciences, Peking University Health Science Center. All experiment protocols were approved by the Animal Use and Care Committee of Peking University, and were in accordance with the Guidelines of Animal Use and Protection from the NIH, USA adopted at Peking University.

2.2 Chemicals (2*R*)-amino-5-phosphonopentanoate (AP5; Sigma-Aldrich, St. Louis, MO) and mibefradil (MIB; Sigma-Aldrich) were each dissolved in sterile deionized water as stock solutions (AP5 at 25 mmol/L; MIB at 10 mmol/L). Both were diluted to appropriate concentrations in Hanks' balanced salt solution (HBSS) just before use. Formaldehyde was diluted to 10 mmol/L just before use.

2.3 Primary culture of hippocampal neurons Hippocampal neurons were prepared from Sprague-Dawley rats on embryonic day 18 after pregnant rats were anesthsized in a sealed glass container with 5% isoflurane mixed with air. Cells were seeded onto coverslips pre-coated with poly-*D*-lysine in a confocal microscopy chamber (Lab-Tek™, Nunc, Thermo Fisher Scientific Inc., Pittsburgh, PA) at a density of 2×10^5 cells/cm^2 and incubated at 37°C under 5% CO_2. After 4 h, the culture medium was replaced with neurobasal medium supplemented with 2% B-27 and 0.5 mmol/L glutamine, and then the medium was half-replaced every 3–4 days. Cultures were used after 7–10 days *in vitro* (DIV).

2.4 Intracellular calcium concentration measurement Primary hippocampal neurons at 7–10 DIV were loaded with 50 μmol/L Fluo-3/AM (Molecular Probes, Life Technologies Corp., Grand Island, NY) diluted in HBSS and incubated for 30 min at 37°C. The neurons were then washed 3 times with indicator-free HBSS to remove the dye. $[Ca^{2+}]_i$ was detected with a confocal laser scanning microscope (Leica Co. Ltd., Germany). Fluo-3/AM was excited at 488 nm, and emitted fluorescence was collected at 515 nm.

The microscope was set to scan every 5 s, and a pause of 10 s was set for drug application. The drugs were carefully applied with pipettes and the added volume was kept at <10% of the original volume so as not to induce fluctuations in the level of the liquid. At the beginning of each experiment, there was a 30-s period to measure basal fluorescence intensity. Then formaldehyde, at a final concentration of 1 mmol/L, was added into the bath solution, and the fluorescence intensity in loaded hippocampal neurons was scanned for 5 min (defined as the first 5-min experimental interval). Antagonists (AP5 or MIB) or HBSS

(as a negative control) were added, followed by another 5-min scan (defined as the second 5-min experimental interval). To test the viability of neurons after formaldehyde perfusion, KCl at a final concentration of 60 mmol/L was added to the bath solution, followed by a 3-min scan. For a positive control, specific agonists of NMDARs (NMDA and glycine, at final concentrations of 250 μmol/L and 10 μmol/L, respectively), instead of formaldehyde, were each added, and the NMDAR antagonist (AP5) was then added at a final concentration of 25 μmol/L.

2.5 Data analysis The basal intensity was set as the average value of six measurements (30-s baseline measurement) of fluorescence intensity. During the first 5-min experimental interval, the average of five fluorescence intensity values around the highest value was used as the intensity after treatment. During the second 5-min experimental interval, the average of five fluorescence intensity values around the lowest value was used to estimate the possible effect of antagonist treatment. All data are expressed as mean ± SEM. One-way ANOVA followed by Tukey's post-test was used for multiple comparisons. Two-tailed unpaired Student's *t*-test was used for comparison between two groups. P <0.05 was considered statistically significant.

3 Results

We first determined whether formaldehyde at a low pathological concentration increases $[Ca^{2+}]_i$ in primary hippocampal neurons at 7–10 DIV. Based on the average concentration of formaldehyde in cancer tissues from patients[7], we used 1 mmol/L as the test concentration. Formaldehyde at this level approximately doubled the $[Ca^{2+}]_i$ (Fig. 1A, B). The subsequent addition of 60 mmol/L KCl induced a sharp increase of neuronal fluorescence intensity, indicating good cell viability during the formaldehyde testing (Fig. 1C). KCl is a classic membrane depolarizing agent, and 60 mmol/L KCl does not induce membrane breakdown according to previous reports[15].

In order to test whether NMDARs are involved in the formaldehyde-induced $[Ca^{2+}]_i$ increase in hippocampal neurons, the NMDAR antagonist AP5 (25 μmol/L) was given 5 min after formaldehyde (1 mmol/L) perfusion. The Ca^{2+} fluorescence intensity of hippocampal neurons declined by ~22%, significantly lower than the value after formaldehyde treatment (Fig. 2A, D).

As positive controls, the NMDAR agonists NMDA (250 μmol/L) and glycine (10 μmol/L) were added instead of formaldehyde. We found that 25 μmol/L AP5 reduced the fluorescence intensity by 60% when given after the NMDAR agonists (Fig. S1), indicating that 25 μmol/L AP5 was sufficient for NMDAR blockade. These results suggested that the increased fluorescence intensity in hippocampal neurons induced by formaldehyde is partly attributable to NMDARs.

To exclude the possibility that the reduced fluorescence intensity was induced by fluctuations in the liquid level due to antagonist perfusion, we used 10 μL HBSS (equal volume to the added antagonist) as a negative control. We found that HBSS did not influence the fluorescence intensity (Fig. S1), suggesting that it was the pharmacological effect of AP5 that led to the reduced fluorescence intensity.

In fact, other than high Ca^{2+} permeability, NMDAR activation is voltage-dependent and can be blocked by Mg^{2+} at resting membrane potential[16,17]. The membrane potential must be depolarized to −40 mV before NMDARs are relieved from this blockade. In the present study of neurons in the resting state, NMDARs in the hippocampal neurons, in large part, would have been blocked by the Mg^{2+} in HBSS. So, other factors may help relieve this blockade. Therefore, we tested T-type Ca^{2+} channels, which are characterized by activation at a low voltage.

T-type Ca^{2+} channels are widely expressed in the central nervous system including the hippocampus, and their activation threshold is close to the resting membrane potential of −60 mV[12]. We first determined whether T-type Ca^{2+} channels are activated by 1 mmol/L formaldehyde. We added MIB, a specific blocker of T-type Ca^{2+} channels, at a final concentration of 1 μmol/L (in a volume of 2 μL), which was much higher than its half-maximal inhibitory concentration in the hippocampus[18]. When MIB was given after 1 mmol/L formaldehyde perfusion, the Ca^{2+} fluores-

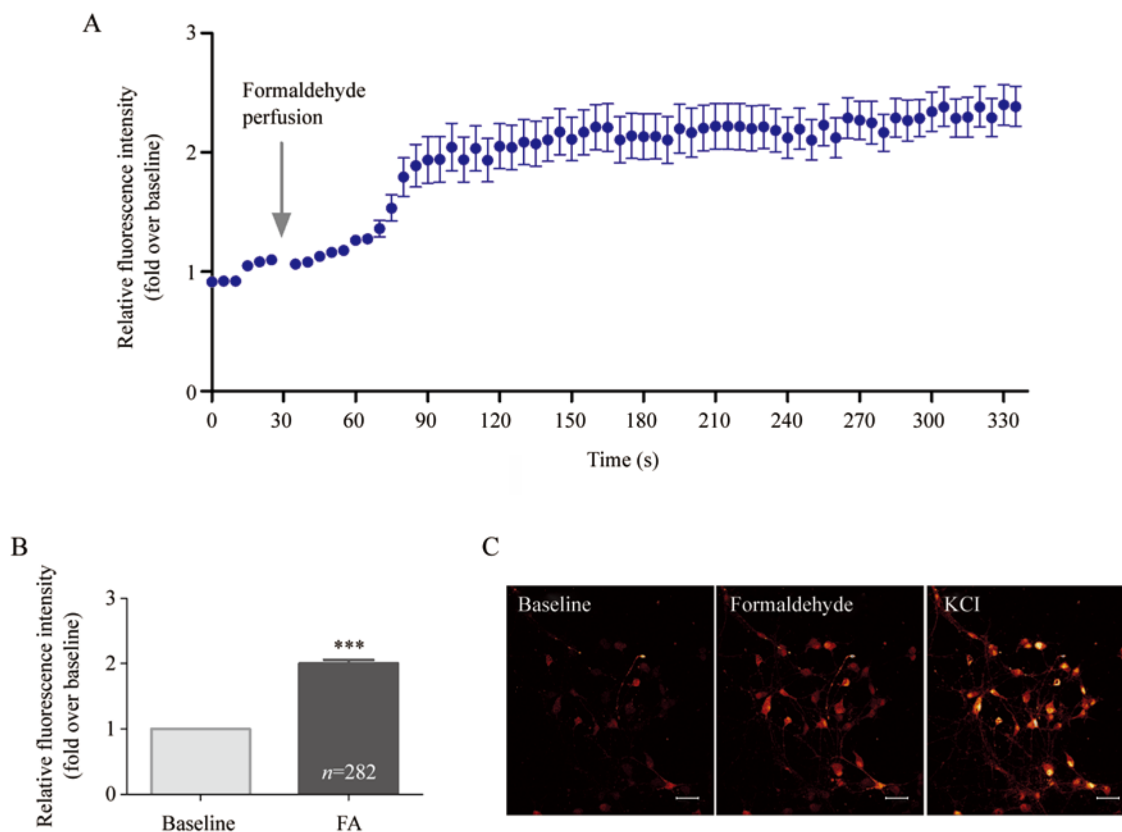

Fig. 1. Formaldehyde at 1 mmol/L increases intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in primary cultured hippocampal neurons. A: Time-course of Fluo-3/AM fluorescence intensity changes indicative of $[Ca^{2+}]_i$ after formaldehyde application. B: Statistical analysis of fluorescence intensity changes. Formaldehyde (FA) increased the intensity compared with baseline. $^{***}P$ <0.001, unpaired *t*-test. C: Representative confocal images of hippocampal neurons loaded with Fluo-3/AM for 30 min (baseline), after perfusion with formaldehyde at 1 mmol/L, followed by KCl at 60 mmol/L. Scale bars, 40 µm.

cence intensity showed a ~25% reduction (Fig. 2B, D).

In addition, a combination of 25 µmol/L AP5 and 1 µmol/L MIB reduced the formaldehyde-induced fluorescence intensity by 31%, which was significantly greater than that found after AP5 or MIB administration alone (Fig. 2C, D). These results suggested that both NMDARs and T-type Ca^{2+} channels participate in the formaldehyde-induced increase of $[Ca^{2+}]_i$ in hippocampal neurons.

To find out which, NMDARs or T-type Ca^{2+} channels, plays a role in initiating the formaldehyde-induced Ca^{2+} influx, we administered AP5 or MIB 3 min before formaldehyde (1 mmol/L) perfusion. Pre-treatment with AP5 at 25 µmol/L, and even 50 µmol/L, did not inhibit the formaldehyde-induced increase of fluorescence intensity, but inhibited the KCl-induced increase in a concentration-dependent manner (Fig. 3). This result suggested that NMDARs are not involved in the initiation of the formaldehyde-induced increase of $[Ca^{2+}]_i$.

In contrast, pre-treatment with MIB (1 µmol/L) significantly inhibited the formaldehyde-induced increase of $[Ca^{2+}]_i$ (Fig. 4). Only a 38% increase relative to the basal fluorescence intensity was found, which was significantly lower than the 97% increase after formaldehyde perfusion without MIB pre-treatment. Meanwhile, combined pre-treatment with AP5 (even at 50 µmol/L) and MIB (1 µmol/L) significantly inhibited the formaldehyde-induced increase of $[Ca^{2+}]_i$ (40% increase relative to the basal fluorescence intensity, similar to that of pre-treatment with MIB alone).

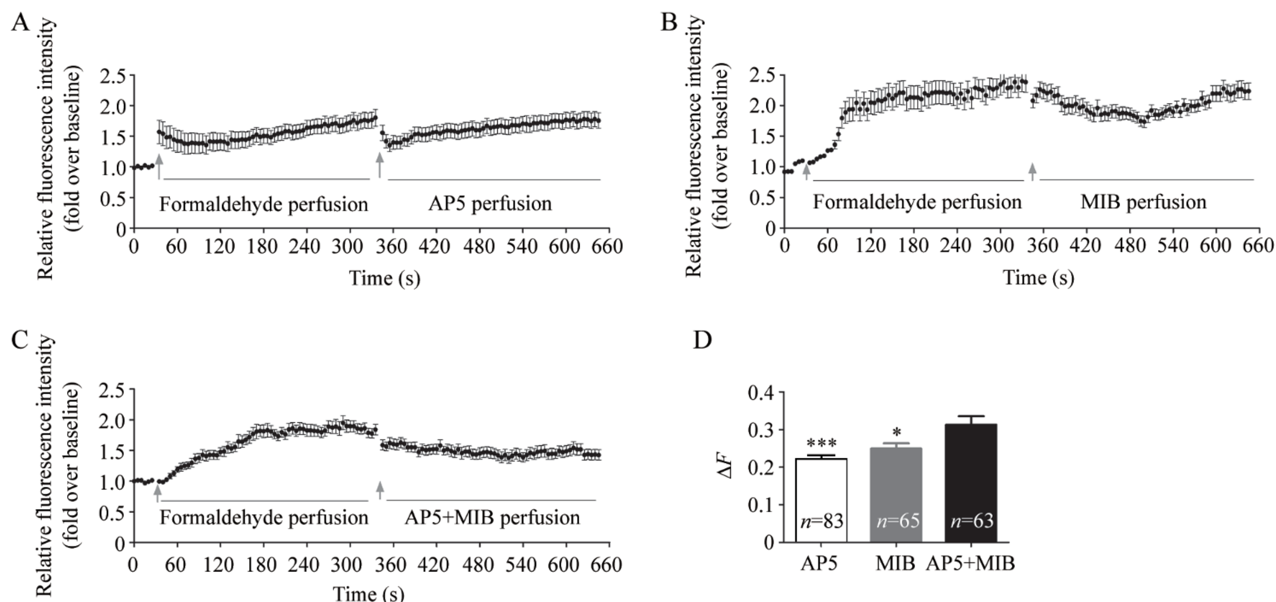

Fig. 2. An NMDAR antagonist (AP5) and the T-type Ca^{2+} channel blocker mibefradil (MIB) inhibit the formaldehyde-induced increase of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in primary cultured hippocampal neurons. A–C: Time-courses of Fluo-3/AM fluorescence intensity changes after formaldehyde perfusion followed by AP5 (A), MIB (B) and combined treatment (C). D: Statistical analysis of fluorescence intensity changes. The combination of AP5 and MIB had a greater inhibitory effect than AP5 or MIB alone. $\Delta F = (F_1-F_2)/F_1$, in which F_1 represents the averaged value of five fluorescence intensity values around the highest value after 1 mmol/L formaldehyde perfusion, and F_2 represents the averaged value of five fluorescence intensity values around the lowest value after AP5 or MIB treatment. *P <0.05, ***P <0.001, one-way ANOVA followed by Tukey's post-tests.

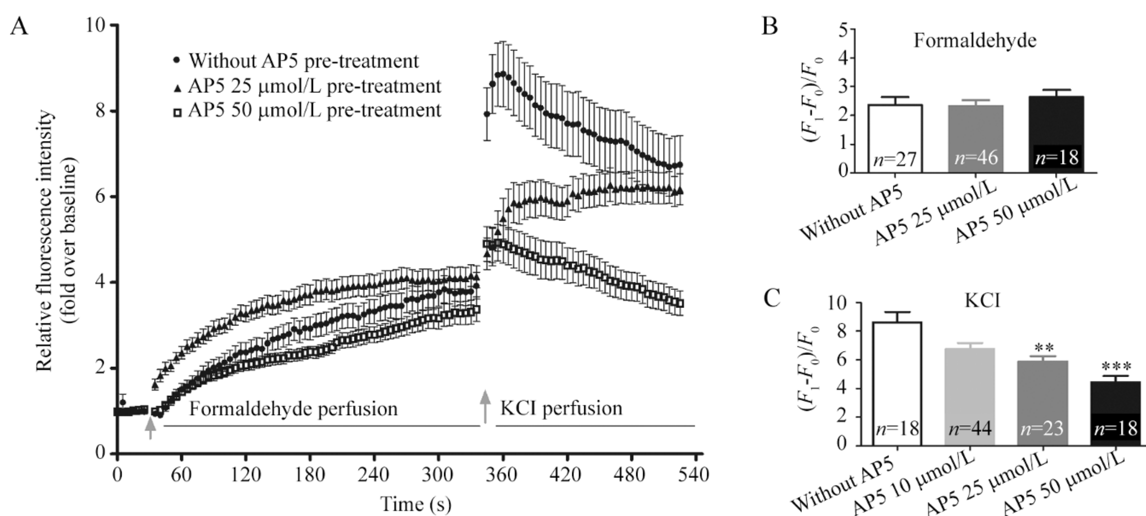

Fig. 3. Effects of NMDAR antagonist (AP5) pre-treatment on formaldehyde-induced increase of $[Ca^{2+}]_i$ in hippocampal neurons. A: Time-course of Fluo-3/AM fluorescence intensity changes. AP5 was added 3 min before formaldehyde perfusion; KCl was added 5 min after formaldehyde perfusion. B: Statistical analysis of fluorescence intensity changes after formaldehyde perfusion with AP5 pre-treatment. C: Statistical analysis of fluorescence intensity changes after KCl perfusion. AP5 did not have any effect on the formaldehyde-induced increase of fluorescence intensity (B) although it inhibited the KCl-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner (C). F_1 represents the averaged value of the five fluorescence intensity values around the highest value after formaldehyde or KCl perfusion, while F_0 represents the averaged value of the fluorescence intensity during the baseline 30-s scans. **P <0.01, ***P <0.001, one-way ANOVA followed by Tukey's post-test.

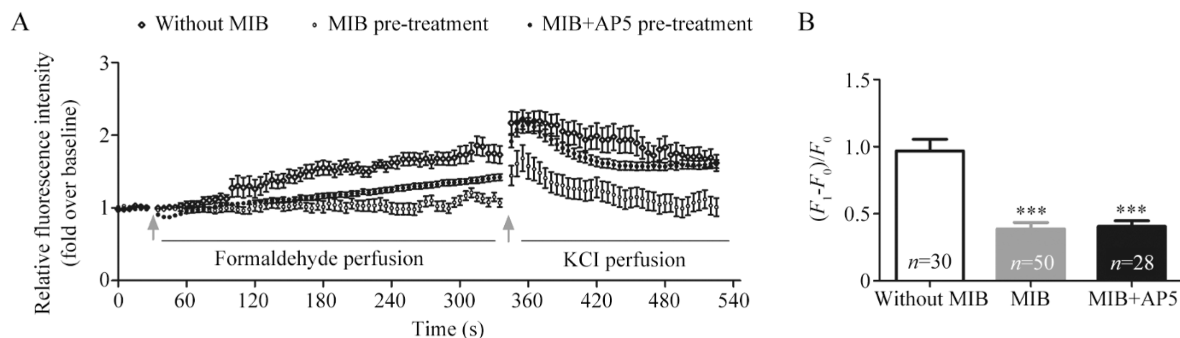

Fig. 4. Effects of pre-treatment with mibefradil (MIB) alone or MIB plus AP5 on formaldehyde-induced increase of $[Ca^{2+}]_i$ in cultured hippocampal neurons. A: Time-course of Fluo-3/AM fluorescence intensity changes. MIB or a combination of MIB plus AP5 was added 3 min before formaldehyde perfusion; KCl was added 5 min after formaldehyde perfusion. B: Statistical analysis of fluorescence intensity changes after formaldehyde perfusion with MIB or MIB plus AP5 pre-treatment. MIB alone inhibited the formaldehyde-induced increase of fluorescence intensity. The combination of MIB with AP5 did not enhance the MIB inhibitory effect. ******P* <0.001, one-way ANOVA followed by Tukey's *post hoc* test.

These results indicated that T-type Ca^{2+} channels, but not NMDARs, play a role in initiating the Ca^{2+} influx into hippocampal neurons after formaldehyde treatment.

4 Discussion

In the present study, we found that formaldehyde perfusion at a low pathological concentration increased intracellular Ca^{2+} fluorescence intensity in primary cultured hippocampal neurons. By using NMDAR antagonists and T-type Ca^{2+} channel blockers, we further showed that this increase of $[Ca^{2+}]_i$ was mediated by NMDARs and T-type Ca^{2+} channels, and in addition, the latter participated in the initiation of this increase.

Formaldehyde is a generally known toxin. Formalin, which is formaldehyde in solution, is commonly used to establish pain models in rats and mice when injected into the paw[7]. In fact, formaldehyde exists within the body at low concentrations. This endogenous formaldehyde is a consistent metabolic product of methylation and demethylation, and can be detected in various tissues and body fluids[19]. Its generation and elimination are closely associated with the methionine-homocysteine cycle[20]. The physiological concentration of endogenous formaldehyde in human blood is ~0.1 mmol/L[5]. It can even pass through the blood-brain barrier[21] and damage the central nervous system.

In our previous study, we found that the formaldehyde concentration is as high as ~1 mmol/L in tumor tissues from cancer patients[7]. Breast cancer tissue-derived formaldehyde is elevated in the bone marrow when the cancer cells metastasize into bone. Tissue-derived endogenous formaldehyde even at such a low concentration can induce pain behavior[7,22]. Previous studies have demonstrated that formaldehyde exposure at 0.01–0.1% (~3.3 to ~33 mmol/L) induces mis-folding and aggregation of tau protein in hippocampal neurons[9], which is essential to beta-amyloid-induced neurodegeneration[23]. Cognitive impairments in senile dementia have also been associated with elevated endogenous formaldehyde concentration[10]. These reports suggest that the endogenous formaldehyde produced in tissues at low concentrations plays a pathological role in neurons. In the present study, formaldehyde perfusion at 1 mmol/L induced elevation of $[Ca^{2+}]_i$ in hippocampal neurons (Figs. 1 and 2), providing direct evidence that low levels of formaldehyde affect primary cultured hippocampal neurons. These results also give preliminary evidence for the participation of NMDARs and T-type Ca^{2+} channels in the formaldehyde-induced increase of $[Ca^{2+}]_i$ in hippocampal neurons.

Ca^{2+} in the cytoplasm of a neuron plays a pivotal role in controlling universal cellular events, such as hormone and peptide secretion, and even gene transcription and cell proliferation. Neuronal activation is the first and most important consequence of Ca^{2+} entry. The NMDAR is the

predominant receptor-operated Ca^{2+} channel[24], and the T-type Ca^{2+} channel is a voltage-dependent channel with a low threshold for activation. T-type Ca^{2+} channel-mediated Ca^{2+} spikes are important for neuronal excitation[13], and the transient Ca^{2+} current then triggers burst firing in adult mammalian neurons[14].

In our present study, using primary cultured hippocampal neurons as a model, we showed that the NMDAR antagonist AP5 and T-type Ca^{2+} channel blocker MIB inhibited the formaldehyde-induced increase of $[Ca^{2+}]_i$ (Fig. 2). This provides direct evidence that NMDARs and T-type Ca^{2+} channels are involved in the formaldehyde-induced increase of $[Ca^{2+}]_i$ in hippocampal neurons.

Meanwhile, we noted that the combination of AP5 and MIB inhibited only about 30% of the formaldehyde-induced increase of $[Ca^{2+}]_i$ (Fig. 2C, D). These results suggest that NMDARs and T-type Ca^{2+} channels play important roles in the formaldehyde-induced influx of $[Ca^{2+}]_i$ but there must be additional mechanisms.

Excessive Ca^{2+} influx through NMDARs induces neuronal excitotoxicity. For example, NMDARs are closely associated with neurodegeneration[25], and have been shown to be potential therapeutic targets for Parkinson's disease[26]. To some extent, our results provide an explanation for possible formaldehyde excitotoxicity to neurons.

It is well known that NMDARs are blocked by extracellular Mg^{2+} at resting membrane potential, and the blockade is relieved when the membrane potential is depolarized to −40 mV[17]. In the present study, in resting neurons, NMDARs in hippocampal neurons in buffer would be largely blocked by Mg^{2+}. T-type Ca^{2+} channels activate at low voltages. The present study showed that T-type Ca^{2+} channels initiated the formaldehyde-induced Ca^{2+} entry into hippocampal neurons before NMDARs were activated (Fig. 4). We speculate that when formaldehyde is applied to the cell surface of hippocampal neurons, the T-type Ca^{2+} channels are activated first. A large amount of Ca^{2+} flows into the neurons through the T-type Ca^{2+} channels, which then depolarizes the neuronal membrane. When the membrane potential is depolarized to −40 mV or higher, the blocking effect of Mg^{2+} on NMDARs is relieved and they can be fully activated.

In summary, using primary cultured hippocampal neurons as a model, we found that formaldehyde at a low concentration induced an increase of $[Ca^{2+}]_i$. More interestingly, membrane NMDARs and T-type Ca^{2+} channels were important mediators of this increase. These results provide an explanation for the physiological and pathological effects of tissue-derived endogenous formaldehyde in neuron activation.

Supplemental Data: Supplemental Data include one figure and can be found online at http://www.neurosci.cn/epData.asp?id=47.

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