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NMDA and AMPA receptors mediate intracellular calcium increase in rat cortical astrocytes[1]

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KEY WORDS glutamate; astrocytes; NMDA receptors; AMPA receptors; calcium

ABSTRACT

AIM: To study the effect of glutamate on the intracellular calcium signal of pure cultured rat astrocytes and the role of *N*-methyl-*D*-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors in the procedure. **METHODS:** The fluorescence of calcium was measured by Fura-2/AM (F_{345}/F_{380}). **RESULTS:** *L*-Glutamate induced $[Ca^{2+}]_i$ increase in most of the cells in concentration- and time-dependent manner. NMDA 50 mmol/L induced the fluorescence increase by almost three to four times, while the effect of AMPA 50 mmol/L was just half of that of *D*-(-)-2-amino-5-phosphonopentanoic acid (*D*-AP-5; a selective antagonist of the NMDA receptor). 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, a selective antagonist of the AMPA receptor) abolished the effects of NMDA and AMPA, respectively. *D*-AP-5 and CNQX simultaneously or respectively attenuated the effect of *L*-glutamate at different degrees, but could not abolish it entirely. **CONCLUSION:** Glutamate modulated intracellular Ca^{2+} of pure cultured rat astrocytes through different pathways. The activation of NMDA and AMPA receptors took part in the complex mechanisms.

INTRODUCTION

Almost 50 % of cells in central nervous system are astrocytes. They play an important role in normal physiological activity and have intimate relationship with neurons. There is a close bidirectional communication existing between neurons and astrocytes[1]. Glutamate, as the most important excitatory transmitter in central nervous system, is proved to be a crucial bridge between astrocytes and neurons. Astrocytes responded to glutamate released from neurons by intracellular Ca^{2+} increase under physiological conditions[2]. On the other hand, recent Ca^{2+} imaging studies in cell culture and *in situ* showed that Ca^{2+} elevations in astrocytes induced glutamate release in a calcium-dependent manner[3,4]. Therefore, the modulation of Ca^{2+} elevations in astrocyte by glutamate should aid in understanding the interaction between astrocytes and neurons[5].

The mechanisms of calcium mobilization in astrocytes include: (1) Calcium stores. There are two types of calcium stores within the cultured cortex astrocytes of rats: one is IP_3-sensitive, the other is IP_3-insensitive, and mitochondria could serve as an intracellular calcium buffering system in astrocytes[6,7]. (2) Na^+/Ca^{2+} exchange. It regulated $[Ca^{2+}]_i$ when cytosolic Ca^{2+} increased ten times higher than the resting level. Immunohistochemistry revealed that exchanger molecules distributed in a reticular pattern over the astrocyte surface[8]. (3) Voltage-operated calcium channel and receptor-operated calcium channels. Glutamate receptor

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(GluR) was one of the most important receptors which took part in the astrocytic Ca^{2+} mobilization[9].

There are two classes of glutamate receptors on astrocytes: one is ionotropic receptor linked directly to an ion channel, and the other is metabotropic receptor which induces internal mobilization of Ca^{2+} via inositol phospholipid hydrolysis[10,11]. Glutamate receptor-mediated responses were detected by imaging Ca^{2+} in astrocytes. *N*-Methyl-*D*-aspartate (NMDA) and alpha-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors were found to be expressed in astrocytes through immunocytochemistry methods[12,13]. Intracellular astrocyte calcium played an important role in astrocyte-to-neuron signaling[14,15]. But astrocytes from different animals, different developing periods, even different positions expressed different glutamate receptors. By Northern blot analysis, GluR1 mRNA was the highest in astrocyte cultures from cerebellum and hippocampus and moderate in astrocyte cultures from neocortex and striatum. GluR3 mRNA was detectable in astrocyte cultures from cerebellum and neocortex. GluR2 (the subunit that limits the Ca^{2+} permeability of AMPA receptor) and NR1 mRNA expression were not detected in astrocytes cultured from any brain region examined. *In situ* hybridization studies showed wide expression of GluR1 mRNA in cultured astrocytes, but GluR2 and GluR3 mRNAs were near background levels[16]. The AMPA type-glutamate receptor channels without the GluR2 (GluR-B) subunit were characterized by high Ca^{2+} permeability[17].

In this study, we investigated effects of NMDA and AMPA receptors on type-1 astrocytes from cortex of neonatal rats on intracellular Ca^{2+}.

MATERIALS AND METHODS

Chemicals and drugs Fetal bovine serum, DMEM/F12 were purchased from Gibco Company. HEPES, NMDA, AMPA, *D*-(-)-2-Amino-5-phosphonopentanoic acid (*D*-AP-5; a selective antagonist of NMDA receptor), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, a selective antagonist of AMPA receptor), *L*-glutamate, poly-lysine, trypsin, egtazic acid, and thapsigargin were purchased from Sigma Company. Fura-2/AM was purchased from Molecular Probe Company. Other ordinary drugs were from local chemical drug companies.

Cell culture Sprague-Dawley rats (1-3 d postnatal) were obtained from the Experimental Animal Center of Tongji Medical School, Huazhong University of Science and Technology. All experiments were carried out under the National Animal Protection Protocol. The rats were decapitated under ether anaesthesia. Cortex slice of 500-800 μm thick was cut. The slices were treated with 0.25 % trypsin and incubated in culture medium saturated with 95 % O_2 and 5 % CO_2 at room temperature (21-23 ºC) for 30 min. Culture medium consisted of 78 % DMEM/F12, 10 % fetal calf serum, 1 % benzylpenicillin, and streptomycin 100 mg/L. Cells were maintained in an incubator of 10 % CO_2 at 37 ºC. Seven to nine days later, the culture bottles were put in 37 ºC vibratory device for 15 to 18 h, then passaged every week, and plated on poly-lysine-coated coverslip at cell density of $5\times10^8\ L^{-1}$. Coverslips incubated for 3-7 d were used. The cytoimmunochemical results showed that 98 % of the cells were glial fibrilary acidic protein-positive glial cells. Most of the cells were type-1 astrocytes with multiple dendrites. So we chosed type-1 astrocytes as research samples.

Fura-2 loading The cell-bearing coverslips were rinsed three times with buffer solusion without calcium before loading and then incubated with Fura-2/AM (2-5 μmol/L) at 37 ºC for 30 min. The buffer solution contains (mmol/L): NaCl 140, KCl 5.0, $MgSO_4$ 1, *D*-glucose 10, 1,4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10, egtazic acid 100 μmol/L (pH=7.3).

Intracellular Ca^{2+} measurement The fluorescent microscopy is constructed around an Axiovert 100 (Zeiss, Germany). Excitation wave length was set at 345 nm or 380 nm by monochromator system (TILL, Photonics, Germany) controlled by computer. The fluorescence signals were collected by a photomultipler (Hammamashu R928) and converted to voltage values. X-CHART (Heka, Germany) was used to calculate and monitor the intracellular $[Ca^{2+}]_i$. The fluorescence ratio of F_{345}/F_{380} was used to reflect the fluctuation of $[Ca^{2+}]_i$[18].

Solutions The bath solution in most experiments was (in mmol/L): NaCl 140, HEPES 10, KCl 2, *D*-glucose 10, $MgCl_2$ 2, $CaCl_2$ 2.5, pH=7.3, with Osmolarity=320±5 mOsm/L. The solution without calcium was the same as above by replacing $CaCl_2$ with $MgCl_2$.

RESULTS

Glutamate induced astrocytic $[Ca^{2+}]_i$ increase The response of astrocytic $[Ca^{2+}]_i$ to glutamate was variable, about 83 % of cells had obvious response to the application of *L*-glutamate (n=120), while others

had mild response, and some cells exhibited calcium oscillation (data not shown).

Glutamate elevated $[Ca^{2+}]_i$ in a time- and concentration-dependent manner The magnitude of fluorescence increase induced by glutamate was related to simulation time. When glutamate 100 μmol/L was applied to stimulate the same cell at intervals at least 5 min to avoid receptor desensitization, the longer the stimulation time was, the higher the fluorescence was. As the response time became longer, the decline of fluorescence became slower (*n*=7, Fig 1A).

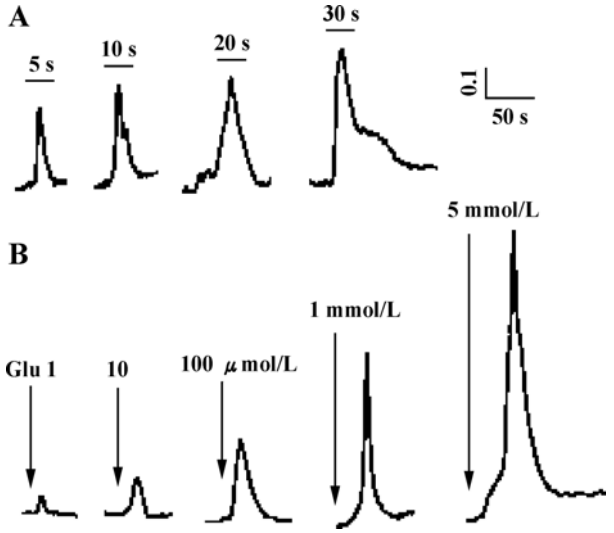

Fig 1. Effect of *L*-glutamate (Glu) on $[Ca^{2+}]_i$ of astrocytes in normal extracellular solution. A) Glu 100 μmol/L was applied for 5 s, 10 s, 20 s, and 30 s on the same cell at intervals at least 5 min to avoid receptor desensitization; B) Glu 1 μmol/L−5 mmol/L was applied on the same cell for 10 s at intervals at least 5 min to avoid receptor desensitization.

Different concentrations of glutamate were applied to stimulate the same cell for 10 s at least for 5 min to avoid receptor desensitization. Glutamate induced Ca^{2+} increase in a concentration-dependent manner. When the concentration was 50 mmol/L, the elevated $[Ca^{2+}]_i$ reached a plateau and did not decline to baseline, which was followed by cell death. The EC_{50} was about 5 mmol/L in normal calcium extracellular solution (Fig 1B).

Effect of NMDA receptor on astrocytes response to glutamate Of all the cells respond to glutamate, the response of 87 % (*n*=66) cells induced by glutamate 1 mmol/L were diminished by *D*-AP-5 100 μmol/L, but could not be abolished entirely (Fig 2). It demonstrated that effect of glutamate on astrocytic

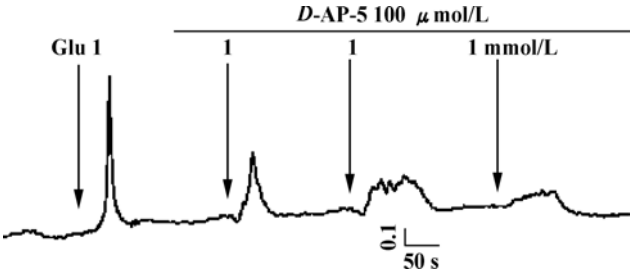

Fig 2. Effect of *D*-AP-5 on the increase of astrocytic $[Ca^{2+}]_i$ induced by glutamate.

calcium signal was partially through activation of NMDA receptors.

About 72 % of all the cells responded to NMDA with $[Ca^{2+}]_i$ increase (*n*=50). The response induced by NMDA 50 μmol/L could be almost abolished by *D*-AP-5 100 μmol/L, and it recovered after a long enough interval (Fig 3), which indicated that the activation of NMDA-receptor took part in astrocytic calcium signal modulation.

Effect of non-NMDA receptor on response of astrocytes to glutamate Of all the cells respond to glutamate, the response of 83 % (*n*=70) cells induced by glutamate 1 mmol/L was diminished by CNQX 45 μmol/L, but could not be abolished entirely (Fig 4).

About 81 % of all the cells responded to AMPA with $[Ca^{2+}]_i$ increase (*n*=56). The response induced by AMPA 50 μmol/L was markedly diminished by CNQX 45 μmol/L, and it recovered after a long enough interval (Fig 5). It demonstrated that the activation of AMPA-receptor took part in astrocytic calcium signal modulation.

When CNQX and *D*-AP-5 was applied simultaneously on the same cell (*n*=23), the increased $[Ca^{2+}]_i$ was inhibited significantly, but could not be abolished completely (Fig 6, 7). It suggested that perhaps there were other mechanism such as metabotropic receptor might take part in the reaction induced by glutamate.

Effect of extracellular Ca^{2+} in the response of astrocyte to glutamate In extracellular solution without calcium or after Ca^{2+}-chelator egtazic acid 2 mmol/L was added in extracellular solution, the response of astrocytes to glutamate became weaker (Fig 8, *n*=22). In normal extracellular solution, the elevation of astrocytic $[Ca^{2+}]_i$ induced by glutamate could be inhibited entirely after stimulation with thapsgargin (Fig 9). It shows that response of glutamate is extracellular-Ca^{2+} independent, and there is other mechanism except

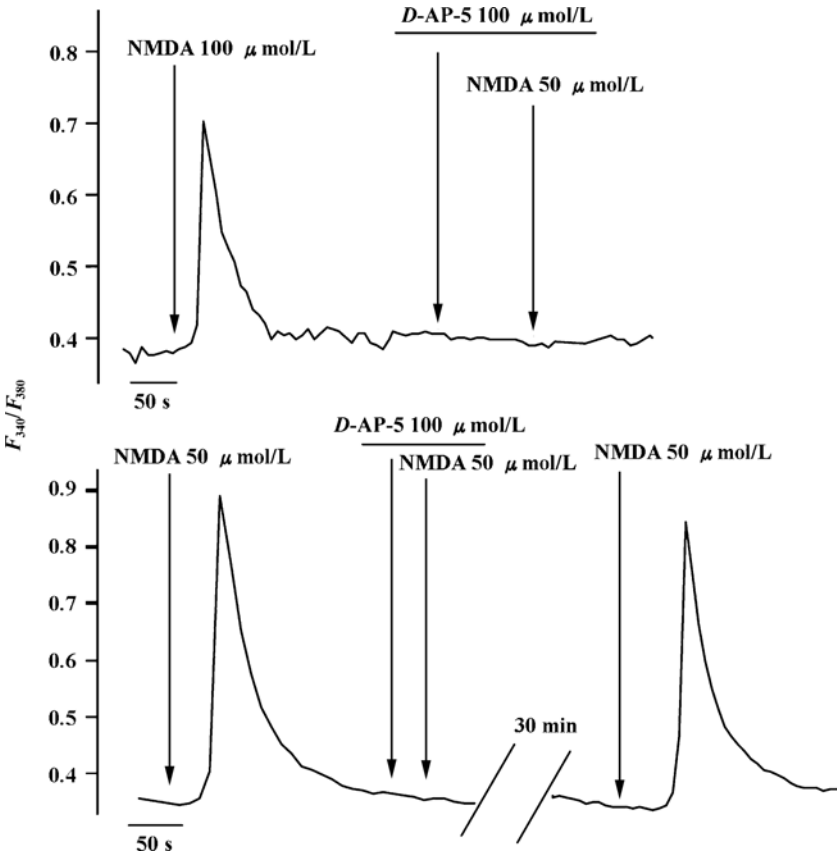

Fig 3. Effect of NMDA on astrocytic $[Ca^{2+}]_i$ in normal extracellular solution.

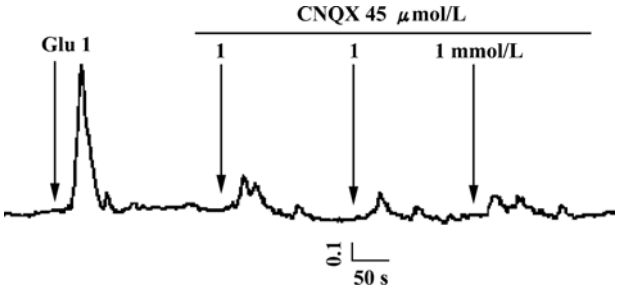

Fig 4. Effect of CNQX on increase of astrocytic $[Ca^{2+}]_i$ induced by glutamate.

Na^+/Ca^{2+} exchange.

DISCUSSION

When cerebral ischemia or damage occurr, glutamate receptors are activated excessively and extracellular glutamate is elevated rapidly[19-21]. Increase in the concentration of intercellular glutamate protected astrocytes during this process. Concentration and diffusion of glutamate in the extracellular space are associated with the degree of astrocytic coverage to neurons[22,23]. Previous work discovered that glutamate reuptake by astrocytes existed not only under pathological states but also under physiological states[2,24-26].

Non-NMDA receptors contributed to partial elevation of $[Ca^{2+}]_i$. CNQX , a potent competitive antagonist of the AMPA/Kainate (non-NMDA) receptor, decreased the elevation of $[Ca^{2+}]_i$ induced by glutamate. Consisted with our results, some evidence showed that astrocytes expressed Ca^{2+}-permeable AMPA receptors[2,11,27]. Aactivation of AMPA receptors in astrocytes caused increase in $[Ca^{2+}]_i$ through the reverse mode operation of the Na^+/Ca^{2+} exchanger with an associated release of Ca^{2+} from intracellular stores[27,28]. Other conflicting results demonstrated that activation of AMPA and NMDA receptor did not affect intracellular calcium stores[29].

Smith *et al* proposed that astrocytes exhibited three transmembrane Ca^{2+} influx pathways: voltage-gated Ca^{2+} channels (VGCCs), AMPA class of glutamate receptors, and Na^+/Ca^{2+} exchangers[28]. But our results was not the same as it completely. Immunocytochemistry and physiology proved that astrocytes expressed NMDA receptors[13,30]. When *D*-AP-5, a selective NMDA receptor antagonist, was applied, response of glutamate

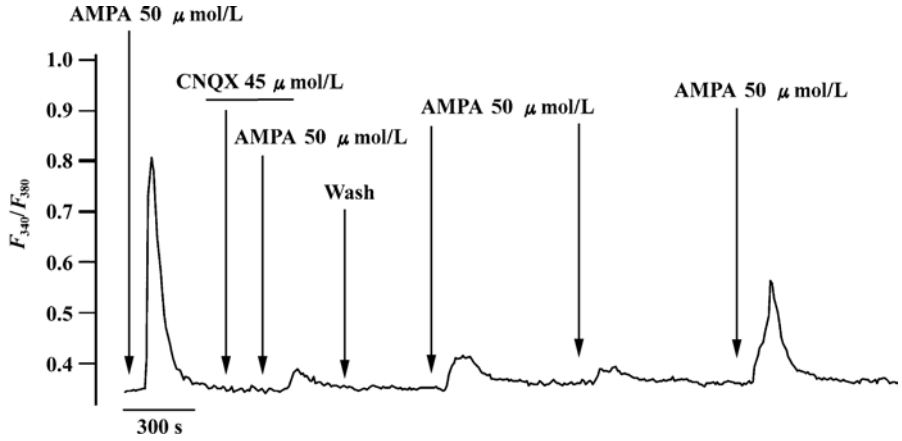

Fig 5. Effect of AMPA on increase of astrocytic $[Ca^{2+}]_i$ in normal extracellular solution.

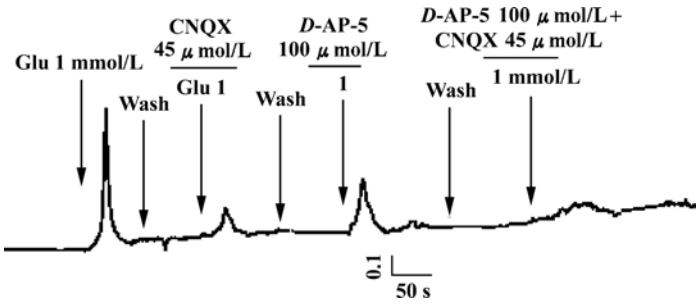

Fig 6. Effect of CNQX and *D*-AP-5 on increase of astrocytic $[Ca^{2+}]_i$ induced by glutamate in normal extracellular solution.

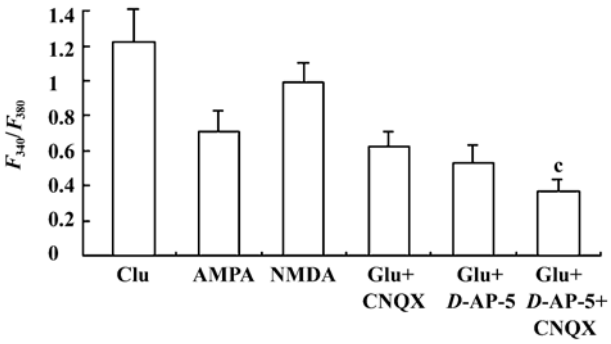

Fig 7. Comparison of the responses of astrocytes to Glu 1 mmol/L, AMPA 50 μmol/L, NMDA 50 μmol/L, Glu 1 mmol/L+CNQX 45 μmol/L, Glu 1 mmol/L+*D*-AP-5 100 μmol/L, and Glu 1 mmol/L+CNQX 45 μmol/L+*D*-AP-5 100 μmol/L. *n*=23. Mean±SD. $^cP<0.01$ *vs* Glu+CNQX group.

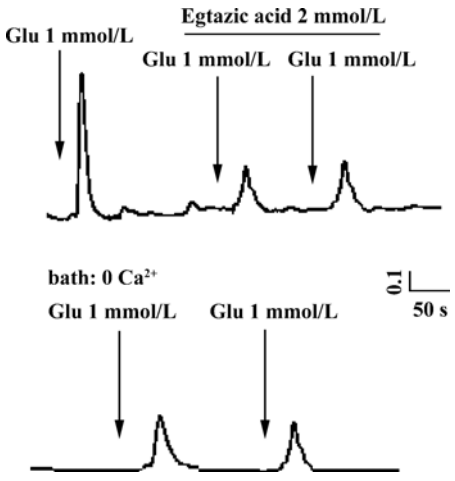

Fig 8. Effect of *L*-glutamate on $[Ca^{2+}]_i$ of astrocytes after addition of egtazic acid or in extracellular solution without calcium.

was abolished. It is an indirect proof of existence of NMDA receptor and its effects on $[Ca^{2+}]_i$ in astrocytes. The mechanism of NMDA receptor in astrocytic calcium mobilization is not clear yet. Our results conflicted with some previous reports, so we speculated that the difference was mainly caused by the cultured cells from different positions or at different developmental periods.

Glutamate can induce cytosolic Ca^{2+} increase in primary cultured astrocytes in time-dependent and con-

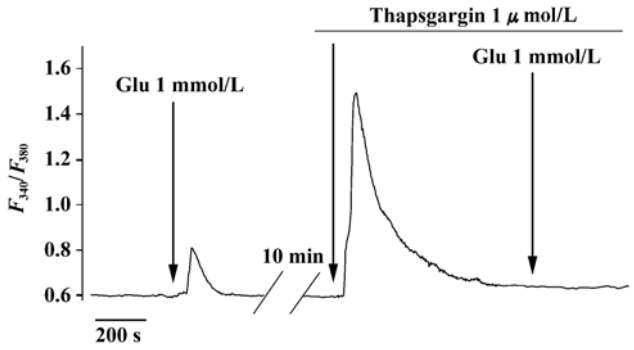

Fig 9. Effect of thapsgargin 1 μmol/L on astrocytic $[Ca^{2+}]_i$ increase induced by glutamate 1 mmol/L in extracellular solution without calcium.

centration-dependent manner. It not only activated non-NMDA and NMDA receptors, but also induced intracellular calcium mobilization. It enhanced the generation of IP_3 [31,32], the later could activate IP_3-insensitive calcium stores. When NMDA and AMPA receptor are blocked simultaneously by their antagonists *L*-AP-5 and CNQX, respectively, glutamate can still stimulate astrocytic $[Ca^{2+}]_i$ increase slightly. There were conflicting views about whether activation of NMDA and AMPA receptors could affect astrocytic calcium stores, and whether they are extracellular Ca^{2+}-dependent. When extracellular Ca^{2+} was chelated or absent, the response of glutamate still existed though much weaker than that in normal extracellular solution. But the response could be inhibited by thapsgargin. So we deduced that glutamate could induce the release of Ca^{2+} from calcium stores.

Above all, glutamate triggered a complex response in some astrocytes consisting of Ca^{2+} mobilization from intracellular stores and also Ca^{2+} influx by activation of NMDA and non-NMDA receptors .

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