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Effects of allitridi on intracellular Ca^{2+} concentration in isolated rat brain cells

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KEY WORDS brain; cultured cells; allitridi; intracellular fluid; calcium; Fura-2; norepinephrine; sodium glutamate

ABSTRACT

AIM: To study actions of allitridi extracted from garlic on intracellular calcium in isolated rat brain cells. **METHODS**: Brain cells were isolated from newborn rat brain with Fura 2-AM measurements of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). **RESULTS**: Allitridi 1 - 100 $\mu mol \cdot L^{-1}$ concentration-dependently blocked increases of $[Ca^{2+}]_i$ caused by potassium chloride and sodium glutamate (Glu) with IC_{50} of 59.7 and 69.9 $\mu mol \cdot L^{-1}$ respectively. Allitridi 100 $\mu mol \cdot L^{-1}$ blocked norepinephrine (Nor)-induced $[Ca^{2+}]_i$ elevation. **CONCLUSION**: Allitridi is an effective agent for blocking the $[Ca^{2+}]_i$ increase caused by potassium chloride, Nor and Glu.

INTRODUCTION

Allitridi extracted from *Allium sativum* L. has traditionally been used for the treatment of infections and cardiovascular diseases, etc[1]. Allitridi ameliorated cerebral infarction, especially in atherosclerotic cerebral arteries[2]. We previously showed that allitridi protected cerebral ischemia in animals[3], and it improved the rat cerebral microcirculation. Allitridi might be a calcium antagonist[4]. The present study was to explore the mechanism of $[Ca^{2+}]_i$ reduction induced by allitridi.

MATERIALS AND METHODS

Materials Allitridi was purchased from Tianqing Pharmaceutical Factory (Jiangsu Province). Ionomycin, trypsin, norepinephrine bitartrate (Nor), sodium glutamate (Glu), and Fura 2-AM were obtained from Sigma Chemical Co (St Louis MO).

Cell isolation Brain cells were isolated as described[5] with some modifications. Newborn (1-3 d) Sprague Dawley rats (Jiangsu Laboratory Animal Center) were decapitated in a Petri dish on ice. The isolated brain was rinsed with ice-cold Ca^{2+}- and Mg^{2+}-free Hanks' solution containing NaCl 137, KCl 5, glucose 5.6, and HEPES 10 $mmol \cdot L^{-1}$ (pH 7.2-7.4). Having been washed with Ca^{2+}- and Mg^{2+}-free Hanks' solution, the brain was cut into 3-mm^3 piece, and placed in a 10-mL flask containing 0.125 % trypsin and egtazic acid 0.5 $mmol \cdot L^{-1}$ in Ca^{2+}- and Mg^{2+}-free Hanks' solution. The flask was shaken at 37 ℃ for 20 min. Trypsinization was discontinued by adding ice-old Hanks' solution 10 mL containing 10 % bovine serum. Tissue pieces were mechanically dissociated by gently triturating 10-15 times with a polished pipette. The isolated brain cells were filtered through nylon mesh (200 mesh, hole width 95 μm) and collected in a flask. Cells were centrifuged twice at 100 × g for 3 min each. The supernatant was decanted and the cells were resuspended in Hanks' solution containing NaCl 137, $CaCl_2$ 1.3, $MgCl_2$ 0.5, KCl 5, glucose 5.6, and HEPES 10 $mmol \cdot L^{-1}$ (pH 7.4). Trypan blue staining showed a 90 % - 95 % cellular viability rate. The cell suspension was further diluted to 8 mL with Hanks' solution and placed at 37 ℃ for 5 min. A final concentration of Fura 2-AM 5 $\mu mol \cdot L^{-1}$ dissolved in Me_2SO was added. The cells were loaded with Fura 2-AM in water bath for 30 min. The Fura 2-AM-loaded cells were centrifuged at 100 × g for 30 min. The cells were resuspensed in Hanks'

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solution 4 mL containing 0.2 % bovine serum albumin resulting in approximately 5×10^7 cells·L^{-1} which were stored in ice. Prior to measurement, suspensions of cells were incubated at 37 ℃ for 5 min.

$[Ca^{2+}]_i$ was measured with the ratio metric fluorescent dye Fura 2-AM using AR-CM-MIC cation measurement system (Spex Corp, USA) at λ_{ex} 340 nm and 380 nm, λ_{em} 505 nm, as described by Grynkiewicz *et al*[6]. $[Ca^{2+}]_i$ was calculated according to the formular with K_d of 224 nmol·L^{-1}:

$[Ca^{2+}]_i = K_d \times (sb_1/sb_2) \times (R - R_{min})/(R_{max} - R)$ (nmol·L^{-1}). $R = F_{340}/F_{380}$; $R_{min} = F_{340min}/F_{380min}$; $R_{max} = F_{340max}/F_{380max}$; $sb_1/sb_2 = F_{380max}/F_{380min}$; F refers to fluorescence, R_{min} and R_{max} were obtained by adding ionomycin and egtazic acid, the parameters mentioned above were given, $[Ca^{2+}]_i$ was automatically calculated by the computer. Allitridi was added 15 min before the fluorescence measurement while KCl, Nor, and Glu were added immediately prior to the fluorescent determinations.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared using t test.

RESULTS

Allitridi on resting $[Ca^{2+}]_i$ In a Ca-free medium containing egtazic acid 0.1 mmol·L^{-1}, rat neuronal cells contained (76 ± 12) nmol·L^{-1} of $[Ca^{2+}]_i$. The levels were (105 ± 14), (136 ± 16), (172 ± 23), and (254 ± 18) nmol·L^{-1} in the presence of extracellular Ca^{2+} 0.01, 0.1, 1, and 2 mmol·L^{-1}, respectively. $[Ca^{2+}]_i$ was dependent on the extracellular Ca^{2+} concentration. Preincubation with allitridi 100 μmol·L^{-1} for 15 min in Hanks' solution containing Ca^{2+} 0, 0.01, 0.1, 1, and 2 mmol·L^{-1} had no significant effect on the passive diffusive flux through the cytoplasmic membrane of the brain cells (Tab 1).

Allitridi on KCl-induced $[Ca^{2+}]_i$ elevation In a Ca-free medium, KCl 20, 40, and 80 mmol·L^{-1} did not affect the $[Ca^{2+}]_i$, while the brain cells were exposed to high K^+ in Hanks' solution containing Ca^{2+} 1.3 mmol·L^{-1}, the $[Ca^{2+}]_i$ increased rapidly and concentration-dependently. KCl 20, 40, and 80 mmol·L^{-1} increased the $[Ca^{2+}]_i$ by 16 %, 53 %, and 153 % respectively, allitridi 1 μmol·L^{-1} did not change the $[Ca^{2+}]_i$, while pretreatment with allitridi 10, 25, 50, 100 μmol·L^{-1}, could decrease extracellular high KCl (80 mmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation with IC_{50} of 59.7 μmol·L^{-1} (Tab 2).

Tab 1. Effects of allitridi (100 μmol·L^{-1}) on resting $[Ca^{2+}]_i$ in isolated rat brain cells in different extracellular Ca^{2+} concentration medium. $n = 5$. $\bar{x} \pm s$.

$[Ca^{2+}]_e$/mmol·L^{-1}	Control	Allitridi-treated
0	76 ± 12	67 ± 17
0.01	105 ± 14	107 ± 38
0.1	136 ± 16	143 ± 35
1.0	172 ± 23	156 ± 29
2.0	254 ± 18	245 ± 37

Tab 2. Effects of allitridi on KCl actions on $[Ca^{2+}]_i$ of isolated rat brain cells in normal Ca-medium. $n = 5$. $\bar{x} \pm s$. ${}^cP < 0.01$ vs no KCl. ${}^eP < 0.05$, ${}^fP < 0.01$ vs KCl alone.

KCl/ mmol·L^{-1}	$[Ca^{2+}]_i$/ nmol·L^{-1}	Allitridi/ μmol·L^{-1}	Allitridi-treated $[Ca^{2+}]_i$/ nmol·L^{-1}
0	220 ± 19	100	218 ± 45
20	256 ± 18	100	242 ± 19
40	337 ± 23^c	100	276 ± 27^e
80	556 ± 34^c	1	544 ± 35
80	556 ± 34^c	10	535 ± 26
80	556 ± 34^c	25	500 ± 23^e
80	556 ± 34^c	50	387 ± 29^f
80	556 ± 34^c	100	307 ± 24^f

Allitridi on Nor-induced $[Ca^{2+}]_i$ elevation In Ca^{2+}-free solution, Nor 0.01, 0.1, 1, and 10 μmol·L^{-1} increased the $[Ca^{2+}]_i$ by 22 %, 34 %, 68 %, and 80 %, respectively, allitridi 100 μmol·L^{-1} inhibited the Nor (0.01 - 10 μmol·L^{-1})-induced $[Ca^{2+}]_i$ elevation by -11 %, 7 %, 7 %, and 21 %, respectively. When exposed to extracellular Ca^{2+} of 1.3 mmol·L^{-1}, Nor (0.01 - 10 μmol·L^{-1}) increased the $[Ca^{2+}]_i$ by 43 %, 79 %, 98 %, and 114 %, respectively, allitridi 100 μmol·L^{-1} inhibited the Nor-induced $[Ca^{2+}]_i$ elevation by 10 %, 23 %, 33 %, and 44 %, respectively. Low concentration of allitridi (1 - 10 μmol·L^{-1}) did not show apparent

effects on Nor-induced $[Ca^{2+}]_i$ elevation (Tab 3).

Tab 3. Effects of allitridi (100 μmol·L^{-1}) on Nor actions on $[Ca^{2+}]_i$ of isolated rat brain cells in normal Ca *vs* Ca-free medium. $n=5$. $\bar{x} \pm s$. $^cP<0.01$ *vs* no Nor. $^eP<0.05$, $^fP<0.01$ *vs* Nor alone.

Nor/ mmol·L^{-1}	Normal Ca-medium	Allitridi-treated	Ca-free medium	Allitridi-treated
0	220 ± 19	218 ± 45	76 ± 12	67 ± 17
0.01	314 ± 25^c	289 ± 26	92 ± 9	88 ± 13
0.1	394 ± 20^c	340 ± 31^e	102 ± 7^c	93 ± 14
1.0	435 ± 22^c	360 ± 31^f	127 ± 19^c	107 ± 22
10	471 ± 22^c	371 ± 26^f	138 ± 12^c	108 ± 17^e

Allitridi on Glu-induced $[Ca^{2+}]_i$ elevation

In Ca^{2+}-free solution, Glu had no obvious effect on $[Ca^{2+}]_i$, while when exposed to extracellular Ca^{2+} of 1.3 mmol·L^{-1}, Glu 10 μmol·L^{-1} increased the $[Ca^{2+}]_i$ from (220 ± 19) to (477 ± 47) nmol·L^{-1}, indicating that the $[Ca^{2+}]_i$ elevation induced by Glu was mainly dependent on extracellular Ca^{2+}, $MgCl_2$ 5 mmol·L^{-1} inhibited the $[Ca^{2+}]_i$ elevation by 93 % [to (238 ± 12) nmol·L^{-1}], Glu 0.01 – 10 μmol·L^{-1} concentration-dependently elevated the $[Ca^{2+}]_i$. Allitridi 1 – 100 μmol·L^{-1} dose-dependently inhibited the $[Ca^{2+}]_i$ with IC_{50} of 69.9 μmol·L^{-1} (Tab 4).

Tab 4. Effects of allitridi on Glu actions on $[Ca^{2+}]_i$ of isolated rat brain cells in normal Ca-medium. $n=5$. $\bar{x} \pm s$. $^cP<0.01$ *vs* A. $^eP<0.05$, $^fP<0.01$ *vs* E.

Group	Glu/ mmol·L^{-1}	Allitridi/ μmol·L^{-1}	$[Ca^{2+}]_i$/ nmol·L^{-1}
A	0	0	220 ± 19
B	0.01	0	234 ± 31
C	0.1	0	298 ± 20^c
D	1	0	382 ± 22^c
E	10	0	477 ± 47^c
F	100	0	554 ± 29^c
G	10	1	456 ± 17
H	10	10	438 ± 39
I	10	25	404 ± 34^e
J	10	50	351 ± 39^f
K	10	100	323 ± 28^f

DISCUSSION

Our results demonstrate that allitridi reduced $[Ca^{2+}]_i$ via 2 mechanisms: a) by blocking Ca^{2+}-influx from the extracellular space, and b) by blocking Ca^{2+}-release from the intracellular Ca^{2+} stores.

Cytosolic calcium is a very important second messenger, which mediates a lot of physiological or pharmacological reactions and regulates cellular functions. Abnormality of calcium metabolism in brain tissues can produce vasospasm, seisures, edema, etc[7,8]. Calcium overload plays a central role in both acute and especially delayed neuronal damage in ischemia brain tissues. Ca^{2+} fluxes from the extracellular fluid into the intracellular space may trigger irreversible injury[9-11] and enhance the breakdown of protein and lipids thus results in cell damage. Ischemia causes increased Ca^{2+} fluxes into cells because the depolarization and transmitter release could open the voltage-sensitive Ca^{2+} channels and the receptor-operated Ca^{2+} channels. Our study suggested that allitridi had inhibitory effects on the $[Ca^{2+}]_i$ elevation induced by KCl, Nor, and Glu. In conclusion, allitridi was shown to reduce the Ca^{2+} fluxes *via* either voltage-sensitive Ca^{2+} channels blockade or receptor-operated Ca^{2+} channels blockade at a higher dosage, the inhibitory effects of allitridi on $[Ca^{2+}]_i$ elevation are beneficial to its protection of brain against cerebral ischemia.

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大蒜新素 脑细胞 细胞内液
钙 Fura-2

大蒜新素对分离大鼠脑细胞内游离 Ca^{2+} 的影响

R965.2

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关键词 脑; 培养的细胞; 大蒜新素; 细胞内液; 钙; Fura-2; 去甲肾上腺素; 谷氨酸钠

目的: 观察大蒜新素对不同刺激剂所致分离大鼠脑细胞内游离钙的影响. **方法**: 以 Fura 2-AM 为细胞内游离钙的荧光指示剂, 用 AR-CM-MIC 阳离子测定系统, 直接测定了分离新生大鼠脑细胞内游离钙($[Ca^{2+}]_i$)值, 观察了大蒜新素的影响. **结果**: 大蒜新素对脑细胞静息$[Ca^{2+}]_i$无明显影响, 大蒜新素 1-100 $\mu mol \cdot L^{-1}$能剂量依赖性地抑制高 K^+ 和谷氨酸引起的$[Ca^{2+}]_i$ 升高, 其中 IC_{50}分别为 59.7 和 69.9 $\mu mol \cdot L^{-1}$, 高剂量大蒜新素 100 $\mu mol \cdot L^{-1}$能抑制去甲肾上腺素引起的$[Ca^{2+}]_i$ 升高. **结论**: 大蒜新素对高 K^+、去甲肾上腺素及谷氨酸引起的$[Ca^{2+}]_i$ 升高的抑制作用可能是其抗脑缺血作用机制之一.

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读者注意

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