

from inositol 1, 4, 5-trisphosphate-sensitive Ca^{2+} store by anti-depressant drugs in cultured neurons of rat frontal cortex.

J Neurochem 1993; 60: 595 - 601.

9 Berridge MJ. Inositol trisphosphate and calcium signalling.

Nature 1993; 361: 315 - 25.

10 Wakade TD, Bhave SV, Bhave A, Przywara DA, Wakade AR.

Ca^{2+} mobilized by caffeine from the inositol 1, 4, 5-trisphosphate-insensitive pool of Ca^{2+} in somatic regions of sympathetic neurons does not evoke [3H]norepinephrine release.

J Neurochem 1990; 55: 1806 - 9.

11 Barish M. Increases in intracellular calcium ion concentration

during depolarization of cultured embryonic xenopus spinal neurons. J Physiol (Lond) 1991; 444: 545 - 65.

12 Simpson PB, Challiss RAJ, Nahorski SR.

Involvement of intracellular stores in the Ca^{2+} responses to *N*-methyl-*D*-aspartate and depolarization in cerebellar granule cells.

J Neurochem 1993; 61: 760 - 3.

胞内储备钙参与 3, 4-二氨基吡啶 诱发去甲肾上腺素释放¹

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关键词 去甲肾上腺素; 海马; 3, 4-二氨基吡啶; 利血平; BAPTA; 丹曲林; 地昔帕明

目的: 用大鼠海马脑片研究 3, 4-二氨基吡啶 (DAP) 诱发去甲肾上腺素胞外钙-不依赖释放的机制。 **方法:** 大鼠海马脑片用 [3H]NE 孵育后, 进行表面灌流, 测 [3H]NE 释放。 **结果:** 在胞外无钙条件下, DAP 能显著加强 [3H]NE 释放, 当用利血平使囊泡 [3H]NE 排空, 则 DAP 作用消失, 用高浓度地昔帕明刺激 IP_3 -敏感的胞内 Ca^{2+} 储备库, 能有力地增强 [3H]NE 释放, 而高浓度咖啡因对 [3H]NE 释放只有很微弱的作用, 丹曲林钠对 DAP 诱发 [3H]NE 释放无任何抑制作用。 **结论:** 在胞外无钙条件下, DAP 通过 IP_3 -敏感的 Ca^{2+} 储备库释放 Ca^{2+} , 从而诱发囊泡内的去甲肾上腺素释放。

Effects of methylflavonolamine on free intracellular calcium in isolated embryonic rat brain cells

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KEY WORDS methylflavonolamine; Fura-2; Calcium; flunarizine; glutamate; brain; cultured cells

AIM: To observe the effects of methylflavonolamine (MFA) on free intracellular calcium concentration ($[Ca^{2+}]_i$) of isolated embryonic rat brain cells in presence and absence of high extracellular potassium and *L*-glutamate.

METHODS: $[Ca^{2+}]_i$ was measured in a spectrofluorometer by preloading the cells with calcium sensitive fluorescent indicator Fura 2-AM.

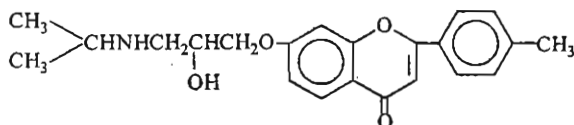
RESULTS: Resting $[Ca^{2+}]_i$ was 197 ± 20 nmol/L ($n = 44$) in the presence of Ca^{2+} 1.3 mmol

$\cdot L^{-1}$ in Hanks' solution. MFA 0.15 mmol $\cdot L^{-1}$ had no effect on the resting $[Ca^{2+}]_i$. When extracellular Ca^{2+} was 1.3 mmol $\cdot L^{-1}$, MFA ($0.03 - 0.3$ mmol $\cdot L^{-1}$) concentration-dependently inhibited the $[Ca^{2+}]_i$ elevation induced by high extracellular potassium, with an IC_{50} value of 0.14 (95 % confidence limits: $0.05 - 0.42$) mmol $\cdot L^{-1}$. At higher concentration ($0.15 - 0.30$ mmol $\cdot L^{-1}$), MFA decreased *L*-glutamate-induced $[Ca^{2+}]_i$ elevation, with an IC_{50} of 0.20 (95 % confidence limits: $0.01 - 3.40$) mmol $\cdot L^{-1}$. **CONCLUSION:** MFA inhibited Ca^{2+} influx through voltage-dependent calcium channel and, at higher concentration, through receptor-operated calcium channel in the embryonic rat brain cells.

Methylflavonolamine (MFA) inhibits thrombus formation in rats and rabbits *in vitro*⁽¹⁾ and contractions induced by noradrenaline, calcium, and potassium in rabbit aortic strips⁽²⁾ and ileum⁽³⁾. These actions of MFA suggest a calcium antagonism. But its direct actions on free intracellular calcium ($[Ca^{2+}]_i$) have not been reported. In this study, we observe the effect of MFA on $[Ca^{2+}]_i$ in isolated embryonic rat brain cells.

MATERIALS AND METHODS

MFA, a white powder which was odorless, bitterish, and soluble in water, alcohol, ethyl ether and Me_2SO , was synthesized by Shanghai Institute of Pharmaceutical Industry. It was dissolved in ion-free water. Fura 2-AM was product of Institute of Materia Medica, Chinese Academy of Medical Sciences. Flunarizine (Flu) was a product of Xi-an Janssen Pharmaceutical Ltd. *L*-Glutamic acid (Glu) was a Sigma product. KCl and $MgCl_2$ were products of Beijing Chemical Factory, their purities are over 98%. Other chemicals were AR.



Methylflavonolamine (M_r 367)

Brain cells were isolated from embryonic rats (gestation 15–18 d) according to the method of reference⁽⁴⁾ with some modifications. The isolated brain was rinsed with ice-cold Hanks' solution, pH 7.4, containing ($mmol \cdot L^{-1}$): NaCl 137, KCl 5.0, $MgSO_4 \cdot 7H_2O$ 0.8, Na_2HPO_4 0.6, KH_2PO_4 0.4, $NaHCO_3$ 3.0, glucose 5.6, and HEPES 10.0. Vessels and meninges were removed. Following a wash step with Hanks' solution, the brain was gently triturated with a polished pipette. The brain cells were filtered through nylon mesh (200 mesh, hole size 95 μm) and collected in a flask. Cells were centrifuged at $300 \times g$ for 5 min twice. The cells were resuspended in warm Hanks' solution (pH 7.4, 37 $^{\circ}C$). An aliquot of cell suspensions was taken; trypan blue staining consistently showed 90%–95% cellular viability rates. The cell suspension was diluted to 10^9 cells $\cdot L^{-1}$ with Hanks' solution and shaken at 37 $^{\circ}C$ for 5 min. Fura 2-AM $5 \mu mol \cdot L^{-1}$ in Me_2SO was added to one aliquot of cell suspension and the same volume of Me_2SO was added to the other aliquot as control. The cell suspensions were shaken at 37 $^{\circ}C$ for 45 min. The Fura-2-loaded cells and control cells were centrifuged at $300 \times g$ for 5 min twice. The cells were resuspended in warm Hanks' solution containing 0.2% bovine serum albumin, to 10^9 cells $\cdot L^{-1}$ and were incubated at

37 $^{\circ}C$ for 2–3 min prior to measurement.

A RF-540 spectrofluorophotometer (Shimadzu) was used for fluorescence determinations (λ_{ex} : 340 nm, λ_{em} : 490 nm). $[Ca^{2+}]_i$ was calculated with K_d of $224 \text{ nmol} \cdot L^{-1(5)}$: $[Ca^{2+}]_i = K_d \times (F - F_{min}) / (F_{max} - F)$. The maximal fluorescence (F_{max}) was determined by final concentration of 0.1% Triton X-100 added. The minimal fluorescence (F_{min}) was determined by using final concentration of egtazic acid $8 \text{ mmol} \cdot L^{-1}$ (pH > 8.5) to deplete calcium. Autofluorescence was determined on control cells without Fura 2-AM.

Statistical analysis of change in $[Ca^{2+}]_i$ was determined by *t*-test or ANOVA. IC_{50} was calculated by linear regression analysis after variable transformation.

RESULTS

After Fura-2 loading, the peak of excitation spectrum in isolated rat brain cells was shifted to 340 nm. When addition of Triton X-100 (final concentration 0.1%) to lyse cells, the spectrum peak in 340 nm increased. By addition of egtazic acid (final concentration $8 \text{ mmol} \cdot L^{-1}$, pH > 8.5), the spectrum of Fura-2 revealed a peak at 370–380 nm.

MFA on resting $[Ca^{2+}]_i$ The resting $[Ca^{2+}]_i$ was $197 \pm 20 \text{ nmol} \cdot L^{-1}$ ($n = 44$) in Hanks' solution with Ca^{2+} $1.3 \text{ mmol} \cdot L^{-1}$. $[Ca^{2+}]_i$ were 95 ± 11.3 , 108 ± 10.3 , 136 ± 11.5 , 145 ± 11.5 ($nmol \cdot L^{-1}$) in the presence of extracellular Ca^{2+} 0, 0.01, 0.1, 1.0 $mmol \cdot L^{-1}$, respectively. Preincubated with MFA $0.15 \text{ mmol} \cdot L^{-1}$ in Hanks' solution for 10 min, MFA did not cause a significant change in $[Ca^{2+}]_i$.

MFA on KCl-induced $[Ca^{2+}]_i$ changes Addition of KCl 25, 50, and 75 $mol \cdot L^{-1}$ in cell suspensions containing Ca^{2+} $1.3 \text{ mmol} \cdot L^{-1}$ $[Ca^{2+}]_i$ increased rapidly and concentration dependently. The net increases of $[Ca^{2+}]_i$ were 87 ± 8 , 269 ± 23 , and $320 \pm 25 \text{ nmol} \cdot L^{-1}$ respectively. MFA (0.03–0.3 $mmol \cdot L^{-1}$) inhibited the KCl ($50 \text{ mmol} \cdot L^{-1}$)-induced $[Ca^{2+}]_i$ elevation by 12%, 30%, 38%, 58%, and 87% respectively, with an IC_{50} of 0.14 (95% confidence limits: 0.05–0.42) $mmol \cdot L^{-1}$. The competitive voltage-dependent calcium channel antagonist Flunarizine (Flu) was tested as positive control. ANOVA showed a significant concentration dependent inhibition by MFA or Flu on KCl-induced $[Ca^{2+}]_i$ elevation (Fig 1A).

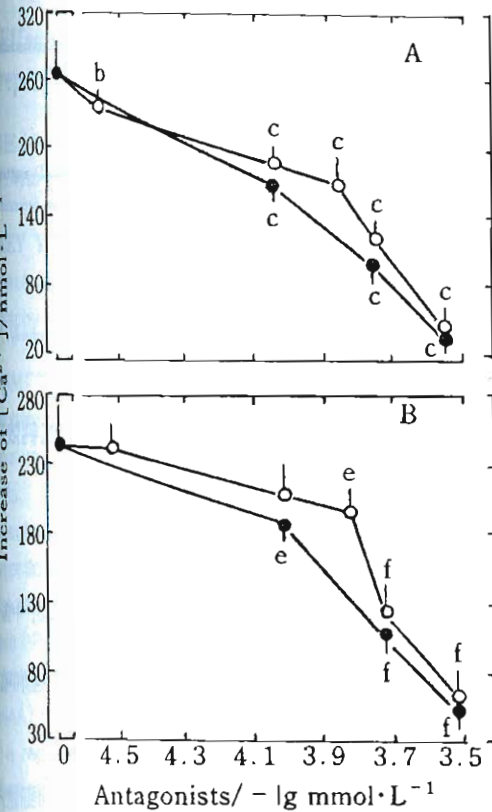


Fig. 1. Effects of methylflavonolamine (MFA ○), flunarizine (A: Flu ●) and MgCl_2 (B: ●) on the increase of free intracellular calcium in isolated brain cells of embryonic rats. $n = 5 - 7$ cell suspensions (each was pooled from 10 embryonic rats and assayed in triplicate). A) * $P < 0.05$, † $P < 0.01$ vs KCl ($50 \text{ mmol} \cdot \text{L}^{-1}$)-induced group. B) * $P < 0.05$, † $P < 0.01$ vs Glu ($100 \text{ mol} \cdot \text{L}^{-1}$)-induced group.

MFA on Glu-induced changes of $[Ca^{2+}]_i$

L -Glu 100, 200, and $300 \mu\text{mol} \cdot \text{L}^{-1}$ increased $[Ca^{2+}]_i$ 241 ± 30 , 330 ± 30 , and $367 \pm 38 \text{ nmol} \cdot \text{L}^{-1}$, respectively. Addition of MFA $0.1 \text{ mmol} \cdot \text{L}^{-1}$ prior to L -Glu had no significant effect on the increase of $[Ca^{2+}]_i$. MFA $0.15 - 0.3 \text{ mmol} \cdot \text{L}^{-1}$ attenuated Glu ($100 \mu\text{mol} \cdot \text{L}^{-1}$)-induced elevation of $[Ca^{2+}]_i$ by 19%, 49%, and 77%, respectively, with an IC_{50} of 0.20 (95% confidence limits: 0.01 - 3.40) $\text{mmol} \cdot \text{L}^{-1}$. The N -methyl-D-aspartate (NMDA) receptor antagonist MgCl_2 was tested as control. ANOVA showed a concentration-dependent inhibition on Glu-induced $[Ca^{2+}]_i$ elevation by MFA or MgCl_2 (Fig 1B).

DISCUSSION

Isolated brain cells are simple materials for the

experiment, but lower cellular viability rate and the leak of Fura 2-AM are the shortcoming of the method, so we used trypan blue staining all through experiment to examine cellular viability and added bovine serum albumin in Hanks' solution to inhibit leak of fluorescent indicator.

The resting $[Ca^{2+}]_i$ value in our study is closed to the reported value of $[Ca^{2+}]_i$ in isolated newborn rat brain cells⁽⁷⁾, and also in the range of value for $[Ca^{2+}]_i$ in neurons⁽⁶⁾. The result is a good evidence that calcium homeostatic, cellular structural and functional integrity are well preserved in isolated embryonic rat brain cells. Our results suggest that MFA has no apparent effect on passive diffusible flux of Ca^{2+} through the resting cytosolic membrane in rat brain cells. The effect of MFA on resting $[Ca^{2+}]_i$ was similar to those of verapamil and diltiazem⁽⁸⁾.

Raising extracellular potassium caused depolarization of membrane to a certain extent which opened voltage dependent calcium channel (VDC). We found that various levels of KCl-induced $[Ca^{2+}]_i$ increased rapidly, the response is closed to that of newborn rat brain cells⁽⁷⁾, but weaker than that of synapsomes⁽⁹⁾. The difference may be attributed to regulation ability of membrane. MFA inhibited KCl-induced $[Ca^{2+}]_i$ elevation, it is inferred that MFA decreased the $[Ca^{2+}]_i$ elevation through blocking VDC in brain cells of rats.

With regard to L -Glu-induced $[Ca^{2+}]_i$ change in brain cells, there has been a general agreement reached by the observation that Glu-sensitive receptor operated calcium channel in nerve cells⁽¹⁰⁾. Glu caused $[Ca^{2+}]_i$ elevation significantly in our study, the response is similar to that reported⁽¹¹⁾. The inhibitory effect of MFA on Glu-induced $[Ca^{2+}]_i$ elevation was less potent than on the KCl-induced. Although there are different subtypes of Glu-sensitive ROC, the effect of NMDA-type ROC on Glu-induced $[Ca^{2+}]_i$ elevation is critical⁽¹²⁾. Since the activation of NMDA-type ROC relies on membrane depolarization, the inhibitory effect of MFA on Glu-induced $[Ca^{2+}]_i$ elevation is directly through ROC or indirectly through VDC.

Loss of calcium homeostasis was responsible for

the ischemia reperfusion injury^[13]. The inhibitory effects of MFA on VDC and/or ROC in brain cells may contributed to its protective effects on cerebral ischemia reperfusion injury. In conclusion, our study showed that MFA inhibited $[Ca^{2+}]_i$ elevation induced by KCl and Glu directly in brain cells of embryonic rats. The effects are basis of its pharmacologic action.

REFERENCES

- 1 Wu YJ, Zhou EF, Hao YB, Tang YZ, Wan BS. Effects of 4'-methyl-7-(2-hydroxy-3-isopropylaminopropoxy)-flavone hydrochloride on experimental thrombus formation in rats and rabbits. *Acta Pharm Sin* 1986; **21**: 774 - 7.
- 2 Zhang MS, Zhou EF. Methylflavonolamine hydrochloride inhibits contraction induced by noradrenaline, calcium and potassium in rabbit isolated aortic strips. *Br J Pharmacol* 1988; **94**: 1184 - 8.
- 3 Long P, Yan CH, Zhang MS, Zhou EF. Effects of methylflavonolamine hydrochloride on contractility of isolated rabbit ileum. *Acta Pharmacol Sin* 1992; **13**: 63 - 5.
- 4 Dildy JE, Leslie SW. Ethanol inhibits NMDA-induced increase in free intracellular Ca^{2+} in dissociated rat brain cells. *Brain Res* 1989; **499**: 383 - 7.
- 5 Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**: 3440 - 50.
- 6 Nachshen DA. Regulation of cytosolic calcium concentration in presynaptic nerve endings isolated from rat brain. *J Physiol (Lond)* 1985; **363**: 87 - 101.
- 7 Zeng WZ, Liu TP. Effects of tetrandrine on free intracellular Ca^{2+} in isolated rat brain cells. *Acta Pharmacol Sin* 1993; **14**: 397 - 400.
- 8 Kanaide H, Kobayashi S, Nishimura J, Hasegawa M, Shogakiuchi Y, Matsumoto T, *et al.* Quin-2 microfluorimetry and effects of verapamil and diltiazem on calcium release from rat aorta smooth muscle cells in primary culture. *Circ Res* 1988; **63**: 16 - 26.
- 9 Gao H, Feng YP. Estimation of free calcium level within synaptosomes by using Fura-2 and the effect of calcium channel agonist and antagonist. *Acta Pharm Sin* 1993; **28**: 404 - 9.
- 10 Manev H, Costa E, Wroblewski JI, Guidotti A. Over stimulation of excitatory amino acid receptor and antagonistic pathway to neurotoxicity. *Prog Physiol Sci* 1991; **22**: 107 - 10.

- 11 Che JT, Zhang JT, Qu ZW, Peng XQ. Effects of tetrandrine on free intracellular Ca^{2+} in embryonic brain cells. *Acta Pharm Sin* 1994; **29**: 492 - 6.
- 12 Cox JA, Lysko PG, Henneberry RC. Excitatory amino acid neurotoxicity at the N-methyl-D-aspartate receptor in cultured neurons: role of the voltage-dependent magnesium block. *Brain Res* 1989; **499**: 267 - 72.
- 13 Schanne FAX, Kane A, Young EE, Faber J. Calcium dependence of toxic death: a final common pathway. *Science* 1979; **206**: 700 - 2.

甲基黄酮醇胺对分离的胎鼠脑细胞内游离钙浓度的影响

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关键词 甲基黄酮醇胺; Fura-2; 钙; 氟桂利嗪; 谷氨酸; 脑; 培养的细胞

目的: 观察甲基黄酮醇胺 (MFA) 对胎鼠脑细胞内游离钙浓度在静息以及激动剂存在时的作用。方法: 用钙离子荧光染料 Fura-2-AM 负载后, 测定分离的胎鼠脑细胞内游离钙浓度 ($[Ca^{2+}]_i$) 及其变化。结果: 在含钙 $1.3 \text{ mmol} \cdot \text{L}^{-1}$ 的 Hanks 液中, $[Ca^{2+}]_i$ 为 $197 \pm 20 \text{ nmol} \cdot \text{L}^{-1}$ ($n=44$)。MFA $0.15 \text{ mmol} \cdot \text{L}^{-1}$ 对静息脑细胞内钙浓度无明显影响。在细胞外钙 $1.3 \text{ mmol} \cdot \text{L}^{-1}$ 条件下, MFA ($0.03 - 0.3 \text{ mmol} \cdot \text{L}^{-1}$) 浓度依赖性地抑制高钾去极化导致的 $[Ca^{2+}]_i$ 升高, IC_{50} 为 0.14 (95% 可信限: $0.05 - 0.42$) $\text{mmol} \cdot \text{L}^{-1}$ 。在较高浓度时 MFA ($0.15 - 0.3 \text{ mmol} \cdot \text{L}^{-1}$) 也可抑制谷氨酸兴奋所引起的 $[Ca^{2+}]_i$, IC_{50} 为 0.20 (95% 可信限 $0.01 - 3.40$) $\text{mmol} \cdot \text{L}^{-1}$ 。结论: MFA 抑制高钾去极化引起的 $[Ca^{2+}]_i$ 升高, 在较高浓度时也抑制谷氨酸兴奋所致的 $[Ca^{2+}]_i$ 升高。