Effects of hyperin on free intracellular calcium in dissociated neonatal rat brain cells

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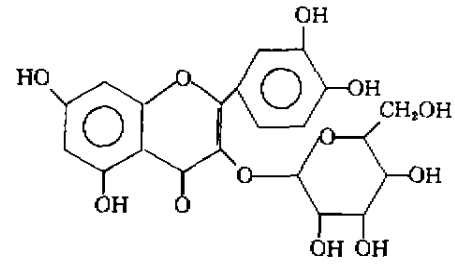
KEY WORDS hyperin; brain; cultured cells; Fura-2; calcium; norepinephrine; sodium glutamate; serotonin

ABSTRACT

AIM: To study the effects of hyperin (Hyp) on free intracellular calcium concentration ($[Ca^{2+}]_i$) of brain cells. **METHODS:** The neonatal rat brain cells were dissociated. $[Ca^{2+}]_i$ in presence and absence of extracellular high K^+, *L*-glutamic acid (Glu), 5-hydroxytryptamine (5-HT), and norepinephrine (NE) were assayed with Fura 2-AM. **RESULTS:** The resting $[Ca^{2+}]_i$ in Hanks' solution ($CaCl_2$ 1.3 mmol·L^{-1}) was (208 ± 12) nmol·L^{-1} (n = 17). Hyp had no significant effects on the resting $[Ca^{2+}]_i$. Hyp 1.0, 4.0, and 16.0 μmol·L^{-1} markedly inhibited the increase of $[Ca^{2+}]_i$ evoked by K^+ 50 mmol·L^{-1} in a concentration-dependent manner. Hyp 16.0 μmol·L^{-1} inhibited the increases of $[Ca^{2+}]_i$ induced by NE 1, 2, 4, and 8 μmol·L^{-1}. Hyp (16.0 μmol·L^{-1}) also markedly attenuated 5-HT and Glu-induced increase of $[Ca^{2+}]_i$. **CONCLUSION:** Hyp possessed inhibitory effects on influx of Ca^{2+} in the neonatal rat brain cells.

INTRODUCTION

Hyperin (hyperoside: quercetin-3-*O*-galactoside; Hyp), a flavonal glycoside, was extracted from Chinese herb *Abelmoschus manihot L*. Our previous studies showed that Hyp inhibited inward flow of calcium ion in cardiomyocyte[1] and had protective effects against cerebral ischemia-reperfusion injury in mice[2] and rats[3]. It is obvious that Hyp possesses potential clinical values in preventing and treating ischemic-reperfusion injuries. It has been considered that its protective effects may be related to the reduction of free intracellular calcium concentration ($[Ca^{2+}]_i$). In the present study, effects of Hyp on $[Ca^{2+}]_i$ level in dissociated brain cells of neonatal rats were observed.



3,3',4',5,7-Pentahydroxyflavone-3-*O*-galactoside

MATERIALS AND METHODS

Hyp, a yellow powder, mp 230 - 231 ℃, purity >95 % (Anhui Institute of Medical Science); dimethyl sulfoxide (Beijing Chemical Factory); RPMI-1640 medium (Gibco); Fura 2-AM (Shanghai Institute of Physiology, Chinese Academy of Sciences) dissolved in Me_2SO; 5-HT (Sigma); *L*-glutamic acid (Glu, Sigma). All other chemicals were AR.

Preparation of brain cells[4] Sprague-Dawley neonatal rats (n = 120, Grade Ⅱ, Certificate № AHEA-01, 1 - 3-d old) were

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Received 1997-11-24 Accepted 1998-08-17

ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1999 Jan; 20 (1)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

killed, the brain was isolated and rinsed with ice-cold Hanks' solution containing: NaCl 137; KCl 5.0; $CaCl_2$ 1.3; $MgSO_4 \cdot 7H_2O$ 0.8; Na_2HPO_4 0.6; KH_2PO_4 0.4; $NaHCO_3$ 3.0; glucose 5.6 mmol·L^{-1}; pH 7.4. Blood vessels and meninges were removed carefully. Following a wash step with ice-cold Hanks' solution, the brain was cut into about 1 mm^3 fragments which were digested with trypsin 1.25 g·L^{-1} at 37 ℃ for 15 min. Twice volume of ice-cold RPMI-1640 medium (containing 10 % fetal calf serum) was used to end the digestion. The brain cells were filtered through nylon mesh (200 mesh). The cells were centrifuged (300 × g, 5 min), washed twice and resuspended in RPMI-1640 medium. Trypan blue exclusion method showed that cellular viability rates were above 90 %.

Measurement of Ca^{2+} [4,5] Fura 2-AM was added into the cell suspensions (final concentration = 5 μmol·L^{-1}). Having been incubated at 37 ℃ for 40 min to load Fura 2, the cells suspensions were preincubated with Hyp for 10 min and incubated at 37 ℃ for 3 min prior to measurement.

The fluorescence (F) was determined by a Hitchi 650 – 60 fluorescence spectrophotometer (λ_{ex}: 340 nm, λ_{em}: 500 nm). At the end of the analysis, the final concentration of 0.1 % Triton X-100 was added to measure the maximal fluorescence (F_{max}), the final concentration of egtazic acid 10 mmol·L^{-1} was added to measure minimum fluorescence (F_{min}). The values of $[Ca^{2+}]_i$ were calculated: $[Ca^{2+}]_i = K_D \times (F - F_{min})/(F_{max} - F)$, the apparent dissociation constant (K_D) was 224 nmol·L^{-1}.

Statistical method All data were analyzed by t-test.

RESULTS

Effects of Hyp on the resting $[Ca^{2+}]_i$ The resting $[Ca^{2+}]_i$ in dissociated neonatal rat brain cells was (208 ± 12) nmol·L^{-1} in Hanks' solution ($CaCl_2$ 1.3 mmol·L^{-1}); Hyp 1.0, 4.0, and 16.0 μmol·L^{-1} had no significant effect on the resting $[Ca^{2+}]_i$ (Tab 1).

Tab 1. Effects of Hyp on the resting and KCl-induced increases of $[Ca^{2+}]_i$ in dissociated neonatal rat brain cells. Number of cell suspensions (each was pooled from 5 neonatal rats and assayed in triplicate) is in parentheses. $\bar{x} \pm s$.
$^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control; $^fP < 0.01$ *vs* resting.

Group	Concentration/ μmol·L^{-1}	$[Ca^{2+}]_i$/nmol·L^{-1} resting	K^+ 50 mmol·L^{-1}
Control	–	208 ± 12(17)	517 ± 59(9)^f
Hyp	1.0	209 ± 8(4)^a	430 ± 31(4)^b
	4.0	208 ± 8(5)^a	347 ± 19(5)^c
	16.0	206 ± 8(20)^a	295 ± 42(8)^c
Nim	5.0	204 ± 10(4)^a	315 ± 38(4)^c

Effects of Hyp on KCl-induced increase of $[Ca^{2+}]_i$ KCl 50 mmol·L^{-1} increased the $[Ca^{2+}]_i$ in the brain cells, the net increase of $[Ca^{2+}]_i$ was 309 nmol·L^{-1}; Hyp (1.0, 4.0, and 16.0 μmol·L^{-1}) markedly inhibited this elevation in a concentration-dependent manner, its inhibitory rates were 28.2 %, 55.0 %, and 71.8 %, respectively. Similar results were obtained with nimodipine (Nim).

Effects of Hyp on NE-evoked increase of $[Ca^{2+}]_i$ Addition of NE 1, 2, 4, and 8 μmol·L^{-1} in cells suspensions, the $[Ca^{2+}]_i$ was increased concentration-dependently. Hyp (16.0 μmol·L^{-1}) markedly attenuated the NE (1 – 8 μmol·L^{-1})-induced increase of $[Ca^{2+}]_i$, its inhibitory rates for 1, 2, 4, and 8 μmol·L^{-1} NE were 46.4 %, 53.2 %, 44.0 %, and 51.2 %, respectively (Fig 1).

Effects of Hyp on sodium glutamate and 5-HT-induced increase of $[Ca^{2+}]_i$ Both of sodium glutamate (pH 7.8) 150 μmol·L^{-1} and 5-HT 1.5 μmol·L^{-1} markedly increased $[Ca^{2+}]_i$ (Tab 2).

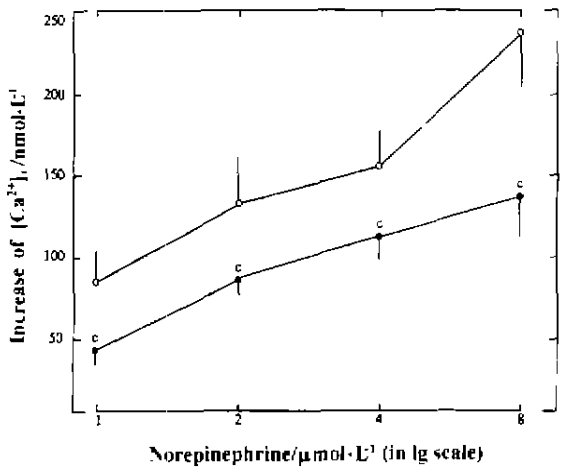

Fig 1. Effect of Hyp on NE-envoked increase of $[Ca^{2+}]_i$ in dissociated neonatal rat brain cells. $n = 5$ cell suspensions. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Tab 2. Effects of Hyp on 5-HT and Glu-induced increases of $[Ca^{2+}]_i$ in dissociated neonatal rat brain cells. $\bar{x} \pm s$. n is the number of cell suspensions. $^cP < 0.01$ *vs* control.

Group	Concentration/ μmol·L^{-1}	n	$[Ca^{2+}]_i$/nmol·L^{-1} 5-HT	Glu
Control	-	5	431 ± 27	440 ± 20
Hyp	16.0	5	321 ± 22^c	306 ± 25^c
Nim	5.0	4	340 ± 30^c	348 ± 19^c

Hyp 16.0 μmol · L^{-1} inhibited 5-HT and Glu-induced increases of $[Ca^{2+}]_i$, the inhibitory rates were 49.3 % and 58.1 %, respectively. Nim (5.0 μmol · L^{-1}) also inhibited 5-HT and Glu-induced increases of $[Ca^{2+}]_i$.

DISCUSSION

The resting $[Ca^{2+}]_i$ value of dissociated newborn rat brain cells in our study is close to the value of $[Ca^{2+}]_i$[6]. Our results showed that Hyp (1.0, 4.0, 16.0 μmol · L^{-1}) did not influence the resting $[Ca^{2+}]_i$ in neonatal rat brain cells.

High K^+ could cause depolarization of membrane and open voltage-dependent calcium channel (VDC)[4,7]. Hyp (1.0 – 16.0 μmol · L^{-1}) markedly and concentration-dependently inhibited 50 mmol · L^{-1} KCl-induced $[Ca^{2+}]_i$ elevation in brain cells. This finding corresponded to our previous report that Hyp inhibited ^{45}Ca influx in atrium preparation in mice. The result suggested that Hyp blocked VDC to attenuate KCl-induced increase of $[Ca^{2+}]_i$ in nerve cells.

The regulation of G proteins on Ca^{2+} channels has been an active area of research. G proteins not only regulated Ca^{2+} channels indirectly via cytoplasmic second message, but also act directly on Ca^{2+} channels[7]. Since G proteins couple a variety of receptors to Ca^{2+} channels, NE, 5-HT *etc* can activate the proper G protein-coupled receptors to open Ca^{2+} channels. Hyp inhibited NE and 5-HT-induced increase of $[Ca^{2+}]_i$. Results suggested that Hyp inhibited Ca^{2+} influx induced by activation of G protein-coupled receptors (5-HT and α-adrenergic receptors).

N-methyl-*D*-aspartate (NMDA) receptors found on neurons were important in the control of nerve activity. Activation of NMDA receptors led to the opening of Ca^{2+} channels[8,9]. Although there are different subtypes of Glu-sensitive Ca^{2+} channels, the effects of NMDA receptor-linked Ca^{2+} channels on Glu-induced $[Ca^{2+}]_i$ elevation are critical. In our study, Glu caused $[Ca^{2+}]_i$ increase and Nim inhibited this elevation, the responses were similar to Lu's report[10]. Hyp 16.0 μmol · L^{-1} markedly inhibited Glu-induced increase of $[Ca^{2+}]_i$. These inhibitory effects suggested that Hyp could also block NMDA receptor-linked Ca^{2+} channels to decrease $[Ca^{2+}]_i$ increase in nerve cells.

In summary, Hyp possesses the inhibitory effects on influx of Ca^{2+} in neonatal rat brain cells.

ISSN 0253-9756 Acta Pharmacologica Sinica 中国药理学报 1999 Jan; 20 (1)
E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

REFERENCES

1 Chen ZW, Ma CG, Fang M, Xu SY.
The blocking effect of hyperin on the inward flow of calcium ion.
Acta Pharm Sin 1994; 29: 15 – 9.

2 Chen ZW, Ma CG.
The protective effect of hyperin and quercetin on the cerebral ischemia injury in mice.
Natural Prod Res Develop 1997; 9 (2): 21 – 3.

3 Chen ZW, Ma CG, Zhao WZ. Protective effect of hyperin against cerebria-reperfusion injury.
Acta Pharm Sin 1998; 33: 14 – 7.

4 Li M, Wang JF, Han JS, Zhang JT. Measuremant of intracellular free Ca^{2+} concentration in dissociation rat brain cells: using Fura-2/AM.
Acta Pharm Sin 1991; 26: 890 – 4.

5 Willhart K, Christof S. Intracellular free calcium concentration in rat anterior pituitary cells as indicated by fura-2: effect of arginine-vasopressin.
Naunyn Schmiedebergs Arch Pharmacol 1987; 336: 321 – 6.

6 Dildy JE, Leslie SW.
Ethanol inhibits NMDA-induced increase in free intracellular Ca^{2+} in dissociated brain cells.
Brain Res 1989; 499: 383 – 7.

7 Hartzell HC, Fischmeister R.
Direct regulation of cardiac Ca^{2+} channels by G proteins: neither proven nor necessary?
Trends Pharmacol Sci 1992; 13: 380 – 5.

8 Duan WZ, Tang YZ.
Effects of methylflavonolamine on free intracellular calcium in isolated embryonic rat brain cells.
Acta Pharmacol Sin 1996; 17: 305 – 8.

9 Manev H, Costa E, Wroblewski JT, Guidotti A.
Over stimulation of excitatory amino acid receptor and antagonistic pathway to neurotoxicity.
Prog Physiol Sci 1991; 22: 107 – 10.

10 Lu YM, Zhang JT, Zhao FQ, Li F. Effects of nimodipine on *l*-glutamate-induced seizures and Ca^{2+} influx in hippocampus in freely moving rats.
Acta Pharmacol Sin 1991; 12: 297 – 300.

11 Duan WZ, Liang YQ, Tang YZ.
Protection of Zinc sulfate on acute cerebral ischemia reperfusion injury in rats.
Chin Pharmacol Bull 1997; 13: 39 – 42.

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金丝桃苷对分离的新生大鼠脑细胞内游离钙浓度的影响

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关键词 金丝桃苷；脑；培养的细胞；Fura-2；钙；去甲肾上腺素；谷氨酸钠；血清素

目的：研究金丝桃苷（Hyp）对新生大鼠的脑细胞内游离钙浓度的作用。**方法**：分离新生大鼠的脑细胞；用钙离子荧光指示剂 Fura-2 测定静息和激动剂存在时新生鼠脑细胞内游离钙浓度。**结果**：在 $CaCl_2$ 1.3 mmol·L^{-1}的 Hanks' 液中、静息 $[Ca^{2+}]_i$ 为 (208 ± 12) nmol·L^{-1} (n = 17)、Hyp 对静息 $[Ca^{2+}]_i$ 无明显影响；Hyp 1.0, 4.0, 16.0 μmol·L^{-1}呈浓度依赖性显著抑制 KCl 50 mmol·L^{-1}致 $[Ca^{2+}]_i$ 增高；Hyp 16.0 μmol·L^{-1}显著抑制去甲肾上腺素 1, 2, 4 和 8 μmol·L^{-1}诱发的 $[Ca^{2+}]_i$ 的增高；Hyp 16.0 μmol·L^{-1}还可显著抑制谷氨酸和 5-羟色胺致 $[Ca^{2+}]_i$ 增高。**结论**：Hyp 对新生大鼠脑细胞钙内流有阻滞作用。

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