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Inhibitory effects of melatonin on free intracellular calcium in mouse brain cells

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KEY WORDS melatonin; calcium; aging; brain; synaptosomes; cerebral cortex; neurons; Bay-K-8644; potassium chloride; sodium glutamate

ABSTRACT

AIM: To study the effects of melatonin (Mel) on cortical intrasynaptosomal calcium concentration in old mice and on $[Ca^{2+}]_i$ elevation induced by Bay-K-8644, KCl, and sodium *l*-glutamate in isolated brain cells of neonatal mouse, and to determine the antiaging mechanism of Mel. **METHODS:** $[Ca^{2+}]_i$ was measured in an RF-5000 recording spectrofluorophotometer by preloading the synaptosomes or cells with Fura 2-AM. **RESULTS:** Long term of administrating Mel inhibited the overload of $[Ca^{2+}]_i$ in old mouse cerebral cortex. The $[Ca^{2+}]_i$ in both high (20 $mg \cdot L^{-1}$) and low dose (1 $mg \cdot L^{-1}$) of Mel groups was reduced from (434 ± 32) $nmol \cdot L^{-1}$ (the older control group) to (330 ± 41) and (313 ± 56) $nmol \cdot L^{-1}$, respectively, $P < 0.01$. Mel 0.01, 0.1, 1, and 3 $\mu mol \cdot L^{-1}$ remarkably reduced $[Ca^{2+}]_i$ elevations in isolated newborn mouse brain cells induced by Bay-K-8644, KCl, and Glu. **CONCLUSION:** The inhibitory effect of Mel on neuronal $[Ca^{2+}]_i$ overload is involved in its antiaging effect.

INTRODUCTION

Melatonin (Mel, *N*-acetyl-5-methoxyltryptamine) is a hormone secreted mainly by the pineal body. Many investigators have studied the pineal body and Mel in the processes of both aging and age-related diseases[1]. The interests are from the importance of Mel in a number of biological functions and the fact that Mel production in the organism is gradually lost throughout life[2]. Exogenously administering Mel could prolong life, postpone aging, and reduce the age-related diseases[3]. $[Ca^{2+}]_i$ overload would result in irreversible injury and is a common pathway for cell death, particularly in the central neurons. The rise of $[Ca^{2+}]_i$ increases the free radical production, and the accumulation of free radicals causes the increase of $[Ca^{2+}]_i$. A prominent theory of aging is that the rate of aging attributes to accumulated free radical damage[4]. The damages may be more obvious in the central nervous system, which is highly susceptible to oxygen-based radicals, and may be especially important in aging. The purpose of this study was to explore the antiaging mechanism of Mel.

MATERIALS AND METHODS

Mel ($C_{13}H_{16}N_2O_2$, FW 232.3, purity > 99 %, light sensitive desiccate, stored at < 0 ℃) was synthesized by Institute of Materia Medica, Chinese Academy of Medical Sciences. RF-5000 recording spectrofluorophotometer (Shimadzu Co, Japan); XL-90 ultracentrifugator (Beckman, Germany); SCR20BA-centrifugator (Hitachi Koki Co, Japan); Fura 2-AM, Triton X-100, egtazic acid, Me_2SO, and sodium *l*-glutamate (Glu, Sigma, USA); HEPES and

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Bay-K-8644 (Boehringer, Germany); DMEM medium (Gibco-BRL, USA). Other chemicals were of AR.

Old (15 months) and newborn (3 - 5 d) Kunming mice were provided by the Animal Center of Chinese Academy of Medical Sciences (Grade Ⅱ, Certificate № 01-3001).

Artificial cerebro-spinal fluid (ACSF): NaCl 132, KCl 3, $MgSO_4 \cdot 7H_2O$ 1, NaH_2PO_4 1, glucose 10, HEPES 10, and $CaCl_2$ 1 $mmol \cdot L^{-1}$, pH 7.4.

$MgSO_4$- and NaH_2PO_4-free ACSF: NaCl 132, KCl 3, glucose 10, HEPES 10, and $CaCl_2$ 1 $mmol \cdot L^{-1}$, pH 7.4.

Hanks' solution: NaCl 137, KCl 5, $CaCl_2$ 1.3, $MgSO_4 \cdot 7H_2O$ 0.8, Na_2HPO_4 0.6, KH_2PO_4 0.4, $NaHCO_3$ 3, glucose 5.6, and HEPES 2 $mmol \cdot L^{-1}$, pH 7.4.

Preparation of old mouse cerebral cortex synaptosome Old mice (15 months) of either sex weighing 44 g ± *s* 4 g were randomly divided into the low dose treated ($n = 10$), high dose treated ($n = 10$), and the control ($n = 10$) groups. Treated group: after the addition of Mel 1 $mg \cdot L^{-1}$ (low dose) and 20 $mg \cdot L^{-1}$ (high dose) to the drinking water of mice on a nightly basis for 2 months, they were injected ip Mel 0.5 $mg \cdot kg^{-1}$ and 2 $mg \cdot kg^{-1}$ for 1 month. Control group received the same amount of vehicle (NS).

Cortical synaptosomes were isolated from old mice[5,6] with some modifications. The homogenate was centrifuged at 4 ℃, 1000 × *g* for 10 min. Supernatant was placed in the gradient density solution consisting of sucrose solution 0.8 and 1.2 $mol \cdot L^{-1}$ and ultracentrifuged at 4 ℃, 150 000 × *g* for 50 min. The suspension layer between sucrose solution 1.2 and 0.8 $mol \cdot L^{-1}$ was collected and centrifuged at 4 ℃, 20 000 × *g* for 30 min. The pellets were resuspended in ACSF. The suspension was centrifuged at 4 ℃, 9500 × *g* for 10 min. The sediments were resuspended in 5 mL of ACSF. The samples were stored at 0 - 4 ℃. The determination of protein was performed[7]. The content of protein was 1 $g \cdot L^{-1}$.

Preparation of neonatal mouse brain cells Brain cells were prepared according to the method[8,9] with some modifications. Newborn (3 - 5 d) Kunming mouse brain cells were filtered through nylon mesh (200 mesh, hole size 95 μm) and collected in a flask. Cells were centrifuged at 300 × *g* for 5 min twice. The supernatant was decanted and the cells were resuspended in warm Hanks' solution. An aliquot of cell suspension was taken, trypan blue staining consistently showed 90 % - 95 % cellular viability rate. The suspension was diluted to 2×10^6 cells $\cdot L^{-1}$ with Hanks' solution and shaken at 37 ℃ for 5 min.

The experiment was divided into 2 groups. Control group: 5 min after the resting $[Ca^{2+}]_i$ was determined, final concentrations of KCl 50 $mmol \cdot L^{-1}$, Glu 10 $mmol \cdot L^{-1}$, and Bay-K-8644 0.1 $mmol \cdot L^{-1}$ were added, and 5 min later, $[Ca^{2+}]_i$ was determinated. Treated group: The resting $[Ca^{2+}]_i$ was determined. After 5 min Mel (final concentrations 0.01, 0.1, 1, and 3 $\mu mol \cdot L^{-1}$) were given to observe the changes of $[Ca^{2+}]_i$. Five minutes after Mel administration, the above calcium agonists were added to observe the elevation of $[Ca^{2+}]_i$.

Fura 2-AM-loaded samples Fura 2-AM (a Ca^{2+} fluorescent indicator) 5 $\mu mol \cdot L^{-1}$ in Me_2SO was added to an aliquot of suspension. The suspension was shaken at 37 ℃ for 45 min, then centrifuged at 13 000 × *g* (synaptosomes) or 300 × *g* (cells) for 5 min twice. The pellets or cells were resuspended in $MgSO_4$- and NaH_2PO_4-free ACSF (synaptosomes) or warm Hanks' solution (cells), respectively for detection.

Measurement of $[Ca^{2+}]_i$ An RF-5000 recording spectrofluorophotometer was used for fluorescence determination. The condition of

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measurement: alternate λ time scan: λ_{ex} A = 340 nm, λ_{ex} B = 380 nm, λ_{em} A = 500 nm, λ_{em} B = 500 nm; bandwidth (Ex) 5 nm, bandwidth (Em) 10 nm; time interval: 2 s; response: 0.02 s; sensitivity: high; temperature: (37 ± 1) ℃.

After Fura-2 loading, the peak of excitation spectrum in samples was shifted from 380 nm to 340 nm. When addition of Triton X-100 (final concentration 0.1 %) to lyse the plasmatic membranes, the spectrum peak in 340 nm increased. By addition of egtazic acid (final concentration 5 $mmol \cdot L^{-1}$, pH > 8.5), the spectrum of Fura-2 released a peak at 370 - 380 nm.

Calculation of $[Ca^{2+}]_i$ $[Ca^{2+}]_i = K_d \times (R - R_{min})/(R_{max} - R) \times (sf_2/sb_2)$. K_d was the dissociation constant of Fura-2 for Ca^{2+} and assumed to be 224 $nmol \cdot L^{-1}$ at 37 ℃. R was the ratio of corrected fluorescence signals at 340 and 380 nm. R_{max} (maximal fluorescence) was the ratio obtained by final concentration of 0.1 % Triton X-100 added. R_{min} (minimal fluorescence) was the ratio of the corrected signals obtained by using final concentration of egtazic acid 5 $mmol \cdot L^{-1}$ (pH > 8.5) to deplete calcium. sf_2 and sb_2 represented the emission intensities at 380 nm excitation in saturated and calcium-free conditions, respectively.

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

RESULTS

Resting $[Ca^{2+}]_i$ of cortical intrasynaptosomes in old mice Cortical intrasynaptosomal free calcium concentration in old mice [15 months, (434 ± 32) $nmol \cdot L^{-1}$] was higher than that in adult mice [3 - 6 months, (265 ± 59) $nmol \cdot L^{-1}$]. Compared with the older control group, the $[Ca^{2+}]_i$ in both high and low dose of Mel groups reduced obviously [(330 ± 41) and (313 ± 56) $nmol \cdot L^{-1}$, $P < 0.01$], but was still hgher than that in adult mice ($P < 0.05$). There was no significant difference in $[Ca^{2+}]_i$ between 2 treated groups ($P > 0.05$).

Inhibitory effects of Mel on $[Ca^{2+}]_i$ elevation by KCl, Glu, and Bay-K-8644 Mel did not cause a significant change in resting $[Ca^{2+}]_i$ ($P > 0.05$). In comparison with the control, Mel 0.01, 0.1, 1, 3 $\mu mol \cdot L^{-1}$ remarkably reduced $[Ca^{2+}]_i$ elevations induced by KCl, Glu, and Bay-K-8644 ($P < 0.01$) (Tab 1).

Tab 1. Effects of melatonin on 3 calcium agonists-induced $[Ca^{2+}]_i$ ($nmol \cdot L^{-1}$) in isolated brain cells of neonatal mice. $n = 5$ cell suspensions (each suspension was pooled from 6 newborn mice). $\bar{x} \pm s$. [a]$P > 0.05$, [c]$P < 0.01$ vs resting $[Ca^{2+}]_i$. [f]$P < 0.01$ vs Bay-K-8644 (0.1 $mmol \cdot L^{-1}$) group. [i]$P < 0.01$ vs KCl (50 $mmol \cdot L^{-1}$) group. [l]$P < 0.01$ vs Glu (1 $mmol \cdot L^{-1}$) group.

Group	Control	Melatonin/$\mu mol \cdot L^{-1}$		
		0.01	0.1	1
Resting	204 ± 36	210 ± 37[a]	211 ± 36[a]	211 ± 39[a]
Bay-K-8644	441 ± 40[c]	360 ± 32[f]	318 ± 35[f]	312 ± 40[f]
KCl	334 ± 37[c]	259 ± 29[i]	243 ± 25[i]	233 ± 24[i]
Glu	279 ± 23[c]	222 ± 22[l]	218 ± 22[l]	213 ± 23[l]

DISCUSSION

The reduction in Mel with age may be contributory to aging and the onset of age-related diseases. Mel has beneficial effects in terms of aging and age-related diseases, may be an antiaging hormone. The view is based on the recent observations that Mel is the most potent hydroxyl radical scavenger thus far discovered[10]. Besides its ability to directly scavenge the highly toxic hydroxyl radical, Mel also promotes the activity of the antioxidative enzyme glutathione peroxidase, thereby further reducing oxidative damage. Thus, Mel preferentially affords antioxidant protection to the brain[11]. In some studies where animals have been supplemented with exogenous Mel

throughout life, life span has been increased by 25 %[12]. Using several different mouse strains, giving Mel in the drinking water nightly prolongs life, postpones aging, and maintains the animals in a more youthful state[13]. The gonadotropin-releasing hormone (GnRH) stimulated LH release by increasing $[Ca^{2+}]_i$ in neonatal rat pituitary cells. Mel inhibited the GnRH-induced elevation of $[Ca^{2+}]_i$ in a dose-dependent manner. Mel 100 $nmol \cdot L^{-1}$ inhibited GnRH-induced increase of $[Ca^{2+}]_i$ by 40 %. It is concluded that Mel may act by inhibiting Ca^{2+} influx. Mel may inhibit the GnRH-induced increase in $[Ca^{2+}]_i$ through a mechanism involving a pertussis toxin-sensitive G-protein[14]. Mel also averted the amyloid β protein (Aβ)-induced increases in $[Ca^{2+}]_i$ and lipid peroxidation. Mel was remarkably effective in preventing death of cultured neuroblastoma cells as well as oxidative damage and $[Ca^{2+}]_i$ increases induced by cytotoxic fragment of Aβ. The importance of this finding was that, in contrast to conventional antioxidants, Mel had a proposed physiological role in the aging process[15].

Our study showed that Mel inhibited $[Ca^{2+}]_i$ elevations induced by Bay-K-8644, KCl, and Glu directly in brain cells of newborn mice and the overload of $[Ca^{2+}]_i$ in old mouse cerebral cortex synaptosomes, suggesting that the reduction of $[Ca^{2+}]_i$ overload may be one of the antiaging mechanisms of Mel. It is possible that Mel reduced $[Ca^{2+}]_i$ overload in old mouse cerebral cortex by inhibiting Ca^{2+} influx.

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褪黑激素对小鼠脑细胞内游离钙浓度的抑制作用

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关键词 褪黑激素；钙；衰老；脑；突触体；大脑皮质；神经元；Bay-K-8644；氯化钾；谷氨酸钠

目的：研究褪黑激素(Mel)对老年小鼠大脑皮层突触体内钙含量以及激动剂诱发的新生小鼠脑细胞$[Ca^{2+}]_i$升高的影响，以探讨Mel抗衰老的作用机理. **方法**：钙离子荧光染料Fura 2-AM负载已制备的突触体或细胞，用RF-5000型双波长荧光分光光度计测定$[Ca^{2+}]_i$. **结果**：长期使用Mel抑制老年小鼠大脑皮层Ca^{2+}超负荷，Mel也降低钙通道激活剂Bay-K-8644、高浓度氯化钾(KCl)和谷氨酸钠诱发的分离的新生小鼠脑细胞$[Ca^{2+}]_i$升高. **结论**：Mel对中枢神经元$[Ca^{2+}]_i$超载的抑制作用与其抗衰老作用有关.

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