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Melatonin inhibits nicotinic acetylcholine receptor functions in bovine chromaffin cells

Su-Hyun Jo[1], Seung-Hyun Lee[2], Kyong-Tai Kim[3], and Se-Young Choi[2]*

[1]Department of Physiology, Institute of Bioscience and Biotechnology, BK21 Plus Graduate Program, Kangwon National University School of Medicine, Chuncheon 24341, Republic of Korea
[2]Department of Physiology, Dental Research Institute, Seoul National University School of Dentistry, Seoul 03080, Republic of Korea
[3]Department of Life Sciences, Division of Integrative Bioscience and Biotechnology, Pohang University of Science and Technology, Pohang 37673, Republic of Korea

Melatonin is a neurotransmitter that modulates various physiological phenomena including regulation and maintenance of the circadian rhythm. Nicotinic acetylcholine receptors (nAChRs) play an important role in oral functions including orofacial muscle contraction, salivary secretion, and tooth development. However, knowledge regarding physiological crosstalk between melatonin and nAChRs is limited. In the present study, the melatonin-mediated modulation of nAChR functions using bovine adrenal chromaffin cells, a representative model for the study of nAChRs, was investigated. Melatonin inhibited the nicotinic agonist dimethylphenylpiperazinium (DMPP) iodide-induced cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) increase and norepinephrine secretion in a concentration-dependent manner. The inhibitory effect of melatonin on the DMPP-induced $[Ca^{2+}]_i$ increase was observed when the melatonin treatment was performed simultaneously with DMPP. The results indicate that melatonin inhibits nAChR functions in both peripheral and central nervous systems.

Keywords: Melatonin, Nicotinic receptors, Calcium signaling, Neurotransmitter agents

Introduction

Melatonin is a neurotransmitter that exhibits various physiological functions ranging from circadian rhythm regulation to cancer cell metabolism [1,2]. Mainly synthesized from 5-hydroxytryptamine through serotonin N-acetyltransferase in the pineal gland, melatonin is secreted based on circadian rhythm and regulates whole body rhythmicity [3]. Melatonin also participates in cell protection in conditions such as ischemia by blocking cell death and inhibiting autophagy [4,5]. The cell-protective role of melatonin has been considered to occur when high concentrations of melatonin act as antioxidants. Recently, crosstalk between melatonin and other signaling substances has been observed mainly in the central nervous system [6,7].

Nicotinic acetylcholine receptors (nAChRs) are expressed in several oral tissues including submandibular ganglion cells [8], gingival epithelial cells [9], gingival keratinocytes [10], and gingival fibroblasts [11]. nAChRs have also been implicated in the development of maxillofacial skeletal muscle [12] and ameloblasts [13]. Therefore, crosstalk between melatonin and nAChRs is a subject of interest. However, how melatonin modulates nAChR functions remains unclear. Thus, understanding the mechanism of nAChR inhibition by melatonin is

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***Correspondence to:** Se-Young Choi, E-mail: sychoi@snu.ac.kr https://orcid.org/0000-0001-7534-5167

important for understanding the neuroprotective role of melatonin.

In this study, the effects of melatonin on neurotransmitter secretion by nAChRs were investigated in a peripheral nervous system model in which the nAChR acts as an excitatory neurotransmitter. Subsequently, bovine adrenal chromaffin cells with a nAChR-mediated neurotransmitter secretion system were used. Chromaffin cells act as postganglionic cells that secrete norepinephrine by receiving preganglionic cholinergic inputs from sympathetic systems [14]. Thus, the neurotransmitter secretion modulated by nAChRs has been intensively studied using chromaffin cells [15,16]. In the present study, crosstalk between melatonin and nAChRs was investigated to understand the peripheral neuromodulating effects of melatonin.

Materials and Methods

1. Materials

Melatonin, dimethylphenylpiperazinium (DMPP), and sulfinpyrazone were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fura-2 pentaacetoxymethyl ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR, USA). [^{3}H] Norepinephrine was purchased from PerkinElmer NEN (Boston, MA, USA). DMEM/F-12 and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA). Bovine calf serum and horse serum were obtained from HyClone (Logan, UT, USA).

2. Chromaffin cell preparation

Chromaffin cells were isolated from bovine adrenal medulla using the two-step collagenase digestion as previously described [17,18]. For the [^{3}H]norepinephrine secretion assay, isolated bovine chromaffin cells were plated in 6-well plates at a density of 5×10^5 cells per well. For the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) measurement, cells were transferred to 100-mm culture dishes (1×10^7 cells per dish). The cells were maintained in DMEM/F-12 containing 10% bovine calf serum and 1% penicillin/streptomycin. The cells were incubated in a humidified atmosphere of 95% room air and 5% CO_2. The culture medium was changed every three days.

3. Measurement of [^{3}H]norepinephrine secretion

[^{3}H]norepinephrine secretion from chromaffin cells was measured as previously described [18]. In brief, cells were loaded with [^{3}H]norepinephrine (1 μCi/mL) in DMEM/F-12 for 1 hour at 37℃ in a humidified atmosphere of 95% room air and 5% CO_2. The cells were incubated in Locke's solution (154 mmol/L NaCl, 5.6 mmol/L KCl, 10 mmol/L glucose, 2.2 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, and 5 mmol/L HEPES buffer adjusted to pH 7.4) to measure the basal secretion. The cells were stimulated with DMPP for 15 minutes and the medium was used to analyze the secreted [^{3}H]norepinephrine. Residual/total [^{3}H] norepinephrine was extracted by adding 10% trichloroacetic acid. The radioactivity was measured using a scintillation counter. The amount of [^{3}H]norepinephrine secreted was calculated as the percentage of total [^{3}H]norepinephrine content.

4. Free Ca^{2+} concentration measurement

The $[Ca^{2+}]_i$ was determined using the fluorescent Ca^{2+} indicator Fura-2/AM, as previously reported [19]. Briefly, the bovine chromaffin cell suspension was incubated in serum-free DMEM/F-12 media with 4 μM Fura-2/AM for 60 minutes at 37℃ under continuous stirring. Sulfinpyrazone (250 μM) was added to all solutions to prevent Fura-2 leakage. Fluorescence ratios were monitored with dual excitation at 340 and 380 nm and emission at 500 nm. Calibration of the fluorescent signal in terms of $[Ca^{2+}]_i$ was performed [20] using the following equation:

$$[Ca^{2+}]_i = K_D[(R - R_{min})/(R_{max} - R)](Sf_2/Sb_2)$$

where R is the ratio of fluorescence emitted by excitation at 340 and 380 nm. Sf_2 and Sb_2 are the proportionality coefficients at 380 nm excitation of Ca^{2+}-free Fura-2 and Ca^{2+}-saturated Fura-2, respectively. R_{min}, the minimal fluorescence ratio, was measured at a condition of 4 mmol/L EGTA, 30 mmol/L Trizma base, and 0.1% Triton X-100. Then R_{max}, the maximal fluorescence ratio, was measured at a condition of 10 mmol/L Ca^{2+}.

5. Data analysis

All quantitative data are expressed as means ± standard error of the mean. Data were analyzed using Origin software (Origin Lab, Northampton, MA, USA). The results were analyzed using the unpaired Student's t-test and $p < 0.05$ was considered statistically significant.

Results

To examine the regulation of nAChR functions in melatonin, first, the effects of melatonin on nAChR-induced norepinephrine secretion in bovine adrenal chromaffin cells were examined. The nAChR-specific agonist, DMPP, successfully induced the preloaded norepinephrine secretion. Preincubation with melatonin inhibited DMPP-mediated norepinephrine secretion in a concentration-dependent manner (Fig. 1).

The catecholamine secretion is triggered by elevated $[Ca^{2+}]_i$ level. The effects of melatonin on DMPP-induced $[Ca^{2+}]_i$ increase was investigated. DMPP evoked an increase in $[Ca^{2+}]_i$ in Fura-2-loaded bovine adrenal chromaffin cells. Under this condition, melatonin inhibited DMPP-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner (Fig. 2). In addition, the inhibitory effect of DMPP-induced $[Ca^{2+}]_i$ increase was more pronounced with the melatonin analogue, 2-iodomelatonin.

Next, whether melatonin acts directly on nAChRs or by activating melatonin-specific receptors was examined. The time course of melatonin-mediated inhibition of DMPP-induced $[Ca^{2+}]_i$ increase was investigated. Melatonin simultaneously suppressed the inhibition of the DMPP-induced $[Ca^{2+}]_i$ increase when preincubated with melatonin for 30 seconds (Fig. 3). The result indicates melatonin does not require preincubation to exert an inhibitory effect and may directly inhibit nAChRs.

Discussion

In this study, the effect of melatonin on the regulation of nAChR functions was investigated to understand crosstalk between melatonin and nAChRs. Results showed preincubation with melatonin inhibited DMPP-mediated norepinephrine secretion, and melatonin inhibited DMPP-mediated $[Ca^{2+}]_i$ in-

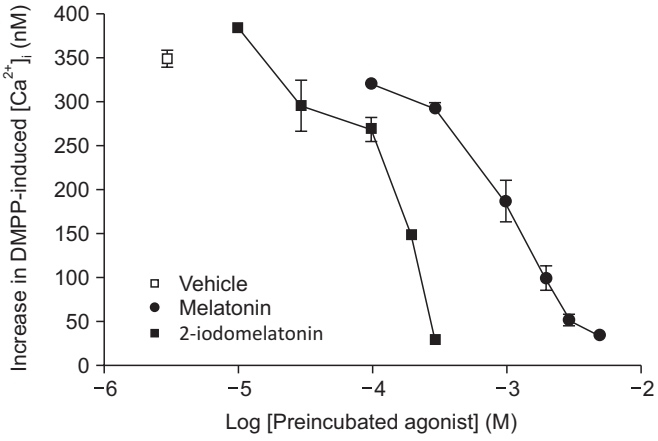

Fig. 2. Melatonin inhibits DMPP-evoked $[Ca^{2+}]_i$ increase in bovine adrenal chromaffin cells. Fura-2-loaded cells were treated with 20 μM DMPP in the presence of the indicated concentrations of melatonin, 2-iodomelatonin, or vehicle. Peak levels of DMPP-induced Ca^{2+} influx were quantitatively analyzed and depicted as % of the DMPP-induced $[Ca^{2+}]_i$ increase. Each point is the mean ± standard error of the mean (n = 3–5).
DMPP, dimethylphenylpiperazinium; $[Ca^{2+}]_i$, free Ca^{2+} concentration.

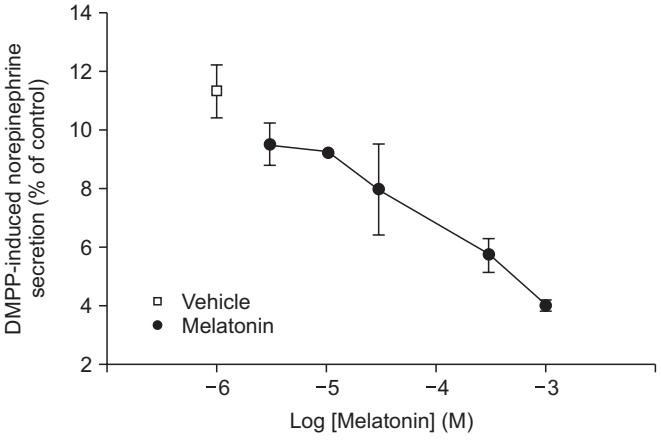

Fig. 1. The effects of melatonin on [^{3}H]norepinephrine secretion in bovine adrenal chromaffin cells. [^{3}H]Norepinephrine-loaded chromaffin cells were treated with 20 μM dimethylphenylpiperazinium (DMPP) in the presence of the indicated concentrations of melatonin or vehicle. The secreted [^{3}H]norepinephrine is expressed as % of total [^{3}H]norepinephrine. Each point is the mean ± standard error of the mean (n = 3).

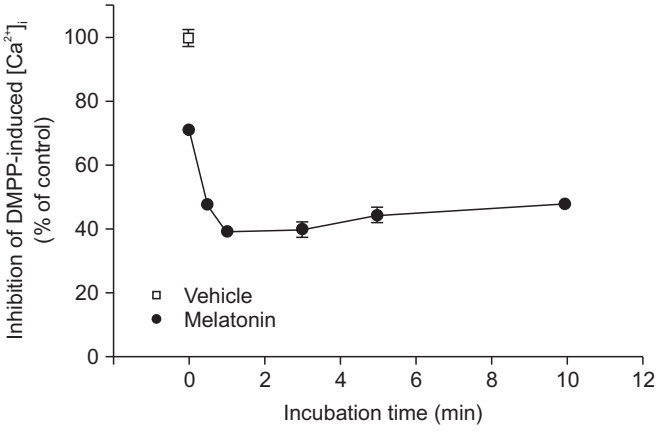

Fig. 3. Time course of melatonin-mediated inhibition of DMPP-evoked $[Ca^{2+}]_i$ increase. Fura-2-loaded cells were treated with 20 μM DMPP after preincubation with 3 μM melatonin, or vehicle, for the indicated time. Peak levels of DMPP-induced Ca^{2+} influx were quantitatively analyzed and depicted as % of the DMPP-induced $[Ca^{2+}]_i$ increase. Each point is the mean ± standard error of the mean (n = 3–6).
DMPP, dimethylphenylpiperazinium; $[Ca^{2+}]_i$, free Ca^{2+} concentration.

crease in bovine adrenal chromaffin cells.

Melatonin controls cell functions in various ways. Crosstalk between melatonin and nAChRs can be divided into the following two types: (1) Melatonin directly inhibits nAChRs. Melatonin has direct binding affinity to nAChRs in the noradrenergic nerve terminal of rat vas deferens [21] and guinea pig submucous plexus [22]. In addition, melatonin was shown to exert a neuroprotective effect by inhibiting alpha-7 nAChR in organotypical hippocampal cultures [23]. (2) Melatonin modulates via melatonin-specific G protein-coupled receptors. In general, similar to amino acid derivative neurotransmitters, melatonin activates melatonin-specific G protein-coupled receptors, MT1 and MT2 [24]. In cerebellar granule cells, picomolar melatonin inhibited nAChR-mediated cation current through MT1 and MT2 receptors [25]. However, the detailed mechanism of how melatonin regulates other components, including the peripheral nervous system, remains unclear. Therefore, identifying molecular targets that receive the neuroactive effects of melatonin is important to better understand the effects of complex and diverse functions of melatonin. In the present study, the dose curve and time course of the inhibitory effects of melatonin on melatonin-induced $[Ca^{2+}]_i$ increase was investigated, and results showed inhibition was in a relatively high concentration range within a very short time period (i.e. within several seconds). These results indicate the melatonin-mediated nAChR inhibition in chromaffin cells may be caused by a direct inhibition independent of G protein-coupled receptors. This direct action was observed in other mechanistic effects of melatonin, and we recently showed the voltage-sensitive calcium channels inhibitory effect of melatonin acts in a melatonin-specific receptor-independent manner [26].

nAChRs are expressed in submandibular ganglion cells and regulate salivary secretion [8]. In addition, nAChRs are expressed in maxillofacial skeletal myocytes to regulate tensile stress [12]. In particular, the nAChRs present in ameloblasts are involved in enamel formation by differential expression during the tooth morphogenesis stage [13]. Therefore, the effect of melatonin on oral functions is very important in dental science. Recently, neuromodulation of nAChRs in the central nervous system has received much clinical attention. Choline esterase inhibitors, which block the degradation of acetylcholine and increase its concentration, have been shown to improve cognitive function in Alzheimer's disease and mild cognitive impairment; nAChRs play an important role in this mechanism [6,27]. Therefore, the results from this study will not only contribute to a better understanding of the physiology of melatonin and nAChRs, but also reaffirm the possibility for various clinical applications of melatonin as a nAChR modulator.

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Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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