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Effects of scoparone on dopamine release in PC12 cells

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ABSTRACT

The effects of scoparone on dopamine release in PC12 cells were investigated. Scoparone at 50–200 μM increased dopamine release into the culture medium. However, the released levels of dopamine by scoparone were not altered in the absence of extracellular Ca^{2+} and by adenylyl cyclase inhibitor MDL-12,330A. Scoparone increased phosphorylation of PKA, CaMK II and synapsin I. Scoparone also enhanced K^+-induced levels of dopamine release by CaMK II phosphorylation. These results suggest that scoparone increases dopamine release by synapsin I phosphorylation via activation of PKA and CaMK II, which are mediated by cyclic AMP levels and Ca^{2+} influx.

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1. Introduction

Degeneration of striatal dopaminergic neurons mediated by the genetic and various environmental factors causes Parkinson's disease, one of the major aging-related neurodegenerative diseases [1]. It has been proposed that regulation of dopaminergic neuronal cell survival and activation of dopamine biosynthesis and neurite outgrowth could potentially relieve the symptoms of Parkinson's disease. The exocytosis function of synapses might also underlie the altered neuronal activity associated with neurodegenerative diseases and phenomena like learning and memory [2].

The release of dopamine from nerve terminals by exocytosis is closely dependent on extracellular Ca^{2+} levels [3] and this process appears to be regulated by the presynaptic actin cytoskeleton [4]. Synapsin I, a synaptic vesicle-associated protein, regulates interactions between synaptic vesicles and the cytoskeleton through its phosphorylation by protein kinase A (PKA) and Ca^{2+}/calmodulin kinase II (CaMK II) [5,6]. The increases in the levels of Ca^{2+} and/or cyclic AMP facilitate neurotransmitter release including dopamine via regulation of synapsin phosphorylation [6]. In addition, elevated K^+ concentration (56 mM) increases intracellular Ca^{2+} levels due to membrane depolarization and induces the release of dopamine in PC12 cells [7]. High K^+ (56 mM) also induces CaMK II phosphorylation [8].

Scoparone (6,7-dimethoxycoumarin) is a major component isolated from the stem bark of *Liriodendron tulipifera* (Magnoliaceae), which exhibits immunosuppressive, vasodilating and anti-inflammatory activities [9,10]. Scoparone is also known to be a phytoalexin [11], which is frequently produced in stressed and infected tissues of plants, and which plays an important role in pathogen resistance.

Recently, it was reported that scoparone induces neurite outgrowth and dopamine biosynthesis, and also protects L-3,4-dihydroxyphenylalanine (L-DOPA)-induced cytotoxicity in PC12 cells, which are mediated by the signaling pathways of cyclic AMP and Ca^{2+} [12,13]. Cyclic AMP regulates tyrosine hydroxylase (TH) activity and its gene expression by activating PKA in dopaminergic neurons and rat adrenal pheochromocytoma, PC12, cells [14]. Cyclic AMP also protects against oxidative stress-induced apoptotic PC12 cell death [15]. Intracellular Ca^{2+} stimulates conventional protein kinase C (PKC) and CaMK II, which also regulates TH gene expression and neuronal cell survival and death [16].

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PC12 cells have been extensively used as an *in vitro* model for dopamine biosynthesis and release, signal transduction and neuronal differentiation [17]. In this study, to further elucidate the functions of scoparone, the effects of scoparone on dopamine release were investigated using undifferentiated PC12 cells.

2. Experimental

2.1. Chemicals

L-DOPA, dopamine, MDL-12,330A hydrochloride, N-2-hydroxyethyl-piperazine-N′-2-ethanesulfonic acid (HEPES) and poly-L-lysine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cyclic AMP enzyme immunoassay system kit was purchased from Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). FLUO4/AM was purchased from Molecular Probes (Eugene, OR, USA). Primary antibodies against synapsin, phosphor-synapsin (Ser 9) and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). RPMI 1640, fetal bovine serum (FBS), horse serum (HS), penicillin and streptomycin were obtained from Gibco (Grand Island, NY, USA). All other chemicals were of reagent grade.

2.2. Cell culture and determination of dopamine release

PC12 cells were grown in RPMI 1640 supplemented with 10% heat-inactivated HS, 5% heat-inactivated FBS, 100 Units/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere with 5% CO_2 at 37 °C [17]. PC12 cells were treated with Krebs–Ringer–HEPES (KRH) solution [135 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH adjusted to 7.4 with NaOH)] in the presence or absence of scoparone at 37 °C. For the K^+-induction experiments, KRH solution was substituted by high K^+ (56 mM)-KRH solution (NaCl concentration was reduced proportionally to maintain osmolarity). For the Ca^{2+}-depletion experiments, Ca^{2+}-free KRH solution containing 1 mM EGTA was used. The samples were centrifuged at 12,000 rpm for 5 min and the released levels of dopamine in the supernatant were determined by an HPLC system (Toso, Tokyo, Japan) as previously described [13,18].

2.3. Determination of cyclic AMP and Ca^{2+} levels

Intracellular levels of cyclic AMP were measured by an immunoassay system kit using a Bauty Diagnostic microplate reader (Molecular Devices, Sunnyvale, CA, USA). Intracellular Ca^{2+} levels were measured using a fluorescent calcium chelator (FLUO4/AM) and a confocal microscopy system at excitation and emission wavelengths of 488 and 522 nm, respectively (LEICA Microsystems GmbH, Heidelberg, Germany).

2.4. Assay for PKA and CaMK II phosphorylation

Phosphorylation of PKA and CaMK II were measured by a non-radioactive PKA assay kit (Stressgen, Victoria, BC, Canada) and CaMK II assay kit (CycLex Co., Nagano, Japan), respectively, according to the supplier's instruction. The final solutions were determined using the aforementioned microplate reader at 450 nm.

2.5. Western blot analysis of synapsin

Activation of synapsin, phosphor-synapsin (Ser 9) and β-actin were analyzed by Western blot. Aliquots of the lysates (30 μg) from each protein sample were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were probed with 1:1000 dilutions of the particular primary antibody and the secondary antibody in blocking buffer (3% nonfat dry milk). The blots were then incubated with an enhanced chemiluminescence substrate solution (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and visualized with radiographic film.

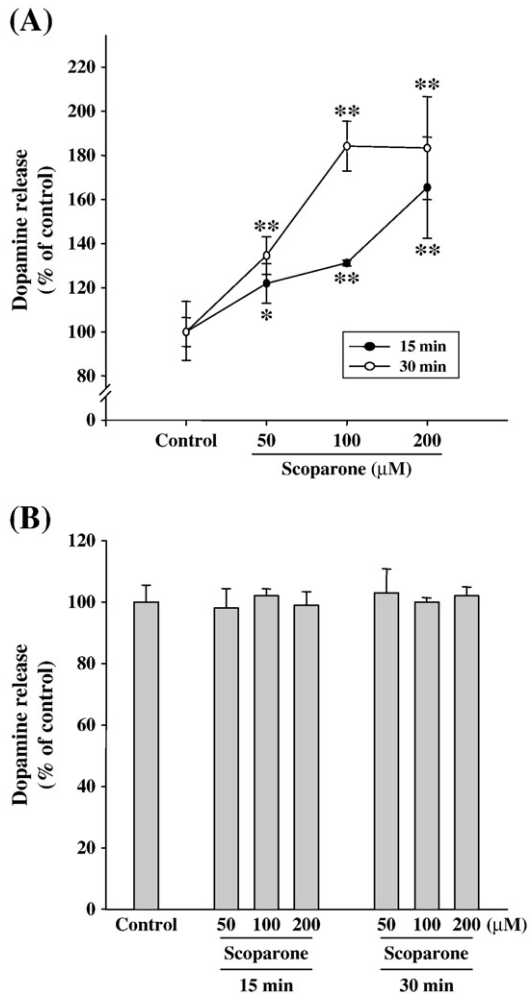

Fig. 1. Effects of scoparone on basal dopamine release from PC12 cells in the presence (A) and absence (B) of extracellular Ca^{2+}. PC12 cells were treated with 50, 100 or 200 μM scoparone and incubated at 37 °C for 15 or 30 min. The extracellular levels of dopamine in the cultured media were measured by an HPLC method. The control level of dopamine release was 0.55 ± 0.04 nmol/ml medium. The results are expressed as the percentages of the control levels and represent means ± S.E.M. of four experiments. Significantly different from control values: $^*p<0.05$; $^{**}p<0.01$ (ANOVA followed by Tukey's test).

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