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Inhibitory effect of donepezil on bradykinin-induced increase in the intracellular calcium concentration in cultured cortical astrocytes

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ABSTRACT

Donepezil is a potent and selective acetylcholinesterase inhibitor developed for the treatment of Alzheimer's disease. In the present study, we investigated the responses of astrocytes to bradykinin, an inflammatory mediator, and the effect of donepezil on these responses using cultured cortical astrocytes. Bradykinin induced a transient increase of intracellular calcium concentration ($[Ca^{2+}]_i$) in cultured astrocytes. Bradykinin-induced $[Ca^{2+}]_i$ increase was inhibited by the exposure to thapsigargin, which depletes Ca^{2+} stores on endoplasmic reticulum, but not by the exclusion of extracellular Ca^{2+}. Twenty four hours pretreatment of donepezil reduced the bradykinin-induced $[Ca^{2+}]_i$ increase. This reduction was inhibited not only by mecamylamine, a nAChR antagonist, but also by PI3K and Akt inhibitors. In addition, donepezil inhibited bradykinin-induced increase of the intracellular reactive oxygen species level in astrocytes. These results suggest that donepezil inhibits the inflammatory response induced by bradykinin via nAChR and PI3K-Akt pathway in astrocytes.

1. Introduction

Alzheimer's disease (AD), one of the most common diseases presenting dementia, is a progressive neurodegenerative disorder characterized by cognitive deficit, and inflammation in the brain is involved in the pathogenesis.[1–4] In inflammatory condition, it is reported that glial cells, such as astrocytes and microglia, are abnormally activated.[5,6] Abnormal activation of glial cells contributes to the pathogenesis of various neurodegenerative disorder, such as AD and Parkinson's Disease.[1–4,7]

Astrocytes are the most abundant cells in central nervous system (CNS), and play important roles in the maintenance of neuronal activity through the release of neurotrophic factors, the maintenance of ion gradient such as extracellular K^+, the construction of blood brain barrier.[8] However, recent study revealed that in pathological condition, astrocytes were abnormally activated and could be deleterious to adjacent neurons through the release of various inflammatory cytokines.[9] Therefore, inhibition of the abnormal activation of astrocytes is supposed to be a therapeutic strategy for several CNS diseases involving inflammation.

Bradykinin, an inflammatory mediator, is produced in the early stage of inflammation and induces the expressions of several inflammatory genes in the brain.[10–12] Particularly, in astrocytes, bradykinin induces a variety of responses such as a transient increase of intracellular calcium concentration ($[Ca^{2+}]_i$),[13] the expression of matrix metalloprotease-9 (MMP-9)[10] and cyclooxygenase-2 (COX-2),[11] and release of interleukin-6 (IL-6)[12] and glutamate.[14] In addition, it has been previously reported that the cleavage of high molecular weight kininogen, the precursor of bradykinin, was increased in the cerebrospinal fluid of AD patients,[15,16] and that bradykinin receptor antagonists ameliorated the cognitive deficits in AD model mice.[17–19] Accordingly, it can be speculated that inflammation induced by bradykinin in astrocytes is involved in the pathogenesis of AD.

Donepezil is a potent and selective acetylcholinesterase inhibitor developed for the treatment of AD,[20] and has been reported to have some effects other than acetylcholinesterase inhibition.[21–23] We previously reported that donepezil protected cultured cortical

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neurons against glutamate neurotoxicity via nicotinic acetylcholine receptors and phosphatidylinositol-3 kinase (PI3K)-Akt pathway.[22] Other group reported that donepezil inhibited the production of inflammatory cytokines induced by the treatment of amyloid-β (Aβ) oligomer in cultured microglia.[23] However, the effect of donepezil on the function in astrocyte has not been elucidated.

In the present study, to elucidate the effect of donepezil on the function in astrocyte, we investigated the responses of astrocytes to bradykinin and the effects of donepezil on these responses using cultured cortical astrocytes.

2. Materials and methods

2.1. Materials

Eagle's minimum essential medium (MEM) was purchased from Nissui Pharmaceutical (Tokyo). Fetal bovine serum was obtained from JRH Biosciences (Lenexa, KS, USA).

Drugs and sources were as follows: Glucose, glutamine, glutamate, HEPES, $NaHCO_3$, hydrochloric acid (HCl), trypsin (Nacalai Tesque Kyoto); dihydro-β-erythroidine hydrobromide and methyllycaconitine citrate (ResearchBiochemicals International, Natick, MA); mecamylamine hydrochloride, and LY294002, Akt inhibitor, and AG490 (Calbiochem, Darmstadt, Germany); bradykinin, des-Arg^9-[Leu^8]-Bradykinin, and HOE140 (peptide institute. Inc., Osaka); thapsigargin (alomone labs); donepezil hydrochloride (Eisai Co. Ltd, Tsukuba).

2.2. Animals

Experiments were carried out using postnatal day 1 pups of Wistar rats for cortical astrocyte monoculture, which were purchased from Japan SLC (Shizuoka). All animal studies were conducted in accordance with the Ethical Guidance of the Kyoto University Animal Experimentation Committee and the Guidance of The Japanese Pharmacological Society. All procedures were approved by the Animal Research Committee, Graduate School of Pharmaceutical Science, Kyoto University.

2.3. Preparation of cultured cortical astrocytes

Cultured cortical astrocytes were prepared from postnatal day 1 pups of Wistar rats according to previously described procedures with slight modification.[24] In brief, the cells were dissociated from the cerebral cortices of neonates, filtered through a cell strainer (100 μm), and plated on uncoated 75 cm^2 flasks. Cultures were incubated in Eagle's MEM supplemented with 10% heat-inactivated fetal bovine serum, glutamine (2 mM), glucose (total 11 mM), $NaHCO_3$ (24 mM), and HEPES (10 mM) at 37 °C in a humidified 5% CO_2 atmosphere. After the cells became confluent, non-astrocyte cells such as microglia and oligodendrocytes were removed by shaking at 400 rpm for 10 min followed by changing the medium, and 2 h after the medium change, flasks were shaken at 250 rpm for 17–19 h on the orbital shaker. Astrocytes were detached using 0.25% trypsin and reseeded on 60-mm dishes, 6-well plates or 24-well plates. The astrocytes were used for experiments at least 1 week after plating. The purity of astrocytes was >95% as determined by immunostaining with anti-GFAP antibody.

2.4. Reverse transcription-PCR

Total RNA was extracted from cultured astrocytes in a 60 mm dish using a ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, USA). Using total RNA, two-step reverse transcription-PCR was performed with the PrimeScript RT-PCR Kit (Takara Bio, Kusatsu) using a PCR Thermal Cycler Dice Gradient (TP600; Takara Bio). The pairs of primers used for amplification were as follows: 5′-CCGGACCACAGCTGGATTTG-3′ and 5′-TCTTCTAGGGCACTCTCGCA-3′ for B_1 receptors; 5′-AGGACGATCCT-CACTCGTCT-3′ and 5′-CATTTCAATGCCCAGAGAGGC-3′ for B_2 receptors; The temperature cycles were 95 °C for 2 min, followed by 35 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s. Amplified PCR products were electrophoresed in a 2% TAE agarose gel and visualized under UV light with 0.1 μg/ml ethidium bromide by using ChemiDoc XRS (Bio-Rad, Hercules, CA, USA). Images were captured with Quantity One software (Bio-Rad).

2.5. Intracellular Ca^{2+} imaging

Intracellular Ca^{2+} concentrations were measured with a Ca^{2+}-sensitive fluorescent dye, fura2-acetoxymethylester (fura2-AM), on a fluorescence imaging system (ARGUS/HiSCA, Hamamatsu Photonics K.K., Shizuoka) according to the methods. Cortical astrocytes cultured on glass coverslips were incubated in Krebs-Ringer buffer (137 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.5 mM $CaCl_2$, 25 mM D-(+)-glucose, 10 mM HEPES, pH 7.4) containing 5 μM fura2-AM and 0.01% cremophorEL (polyoxethylated castor oil) for 30 min at 37 °C and then rinsed with buffer. The cells were alternatively illuminated with light (wavelengths of 340 and 380 nm) at an interval of 2 s and the emission was measured at 500 nm. In this study, peak amplitude was used as an index of $[Ca^{2+}]_i$ increase. Peak amplitude was calculated by subtracting the average value of fluorescence ratio (340 nm/380 nm) in the first 1 min of the measurement from the maximum value of fluorescence ratio (340 nm/380 nm) after drug application.

2.6. Western blotting

Treated cells were washed twice with cold Tris-buffered saline, harvested using a cell scraper, and lysed in buffer containing 20 mM Tris (pH 7.0), sodium 25 mM β-glycerophosphate, 2 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1% aprotinin, 2 mM dithiothreito, and 1 mM vanadate on ice. Lysates were centrifuged at 15,000 rpm for 30 min at 4 °C. After normalization of protein concentrations, supernatants were mixed in equal amounts with a sample loading buffer. After denaturation by boiling at 100 °C for 5 min, samples were loaded onto a SDS-polyacrylamide gel, separated electrophoretically, and transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membranes were incubated for 1 h with Tris-buffered saline containing 0.1% Tween 20 and 5% dehydrated skim milk to block nonspecific binding. Subsequently, the membranes were probed with primary antibody [anti-phospho-Akt (No. 9271, 1:1000 dilution, Cell Signaling Technology, Danver, MA, USA)], anti-Akt (No. 9272, 1:1000 dilution, Cell Signaling Technology), and with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution, GE Healthcare, Waukesha, WI, USA) for 1 h. The membrane-bound secondary antibody was detected with an enhanced chemiluminescence detection system (ECL, GE Healthcare). The band intensities were analyzed with computer software, ImageJ 1.33u (National Institute for Health, Bethesda, MD, USA).

2.7. Measurements of intracellular ROS production

Intracellular ROS level was measured using 5-(and-6)-carboxy-2, 7-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA; Molecular Probes, Eugene, OR, USA) in astrocytes preincubated with 5 μM carboxy-H_2DCFDA for 30 min. A rise in the intracellular ROS level was evaluated as the percentage increase in the fluorescence intensity, $(F-F_0)/F_0$, where F_0 and F represent the

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