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Honokiol and magnolol induce Ca^{2+} mobilization in rat cortical neurons and human neuroblastoma SH-SY5Y cells

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Abstract

We examined the intracellular Ca^{2+} response in primary cultured rat cortical neurons and human neuroblastoma SH-SY5Y cells by Fluo 3 fluorescence imaging analysis. In these two kinds of neuronal cells, honokiol and magnolol increased cytoplasmic free Ca^{2+} with a characteristic lag phase. The cytoplasmic free Ca^{2+} increase was independent of extracellular Ca^{2+}, but dependent on activation of phospholipase C and inositol 1,4,5-triphosphate (IP_3) receptors. These results suggest that honokiol and magnolol increase cytoplasmic free Ca^{2+} through a phospholipase C-mediated pathway, and that the release of Ca^{2+} from intracellular stores mainly contributes to the increase in cytoplasmic free Ca^{2+}. Thus, honokiol and magnolol may be involved in a new activation mechanism closely associated with intracellular Ca^{2+} mobilization.

Keywords: Honokiol; Magnolol; Ca^{2+}; Cortical neuron, rat; SH-SY5Y neuroblastoma

1. Introduction

Honokiol and magnolol are the main constituents of the stem bark of *Magnolia obovata* Thunb (Fujita et al., 1973) and *Magnolia officinalis* Rhed (Li, 1985). They exhibit central depressant (Watanabe et al., 1983), muscle-relaxing (Watanabe et al., 1975) and anxiolytic (Maruyama et al., 1998) effects. These effects have been explained by the actions of honokiol and magnolol on $GABA_A$ receptors (Ai et al., 2001; Squires et al., 1999). Additionally, a dihydrogenated derivative of honokiol showed inhibitory activity on the ammonia-induced increase in intracellular Cl^- in cultured rat hippocampal neurons via $GABA_C$ receptors (Irie et al., 2001). Honokiol and magnolol also regulate the release of neurotransmitters, such as acetylcholine (Hou et al., 2000) and 5-hydroxytryptamine (Hsieh et al., 1998; Tsai et al., 1995). Recently, we reported that honokiol and magnolol had neurotrophic properties, such as promotion of neurite outgrowth and neuronal survival under serum-deprived conditions in cultured rat cortical neurons (Fukuyama et al., 2002).

Intracellular Ca^{2+} plays a pivotal role in regulating diverse aspects of cellular function (Berridge et al., 2000). In the nervous system, changes in intracellular Ca^{2+} levels underpin major neuronal process, including transmitter release, excitability, synaptic plasticity, neurite outgrowth and gene expression (Berridge, 1998; Ghosh and Greenberg, 1995). As part of our studies to clarify the neurotrophic mechanism of honokiol and magnolol, we decided to examine the effect of both compounds on Ca^{2+} homeostasis in neurons by using Fluo 3 fluorescence imaging analysis. In this paper, we report that honokiol and magnolol induced intracellular Ca^{2+} mobilization in primary cultured fetal rat neurons and human neuroblastoma SH-SY5Y cells.

2. Materials and methods

2.1. Materials

Honokiol and magnolol were isolated from the cortex of *M. officinalis* Rhed. The purity was determined by high-performance liquid chromatography (single peak) and by nuclear magnetic resonance spectra. 4-(6-Acetoxymethoxy-2,7-dichloro-3- oxo-9-xanthenyl)-4′-methyl-2,2′ (ethylenedioxy)dianiline-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis (acetoxy-

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methyl) ester (Fluo 3-AM) was purchased from Dojindo Molecular Technologies (Kumamoto, Japan). Neurobasal™ culture medium, Dulbecco's modified eagle medium (DMEM), fetal bovine serum, and B27 supplement were purchased from Gibco (NY, USA). Other compounds used were purchased from Sigma (MO, USA) and Wako (Osaka, Japan).

2.2. Cell culture

2.2.1. Primary culture of cortical neurons

Cerebral cortical cells were obtained from fetuses of timed-pregnant Sprague–Dawley rats (SLC, Japan). The E18 dams were killed by cervical dislocation and the fetuses were immediately removed and placed in Dulbecco's phosphate-buffered saline. The fetuses were quickly decapitated, and the brains were removed and cleaned of meninges. The cortical hemispheres were dissected out, minced with forceps, and completely dissociated into a single-cell suspension using trypsin (first 0.25% trypsin 20 min, then DNase I 5 min). The suspension then was washed two times with MEM (15 ml, 5 min × 1000 rpm). Isolated neurons were diluted to 10×10^4 cells/ml, seeded on ϕ15 mm poly-L-lysine-coated coverslips placed in 24-well plate (0.5 ml/well) in Neurobasal™ medium supplemented with B27 (50:1), 0.5 mM glutamine and 50 IU/ml penicillin and 50 μg/ml streptomycin. The cells were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 and were used for experiments between day 2 and 3 in vitro.

2.2.2. Culture of neuroblastoma SH-SY5Y cells

The neuroblastoma SH-SY5Y cells were obtained from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). Cultures were maintained in DMEM supplemented with 10% fetal calf serum, 50 IU penicillin/ml, 50 μg/ml streptomycin at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For the experiments, confluent SH-SY5Y cells were dissociated, washed and seeded as described above for primary cultures of cortical neurons. The cells were used before they were 50% confluent.

2.3. Fluorescence imaging of cytoplasmic free Ca^{2+} in single cells

Cortical neurons and SH-SY5Y cells were loaded with the Ca^{2+}-sensitive fluorescent dye, Fluo 3-AM (1 μM), at 37 °C for 30 min in culture medium. Then, the cells were incubated for an additional 30 min in a Fluo 3-AM-free physiological buffer (composition in mM: NaCl 145, KCl 5, $MgCl_2$ 0.8, $CaCl_2$ 1.8, HEPES 10, D-glucose 10; pH 7.4 adjusted with NaOH) at room temperature (24–26 °C) to remove extracellular traces of the dye and to complete de-esterification. Subsequently, the coverslips were mounted cell-side up in the free bottom of a RC-20H perfusion chamber (Warner Instruments, Hamden, CT, USA), placed on the stage of an inverted microscope (Eclipse TE300, Nikon, Japan). Throughout the experiments, cells were continuously perfused at ~ 1.5 ml/min by means of gravity-driven perfusion system with drug-free or drug-containing buffer solutions. In some cases, a Ca^{2+}-free buffer was used, which contained NaCl 145, KCl 5, $MgCl_2$ 0.8, HEPES 10, D-glucose 10, EGTA 0.1 in mM; pH 7.4 adjusted with NaOH. The dye in the selected cytoplasmic part of cells was excited by wavelength 488 nm and fluorescence images were captured at 530 nm through a 40 × objective lens (S Fluor 40/0.90, Nikon) at 1-s intervals by an intensified CCD camera (C4742-95ER, Hamamatsu, Japan) controlled by a computer. Fluorescence images were analyzed with the Aquacosmos image processing and measuring system (Hamamatsu, Japan). Data are expressed as F/F_0; F is the intensity value obtained during the experiment and F_0 is the baseline intensity value. All fluorescence measurements were performed at room temperature.

3. Results

3.1. Effects of honokiol and magnolol on intracellular Ca^{2+} mobilization in rat cortical neurons

Methacholine, a muscarinic receptor agonist, has been reported to mobilize intracellular Ca^{2+} by activation of the phospholipase C-inositol 1,4,5-triphosphate pathway (Sorimachi et al., 1992). Methacholine (10 μM) induced a rapid transient increase of intracellular Ca^{2+} in primary cultured cortical neurons (Fig. 1B). This is a characteristic of the muscarinic receptor agonist-mobilized Ca^{2+} response (Prothero et al., 2000). This demonstration ensured the reliability of our Ca^{2+}-imaging perfusion system. Honokiol and magnolol were applied to this system to determine the change in the concentration of free Ca^{2+}. They increased the Ca^{2+} concentration in the dose range 5–20 μM (Fig. 2). Compared with methacholine, the honokiol and magnolol in-

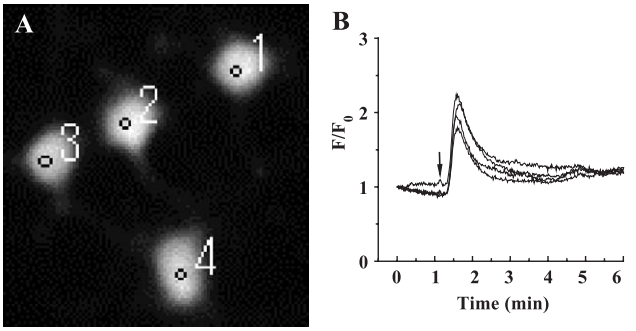

Fig. 1. Demonstration of cortical neurons by fluorescence microscopy and methacholine-induced Ca^{2+} response. (A) Neurons with similar fluorescence intensity were chosen to measure Ca^{2+} change. Fluorescence intensity of numbered cycles in cytoplasm was captured by a computer-controlled digital camera and presented as average. (B) Ca^{2+} responses induced by 10 μM methacholine, a muscarinic receptor agonist. Arrow in the figure shows the start of perfusion buffer containing drugs.

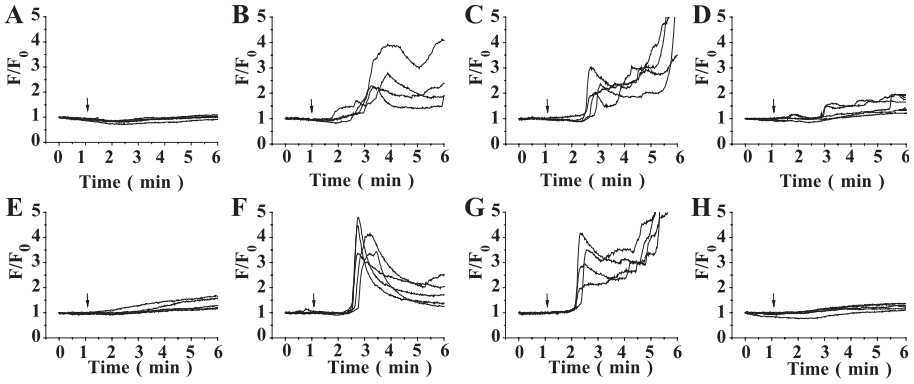

Fig. 2. Cytoplasmic Ca^{2+} increase induced by honokiol and magnolol, and inhibitory effects of U73122 in primary cultured rat cortical neurons. (A), (B) and (C) illustrate the typical responses induced by 5, 10 and 20 μM honokiol, respectively. (E), (F) and (G) illustrate the typical responses induced by 5, 10 and 20 μM magnolol, respectively. (D) and (H) show the increase in intracellular Ca^{2+} in response to 10 μM honokiol and 10 μM magnolol, after a 5-min pre-perfusion with 10 μM U73122. In each group, 7–20 neurons were measured at the same time, 4–6 of which were selected and presented. The viability of SH-SY5Y cells in the U73122-treated group was checked with 10 μM iononmycin. Experiments were performed at least three times and the results were confirmed to be similar. Data presented here came from the same batch of isolated neurons. Arrows in the figure show the start of perfusion of buffer containing drugs.

duced-Ca^{2+} responses had a 30–90-s lag phase. After the lag phase, an initial spike of Ca^{2+} followed by a diminishing phase was observed (Fig. 2A,B,C,E,F,G). Application of 20 μM honokiol and magnolol caused a second increase after the Ca^{2+} had decreased to a level higher than the baseline before drug perfusion (Fig. 2C,G).

3.2. Effects of phospholipase C inhibitor on the honokiol and magnolol-induced Ca^{2+} changes in rat cortical neurons

To identify the source of the increased cytoplasmic free Ca^{2+}, we first used the phospholipase C specific inhibitor, 1-[6-[[17β-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]-hexyl]-1*H*-pyrrole-2,5-dione (U73122) (Jin et al., 1994). IP_3 is a central regulator of intracellular Ca^{2+} mobilization. U73122 can inhibit IP_3 production, thereby preventing intracellular Ca^{2+} mobilization. After pre-incubation with 10 μM U73122 for 5 min before treatment of honokiol and magnolol, the Ca^{2+} response induced by 10 μM honokiol was greatly reduced (Fig. 2D), whereas the Ca^{2+} response induced by 10 μM magnolol was completely eliminated (Fig. 2H).

3.3. Effects of extracellular Ca^{2+} removal on the honokiol and magnolol-induced Ca^{2+} response in rat cortical neurons

To know whether extracellular Ca^{2+} is involved in the effect of honokiol and magnolol, Ca^{2+} was removed from the perfusion buffer and 0.1 mM of the Ca^{2+} chelator EGTA was added to ensure the buffer was Ca^{2+} free. In this Ca^{2+}-free buffer, the Ca^{2+} mobilization still occurred in a dose-dependent manner at concentrations from 2.5 to 20 μM (Fig. 3). Compared with each response in Ca^{2+}-containing buffer,

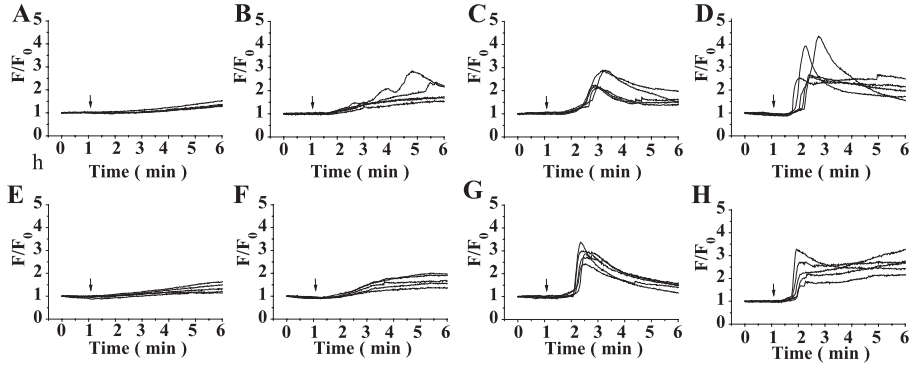

Fig. 3. In Ca^{2+}-free buffer, intracellular Ca^{2+} increased in response to honokiol and magnolol in primary cultured rat cortical neurons. (A), (B), (C) and (D) show the typical Ca^{2+} response of neurons to perfusion with 2.5, 5, 10 and 20 μM honokiol in Ca^{2+}-free buffer, respectively. (E), (F), (G) and (H) show typical Ca^{2+} response of neurons to perfusion with 2.5, 5, 10 and 20 μM magnolol. In each group, 7–20 neurons were measured at the same time, 4–6 of which were selected and presented. Experiments were performed at least three times and the results were confirmed to be similar. Data presented here came from the same batch of isolated neurons. Arrows in the figure show the start of perfusion of buffer containing drugs.

the lowest effective dose shifted to 2.5 μM in the Ca^{2+}-free buffer. It should be noted that the second increase in Ca^{2+} concentration disappeared though the response delay was still observed.

3.4. Honokiol and magnolol-induced Ca^{2+} increase in human neuroblastoma SH-SY5Y cells

Honokiol and magnolol were found to also increase the cytoplasmic Ca^{2+} content of human neuroblastoma SH-SY5Y cells in Ca^{2+} containing buffer (Fig. 4A and E) as well as in Ca^{2+}-free buffer (Fig. 4C and G). U73122 greatly reduced the honokiol-induced Ca^{2+} increase in both Ca^{2+}-containing buffer (Fig. 4B) and Ca^{2+}-free buffer (Fig. 4D), whereas U73122 completely inhibited the magnolol-induced Ca^{2+} increase in Ca^{2+}-containing buffer (Fig. 4F) and Ca^{2+}-free buffer (Fig. 4H). These results are comparable with those observed for the primary cultures of rat cortical neurons.

3.5. Block of the magnolol-induced Ca^{2+} response by pharmacological agents in SH-SY5Y cells

To confirm the identity of the intracellular Ca^{2+} pool from which cytoplasmic Ca^{2+} was released by honokiol and magnolol, the effects of thapsigargin and xestospongin C were examined in SH-SY5Y cells. Thapsigargin is a specific inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase pump (Treiman et al., 1998) and can deplete the endoplasmic reticulum Ca^{2+} store. Pre-perfusion with 10 μM thapsigargin caused an intracellular Ca^{2+} increase which was not affected by later perfusion of 20 μM magnolol (Fig. 5A). Xestospongin C is considered an IP_3 receptor inhibitor (Gafni et al., 1997). Pre-perfusion with xestospongin C for

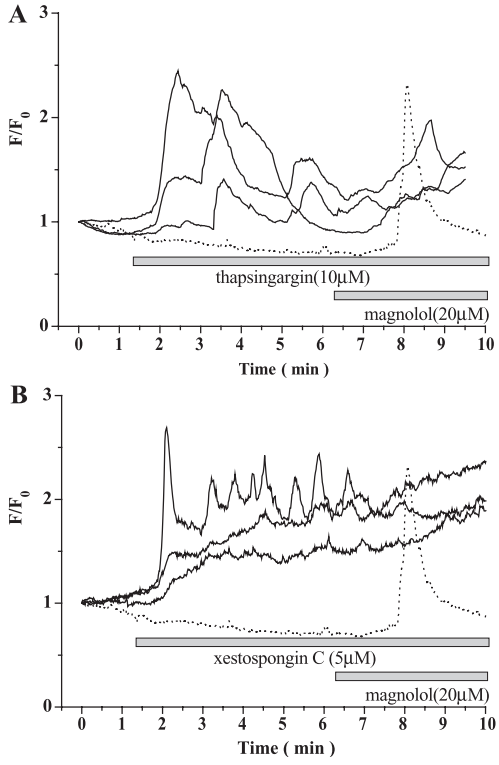

Fig. 5. Effects of thapsigargin and xestospongin C on magnolol-induced Ca^{2+} response in SH-SY5Y cells. (A) Pre-perfusion with 10 μM thapsigargin blocked the 20 μM magnolol-induced Ca^{2+} responses. (B) Pre-perfusion with 5 μM xestospongin C blocked the 20 μM magnolol-induced Ca^{2+} response. In each group, 7–14 SH-SY5Y cells were measured at the same time, 3 of which were selected and presented here. Experiments were performed at least three times and the results were similar. Data presented here came from the same batch of SH-SY5Y cells. Dotted line in the figure is the Ca^{2+} response curve in the control group (without any pharmacological inhibitors), showing the time course after perfusion of 20 μM magnolol stared.

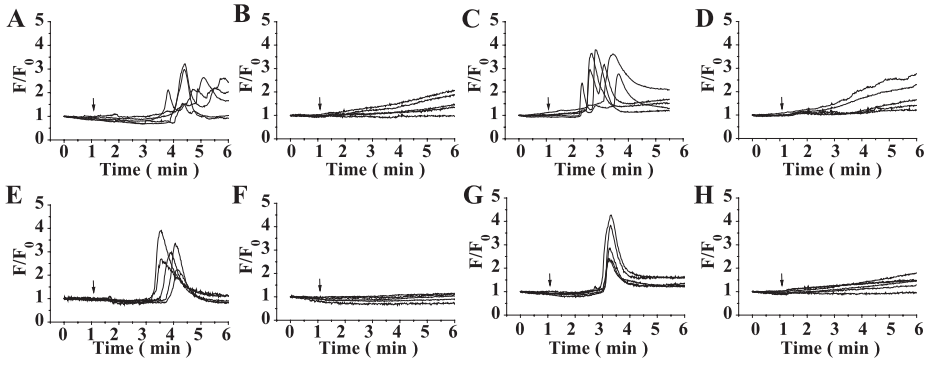

Fig. 4. Intracellular Ca^{2+} mobilization-induced by honokiol and magnolol in human neuroblastoma SH-SY5Y cells. (A) shows typical Ca^{2+} response to treatment with 20 μM honokiol. (B) shows reduced Ca^{2+} response to treatment with 20 μM honokiol after a 5-min pre-incubation with U73122 before treatment with honokiol. (C) shows the typical response to 20 μM honokiol in Ca^{2+}-free buffer. (D) shows the reduced Ca^{2+} response to treatment with 20 μM honokiol and a 5-min pre-incubation with U73122 before treatment with honokiol. (E) shows typical Ca^{2+} response to treatment with 20 μM magnolol. (F) shows reduced Ca^{2+} response to treatment with 20 μM magnolol after a 5-min pre-incubation with U73122 before treatment with magnolol. (G) shows the typical response to treatment with 20 μM magnolol in Ca^{2+}-free buffer. (H) shows the reduced Ca^{2+} response to treatment with 20 μM magnolol and a 5-min pre-incubation with U73122 before treatment with magnolol. The viability of SH-SY5Y cells in the U73122-treated group was checked with 10 μM ionomycin. In each group, 7–20 SH-SY5Y cells were measured at the same time, 4–6 of which were selected and presented. Experiments were performed at least three times and the results were confirmed to be similar. Data presented here came from the same batch of SH-SY5Y cells. Arrows in the figure show the start of perfusion of buffer containing drugs.

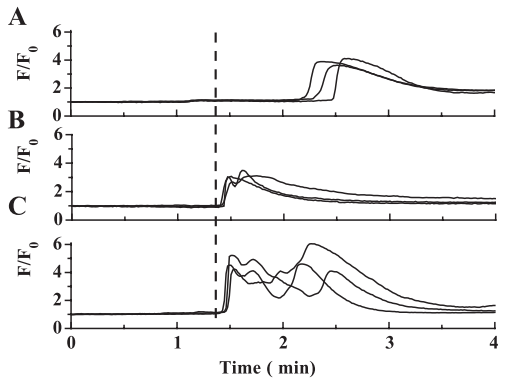

Fig. 6. Comparison of a delayed Ca^{2+} response in SH-SY5Y cells between magnolol and a muscarinic receptor agonist, methacholine. (A) shows the typical Ca^{2+} response to treatment with 20 μM magnolol. (B) shows the typical response to treatment with 10 μM methacholine. (C) shows the Ca^{2+} response to treatment with 20 μM magnolol and 10 μM methacholine at the same time. In each group, 7–20 SH-SY5Y cells were measured at the same time, 3 of which were selected and presented here. Experiments were performed at least three times and the results were similar. Data presented here came from the same batch of SH-SY5Y cells. Dotted line in the figure shows the start of perfusion of buffer containing drugs.

5 min could also cancel the Ca^{2+} response induced by later perfusion of 20 μM magnolol (Fig. 5B).

3.6. Comparison of magnolol and methacholine-induced Ca^{2+} mobilization

For confirmation of the delayed Ca^{2+} response to honokiol and magnolol, a direct comparison of magnolol and methacholine was carried out in SH-SY5Y cells. Magnolol (20 μM) induced a delayed Ca^{2+} increase with a lag of ~ 60 s and methacholine (10 μM) induced a rapid response after perfusion started (Fig. 6A and B). This difference was demonstrated by perfusion of methacholine and magnolol at the same time (Fig. 6C). The fast Ca^{2+} increase was effected by methacholine and was followed by the second Ca^{2+} increase induced by magnolol.

4. Discussion

In the present study, we did not use cell calibration to convert the fluorescence ratio to free Ca^{2+} concentration because the calibration between different batches of cells produced inconsistent results. In order to avoid misleading absolute amounts of Ca^{2+}, we used the fluorescence ratio of F to F_0 to indicate the relative change in Ca^{2+} concentration before and after drug perfusion. The ratio of F to F_0 can reflect the dose–effect relationship between the test sample and the increase in cytoplasmic Ca^{2+}. In fact, higher dose of honokiol and magnolol resulted in a higher ratio change for F to F_0.

Honokiol and magnolol induced a characteristic mobilization of intracellular Ca^{2+} in primary cultured rat cortical neurons and human neuroblastoma SH-SY5Y cells. They have different sensitivities to U73122, and the cell response to magnolol was more synchronous than that to honokiol after a delay (Fig. 2B vs. F; Fig. 4A vs. E). In spite of these differences, the shared characteristics, such as effective dose range, delayed responses and extracellular Ca^{2+} independence, indicate that the same mechanism underlies their pharmacological actions. To overcome the essential problem caused by the heterogeneity of primary cultured cortical neurons when analyzing the Ca^{2+} response of single cells to a drug, we used human neuroblastoma SH-SY5Y cells, which are expected to be a useful model of studies on the detailed temporal and spatial properties, and the mechanism of honokiol and magnolol-induced Ca^{2+} mobilization, because these agents also increased the cytoplasmic free Ca^{2+} concentration in SH-SY5Y cells with a profile comparable with that in the primary cultured neurons.

This is the first demonstration that honokiol and magnolol induce Ca^{2+} mobilization in neuronal cells, although magnolol was reported to elevate cytoplasmic free Ca^{2+} in rat neutrophils (Wang and Chen, 1998). The honokiol or magnolol-induced Ca^{2+} mobilization was inhibited by U73122, thapsigargin and xestospongin C, suggesting that both compounds caused Ca^{2+} release from the intracellular Ca^{2+} store in the endoplasmic reticulum through activation of the phospholipase C-IP_3 pathway. In our study, xestospongin C triggered a fast and sustained Ca^{2+} increase, suggesting that xestospongin C maybe also serve as a Ca^{2+}-mobilizing agent, and is not a specific IP_3 receptor inhibitor as recently found in cultured dorsal root ganglia neurons (Solovyova et al., 2002).

It is very interesting that both compounds induced a characteristically delayed increase in intracellular free Ca^{2+} after drug perfusion, in contrast with the fast Ca^{2+} elevation in response to perfusion of methacholine. Some growth factors also induce a delayed Ca^{2+} response, such as nerve growth factor in PC12 cells (Pandiella-Alonso et al., 1986), epidermal growth factor in A431 cells (Gonzalez et al., 1988) and human diploid fibroblasts (Huang et al., 2000), fibroblast growth factor in NIH-3T3 cells (Pandiella et al., 1989), platelet-derived growth factor in Swiss 3T3 cells (Lopez-Rivas et al., 1987) and human diploid fibroblasts (Huang et al., 2000), and vascular permeability factor in human endothelial cells (Brock et al., 1991), which means that this kind of Ca^{2+} dynamics may play an important role in the regulation of cell growth. Neurotrophins can increase intracellular free Ca^{2+} in neurons through the mobilization of Ca^{2+} from intracellular stores (Lamb and Bielefeldt, 2003), and this intracellular Ca^{2+} mobilization is essential for their functional expression (Egea et al., 2001; Kang and Schuman, 2000). On the other hand, a controlled Ca^{2+} increase itself may serve as a neurotrophic signal, which regulates neurotrophic factor gene expression (West et al., 2001; Finkbeiner, 2000). Thus, intracellular Ca^{2+} mobilization induced by honokiol and magnolol in neurons may be involved in their neurotrophic mechanism.

In conclusion, we found that honokiol and magnolol could induce intracellular Ca^{2+} mobilization in primary cultured rat cortical neurons as well as in human neuroblastoma SH-SY5Y cells with a characteristic profile, presumably through the activation of phospholipase C and IP_3 receptors. It is suggested that a new activation mechanism associated with intracellular Ca^{2+} mobilization is involved in the neurotrophic actions of honokiol and magnolol.

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