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Effects of Hesperidin Are Not Associated with Changes in Basal Synaptic Transmission, Theta-burst LTP, and Membrane Excitability in CA1 Neuron

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Abstract: Hesperidin, the most abundant polyphenolic compound found in citrus fruits, has been known to possess neuroprotective, sedative, and anticonvulsive effects on the nervous system. In a recent electrophysiological study, it was reported that hesperidin induced biphasic change in population spike amplitude in hippocampal CA1 neurons in response to both single spike stimuli and theta-burst stimulation depending on its concentration. However, the precise mechanism by which hesperidin acts on neuronal functions has not been fully elucidated. Here, using whole-cell patch-clamp recording, we revealed that hesperidin did not affect excitatory synaptic activities such as basal synaptic transmission and theta-burst LTP. Moreover, in a current injection experiment, spike number, resting membrane potential and action potential threshold also remained unchanged. Taken together, these results indicate that the effects of hesperidin on the neuronal functions such as spiking activity might not be attributable to either modification of excitatory synaptic transmissions or changes in membrane excitability in hippocampal CA1 neuron.

Key words: hesperidin, hippocampus, long-term potentiation, membrane excitability, whole-cell recording

INTRODUCTION

Hesperidin is a natural polyphenolic flavonoid mainly isolated from citrus fruits. Since its discovery, hesperidin has been widely investigated as a potential therapeutic agent and various pharmacological properties of hesperidin such as antiinflammatory, anticarcinogenic, antihyperlipidemic activities and other miscellaneous effects have been described until now (Emim et al., 1994, Galati et al., 1994, Garg et al., 2001, Monforte et al., 1995). Like other polyphenols found in a wide variety of plants, hesperidin also has an antioxidant activity, protecting cells from damages by reactive oxygen species (Garg et al., 2001, Wilmsen et al., 2005). In addition, previous studies demonstrated that hesperidin might exert its effect on the nervous system by defending neurons from harmful radicals suggesting possible action on neuronal functions like other neuroprotective agents (Cho, 2006, Hwang and Yen, 2008, Lim et al., 2008). More direct effects of hesperidin on neuronal functions came up in the animal study where hesperidin showed sedative and sleep-enhancing effects (Marder et al., 2003). However, physiological mechanisms how hesperidin can play these diverse roles have remained largely unclear.

Of interest, a recent electrophysiological study using slice field recording showed that population spike amplitude was significantly decreased in response to high concentration of hesperidin in hippocampal CA1 region (Dimpfel, 2006). Moreover, hesperidin led to a biphasic change in population spike amplitude after theta-burst stimulation, increasing potentiation of population spike amplitude at low concentration and conversely attenuating potentiation at high concentration. Although this research for the first time presented physiological basis of hesperidin's effect on nervous system, it remained unsolved whether those effects are originated from changes in synaptic transmission or intrinsic excitability because this study only measured population spike amplitude.

In this study, using whole-cell patch-clamp method , we tried to figure out the physiological effects of hesperidin on hippocampal CA1 neurons in more detail. Alterations in basal synaptic transmission, paired-pulse ratio (PPR) and synaptic plasticity such as theta-burst long-term potentiation (LTP) were examined in rat hippocampal CA1 neurons

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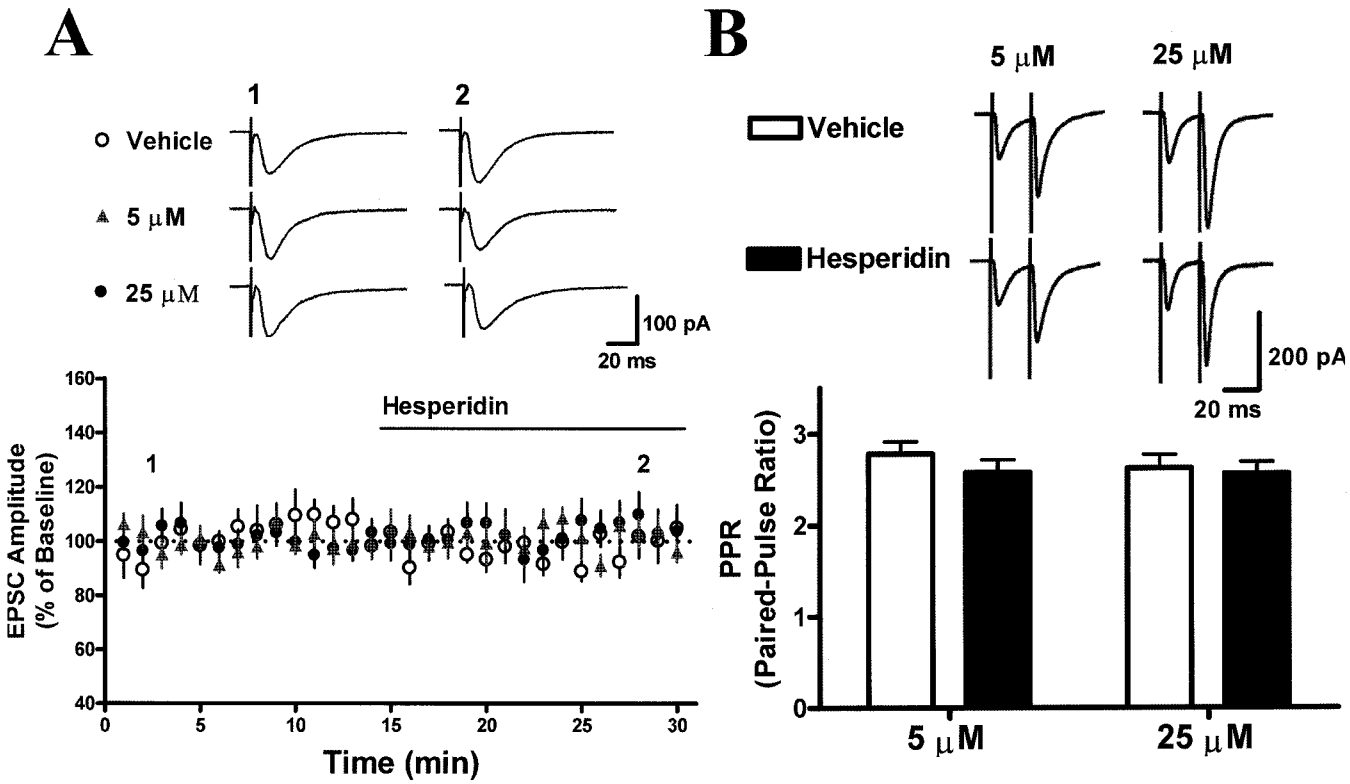

Fig. 1. Effect of hesperidin on basal synaptic transmission. (A) Traces are average of EPSCs during three minutes at indicated time points. After 15 minutes of baseline recording with 0.1% DMSO vehicle, recording solution was changed to that containing hesperidin in hesperidin groups (n=8, n=9, and n=12 in vehicle, 5, 25 μM, respectively). No difference in EPSC amplitude was observed among groups. (B) Paired-pulse ratio (50 msec interval) also remained unchanged with hesperidin treatment. White bar represents PPR under vehicle treatment and black bar represents PPR after hesperidin treatment (n=8 per group). Traces are average of EPSCs during 3 minutes.

with bath application of hesperidin. Also, we probed the possible effect of hesperidin on membrane excitability by measuring spike number and action potential threshold in current injection experiment.

MATERIALS AND METHODS

Slice preparation

Acute hippocampal slices were obtained from 10- to 17-day-old (P10-P17) Sprague-Dawley rats (Orient). These rats were anesthetized with isoflurane by inhalation and decapitated in accordance with the policy and regulation for the care and use of laboratory animals approved by Institutional Animal Care and Use Committee in Seoul National University. Brains were rapidly removed into oxygenated ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM) 124 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgSO_4$, 25 $NaHCO_3$, 1 NaH_2PO_4, 10 glucose. Coronal brain slices of 300 μm were cut using a vibratome (Leica VT 1200S). Slices were incubated in ACSF for at least 1 hour prior to recording. All ACSF solutions were oxygenated with 5 % CO_2 and 95 % O_2 mixed gas.

Whole-cell patch-clamp recording

Whole-cell configuration was made using pipette electrodes with resistance of 3-5 MΩ pulled from micropipette puller (P-97, Sutter Instrument). The internal pipette solution for whole-cell recording contained (in mM) 145 K-gluconate, 5 NaCl, 1 $MgCl_2$, 10 HEPES, 0.2 EGTA, 2 MgATP, 0.1 Na_3GTP, and pH adjusted to 7.2 with KOH. Signals were digitized using Digidata 1440A and amplified by Multiclamp 700B amplifier (Molecular Devices) controlled by Multi-Clamp Commander and pClamp 10 acquisition software (Molecular Devices). Hesperidin (Sigma, H5274) was dissolved in dimethyl sulfoxide (DMSO). Hesperidin or DMSO vehicle were bath applied in ACSF. $GABA_A$ channel blocker, picrotoxin (100 μM), was added to ACSF and CA3 region was removed to eliminate epileptic responses.

RESULTS

Hesperidin does not affect basal synaptic transmission

Two different concentrations of hesperidin, 5 and 25 μM each, were tested in basal synaptic transmission referring to

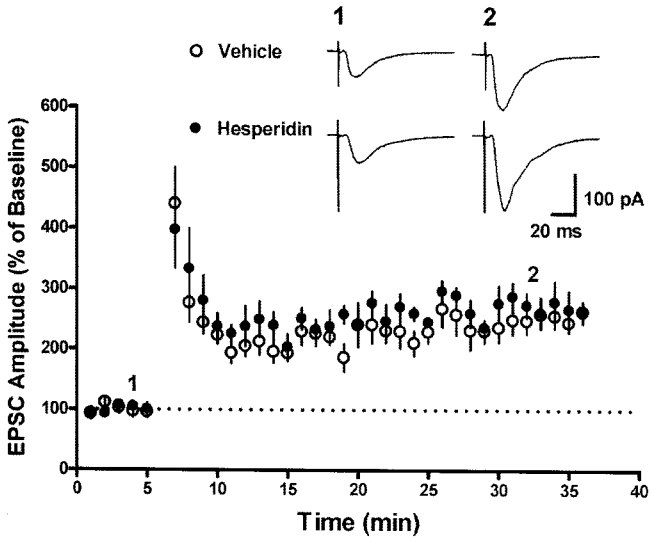

Fig. 2. Theta-burst LTP. High concentration (25 μM) of hesperidin had no effect on theta-burst induced synaptic plasticity (n=7 for vehicle group and n=6 for hesperidin group). The representative recording traces are average of EPSCs during three minutes at indicated time points. Each dot represents the average of three EPSC amplitude data acquired at 0.05 Hz.

the previous report (Dimpfel, 2006). In baseline recording of hesperidin groups, 15 minute-long stable baseline was established with bath-application of vehicle (0.1% DMSO in ACSF) followed by another 15 minutes of baseline recording using recording solution containing 5 or 25 μM of hesperidin. On the other hand, the vehicle group was recorded for 30 minutes without changing recording solution. Both low and high concentration of hesperidin did not elicit alteration of excitatory synaptic responses (Fig. 1A). Paired t-tests comparing EPSC amplitudes before and after hesperidin treatment revealed insignificant differences (P>0.69 in vehicle, P>0.89 at 5 μM, and P>0.65 at 25 μM) and one-way ANOVA did not show significant difference in EPSC amplitudes during the last five minutes among the groups (mean±SEM; 100.54% in vehicle, 100.24% at 5 μM, and 105.77% at 25 μM, $F_{2,26}$=0.2879, P=0.7522).

We also analyzed EPSC kinetics in hesperidin groups, but no changes in 10-90 % rise time (RT) and half-width (HW) were detected with paired t-test. During the first and the last 5 minutes, RT (msec) and HW (msec) were changed from 3.61±0.3 to 3.99±0.3 (P>0.10), from 10.7±0.8 to 12.9±1.3 (P>0.09), respectively, at 5 μM, and changed from 3.70±0.2 to 3.71±0.6 (P>0.97), from 13.1±0.9 to 13.7±1.0 (P>0.48), respectively, at 25 μM. Together with baseline recording, 50 msec interval PPR was examined in the same cell right before and after baseline recording when either vehicle or hesperidin was bath-applied. PPR with hesperidin application was not different from that with vehicle application irrespective of the concentrations of hesperidin (Fig. 1B, P>0.21 and P>0.75 at 5 and 25 μM, respectively, paired t-test). Together, these data suggest that hesperidin does not appear to have an influence on basal synaptic transmission.

Hesperidin has no effect on theta-burst stimulation induced long term potentiation

We next investigated the possible alteration of synaptic plasticity in the presence of hesperidin. As a conditioning protocol, theta-burst stimulation consisting of four trains of stimuli delivered at 10 sec intervals, each train having five stimulus bursts delivered at 5 Hz, with each burst consisting of four pulses at 100 Hz was used. This is similar to the stimulation protocol inducing the biphasic transition of population spike amplitude as reported (Dimpfel, 2006). Either high concentration of hesperidin (25 μM) or 0.1% DMSO vehicle was added in ACSF. After 5 minutes of baseline recording, conditioning stimulus was given to elicit LTP and again theta-burst LTP was unaffected by high concentration of hesperidin (Fig. 2). During the last 5 minutes, EPSC amplitude in vehicle group was potentiated to 253±15% of baseline. Similarly, theta-burst stimulation induced increase in EPSC amplitude to 268±22% of baseline in hesperidin group, showing no difference between vehicle and hesperidin-treated group (P>0.59, unpaired t-test). In summary, theta-burst stimulation in presence of high concentration of hesperidin did not reduce synaptic potentiation.

Hesperidin does not alter membrane excitability of hippocampal CA1 neurons in current injection experiment

Finally, we recorded membrane excitability to examine the effect of hesperidin on firing activity in hippocampus CA1 pyramidal neurons. Current pulses were delivered to cell body via recording electrode from −300 pA to 300 pA, incremented by 100 pA with 10 seconds interval. This test was repeated 3 times with 6 minutes interval. Data were discarded if membrane potential was changed more than 3 mV during recording period as previously described (Staff and Spruston, 2003). Because spike number was significantly decreased 30 minutes after whole-cell formation (data not shown), the recordings from vehicle and hesperidin groups were performed separately in different cells, to minimize factors originated from the diminution of spiking activity over time. In contrast to our assumption that hesperidin may impact on spiking activity, it had virtually no effect on spike number independent of the current intensity injected (Fig. 3A, B, and C). Two-way repeated measures ANOVA analyses revealed insignificant drug effect (vehicle versus hesperidin, $F_{1,17}$<0.21 and P>0.65 for all current intensities) and drug by time interaction (all $F_{2,17}$<3.0 and P>0.06). As previously mentioned, spike number was significantly decreased over time in both groups (time effect, all

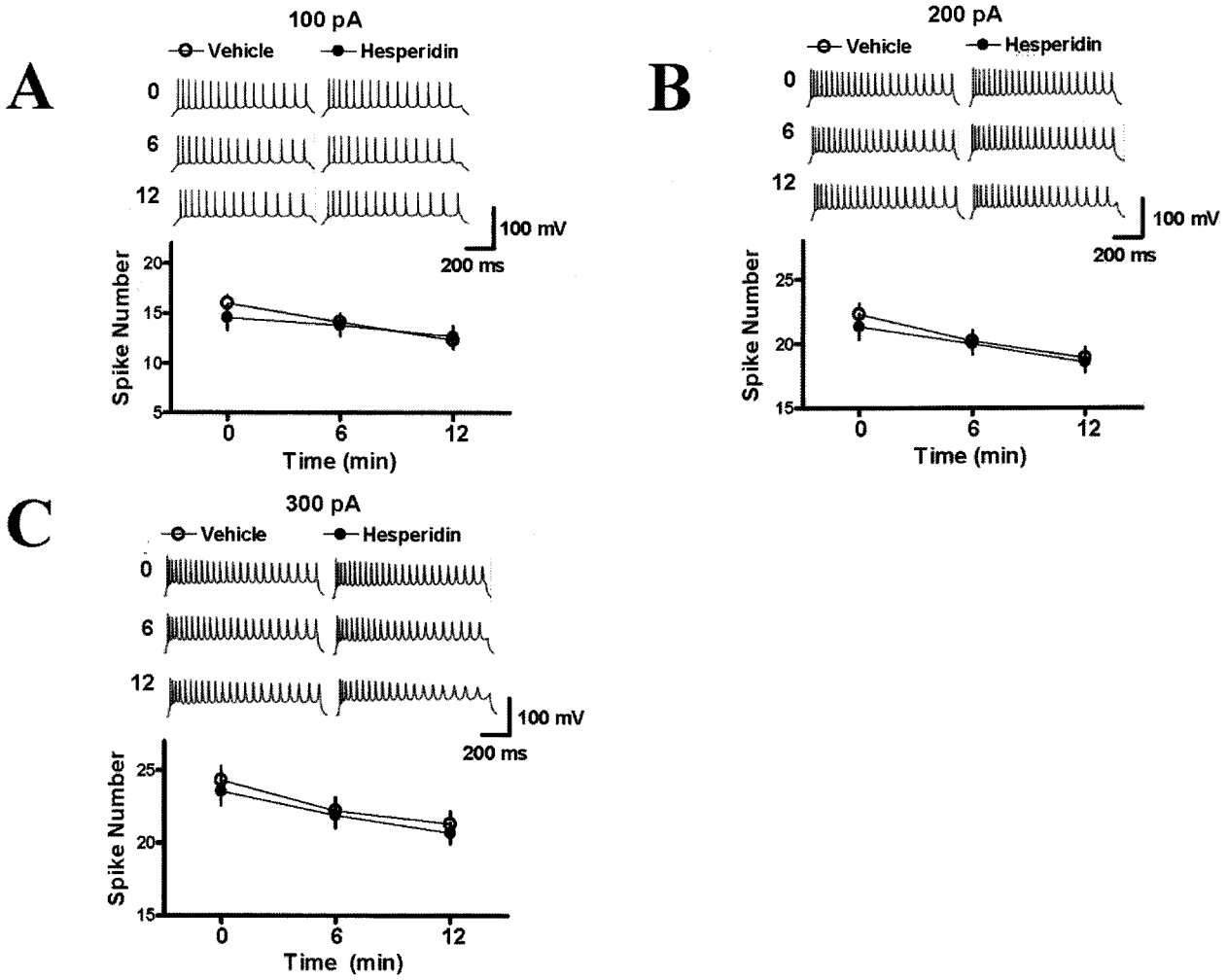

Fig. 3. Time-course of spiking activity measured by current injection experiment. Each cell treated with vehicle (open circle) or hesperidin (closed circle) was tested 3 times (n=10 and n=9, respectively) and high concentration of hesperidin (25 μM) was applied to hesperidin group. Current pulses of 100 pA (A), 200 pA (B), and 300 pA (C) were given to generate action potential. Although spike number was decreased over time (***P<0.0001, time effect, two-way repeated measures ANOVA), no drug effect and drug by time interaction was observed (P>0.65, P>0.06).

$F_{2,17}$>27.7, ***P<0.0001). Moreover, no significant difference in drug effect from both resting membrane potential and action potential threshold was detected (Fig. 4A, B, all $F_{1,17}$<0.65 and P>0.43). Although resting membrane potential was slightly decreased over time only in vehicle treated group (drug by time interaction, $F_{2,17}$=3.6 and *P<0.05), this hyperpolarization did not lead to the substantial difference in membrane potential between vehicle- and hesperidin- treated group (P>0.17 at all time points).

DISCUSSION

In this study, we reported that hesperidin does not affect basal synaptic transmission, PPR, theta-burst LTP and membrane excitability in rat hippocampus CA1. We focused on synaptic activities and intrinsic membrane excitability as plausible candidates to modulate population spike amplitude, since spiking activity could be changed independently by these two different mechanisms (Daoudal and Debanne, 2003). First, modification of synaptic transmission is able to alter magnitude of postsynaptic responses with same synaptic input, and in turn, result in change of firing activity. However, as shown before, we could not find any evidence that hesperidin might have an effect on synaptic modification (Fig. 1A, B). Furthermore, bath application of hesperidin did not lead to any change in synaptic plasticity induced by theta-burst stimulation, although similar protocol elicited the reduction of potentiation of population spike amplitude in hippocampus CA1 (Dimpfel, 2006). These results indicate that physiological effects of hesperidin reported before in neurons can not be explained by the alteration of synaptic activities.

Second, intrinsic excitability, a propensity of firing action potential, also could be related to the change of population

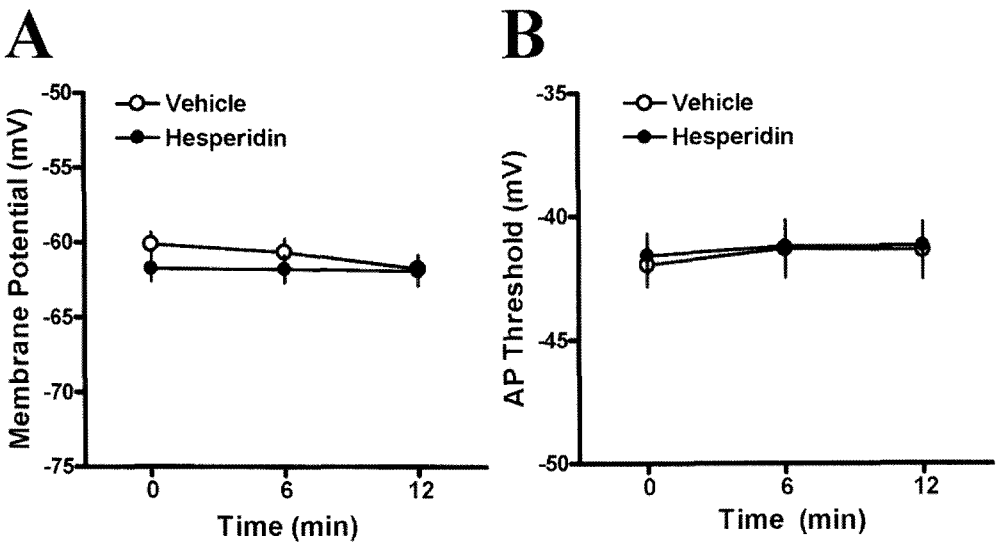

Fig. 4. Time-course of membrane properties during current injection experiment. As figure 3, each cell treated with vehicle (open circle) or hesperidin (closed circle) was tested 3 times (n=10 and n=9, respectively). Hesperidin did not affect resting membrane potential (A) and action potential threshold (B).

spike amplitude. Intrinsic excitability can be altered by modulation of channel activities, impacting on propagation of electrical signals or action potential generation and in turn affecting firing probability. However, as shown in this paper, spike number of hesperidin group did not differ from that of vehicle group in this study (Fig. 3A, B, and C). In addition, resting membrane potential and action potential threshold also remained same (Fig. 4A, B) indicating that effects of hesperidin are independent of membrane excitability at least in terms of threshold and resting membrane potential. Nevertheless, we could not say that hesperidin is ineffective on local intrinsic excitability that is restricted to a certain dendritic region at this stage (Zhang and Linden, 2003). To assess membrane excitability, here we applied current pulses directly into cell body via patch pipette. Although it does not exclude dendritic component, injected current could immediately affect axon hillock generating action potential with a minor loss and could minimize local effect on dendritic region. Thus, the influence of hesperidin on dendritic propagation and coupling with firing of action potential needs to be more investigated. Experimental methods such as measuring EPSP-spike coupling in a single neuron for evaluating firing probability (Daoudal et al., 2002, Staff and Spruston, 2003) or direct dendrite recording could be a way for scrutinizing local dendritic excitability.

Recent studies suggested an involvement of calcium-activated K^+ channels in anticonvulsive and vasorelaxing activities of hesperidin as well as hesperitin, an aglycone of hesperidin (Calderone et al., 2004, Dimpfel, 2006). According to these papers, effects of hesperidin were sensitive to selective antagonist of calcium-activated K^+ channels, iberiotoxin. On the other hand, other types of K^+ channels including A-type channels are unlikely to be involved, as 4-AP, a relatively selective inhibitor of voltage gated K^+ channels, did not block hesperidin effect in field recording. Moreover, $GABA_A$ receptors, in contrast to calcium-activated K^+ channels, does not seem to be required neither although many reports insist that attenuation of $GABA_A$ inhibition is critical mechanism for EPSP-spike potentiation (Chavez-Noriega et al., 1989, Daoudal and Debanne, 2003, Staff and Spruston, 2003). This assumption was originated from the reports that co-administration of picrotoxin and hesperidin did not inhibit behavioral effects, and no interaction between hesperidin and $GABA_A$ receptors was observed in binding assays and oocyte recording with $GABA_A$ receptors (Fernández et al., 2005, 2006). Thus, neither $GABA_A$-dependent inhibitory inputs nor excitatory synaptic transmission as shown in this paper seem to be related with effects of hesperidin. These results supports the idea that local intrinsic excitability in CA1 pyramidal neuron might be associated with effects of hesperidin via affecting channel activity. However, it also remains possible that different mechanisms may act on the other types of neurons and make diverse effects. More recently, opioid receptor and adenosine receptor are also regarded to be associated with sedative effect of hesperidin (Guzmán-Gutiérrez and Navarrete, 2009, Loscalzo et al., 2008), but further study is needed to clarify the detailed mechanisms in the physiological effects of hesperidin.

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REFERENCES

Calderone V, Chericoni S, Martinelli C, Testai L, Nardi A, Morelli I, Breschi MC, and Martinotti E (2004) Vasorelaxing effects of flavonoids: investigation on the possible involvement of potassium channels. *Naunyn Schmiedebergs Arch Pharmacol* 370: 290-298

Chavez-Noriega LE, Bliss TV, and Halliwell JV (1989) The EPSP-spike (E-S) component of long-term potentiation in the rat hippocampal slice is modulated by GABAergic but not cholinergic mechanisms. *Neurosci Lett* 104: 58-64

Cho J (2006) Antioxidant and neuroprotective effects of hesperidin and its aglycone hesperetin. *Arch Pharm Res* 29: 699-706

Daoudal G and Debanne D (2003) Long-term plasticity of intrinsic excitability: learning rules and mechanisms. *Learn Mem* 10: 456-465

Daoudal G, Hanada Y, and Debanne D (2002) Bidirectional plasticity of excitatory postsynaptic potential (EPSP)-spike coupling in CA1 hippocampal pyramidal neurons. *Proc Natl Acad Sci* USA 99: 14512-14517

Dimpfel W (2006) Different anticonvulsive effects of hesperidin and its aglycone hesperetin on electrical activity in the rat hippocampus in-vitro. *J Pharm Pharmacol* 58: 375-379

Emim JA, Oliveira AB, and Lapa AJ (1994) Pharmacological evaluation of the anti-inflammatory activity of a citrus bioflavonoid, hesperidin, and the isoflavonoids, duartin and claussequinone, in rats and mice. *J Pharm Pharmacol* 46: 118-122

Fernández SP, Wasowski C, Loscalzo LM, Granger RE, Johnston GAR, Paladini AC, and Marder M (2006) Central nervous system depressant action of flavonoid glycosides. *Eur J Pharmacol* 539: 168-176

Fernández SP, Wasowski C, Paladini AC, and Marder M (2005) Synergistic interaction between hesperidin, a natural flavonoid, and diazepam. *Eur J Pharmacol* 512: 189-198

Galati EM, Monforte MT, Kirjavainen S, Forestieri AM, Trovato A, and Tripodo MM (1994) Biological effects of hesperidin, a citrus flavonoid. (Note I): antiinflammatory and analgesic activity. *Farmaco* 40: 709-712

Garg A, Garg S, Zaneveld LJ, and Singla AK (2001) Chemistry and pharmacology of the Citrus bioflavonoid hesperidin. *Phytother Res* 15: 655-669

Guzmán-Gutiérrez SL and Navarrete A (2009) Pharmacological exploration of the sedative mechanism of hesperidin identified as the active principle of Citrus sinensis flowers. *Planta Med* 75: 295-301

Hwang S and Yen G (2008) Neuroprotective effects of the citrus flavanones against H2O2-induced cytotoxicity in PC12 cells. *J Agric Food Chem* 56: 859-864

Lim JS, Kim H, Choi Y, Kwon H, Shin KS, Joung I, Shin M, and Kwon YK (2008) Neuroprotective Effects of Berberine in Neurodegeneration Model Rats Induced by Ibotenic Acid. *Animal Cells and Systems* 12: 203-209

Loscalzo LM, Wasowski C, Paladini AC, and Marder M (2008) Opioid receptors are involved in the sedative and antinociceptive effects of hesperidin as well as in its potentiation with benzodiazepines. *Eur J Pharmacol* 580: 306-313

Marder M, Viola H, Wasowski C, Fernandez S, Medina JH, and Paladini AC (2003) 6-methylapigenin and hesperidin: new valeriana flavonoids with activity on the CNS. *Pharmacol Biochem Behav* 75: 537-545

Monforte MT, Trovato A, Kirjavainen S, Forestieri AM, Galati EM, and Lo Curto RB (1995) Biological effects of hesperidin, a Citrus flavonoid. (note II): hypolipidemic activity on experimental hypercholesterolemia in rat. *Farmaco* 50: 595-599

Staff NP and Spruston N (2003) Intracellular correlate of EPSP-spike potentiation in CA1 pyramidal neurons is controlled by GABAergic modulation. *Hippocampus* 13: 801-805

Wilmsen PK, Spada DS, and Salvador M (2005) Antioxidant activity of the flavonoid hesperidin in chemical and biological systems. *J Agric Food Chem* 53: 4757-4761

Zhang W and Linden DJ (2003) The other side of the engram: experience-driven changes in neuronal intrinsic excitability. *Nat Rev Neurosci* 4: 885-900

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