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Different anticonvulsive effects of hesperidin and its aglycone hesperetin on electrical activity in the rat hippocampus in-vitro

Wilfried Dimpfel

Abstract

This study investigated the possibility that hesperidin or hesperetin might interact directly with brain matter in a physiological manner. The effects of both compounds were followed in the in-vitro hippocampus preparation by continuous superfusion in a concentration-dependent manner in the presence of single stimuli and theta-burst stimulation of the Schaffer Collaterals. Hesperidin increased the population spike response at a concentration up to 10 μM, especially after induction of long-term potentiation, but attenuated it significantly at higher concentrations of up to 60 μM. Hesperetin only attenuated the response within the same concentration range. Modulation of the pyramidal cell response in the presence of tetraethylammonium (TEA) and pentylentetrazol on one hand and 4-aminopyridine (4-AP) and bicuculline on the other was influenced in a different way. Whereas hesperidin attenuated the response to 4-AP and bicuculline but not to TEA or pentylentetrazol, hesperetin was able to attenuate the response to TEA and pentylentetrazol, but not to 4-AP or bicuculline. This feature was reproduced and confirmed ex-vivo after repetitive administration of hesperidin and hesperetin in-vivo for one week (500 mg kg^{-1} orally) before in-vitro testing against the challenging effects of 4-AP and TEA. Since the action of hesperidin was sensitive to the presence of iberiotoxin, the involvement of a large conductance calcium-dependent potassium channel might be assumed. In summary, the results provide the possibility for use of both compounds to control pathophysiological disturbances of brain excitability in drug abuse, migraine and epilepsy.

Introduction

Hesperidin (Figure 1) is a naturally occurring flavanone that occurs in citrus and other plants. Hesperidin was first described in 1828 by Lebreton & Brandeo, as reported by Higby (1941). Pharmacological characterization has been reported by Garg et al (2001). A first report on the possible effects of one of the isomers on the central nervous system was published in 2003 by Marder et al. In fresh orange juice its concentration is about 800 mg L^{-1} and it has also been found in the peel (Di Mauro et al 1999). This glycoside is deglycosylated partly in the gut by bacteria to give hesperetin, its so-called aglycone. Hesperidin is used in combination (i.e. with diosmin) for therapeutic control of venous diseases. Its bioavailability is about 25% after oral ingestion (Ameer et al 1996). The mechanism of action has not been elucidated yet. This study investigated the possibility that hesperidin or hesperetin could directly act on brain matter in a specific manner.

For assessment of physiological effects, the model of in-vitro hippocampus slices was used. This model has been used in the past by us to characterize a plant-derived anticonvulsant, losigamone (Dimpfel et al 1995). Electrical stimulation of the Schaffer Collaterals results in activation of the glutamatergic synapses at hippocampal pyramidal cells to give the so-called population spike (pop-spike). The amplitude of this signal reflects the recruitment of the number of pyramidal cells excited by the stimulus. The amplitude therefore reflects synaptic efficacy. Application of a very short-lasting theta-burst stimulus results in higher responses to single-shock stimuli for hours and is called long-term potentiation. This feature has been related to spatial and time-dependent memory processes and is now

University of Giessen c/o NeuroCode AG D 35578 Wetzlar, Sportpark-Str. 9, Germany

Wilfried Dimpfel

Correspondence: W. Dimpfel, University of Giessen c/o NeuroCode AG D 35578 Wetzlar, Sportpark-Str. 9, Germany. E-mail: w.dimpfel@neurocode-ag.com

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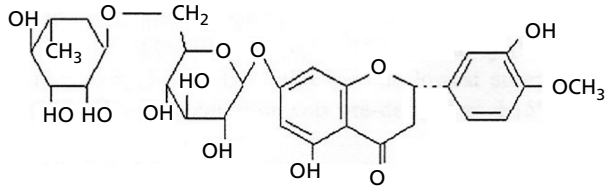

Figure 1 Chemical structure of hesperidin.

extensively used in drug characterization. To compare the effects of hesperidin and hesperetin in this physiological model, both compounds were superfused on to the slices in a continuous manner alone and in the presence of four convulsion-inducing chemicals, namely 4-aminopyridine (4-AP), bicucculine, pentylentetrazol and tetraethyl-ammonium (TEA). Surprisingly, the glycoside and its aglycone acted in a different manner. This specific difference could be confirmed in an ex-vivo design, where both compounds were administrated in-vivo for one week before the in-vitro assessment.

Materials and Methods

Materials

Hesperidin (80% pure) and hesperetin (95% pure) were bought from Sigma-Aldrich (D 82024 Taufkirchen, Germany). TEA and 4-AP were also from Sigma-Aldrich. Iberiotoxin, pentylentetrazol and bicuculline were from RBI/Tocris (D 50933 Cologne, Germany; no purity given). Calcium chloride (98% pure) was from Carl Roth (D 76185 Karlsruhe, Germany).

Experimental methods

The experiments were performed in congruence with German law on the use of animals in research. Hippocampus slices were obtained from 38 adult male Sprague-Dawley rats (Charles River Wiga, Sulzbach, Germany). Rats were kept under a reversed day–night cycle for 2 weeks before the start of the experiments, to allow recording of in-vitro activity from slices during the active phase of their circadian rhythm (Dimpfel 1995). The rats were exsanguinated under ether anaesthesia, the brain was removed in total and the hippocampal formation was isolated under microstereoscopic sight. The mid-section of the hippocampus was fixed to the table of a vibratom (Rhema Labortechnik, Hofheim, Germany) using a cyanoacrylate adhesive, submerged in chilled bicarbonate-buffered saline (artificial cerebrospinal fluid (ACSF) (composition in mM: NaCl 124, KCl 5, $CaCl_2$ 2, $MgSO_4$ 2, $NaHCO_3$ 26, glucose 10) and cut into slices of 400 μm thickness. All slices were preincubated for at least 1 h in Carbogen-saturated ACSF (pH 7.4) in a prechamber before use.

During the experiment, the slices were held and treated in a special superfusion chamber (List Electronics, Darmstadt, Germany) according to Haas et al (1979). The preparation was superfused with ACSF at 180–230 mL h^{-1}. Electrical stimulation (200 μA constant current pulses of 200 μs pulse width) of the Schaffer Collaterals within the CA2 area and recording of extracellular field potentials from the pyramidal cell layer of CA1 was performed according to conventional electrophysiological methods using the Labteam Computer system Neurotool software package (Medisyst GmbH, Linden, Germany). Measurements were performed at 10-min intervals to avoid potentiation mechanisms. Four stimulations, each 20 s apart, were averaged for each time point. After obtaining three stable pre-drug values the perfusion was changed to drug containing ACSF and continued for at least 30 min. The mean amplitude of the three pre-drug signals were averaged and set to 100%. All averaged post-drug changes refer to this reference value.

Long-term potentiation was induced following the methodology of Lynch & Schubert (1980) consisting of a particular theta-stimulation pattern, resembling physiological activation (Larson et al 1986). This stimulation pattern has been recognized as optimal for studying long-term potentiation of synapses in the CA1 region of the hippocampus (Capocchi et al 1992).

Drugs tested were diluted in ACSF to give the final concentration as indicated and tested using 6 different slices. The effects of hesperidin and hesperetin were also tested in the presence of 50 μM 4-AP or 3 mM TEA.

Statistical methods

For statistical analysis the non-parametric Wilcoxon, Mann and Whitney *U*-test has been applied. Critical values of *U* were taken from Milton (1964). Comparisons of amplitude differences in the presence of increasing concentrations of hesperidin or hesperetin were made with respect to baseline values. Antagonism against convulsive compounds or iberiotoxin was evaluated by comparison with values during the challenge with these compounds. Each comparison was based on evaluation of 6 slices.

Results

Action of hesperidin and hesperetin on physiological parameters

The continuous superfusion of hesperidin in the slice preparation resulted in a biphasic action. At a concentration of 5 μM, a minor increase of the population spike amplitude could be registered (not statistically significant). Higher concentrations of 10–60 μM led to a stepwise attenuation of the response to single-shock stimuli (Figure 2) ($P < 0.01$). Stimulation using the theta-burst pattern resulted in the same biphasic action. Firstly, at lower concentrations beyond 10 μM an increase of long-term potentiation was observed ($P < 0.01$), whereas at higher concentration up to 60 μM a considerable depression of the amplitude of the population spike was measured ($P < 0.01$). This attenuation of hesperidin at 40 μM and beyond resulted in even lower values than obtained after single-shock stimulation (Figure 3).

The presence of hesperetin did not provide this biphasic pattern. Instead of an initial increase, attenuation of the population spike amplitude took place between 5 and 60 μM (statistically significant at 10 μM ($P < 0.02$) or higher ($P < 0.01$)). Also, theta-burst stimulation did not produce initial increases

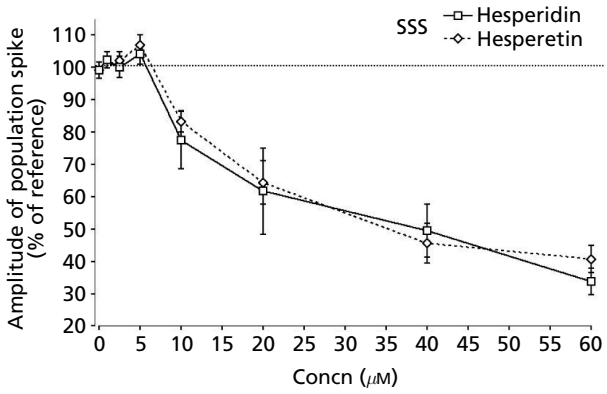

Figure 2 Effect of hesperidin or hesperetin on single-spike population response of rat hippocampal slices. Freshly prepared rat hippocampal slices were superfused with different concentrations of hesperidin or hesperetin. SSS, single-spike stimulation. Amplitude of the population spike is given as percent of the baseline value (reference). Data are given as mean ± s.e.m of n = 6 slices.

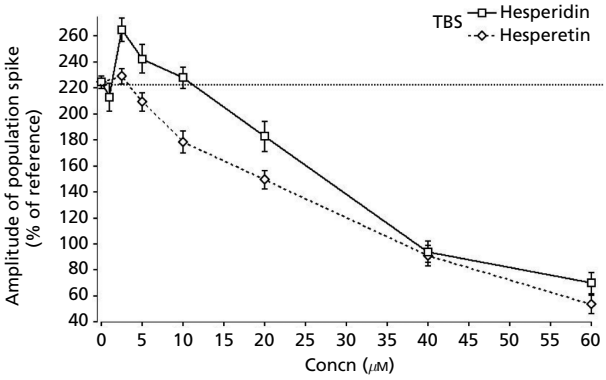

Figure 3 Effect of hesperidin or hesperetin on theta-burst stimulation response of rat hippocampal slices. Freshly prepared rat hippocampal slices were superfused with different concentrations of hesperidin or hesperetin. TBS, theta-burst stimulation. Amplitude of the population spike is given as percent of the baseline value (reference). Data are given as mean ± s.e.m. of n = 6 slices.

but mere attenuation in a concentration-dependent manner between 5 and 60 μM ($P < 0.01$ at 10 μM and higher). Thus, both compounds induced attenuation of synaptic activity within this range of concentrations (Figures 2 and 3).

Action of hesperidin and hesperetin in the presence of convulsants

To test the possibility that hesperidin and hesperetin were able to control pathological changes of excitability, slices were exposed to several chemical compounds known for their ability to induce convulsions in animals – 4-AP, TEA, bicuculline, pentylentetrazol or high concentration of $CaCl_2$. After determination of the changes induced by these chemicals, hesperidin or hesperetin were added by concomitant superfusion. As is documented in Table 1, hesperidin, but not hesperetin, was able to attenuate the increase induced by the presence of 50 μM of 4-AP in a concentration-dependent manner in the concentration range 0–10 μM (statistically significant at 5 and 10 μM). The effects of 50 μM bicuculline were likewise completely attenuated by 40 μM hesperidin but not by hesperetin (Figure 4). The increased spike in the presence of high $CaCl_2$ (4 mM) was only partially antagonized by hesperidin, but completely by hesperetin.

On the other hand, hesperetin attenuated the increases of the population spike as induced by the presence of TEA in a concentration-dependent manner ($P < 0.01$), but had virtually no effect during the presence of 4-AP (Table 1). In addition, the effect of pentylentetrazol (50 μM) or increased presence of $CaCl_2$ (4 mM) was blocked totally by 40 μM hesperetin (Figure 4). Furthermore, the effect of hesperetin was sensitive to iberiotoxin (2 μM), as depicted in Figure 4. Thus, both compounds seem to have a very selective action and differ with respect to their mechanism of action.

Repetitive administration of hesperidin and hesperetin in-vivo

Since there is no data regarding the passage of either of the compounds across the blood–brain barrier in-vivo, both compounds were administered in-vivo for seven days. Two rats received either placebo, or hesperidin or hesperetin at a dosage of 500 mg kg^{-1} orally. After this the rats were processed the next day using the methodology described above. As is depicted in Figure 5, hippocampus slices from rats receiving saline reacted to the convulsants in the known manner. Slices from the two rats that had received hesperidin showed attenuation of the challenge with 4-AP and with TEA. However, slices from rats treated with hesperetin alone showed attenuation of the challenge with TEA, but not with 4-AP. Thus, slices from rats treated with hesperidin showed lower sensitivity to both convulsants, whereas slices from hesperetin-treated rats only showed attenuation of TEA challenge but not to 4-AP challenge in concordance with in-vitro results.

Table 1 Effect of hesperidin and hesperetin on changes in excitability of rat hippocampus slices in the presence of convulsants

4-AP (50 μM)	Hesperidin (μM)	4-AP + Hesperidin
405.8 ± 49.5	2.5	350.5 ± 53.6
384.3 ± 7.2	5	287.5 ± 6.8
370.2 ± 17.6	10	256.0 ± 24.3
4-AP (50 μM)	Hesperetin (μM)	4-AP + Hesperetin
368.6 ± 6.4	5	400.2 ± 6.4
399.6 ± 22.2	10	426.0 ± 32.4
TEA (3 mM)	Hesperidin (μM)	TEA + Hesperidin
432.6 ± 40.4	5	379.0 ± 40.7
406.2 ± 12.7	10	387.5 ± 18.1
TEA (3 mM)	Hesperetin (μM)	TEA + Hesperetin
362.9 ± 30	5	242.0 ± 27.6
344.2 ± 4.7	10	238.1 ± 21.8

Rat hippocampus slices were superfused in the presence of the convulsants 4-aminopyridine (4-AP) or tetraethylammonium (TEA) alone and in combination with hesperidin or hesperetin, respectively. Amplitude of the population spike is given as percent of the baseline value (reference). Values represent means ± s.e.m. from n = 6 slices.

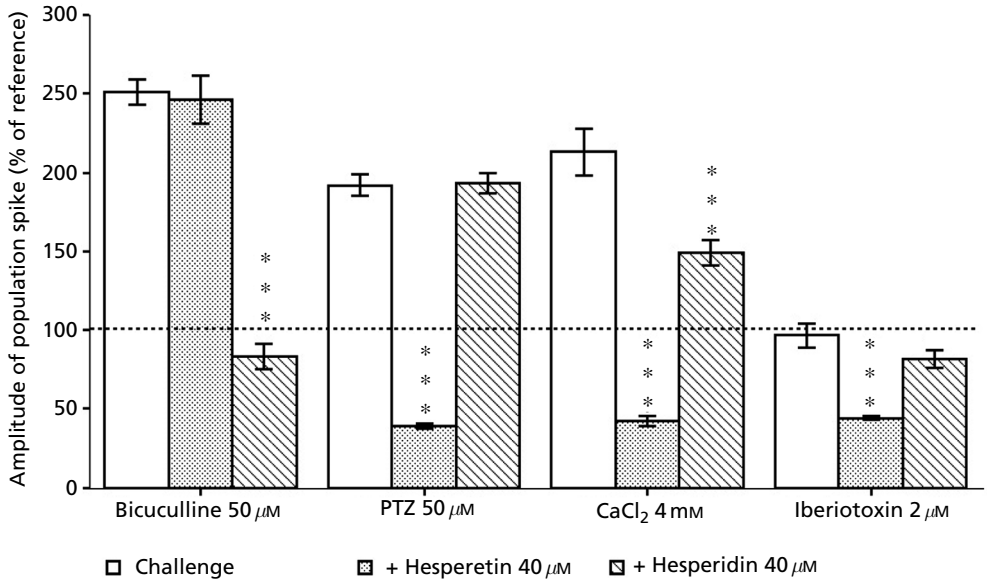

Figure 4 Effect of hesperidin or hesperetin on theta-burst stimulation response of rat hippocampal slices in the presence of convulsants. Freshly prepared rat hippocampal slices were challenged with bicuculline, pentylentetrazol (PTZ), high concentration of calcium or iberiotoxin. Values represent amplitudes of population spikes in percent of the values recorded after single stimuli before the challenge with either of the compounds (baseline = reference). ***$P < 0.01$, vs challenge.

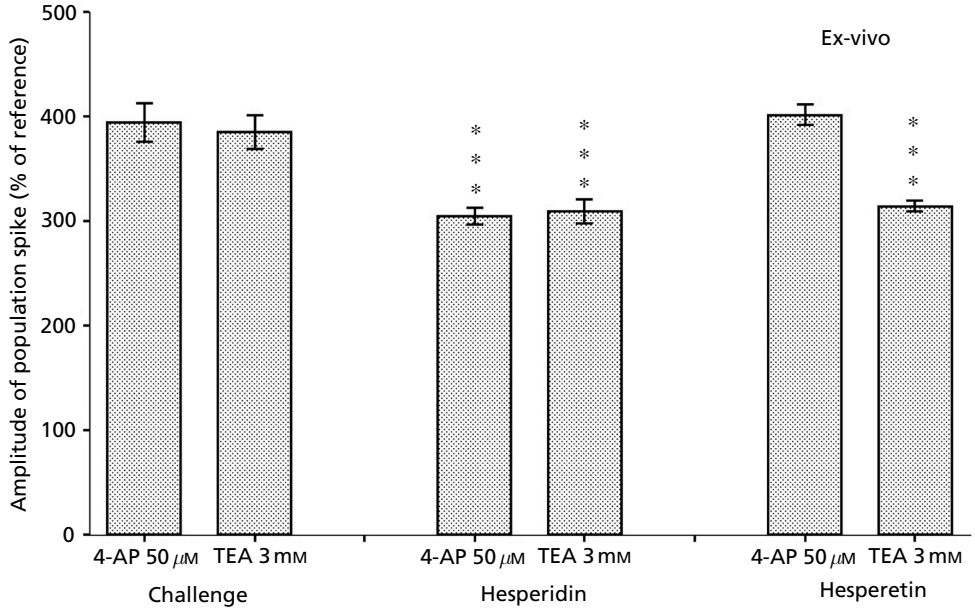

Figure 5 Effect of repetitive administration of hesperidin and hesperetin to rats. Hippocampal slices were prepared from 6 in-vivo treated rats after daily repetitive oral administration of saline, 500 mg kg^{-1} hesperidin or hesperetin (2 rats each). Six slices were prepared and superfused with 4-AP or TEA, respectively, for each of the treatments. Amplitude of the population spike is given as percent of the baseline value (reference). ***$P < 0.01$, vs challenge.

Discussion

Detailed characterization of the central effects of hesperidin and its aglycone hesperetin resulted in the discovery that these compounds had direct effects on the central nervous system that were not identical. Surprisingly, hesperidin was able to antagonize the effects of 4-AP and bicuculline, whereas hesperetin obviously was very effective against the challenge with TEA and pentylentetrazol. Both compounds were able to block the effects of enhanced calcium (hesperetin > hesperidin). Antagonism against TEA by hesperetin has already been observed in a completely different in-vitro model, namely in-vitro isolated aortic rings (Calderone et al 2004). The

question therefore arises as to whether hesperetin acts on one of the calcium-dependent potassium channels within the central nervous system. This interpretation is quite likely, since the synapses between the stimulated Schaffer Collaterals and the hippocampal pyramidal cells use glutamate as transmitter. On the other hand, evidence from the literature suggests a link between the action of glutamate and calcium-activated potassium channels (Isaacson & Murphy 2001). Metabotropic glutamate receptors have also been related to a large-conductance calcium-dependent potassium channel (Holmes et al 1996). Since the effects of both compounds on the population spike amplitude of the hippocampus are sensitive to iberiotoxin (hesperetin < hesperidin), the involvement of a calcium-dependent potassium channel can safely be assumed.

Since this specific antagonism to particular convulsants was also observed after in-vivo repetitive administration, a prophylactic activity against various forms of pathological excitability can be assumed. Treatment with hesperetin only changed the sensitivity to TEA but not to 4-AP. This confirms the selectivity of this compound. The fact that sensitivity to both convulsants changed after in-vivo treatment with hesperidin is in concordance with the fact that in the gut hesperidin is converted partially to hesperetin by bacteria (Lee et al 2004). Thus, sensitivity against both convulsants in the in-vitro preparation could be expected since both compounds are present for action. The results also hint at the in-vivo passage of hesperetin into the central nervous system, since otherwise a change in reactivity of the hippocampal slices to TEA after its direct administration to the rats would not be detectable. This result is in concordance with data published by Youdim et al (2003), who observed uptake of hesperetin by brain epithelial cell lines in tissue culture.

From the results obtained, one could assume anticonvulsive capabilities for both compounds as deduced from the antagonistic activity of hesperidin and hesperetin against 4-AP and TEA, respectively. Since hesperidin is partially converted to hesperetin after administration, it should have broader effects on pathophysiological excitations (i.e., during abstinence from alcohol or other abused drugs). Also, different forms of epilepsy or migraine should be sensitive to the action of these flavanones.

Conclusion

Electrophysiological analysis of the action of hesperidin and its aglycone hesperetin in the rat hippocampus in-vitro provides evidence for selective sensitivity to various convulsive compounds and iberiotoxin. Thus, both natural compounds might be useful in controlling special patho-physiological changes of brain excitation, such as those occurring in drug abuse, migraine or epilepsy.

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