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Arctigenin reduces neuronal responses in the somatosensory cortex via the inhibition of non-NMDA glutamate receptors

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Abstract

Lignans are biologically active phenolic compounds related to lignin, produced in different plants. Arctigenin, a dibenzylbutyrolactone-type lignan, has been used as a neuroprotective agent for the treatment of encephalitis. Previous studies of cultured rat cerebral cortical neurones raised the possibility that arctigenin inhibits kainate-induced excitotoxicity. The aims of the present study were: 1) to analyse the effect of arctigenin on normal synaptic activity in *ex vivo* brain slices, 2) to determine its receptor binding properties and test the effect of arctigenin on AMPA/kainate receptor activation and 3) to establish its effects on neuronal activity *in vivo*. Arctigenin inhibited glutamatergic transmission and reduced the evoked field responses. The inhibitory effect of arctigenin on the evoked field responses proved to be substantially dose dependent. Our results indicate that arctigenin exerts its effects under physiological conditions and not only on hyper-excited neurons. Furthermore, arctigenin can cross the blood-brain barrier and in the brain it interacts with kainate sensitive ionotropic glutamate receptors. These results indicate that arctigenin is a potentially useful new pharmacological tool for the inhibition of glutamate-evoked responses in the central nervous system *in vivo*.

Keywords: arctigenin, ionotropic glutamate receptors, evoked field potentials, rat, brain slices, *in vivo* experiments

Abbreviations: AMPA: α-amino-3-hydroxy-5-methylisoxazole-4-propionate; NMDA: *N*-methyl-D-aspartate; S.E.M.: standard error of means.

1. Introduction

Lignans are important biologically active phenolic compounds related to lignin, produced in different plants, for example in tribe Cynareae, such as Arctium, (Boldizsár et al., 2010a), Cirsium (Boldizsár et al., 2010b) species, Forsythia (Maiada et al, 1990) or Torreya (Zhao et al., 1999) plants. These compounds are present either in free aglycone or in glycosylated form in a wide variety of plants in wooden parts, roots, leaves, flowers, fruits or seeds. Due to their antibacterial, antiviral, antifungal, antioxidant and insecticidal properties they are likely to play an important role in the plants' defence against a variety of pathogenic agents (Harmatha and Dinan 2003; Wink, 2006).

Among lignans, the dibenzylbutyrolactone-type arctigenin (ATG) has special importance. In addition to the neuroprotective activity of arctigenin, it also shows significant anti-tumor (Mervai et al., 2015), antiviral (Hayashi et al., 2010; Zhang et al., 2014), anti-inflammatory (Cho et al., 2004; Zhao et al., 2009; Hyam et al., 2013,) and phytoestrogenic (Turner et al., 2007; Landete, 2012) activities, highlighting the importance of this natural lignan aglycone.

As a neuroprotective agent (Jang et al., 20012; Ma et al., 2010), arctigenin has been used as a neuroprotective medicine in traditional medication for treatment of encephalitis (Saxena and Dhole, 2008; Zhang et al., 2014). Additionally, previous studies of cultured rat cerebral cortical neurones suggested that arctigenin inhibits the excitotoxic effect of kainate by binding to non-NMDA type glutamate receptors (Jang et al., 2002). However, the direct effects of arctigenin on normal neuronal activity have not been established. Arctigenin is a lipophilic molecule that likely to penetrate the blood-brain-barrier, but this has not been demonstrated experimentally. Therefore, the aims of the present study were: 1) to analyse the effect of arctigenin on synaptic activity in *ex vivo* brain slice experiments, 2) to determine its receptor binding properties and effects on AMPA/kainate receptor activation and 3) to establish its penetration through the blood-brain barrier and effects on neuronal activity *in vivo*.

2. Materials and methods

Male Wistar rats (150-280 g for *ex vivo* and 360-525 g for *in vivo* studies, Toxicoop, Hungary) were used for all experiments in accordance with the European Communities Council Directive (86/609/EEC) and with the guidelines of the Eötvös Loránd University Animal Care and Use Committee (p.n.: XIV-I 001/515-4/2012). All possible efforts were

made to minimize animal suffering and to reduce the number of animals used. Rats were maintained in 12 h light/dark cycle and controlled temperature (22±2 °C). Standard pellet food and tap water were available ad libitum.

Arctigenin purchased from Tocris Bioscience (Avonmouth, UK) was dissolved in 3% DMSO containing sterile physiological saline and stored as 1 mM stock solution at -20ºC. All other chemicals were purchased from Sigma-Aldrich (Hungary) if not stated otherwise.

2.1. *Slice preparation and electrophysiological recording*

Electrophysiological recordings were carried out on 64 coronal slices of somatosensory cortex prepared from 18 adult rats as previously described (Világi et al., 2009). Rats were decapitated in deep chloral-hydrate (350 mg/kg) anaesthesia. Brains were quickly removed and 400 μm thick coronal slices were cut by a vibratome. After 1 h regeneration period in the incubation solution, slices were transferred to a Haas-type recording chamber (Experimetria Ltd., Budapest, Hungary) and perfused (2.0 ml/min) with standard artificial cerebrospinal solution (ACSF). The solution was saturated with carbogene (5% CO_2, 95% O_2) at 33±1°C. The composition of the ACSF solution was (in mM): 126 NaCl; 26 $NaHCO_3$; 1.8 KCl; 1.25 KH_2PO_4; 1.3 $MgSO_4$; 2.4 $CaCl_2$; 10 glucose.

Glass microelectrodes filled with 1 M NaCl (8-10 MΩ) were positioned as recording electrodes into the lower part of layer 3 (L3) of the somatosensory cortex, while bipolar tungsten stimulating electrodes were placed directly below the recording electrodes at the border of the white and grey matters. Duration of the stimulation square voltage pulses was 100 μs and the amplitude was gradually varied between threshold and supramaximal values. Signals were amplified by an Axoclamp2A amplifier (Axon Instruments Inc., Union City, CA), digitalized by an A/D converter (NI-6023E, National Instruments, Austin, Texas) and recorded with the SPEL Advanced Intrasys computer program (Experimetria Ltd., Budapest, Hungary). Single-shock stimulation evoked a characteristic field response, which consists of an early (N1) synaptic component (mean latency: 6.1 ms), which was followed by a positive (P1) and a late negative (N2) peaks, while sometimes but not always a previous, non-synaptic, antidromic component also appeared (Fig. 1).

The viability of each slice was tested at the beginning of the recording session. If the peak-to-peak amplitude of the maximal evoked response (N1-P1) was smaller than 1.0 mV, the slice was excluded from the experiments. Slices were continuously stimulated with medium-strength stimuli at a rate of 0.1 Hz. Stimulus threshold (T) was determined 15 min after placing the slices into the recording chamber. Subsequently, 0.1 Hz continuous

stimulation was applied with 2T for 30 min to record control evoked responses, and at the end of this period 10 evoked responses at 2T were stored.

To test the inhibitory effect of arctigenin at three different concentrations, standard perfusion solution was switched to arctigenin containing solution (stock solution was diluted to a final concentration of 1, 10 or 20 μM in the perfusion solution) for 30 min following the first measurement of evoked field potential (EFP) at 2T. At the end of arctigenin application the 10 test responses at 2T were also stored.

Recorded data were analysed using the SPEL (Solution Pack for Experimental Laboratories) Advanced Intrasys computer program (Experimetria Ltd., Budapest, Hungary). Each experimental group contained 7-8 independent records obtained from different animals. The early component was characterized by the peak amplitude of the early monosynaptic N1 component and by peak-to-peak amplitude of N1-P1 polysynaptic waves (Fig. 1A). The amplitude of the second negative (N2), late component was also determined. During the statistical analysis interleaved control and treated groups were compared. In each series of experiments one-way ANOVA was performed followed by Newman-Keuls post hoc test. Homogeneity of variances and normal distribution of data were tested before statistical analysis. Results are presented as mean ± S.E.M and changes were considered to be significant if $p < 0.05$.

2.2. *Receptor ligand binding assay*

Human embryonic kidney (HEK) 293 cells transfected with the GluK1 kainate receptor subunit were maintained and harvested as described previously (Atlason et al., 2010). Membrane fractions were washed three times in binding buffer (50 mM Tris buffered with citric acid pH=7.4) by resuspension and centrifugation (40,000g, 20 min, 4^{o}C). Membrane proteins (100 μg) were incubated for 1 h on ice with 50 nM [^{3}H]kainate (37 MBq/ml; PerkinElmer Life and Analytical Sciences, Waltham, MA) in the absence and in the presence of 0.01-1 mM arctigenin. Non-specific radioligand binding was measured in the presence of unlabelled kainate (1 mM; Tocris Bioscience, Avonmouth, UK). Receptor-ligand complexes were recovered using a filter in a Brandel cell harvester (model M-30; Brandel, Gaithesburg, MD, USA) and washed three times using 4 ml of binding buffer. Radioligand binding was measured using a scintillation counter (LS6500; Beckman, High Wycombe, UK).

2.3. *Cobalt-uptake assay*

Slices were obtained from six animals to test changes in Co^{2+} uptake as a consequence of arctigenin treatment. Slice preparation was carried out in the same way as described under

2.1. The thickness of the slices was, however, 250 μm. From each rat 8-10 slices were stained and evaluated. The procedure was performed as described previously (Pruss et al., 1991). Briefly, slices were incubated in a Ca^{2+} free incubation solution for 5 min at room temperature (~20°C) before placement into an uptake buffer (in mM) (13 sucrose, 57.5 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 12 glucose, 10 HEPES) supplemented with 5 mM $CoCl_2$ and 100 μM kainate. Slices incubated in the same solution without kainate served as background controls. Half of the brain slices were also exposed to either 10 or 20 μM arctigenin in uptake buffer. Incubation lasted for 20 min at 22°C, and then slices were rinsed once in the uptake buffer and incubated in the same buffer containing 2 mM EDTA to remove non-specifically bound Co^{2+}. Following that, slices were rinsed twice with the uptake buffer, and Co^{2+} was precipitated by incubation in a 0.12% $(NH_4)_2S$ solution for 5 min. During this procedure, dark CoS precipitate was formed in the cells. At the end, slices were fixed in 4% paraformaldehyde for 30 min and mounted in glycerin for image analysis.

Co^{2+}-stained slices were placed on an Olympus CH-2 microscope equipped with an Olympus Camedia C4040-Zoom digital camera connected to a PC running AnalySIS 3.2 Docu (Soft Imaging System) software. Images were captured by the CCD camera under the control of AnalySIS 3.2 Docu software. Exposure time was fixed during image acquisition. Images were taken at 4-fold magnification and converted to 8-bit grey scale images. Subsequent densitometry analysis was made by custom written Mathworks Matlab software as follows: perpendicular to the pial surface a rectangular shaped region of interests (ROI) was defined in the dimensions of 450x1800 μm spanning all layers of the neocortex. Optical density (OD) was measured in arbitrary units using 256 grey levels of images within ROIs, followed by an averaging of OD values in each row of pixels in the ROI. OD of a 20x20 pixel area out of the slice was determined in each slide as background and data were always corrected with this OD value. The distance between the pial surface and the corpus callosum was divided into 20 equal divisions for the comparison of corresponding regions in various treatment groups. These divisions were correlated to cortical layers on the basis of previous studies (Skoglund et al., 1996). Original continuous OD distribution was interpolated to the 20 divisions and correlated to cortical layers. In all experiments the corrected OD values of kainate-stimulated (control) slices were expressed as a percentage of corrected OD values of slices without kainate application. The same calculation was performed for arctigenin treated brain slices.

2.4. *Test of the crossing of blood-brain barrier*

Rats (n=4) received 20 mg/kg arctigenin intraperitoneally (i.p.). Physiological saline solution containing arctigenin was injected at body temperature in a volume of 4 ml. After 20 min, rats were sacrificed and perfused transcardially with 150 ml ice cold ACSF to wash out the blood from the brain. Brain were removed, rinsed, weighed and immediately snap-frozen in liquid nitrogen for lyophilisation. Samples were extracted in 2.0 mL methyl alcohol and dialysed to obtain a protein free arctigenin containing extract. Microdialysis probes were hollow fibres (Travenol, cut-off at 5000 Da, o.d. 0.2 mm, length of the active surface: 3 mm) adjusted into 23-Gauge, stainless steel tubing. Methyl alcohol was used for perfusion (2.0 µL/min for 10 min). The 20 µL of arctigenin containing dialysate was analysed by high-performance liquid chromatography (HPLC) using UV detection. The HPLC system consisted of two Pharmacia LKB pumps and a VWM 2141UV detector (Pharmacia LKB Biochrom, Cambridge, UK). Column: GraceSmart RP18 (5_m), 150 x 4.6mm (Grace Davison Discovery Sciences Lokeren, Belgium). Gradient elution was carried out at 20-22ºC. Eluents: eluent A, acetonitrile/0.07M acetic acid 15/85 (v/v), eluent B, acetonitrile/0.07M acetic acid 85/15 (v/v). Gradient: 15% B → 30% B over 5 min, 30% B → 44% B over 7 min, 44% B → 100% B over 5 min. UV detection was carried out at λ = 280 nm. Retention time of arctigenin was 11.48 min.

2.5. *In vivo recording*

Rats (n=6) were anesthetized with urethane (1.2 g/kg, i.p.) and fixed in a stereotaxic frame (David Kopf) with the top of the skull set horizontal to comply with the rat brain atlas of Paxinos and Watson (Paxinos and Watson, 1998). A 4 x 3 mm craniotomy centred 1.5 mm posterior to Bregma and 1.5 mm lateral to the midline was made to expose the hind limb representation area in the right hemisphere. The dura mater was left intact during the whole experiment. Rectal temperature was maintained at 37 °C Arctigenin solutions were also applied i.p. Arctigenin and respective saline solutions were injected at body temperature in a volume of 2 ml in case of the 20 mg/kg dose (n=4) while injection volume was 4 ml in case of the 50 mg/kg dose (n=2). To test possible non-specific effect of the injections, recording sessions started with the application of control saline solutions followed by 1-hour long recording. Then arctigenin was applied and its effect was recorded for 1 h.

Hind limb (HL) area of the primary somatosensory cortex (SI) was localized as described previously (Toth et al., 2008). A 1.1 mm stainless steel reference electrode (Fine Science Tools, USA) was placed above the cerebellum. A 16-channel vertical electrode array was positioned 1-1.2 mm below the dura (Neuronelektród Ltd., Budapest, Hungary) to record

evoked extracellular field potentials (EFPs) from different cortical layers at the same recording site. After insertion of the recording array, two stimulating needle electrodes were inserted into the HL close to the ankle to enable electrical stimulation of the tibial nerve. Stimuli for EFPs recording were square-wave pulses generated by a stimulator (Master8, A.M.P.I., Jerusalem, Israel), and delivered through a Master8 ISO-Flex stimulus isolation unit. The threshold stimulus voltage was defined as the lowest intensity that elicited minimal movement in the hind paw (4.25 V ± 0.28 V). During the whole experiment, stimulus strength twice the threshold was used (2T) with 200 μs duration. Stimulus rate was set to 0.1 Hz and a total of 360 EFPs were recorded in 1 h. EFPs were recorded in 2000 ms long blocks (1000 ms pre-stimulus, 1000 ms post-stimulus periods).

EFP signals were fed to a 16-channel differential amplifier (Supertech Ltd., Pecs, Hungary), conditioned (filter: 0.1 Hz–1000 Hz, gain: 5000x) then digitalized at 3 kHz with 16 bit resolution (Labview; National Instruments, Austin, TX, USA). All data were stored on hard disk for off-line analysis.

Parameters of the EFPs recorded were calculated from layer 3 and layer 5. Single EFPs were averaged in 10 min long blocks where every block contained 60 responses. Amplitude and latency values of the characteristic waves were determined in these blocks and expressed as mean and standard error of the means (S.E.M). The areas under the curves (AUC) were also calculated to take into account the potential changes in the width of the different waves of the EFPs. For this, the baseline of the curves was shifted to zero. Numerical integrate values were calculated in Microsoft Excel using the trapezoidal rule (Burden and Faires, 2004). As duration of the P1 wave in EFPs recorded from layer 3 showed large variations, numerical integrate was calculated between predefined time points of the P1 wave (4 ms and 45 ms). Layer 5 EFPs were highly uniform in shape and in this case integrate was calculated the start and end points of the N1.

Amplitude, latency and AUC differences seen in control vs. arctigenin responses were compared statistically by one-way ANOVA followed by Student-Newman-Keuls post hoc test. Assumption that data are sampled from populations that follow Gaussian distributions was checked by the test of Kolmogorov and Smirnov. Statistical tests were performed using Instat (GraphPad, San Diego, CA, USA). Statistical significance was accepted at the $p < 0.05$ level. At the end of the experiment, position of the recording array was marked using direct current injections, and confirmed by histological analysis.

3. Results

3.1. *Evoked responses in slice experiments*

In control slices, the mean of the peak amplitude of N1 component (Fig. 1A) was -2.03±0.20 mV (n=8) at the end of the 30 min recording (Fig. 2A). While the application of 1 μM arctigenin produced no significant change (-1.57±0.13 mV, p=0.081), the 10 μM and 20 μM arctigenin significantly reduced the amplitude of the N1 component of EFP (-1.27±0.09 mV, p=0.005**; n=7; and -0.86±0.09 mV, p=0.0003***; n=7) at the end of the 30 min incubation period. The peak-to-peak amplitude of the polysynaptic N1-P1 component of the evoked field potential was 2.31±17.7 mV (n=8) at the end of the recording (Fig. 2B). While the application of 1-10 μM arctigenin produced no significant change (1 μM: 1.92±0.18 mV, p=0.243; 10 μM: 1.92±0.18 mV, p=0.066; n=7), 20 μM arctigenin considerably reduced the EFP amplitude of N1-P1 (0.92±0.08 mV, p=0.0024**; n=7) at the end of the 30 min incubation period.

The effect of arctigenin on N2 component of EFP was also tested. In control slices after 30 min -0.65±0.13 mV (n=8) evoked field potential amplitude was measured. Arctigenin applied in 1, 10 and 20 μM concentrations had no significant effect on evoked field potential amplitude (-0.41±0.15 mV, (p=0.805, n=7), -0.20±0.04 mV, (p=0.486, n=7) and -0.12±0.02 mV (p=0.039; n=7), respectively).

3.2. *Receptor binding analysis*

Cell membrane fraction from transfected HEK 293 cells was used to analyse the effect of arctigenin on [^{3}H]kainate binding to GluK1 kainate receptor subunit (Fig. 3) (Atlason et al., 2010). [^{3}H]kainate binding assays performed in the presence of different arctigenin concentrations (0.01-1 mM) revealed a significant reduction in [^{3}H]kainate binding activity to GluK1 at 1 mM (Fig. 3; n=4, $p<0.01$; Student's t-test).

3.3. *Alterations in Co^{2+}-uptake*

Following kainate activation, Co^{2+} crosses the plasma membrane through Ca^{2+} permeable AMPA and kainate receptors (Mayer and Westbrook, 1987). The distribution and relative intensity of the signal produced by CoS precipitation with and without kainate treatment (Fig. 4A) can be compared using densitometry (Fig. 4B). Arctigenin effectively and dose-dependently reduced the AMPA/kainate receptor activation and subsequent Co^{2+} uptake especially in the supragranular layers of somatosensory cortex (Fig. 4B). Overall, OD associated with kainate-induced Co^{2+} uptake was significantly reduced in most layers of the

somatosensory cortex in arctigenin-treated slices (Fig. 4). The most prominent changes of OD was identified in layers 2/3 in the control kainate-treated slices (193.2±23.6%, n=6), where 10 or 20 μM arctigenin application parallel with kainate treatment reduced the changes of OD to 122.3±21.5% and 85.1±31.6%, respectively. The level of decrease was significant in these layers in cases both of 10 and 20 μM arctigenin treatment (n=6-6, $p<0.02$*).

3.4. Arctigenin level in the brain following intraperitoneal application

These measurements were carried out to establish the penetration of arctigenin into the brain tissue through the blood-brain barrier. Substantial amount of arctigenin was detectable in the brain tissue 20 min after 20 mg/kg intraperitoneal application of the lignan, which indicates that it can cross the blood-brain barrier. The average wet brain weight was 1.47±0.11 g (n=4). The arctigenin content was $3.19\pm0.5\text{x}10^{-7}$g/1 g wet brain tissue.

3.5. *Evoked field potentials in vivo*

Tibial nerve stimuli elicited clear EFPs in the cortex both in the control and arctigenin-treated animals. Short latency responses (up to 50 ms) in layer3 EFPs showed a characteristic positive peak (P1), which was highly variable in duration (Fig. 5A, top). EFPs showed slight potentiation in response to the consecutive stimulation during the control recordings. By the end of the one-hour-long recording in layer 3, P1 amplitude increased to 84.66±11.3 μV from the initial 67.22±8.63 μV value seen in the first 10 min long block. Similar increase was seen in the N1 amplitude of layer5 responses, but it was smaller (7.5±43.1 % change; data not shown). After the injection of arctigenin, P1 amplitude significantly decreased in layer 3 (Fig. 2) compared to the value of the last 10 min block of the control recording taken as baseline. The strongest decrease was seen in the first 10 min period following arctigenin application, the mean amplitude was only 59.18 ±12.96 μV which meant 30 % decrease (p=0.623, n=6). The decrease persisted for longer time, the amplitude was 75.6 ±18.17 μV (p=0.623, n=6) 30-min post-injection. In layer5, EFP amplitude did not change significantly after arctigenin injections (data not shown).

There was a tendency to decrease in the AUC values in case of layer3 EFPs after arctigenin injections in the first 20 min but these changes were not statistically significant (Fig. 5B).

4. Discussion

A previous study suggested a direct interaction between arctigenin and kainate receptors in cultured cerebrocortical neurons (Jang et al., 2002). This study demonstrated the neuroprotective effect of arctigenin in an *in vitro* cell culture system using kainate evoked neurodegeneration. As a reduced [^{3}H]kainate binding to neuronal membrane fractions was reported in the presence of arctigenin, the investigators proposed a selective kainate receptor effect. However, it was stated, that arctigenin produced similar effect as the non-NMDA receptor antagonist CNQX (Jang et al., 2002). This finding raised the intriguing possibility that arctigenin may inhibit the electrical activity of neuronal networks *in vivo* in the neocortex.

4.1. *Arctigenin significantly decreases the excitability of brain slices*

In our somatosensory cortex slice experiments the recording electrode was positioned in the layer 3, as the amplitude of EFPs evoked by electrical stimulation of the border of the corpus callosum and the grey matter is the largest in this layer (Abbes et al., 1991). Both AMPA, NMDA and kainate types of glutamate receptors play a role in the development of these evoked responses (Conti and Weinberg, 1999; Huettner, 2003). AMPA/kainate receptors contribute mainly to the development of early component of synaptic responses (Bailey et al., 2001; Campbell et al., 2007). Different types of kainate receptor subunits are widespread in the brain, which are expressed on neurons both pre- and postsynaptically (Ali et al., 2001; West et al., 2007) in hetero-tetrameric subunit assemblies (Chittajallu, 1999). While AMPA receptor density is rather high in the supragranular layers of the somatosensory cortex (Martin et al., 1993; Van Damme et al., 2003), the presence of kainate receptors is also demonstrated both on pyramidal cell dendrites and on interneurons, although, on pyramidal cell of layer 5 and layer 6 its concentration is higher (Bahn et al., 1994; Jabłońska et al., 1998; Bailey, et al., 2001; Zilles et al., 2002). In hippocampal slice preparations it was demonstrated that inhibition of presynaptic kainate receptors on pyramidal cells decrease the glutamate release, which reduce the activation of the postsynaptic cells (Lerma, 2006). Inhibition of postsynaptic kainate receptors also diminishes the membrane depolarization (Lerma, 2006). Our experiments demonstrate that arctigenin directly binds to GluK1 kainate receptor subunits. Also, 10 μM arctigenin effectively reduces the amplitude of the early monosynaptic components of EFPs. The inhibitory effect of arctigenin on the evoked field responses proved to be substantially dose dependent, however, the late component was reduced only by the largest dose of arctigenin. Mainly AMPA/kainate receptor activation underlies the first synaptic components of evoked field potentials, so the inhibitory effect of arctigenin on

synaptic efficacy indicates that it may exerts effect not only at pathophysiological but also at normal physiological condition on AMPA/kainate receptor. The smaller effect of arctigenin on late component is consistent with previous studies, because primarily the NMDA receptor activation is responsible for the development of the late component of EFP, and arctigenin does not seem to affect these receptors (Conti and Weinberg, 1999).

4.2. Arctigenin inhibits AMPA/kainate type ligand-gated ion channel activation

Co^{2+} can cross neuronal plasmamembrane through activated AMPA/kainate receptors instead of Ca^{2+}, accordingly the alteration of Co^{2+} staining refer to modified non-NMDA type ligand gated ion channel activation (Williams et al., 1992). We detected, that kainate-induced Co^{2+} uptake was not uniform along the column perpendicular to the pial surface in the somatosensory area of control slices, in the layer 2/3 is higher than in layer 5 or layer 6. Co^{2+} uptake was effectively reduced in the presence of arctigenin, which was particularly noticeable in the supragranular layers. As kainate can bind not only to kainate receptors, but also to AMPA receptors, we have to take into consideration that AMPA receptors might also be affected by arctigenin (Iino et al, 1990).

4.3. Arctigenin reduces synaptic activity in vivo through its antagonistic effects on glutamate receptors

We have demonstrated that arctigenin can cross the blood-brain barrier and appears in the brain following intraperitoneal administration. The *in vivo* electrophysiological experiments revealed that arctigenin has an antagonistic effect on glutamate-evoked responses, which also indicate that arctigenin can get into the cortex. The inhibitory effect proved to be more prominent in the outer layers of the cortex, which may be due to the differential distribution of different ionotropic glutamate receptors. Our results indicate that glutamatergic transmission can be modulated *in vivo* by arctigenin, which is a new type of glutamate receptor antagonist, and could be a lead compound for the development of neuroprotective drugs. On the basis of our experiments we can conclude that arctigenin may exerts its effect on neurons in physiological conditions not only on neurons overexcited i.e. by kainic acid application. Arctigenin and its derivatives, may lead to improved treatment strategies in different neural diseases like schizophrenia, autism, bipolar disorders, mental retardation together with epileptiform diseases, chronic pain or migraine (Contractor et al., 2011). However, the potentially broader effects of arctigenin on other ionotropic and metabotropic glutamate receptors also need to be considered and investigated in future studies.

5. Conclusions

Several biologically active compounds possess neuroprotective effects. It was proved that arctigenin, a lignan produced by different plants, effectively protects cultured neurons from excitotoxicity via the inhibition of kainate sensitive ionotropic glutamate receptors. Here we demonstrated that arctigenin can cross the blood-brain barrier, reduces the activity of ionotropic glutamate receptors, and inhibits normal electrical responses both in brain slices and in living animals.

Conflict of interests Statement

The authors declare that they have no conflict of interests.

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Figure legends:

Fig. 1. Original records of evoked field potentials (EFPs) in *ex vivo* cortical slice evoked by electrical simulation of the border of the white and grey matter (A) and *in vivo* by electrical stimulation of tibial nerve (B). **Panel A**: The first, early synaptic component was characterized by the monosynaptic N1 component amplitude and by the polysynaptic N1-P1 peak-to-peak amplitude. **Panel B:** The response evoked in cortical layer 3 was characterised by the positive wave (P1) amplitude.

Fig. 2. Arctigenin (ATG) reduced the amplitude both the monosynaptic and polysynaptic part of the early component of the evoked field potentials. The effect of arctigenin on extracellularly recorded evoked potentials was tested in *ex vivo* slice preparations. **Insert** shows original record of a control evoked response (black line) and the effect of application of 20 μM arctigenin on it (gray line). **Panel A:** dose dependent suppression of the early monosynaptic component (N1) of field potentials by ATG at 2T stimulus intensity. ATG was able to reduce significantly the amplitude of N1 component of the evoked response applying in 10 μM and 20 μM concentration (data are presented in absolute value). **Panel B:** suppression of the early, polysynaptic component (N1-P1) of field potentials by ATG was also dose dependent at 2T stimulus intensity. ATG was able to reduce the (N1-P1) component in 20 μM concentration (n=8 in each groups).

**p< 0.005, ***p< 0.0003. Statistical significance was checked by one-way ANOVA, followed by Newman-Keuls post hoc test.

Fig. 3. Arctigenin (ATG) inhibits [^{3}H]kainate binding to GluK1 kainate receptor subunit at 1 mM. Membranes from HEK 293 cells stably transfected with GluK1 were incubated with 50 nM [^{3}H]kainate and varying concentrations of arctigenin, with unlabelled kainate (KA) as positive control. Unlabelled kainate and 1 mM arctigenin significantly inhibited [^{3}H]kainate binding to GluK1. n=3, *p<0.05, **p<0.01; Student's t-test.

Fig. 4. Arctigenin (ATG) suppresses Co^{2+} uptake in supragranular layers of somatosensory cortex. **Panel A** shows representative images of Co^{2+} uptake patterns in treatment groups: first and second columns represent sections without and with kainate (KAIN) application, respectively. Images ATG 10 μM and ATG 20 μM represent arctigenin-

treated sections with together with kainate application. The optical density changes (ΔOD) are shown on **Panel B**. Both concentration of arctigenin reduced the Co^{2+} permeability of cells in supragranular layers, especially in layer 3. L1-6 represent cortical layers. n=6 in each groups, *p<0.05; Student's t-test.

Fig. 5. Characteristics of EFP waves evoked by electrical stimulation of the HL (panel A) and illustration of the AUC calculated in this work (panel B). **Insert** illustrates the evoked response showed a characteristic positive wave (P1) in layer 3. Amplitude of P1 was calculated as a deviation from the pre-stimulus baseline. Black curve shows averaged EFP from the baseline period while grey curve represents averaged EFP after 10 min arctigenin application. Stimulus onsets are marked by arrow. Note that stimulus artefacts were removed from the averaged EFP curves. **Panel A**: changes of P1 wave amplitude after arctigenin application compared to baseline in layer 3 (n=6). Averaged values of the last control block (between 50 and 60 min from the time point of the control injection) were taken as baseline (100 %). Each block plotted represents the average of 60 individual EFPs per rat. Statistical significance was checked by one-way ANOVA followed by Student-Newman-Keuls post hoc test. Significance level: * - $p < 0.05$. Data are expressed as mean and S.E.M. **Panel B**: AUC changes of the EFP in layer 3. For the calculation of AUC values, the baseline of the curves was shifted to zero then the numerical integrate of the ruled area was determined in case of layer3 EFPs. Averaged EFP curves were calculated from single EFPs recorded in the last control block (between 50 and 60 min from the time point of the control injection, n=60 EFPs) then AUC of the averaged EFP was determined. Then the same procedure was applied for the EFPs recorded after the arctigenin injections. Statistical significance was checked by one-way ANOVA followed by Student-Newman-Keuls post hoc test. Data are expressed as mean and S.E.M.