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Apigenin modulates GABAergic and glutamatergic transmission in cultured cortical neurons

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Abstract

Using the patch-clamp technique, we studied the modulation of ionotropic γ-aminobutyric acid (GABA) and glutamate neurotransmission by apigenin, a flavonoid with sedative and antidepressant activity. Apigenin reversibly reduced GABA-evoked currents mediated by $\alpha_1\beta_2\gamma_2$ receptors expressed in HEK293 cells. Amplitude and frequency of spontaneous postsynaptic inhibitory currents (sIPSCs) mediated by $GABA_A$ receptors were also decreased by apigenin in cultured cortical neurons. The flavonoid was almost inactive on α-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA) mediated currents while it reduced *N*-methyl-D-aspartate (NMDA) receptor mediated responses with a half maximal inhibiting concentration (IC_{50}) of 10 μM. The flavonoid inhibited also peak amplitude and frequency of spontaneous postsynaptic excitatory currents (sEPSCs). Finally, apigenin is neuroprotective against glutamate-induced neurotoxicity in cerebellar and cortical neurons in culture. Our data reveal the antagonistic effect of apigenin on GABA and NMDA channels. While the inhibition on GABA receptor cannot explain the effects of the drug in vivo our data on NMDA channels reveal a new target of apigenin. A reduction of the network excitability could thus account for the sedative effects. Furthermore, our data suggest a potential neuroprotective activity of apigenin.

Keywords: Flavonoid; $GABA_A$ receptors; NMDA receptors; Patch clamp; Neurotoxicity

1. Introduction

Flavonoids are important metabolites of vascular plants structurally related to flavone (Fig. 1). They posses different biological activities that range from anti-inflammatory, antioxidant and antitumoral to sedative, anxiolytic and antidepressive (for review, see Middleton et al., 2000; Harborne and Williams, 2000). The flavonoid apigenin is present in different plants (*Matricaria*, *Passiflora*, *Perilla*) and extracts commonly used for their anxiolytic, sedative and antidepressant properties. The search for new drugs to treat mood and anxiety disorders is supported by the fact that compounds currently used, i.e., benzodiazepines and monoaminergic antidepressant, are inefficacious in several cases (20% of major depression and 70% in post-traumatic stress disorder) and produce unwanted side effects. The study of the active compounds of medicinal plants could thus provide new tools for the treatment of these mental disorders. At the end of the 1980s, Medina et al., (1989) demonstrated that some flavonoids isolated from medicinal plants had a certain affinity for benzodiazepine recognition site on $GABA_A$ receptors. These data supported the idea that flavonoids could modulate GABA receptor function similarly to benzodiazepines (Medina et al., 1989), thus explaining the pharmacological effect of some plants.

In particular, it has been shown that in in vitro experiments apigenin can bind to the benzodiazepine receptor (Medina et al., 1990). In vivo studies revealed that apigenin exerts anxiolytic and sedative properties (Salgueiro et al., 1997) and antidepressant activity in the Porsolt test (Nakazawa et al., 2003). Our group confirmed the sedative but not the anxiolytic effect of apigenin (Zanoli et al., 2000). The sedative effect after all could not be ascribed to an

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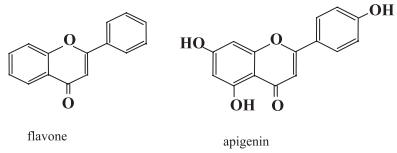

Fig. 1. Chemical structures of flavone and apigenin.

activation of the benzodiazepine receptor since it was not blocked by Ro 15-1788 (flumazenil), a selective antagonist of that site and since in electrophysiological experiments apigenin reduced GABA-evoked currents in cerebellar granule cells in culture (Avallone et al., 2000). In addition, the affinity of apigenin for the benzodiazepine site was too low (EC_{50} 10^{-4} M).

All these data suggest that apigenin do not act on the benzodiazepine site and more likely this compound could affect other neurotransmission systems in the brain.

To better understand the pharmacological activity of apigenin, we investigated its potential ability to modulate GABA and glutamate neurotransmission by performing electrophysiological experiments.

We tested apigenin on $\alpha_1\beta_2\gamma_2$ GABA receptors expressed in HEK293 and on native GABA receptors in cortical neurons in culture. We also studied apigenin effect on spontaneous postsynaptic inhibitory currents (sIPSCs), given their physiological role. Subsequently, we tested apigenin on spontaneous and evoked currents mediated by AMPA receptors, NMDA native receptors and recombinant NR1a/NR2A vs. NR1a/NR2B receptors. Finally, bearing in mind that an overactivation of NMDA receptors mediates glutamate related neurotoxcicity, we investigated apigenin neuroprotective effect on cerebellar and cortical neurons in culture exposed to glutamate.

2. Materials and methods

2.1. Primary cultures of cerebellar granule cells and cortical neurons

Primary culture of cerebellar granule neurons and cortical neurons were prepared from newborn and 7-day-old Sprague–Dawley rats as previously described (Gallo et al., 1987; Luo et al., 2002) following the European Community Guidelines for the use of experimental animals. Briefly, cells from cerebella or cortex were dispersed with trypsin (0.24 mg/ml; Sigma, Milan, IT) and plated at a density of 1.5×10^6 cells/ml on 35-mm Falcon dishes coated with poly-L-lisine (10 μg/ml, Sigma). Granule cells were grown in Basal Eagle's Medium (BSA; Celbio, Milan, IT), supplemented with 10% foetal bovine serum (Celbio), 2mM glutamine, and 100 μg/ml gentamycin (Sigma) and maintained at 37 °C in 5% CO_2. Cortical neurons were also plated in Basal Eagle Medium. After 24 h in vitro the medium was replaced with a half and half mixture of Basal Eagle's Medium (BME) and Neurobasal medium containing 2% B27 supplement, 1% antibiotic, and 0.25% glutamine (Invitrogen). Thereafter, for cortical neurons, half of the medium was replaced twice a week with Neurobasal medium. At second day in vitro (DIV2) for granule cells and DIV 5 for cortical neurons, cytosine arabinofuranoside was added at the final concentration of 10 μM. Recordings were made from DIV 9-11 neurons in culture.

2.2. HEK293 cells culture

HEK293 cells stably transfected with $GABA_A$ $\alpha_1\beta_1\gamma_{2s}$ receptors were purchased from Promochem (UK) and grown at 37 °C and 5% CO_2 in MEM medium (with Earle's salt supplemented with 10% Foetal Bovine Serum and glutamine). One day before experiments, HEK293 confluent dishes were split with trypsin-EDTA 0.05% (Euroclone) and plated at the density of 0.5 million/ml in 2-ml dishes containing coverslips.

2.3. JM4C cells culture

Mouse connective tissue fibroblasts JM4C cells stably transfected with NR1a/NR2A or NR1a/NR2B human NMDA inducible receptors were kindly donated by Dr. Paul Whiting, Merk Sharp & Dohme (UK). They were grown in Dulbecco's Modified Basal Eagle's medium (DMEM) with Na^+ pyruvate, 4500 mg glucose and glutamine. For growing, DMEM was added with geneticin 1 mg/ml (Sigma) and foetal bovine serum 10% (Celbio). One day before experiment, fibroblasts confluent dishes were split with trypsin-EDTA 0.05% (Euroclone) and plated at the density of 0.5 ML/ml in 2 ml dishes containing an inducing DMEM with ketamine (final concentration 0.015%, Gellini International, Italy) and 25 nM dexamethasone (Sigma) to induce NMDA receptors expression.

2.4. Solutions and drugs

The recording chamber was continuously perfused at 5 ml/min with an extracellular medium composed of (mM) NaCl 145, KCl 5, $CaCl_2$ 1, HEPES 5, Glucose 5, Sucrose 20, pH 7.4 with NaOH. NMDA-sEPSCs were recorded at −60 mV in Mg^{2+}-free solution in the presence of 5 μM 2,3-Dihydro-6-nitro-7-sulfamoyl-benzo (F) quinoxaline (NBQX, Tocris) and 50 μM Bicuculline methiodide (BMI, Sigma). GABA-sIPSCs were recorded at −60 mV in the presence of Mg^{2+} 1 mM and of 5 μM NBQX. Intracellular solution contained (mM) KCl 140, $MgCl_2$ 3, EGTA 5, HEPES 5, ATP-Na 2. pH was adjusted to 7.3 with KOH. Glutamate was purchased from Sigma; Ro 15-1788 was purchased from Hoffman-La Roche (CH).

Apigenin was dissolved in dimethyl sulfoxide (DMSO) and diluted at the final concentration in extracellular medium (DMSO f.c. less than 0.1%; equivalent amount of DMSO was added to all control solutions). GABA, NMDA

and glycine were dissolved in the extracellular solution. All drugs were applied directly by gravity through a Y-tube perfusion system (Murase et al., 1989). Drug application had a fast onset and achieved a complete local perfusion of the recorded cell.

2.5. Electrophysiological recordings

Recordings were performed on single neuron after 7–9 days in culture at room temperature, under voltage clamp in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981). Electrodes were pulled from borosilicate glass (Hidelberg, FRG) on a vertical puller (PB-7, Narishige) and had a resistance of 5–7 Ω when filled with KCl internal solution. Currents were amplified with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz, digitised at 10 kHz.

2.6. Toxicity experiments

For neurotoxicity experiments, cerebellar granule cells and cortical neurons were plated in 24-well multiplates. At DIV8, wells were washed once with phosphate-buffered saline (PBS), then incubated for 1 h in sterile Locke's buffer (NaCl 154 mM, KCl 5.6 mM, $CaCl_2$ 2.3 mM, $NaHCO_3$ 3.6 mM, D-glucose 5.5 mM, HEPES 5 mM, pH 7.4) containing 100 μM glutamate and 10 μM glycine with or without apigenin. Control group was treated with buffer solution only. Twenty-four hours later, a solution containing 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT, 3 mg/ml) was added. MTT is converted to an insoluble purple formazan by mitochondrial dehydrogenases of living cells. Formazan was solubilized with isopropanol addition of HCl (333 ml of 37% HCl in 50 ml of isopropanol). Absorbance was measured with Labsystem Multiskan MCC/30 with dual wavelength lecture (570 and 630 nM). Data values are mean±S.E.M.

2.7. Data analysis

Offline data analysis, curve fitting, and figure preparation were performed with Clampfit 8 (Axon Instruments), Microsoft Office 2000, Origin 4.1 (Microcal, Northampton, MA) and Minianalysis (Synaptosoft, Decatur GA) software. After normalisation, a fitting of the dose–response relationship was performed using the logistic equation $\%I_{max}=100/I_{max}\{1+(IC_{50}/[AGO]^{nh})\}$, where I_{max} is the maximal current elicited by the agonist, IC_{50} is the agonist concentration eliciting the half-maximal response and n_h is the Hill's coefficient. Fitting of the decay phase of spontaneous currents was performed using a simplex algorithm for least-squares exponential fitting routines. Decay times of averaged currents derived from fitting to double exponential equations of the form $I(t)=I_f\exp(-t/\tau_f)+I_s\exp(-t/\tau_s)$, where I_f and I_s are the amplitudes of the fast and slow decay components, and τ_f and τ_s are their respective decay time constants used to fit the data. To compare decay time between different conditions we used a weighted mean decay time constant $\tau_w=[I_f/(I_f+I_s)]t_f+[I_s/(I_f+I_s)]$. Data values are mean±S.E.M.

3. Results

Using the patch-clamp technique in the whole cell configuration we tested the flavonoid apigenin on HEK293 cells stably transfected with recombinant $\alpha_1\beta_2\gamma_2$ GABA receptors. As shown in Fig. 2, apigenin reversibly reduced the currents evoked by 5 μM GABA in a dose-dependent way. At the concentration of 30 μM, apigenin reduced GABAergic current amplitude of 35±2% (n=33; Fig. 2). The maximal effect, elicited at 100 μM, was a reduction of 48±4%. From the dose–response curve of apigenin (Fig. 2B), we calculated a half maximal inhibiting concentration (IC_{50}) of 17 μM (p=0.9). The effect elicited by 30 μM apigenin was not blocked by the antagonist of the benzodiazepine recognition site Ro 15-1788 applied at 1 and 5 μM (Fig. 2C).

The effect of apigenin on native receptors expressed in cortical neurons was more pronounced when compared to that observed in recombinant receptors. At 30 μM, apigenin reduced the current evoked by 5 μM GABA of 52±5% (n=8) and the effect was not blocked by 5 μM Ro 15-1788 (−50±4%; n=8).

We tested the effect of apigenin in cultured cortical neurons at DIV 9-11 on sIPSCs that are mediated by synaptic $GABA_A$ receptors activation (GABA-sIPSCs; Fig. 3) since completely blocked by bicuculline. The flavonoid reduced the frequency of spontaneous events

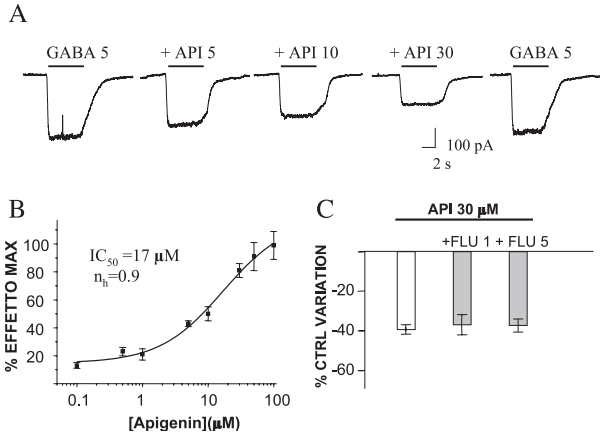

Fig. 2. Apigenin decreases GABA evoked currents on $\alpha_1\beta_2\gamma_2$ recombinant receptors. (A) Whole-cell recording of GABA-evoked currents (5 μM) in the presence of increasing concentrations of apigenin (API; in μM) on $\alpha_1\beta_2\gamma_2$ recombinant receptors stably expressed in HEK293 cells (holding potential V_h=−60 mV). (B) Apigenin dose response curve in percentage of average maximal effect (−48±4%). IC_{50}=17 μM, p=0.9. (D) Average percentage of variation of GABA-evoked currents (5 μM) in the presence of API (30 μM) alone or with flumazenil.

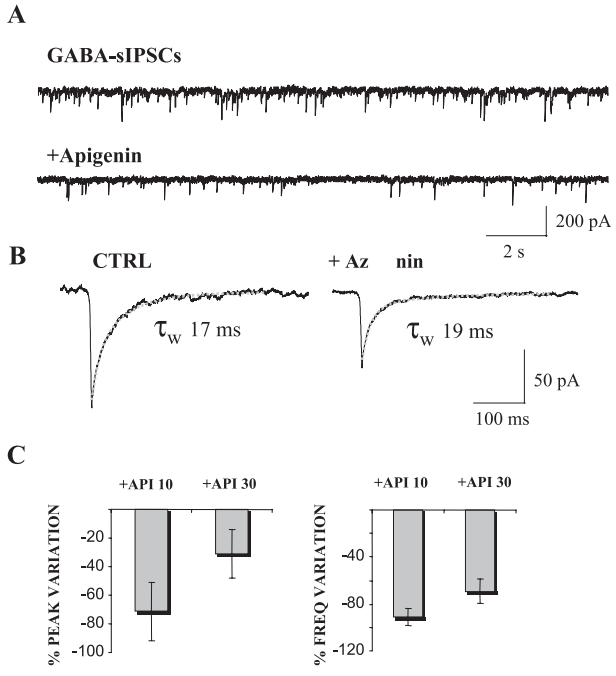

Fig. 3. Apigenin reduces spontaneous IPSCs frequency and amplitude. (A) Whole-cell recording of sIPSCs from a cortical neuron (DIV 10) in control conditions and with apigenin 30 μM (V_h=−60 mV). (B) Averaged sIPSCs with fitting of decay superimposed and indication of the resulting weighted time constant (τ_w). (C) Summary of percentage of peak and frequency variation from control with two different concentrations of apigenin.

from 1.1±0.5 Hz for control to 0.3±0.1 Hz (n=6) at 10 μM. sIPSCs peak amplitude was reduced from 133±16 pA for control to 95±12 pA with 10 μM apigenin (n=6). By the contrary, the sIPSCs decay kinetics measured with the

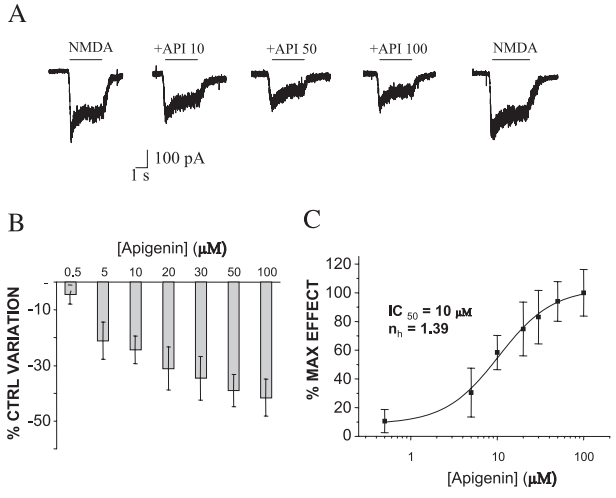

Fig. 4. Apigenin reduces NMDA evoked currents in cultured cortical neurons. (A) Whole-cell recordings of NMDA-evoked currents (100 μM and 10 μM Gly; V_h=−60 mV) in the presence of increasing concentrations of apigenin (API; in μM) on native receptors expressed on cultured cortical neurons (DIV8) B. Mean percentage of variation of NMDA (100 μM) and Gly (10 μM) evoked currents (plateau) in the presence of increasing concentrations of apigenin (in μM). (C) Apigenin dose–response curve in percentage of average maximal effect (−42±7%). IC_{50}=10 μM, p=1.39.

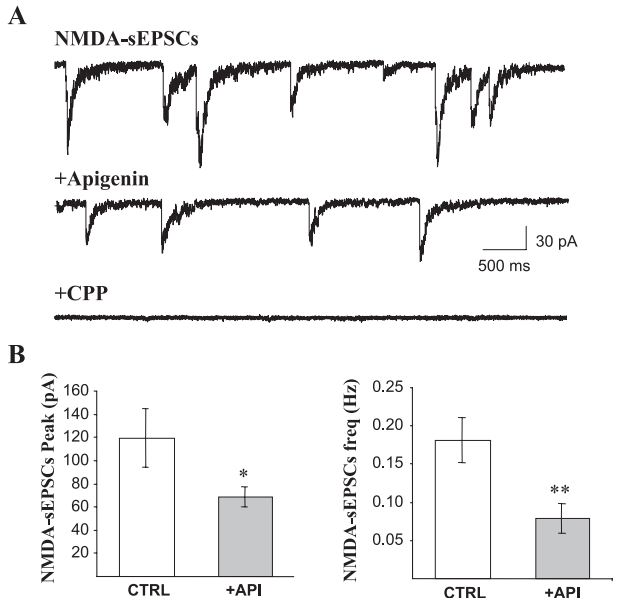

Fig. 5. Apigenin reduces frequency and amplitude of NMDA-mediated sEPSCs (NMDA-sEPSCs). (A) Whole-cell recordings of NMDA-sEPSCs from a cortical neuron (DIV 10; V_h=−60 mV) in control condition (with 20 μM BIC and 5 μM NBQX), with 20 μM API and with 10 μM CPP. (B) Mean NMDA-sEPSCs peak amplitude and frequency in the different conditions.

weighted decay time constant τ_w was not affected being 19±3 ms for control and 16±5 ms with 30 μM apigenin. The percentage of variation of frequency and peak amplitude is summarized in Fig. 3C.

It was reported that the flavonoid quercetin can modulate ionotropic receptors other than GABA (AMPA, nicotinic, serotonin) whereas no data are available on apigenin. We tested apigenin on currents mediated by the activation of AMPA and NMDA glutamate receptors in

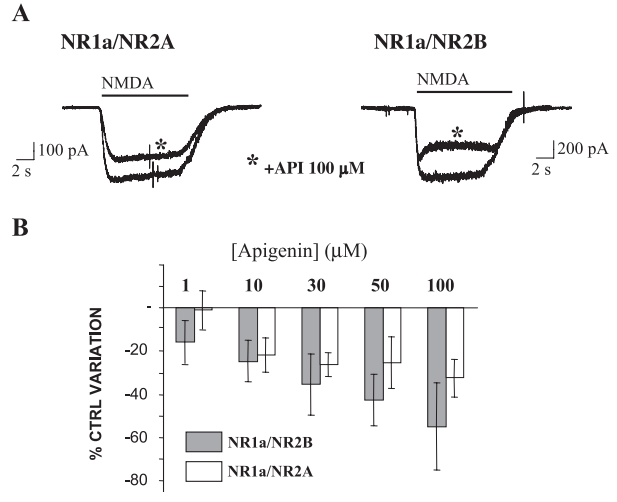

Fig. 6. Apigenin reduces NMDA currents mediated by recombinant NR1a/NR2A and NR1a/NR2B receptors. (A) Representative recordings of NMDA evoked currents in JM4C cells stably transfected with NR1a/NR2A and NR1a/NR2B recombinant receptors (V_h=−60 mV). (B) Histograms summarizing the average percentage variation from control of apigenin.

cultured cortical neurons. Only when applied at high concentrations, apigenin slightly reduced kainic-elicited currents (−13±5% at 100 μM). On the other hand, apigenin at micromolar concentrations reduced NMDA-evoked currents (Fig. 4) in a reversible way. The maximal reduction observed was −42% (100 μM; n=9) and the IC_{50} from the dose–response curve resulted 10 μM (Fig. 4C).

The modulation by apigenin on spontaneous excitatory postsynaptic currents (sEPSCs) exclusively mediated by synaptic NMDA receptors (NMDA-sEPSCs since completely blocked by CPP; Fig. 5) was tested by perfusing the cells with an extracellular solution devoid of Mg^{++} (to allow NMDA channel opening) and the addition of 5 μM NBQX and 20 μM bicuculline (to block AMPA and GABA channels). The presence of 20 μM apigenin significantly reduced frequency and peak amplitudes of NMDA-sEPSCs, as summarized in the histograms of Fig. 5B.

To study a possible NMDA subunit selectivity, we tested apigenin on JM4C cells expressing NR1a/NR2A and NR1a/NR2B recombinant receptors. As shown in Fig. 6, apigenin reduced NMDA-evoked currents mediated by both subunit combinations. At high doses, apigenin exerted a stronger effect on NR2B comprising receptors, although the difference was not statistically significant.

Finally, considering that NMDA receptors are largely involved in glutamate induced neurotoxicity apigenin was studied as a putative neuroprotective agent. In cerebellar granule cells in culture grown in 24-well multiplates glutamate was applied for 1 h alone or with different concentrations of apigenin. Cell viability was measured after 24 h using the MTT test. Apigenin, which was devoid of toxicity per se, reduced in a dose-dependent manner glutamate induced neuronal damage (Fig. 7). On the average, in three different experiments, apigenin reduced glutamate neurotoxicity of 33±9% at 30 μM and of 65±15% at 100 μM.

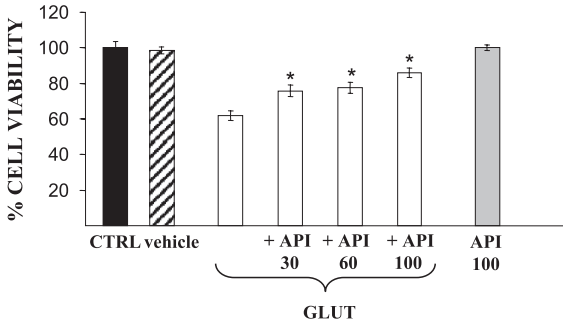

Fig. 7. Apigenin protects against glutamate induced neurotoxicity. Representative experiment showing the percentage of control cell viability measured with the MTT assay 24 h after glutamate treatment (see Materials and methods; vehicle=1% DMSO). Apigenin was tested alone (gray bar) or coapplied at different concentrations with glutamate and glycine. (*p<0.01 t-test vs. glutamate).

4. Discussion

The flavonoid apigenin, present in several plants used for their anxiolytic and antidepressant properties, decreases GABA-evoked currents mediated by $\alpha_1\beta_2\gamma_2$ recombinant receptors expressed in HEK293 cells and by native receptors in cortical cells. These data are in agreement with our previous results on cerebellar granule cells (Avallone et al., 2000) and with those of Goutman et al. (2003) on $\alpha_1\beta_1\gamma_{2s}$ receptors expressed in *Xenopus* oocytes.

Apigenin modulatory effect on $GABA_A$ receptors was not mediated by the benzodiazepine site since flumazenil did not block the flavonoid effect as shown also in in vivo studies where it failed to prevent apigenin sedative effect (Zanoli et al., 2000).

Previous studies on apigenin effect on GABA receptors focused on exogenous applications of GABA acting on both synaptic and extrasynaptic receptors. No results are available about the flavonoid effect neither on synaptic receptors function nor on spontaneous neurotransmitter release.

For this reason, we studied how apigenin could affect frequency, amplitude and decay of sIPSCs. Bath application of apigenin (10 and 30 μM) induced a strong reduction in frequency and peak amplitude of sIPSCs, while the decay kinetics was unaffected. Thus, the inhibitory effect of apigenin on $GABA_A$ receptors was detected also at the level of spontaneous synaptic currents. The strong frequency reduction suggests also a presynaptic effect of the compound or a reduction of the network excitability.

A decrease in the inhibitory neurotransmission, however, could not account for the sedative and antidepressant activity of apigenin reported in vivo. For this reason, we tested the flavonoid on glutamate ionotropic channels.

While the effect observed on AMPA receptors was very weak, apigenin decreased NMDA-evoked currents with a potency similar to that observed on GABA receptors.

Furthermore, apigenin could reduce also NMDA synaptic receptor function and glutamate spontaneous release. Our data on recombinant receptors failed to evidentiate a clear subunit selectivity of apigenin between NR2A and NR2B comprising receptors.

The reduction of NMDA receptor functionality that we report in this paper could account for the reduced sIPSCs frequency mediated by $GABA_A$ receptors through a reduction of the whole network excitability or through a direct effect on NMDA presynaptic receptors on GABAergic terminals (Glitsch and Marty, 1999). The antagonism on glutamatergic NMDA receptors could be predominant over the reduction of $GABA_A$ receptors and produce a reduction of the network excitability. Furthermore, the flavonoid could affect other ionic channels. Indeed, it was reported that some flavonoids can open calcium activated potassium channels (Koh et al., 1994), an effect that could reduce synaptic vesicle release. Goutman et al. (2003) observed that the flavonoid quercetin inhibited not only $GABA_A$ but also α_4-β_2 nicotinic, AMPA-kainate and 5-HT_3 serotonin receptors. These evi-

dences suggest that the effects of different flavonoids in the central nervous system derive from the modulation of multiple neurotransmitter systems.

In the toxicity experiments, apigenin exerted a neuroprotective effect against glutamate induced neurotoxicity. This effect could be explained with the antagonism at NMDA receptors, although we cannot rule out the involvement of other mechanisms. For example, it was demonstrated that apigenin reduced amyloid β-protein neurotoxicity by inactivating the caspase cascade and cytochrome c release, without a clear antioxidant effect. We believe, however, that the effect of apigenin in our experiments is mainly due to the NMDA inhibition since caspase blockade required a prolonged (40 h) exposure to the flavonoid.

Taken together, our data show that apigenin inhibits GABA receptor function also at the synaptic level. Moreover, our data reveal for the first time that apigenin reduces NMDA receptor function. The inhibitory effect on glutamatergic transmission could explain the sedative effects reported in vivo (Avallone et al., 2000) and the neuroprotective effect against glutamate induced damage. Since different NMDA receptor antagonists were effective in many animal models of depression (Skolnick, 1999) this could also account for apigenin antidepressant activity (Nakazawa et al., 2003).

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References

Avallone, R., Zanoli, P., Puia, G., Kleinschnitz, M., Schreier, P., Baraldi, M., 2000. Pharmacological profile of apigenin, a flavonoid isolated from *Matricaria chamomilla*. Biochem. Pharmacol. 59 (11), 1387–1394.

Gallo, V., Kingsbury, A., Balazs, R., Jorgensen, O.S., 1987. The role of depolarization in the survival and differentiation of cerebellar granule cells in culture. J. Neurosci. 7, 2203–2213.

Glitsch, M., Marty, A., 1999. Presynaptic effects of NMDA in cerebellar Purkinje cells and interneurons. J. Neurosci. 19, 511–519.

Goutman, J.D., Waxemberg, M.D., Donate-Oliver, F., Pomata, P.E., Calvo, D.J., 2003. Flavonoid modulation of ionic currents mediated by GABA(A) and GABA(C) receptors. Eur. J. Pharmacol. 461, 79–87.

Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J., 1981. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. Pflugers Arch. 391, 85–100.

Harborne, J.B., Williams, C.A., 2000. Advances in flavonoid research since 1992. Phytochemistry 55, 481–504.

Koh, D.S., Reid, G., Vogel, W., 1994. Activating effect of the flavonoid phloretin on Ca++ activated K+ channels in myelinated nerve fibers of *Xenopus laevis*. Neurosci. Lett. 165, 167–170.

Luo, J.H., Fu, Z., Losi, G., Kim, B.G., Prybylowski, K., Vissel, B., Vicini, S., 2002. Functional expression of distinct NMDA channel subunits tagged with green fluorescent protein in hippocampal neurons in culture. Neuropharmacology 42, 306–318.

Medina, J.H., Pena, C., Levi de Stein, M., Wolfman, C., Paladini, A.C., 1989. Benzodiazepine-like molecules, as well as other ligands for the brain benzodiazepine receptors, are relatively common constituents of plants. Biochem. Biophys. Res. Commun. 165, 547–553.

Medina, J.H., Paladini, A.C., Wolfman, C., Levi de Stein, M., Calvo, D., Diaz, L.E., Pena, C., 1990. Chrysin (5,7-di-OH-flavone), a naturally-occurring ligand for benzodiazepine receptors, with anticonvulsant properties. Biochem. Pharmacol. 40, 2227–2231.

Middleton Jr., E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52, 673–751.

Murase, K., Ryu, P.D., Randic, M., 1989. Excitatory and inhibitory amino acids and peptide-induced responses in acutely isolated rat spinal dorsal horn neurons. Neurosci. Lett. 103, 56–63.

Nakazawa, T., Yasuda, T., Ueda, J., Ohsawa, K., 2003. Antidepressant-like effects of apigenin and 2,4,5-trimethoxycinnamic acid from *Perilla frutescens* in the forced swimming test. Biol. Pharm. Bull. 26, 474–480.

Salgueiro, J.B., Ardenghi, P., Dias, M., Ferreira, M.B., Izquierdo, I., Medina, J.H., 1997. Anxiolytic natural and synthetic flavonoid ligands of the central benzodiazepine receptor have no effect on memory tasks in rats. Pharmacol. Biochem. Behav. 58, 887–891.

Skolnick, P., 1999. Antidepressants for the new millennium. Eur. J. Pharmacol. 375, 31–40.

Zanoli, P., Avallone, R., Baraldi, M., 2000. Behavioral characterisation of the flavonoids apigenin and chrysin. Fitoterapia 71, S117–S123.