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CHRYSIN (5,7-DI-OH-FLAVONE), A NATURALLY-OCCURRING LIGAND FOR BENZODIAZEPINE RECEPTORS, WITH ANTICONVULSANT PROPERTIES

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Abstract—Chrysin (5,7-di-OH-flavone) was identified in *Passiflora coerulea* L., a plant used as a sedative in folkloric medicine. Chrysin was found to be a ligand for the benzodiazepine receptors, both central ($K_i = 3\,\mu M$, competitive mechanism) and peripheral ($K_i = 13\,\mu M$, mixed-type mechanism). Administered to mice by the intracerebroventricular route, chrysin was able to prevent the expression of tonic–clonic seizures induced by pentylenetetrazol. Ro 15-1788, a central benzodiazepine receptor antagonist, abolished this effect. In addition, all of the treated mice lose the normal righting reflex which suggests a myorelaxant action of the flavonoid. The presence in *P. coerulea* of benzodiazepine-like compounds was also confirmed.

A variety of chemical compounds, not structurally related to the benzodiazepines (BZD), bind to BZD receptors present in the nervous tissue (see Ref. 1 for review). The biflavonoid amentoflavone was an unexpected addition to this list [2], although its pharmacological properties were not explored.

We recently screened eleven non-flowering plants known to contain biflavonoids and three flowering plants, used as sedatives in folkloric medicine, looking for BZD receptor ligands [3], and demonstrated the relatively common occurrence of BZD-like molecules as well as other ligands of the mentioned receptors.

In the present work we have examined in more detail the composition of *Passiflora coerulea* L. [4] and have been able to isolate and identify 5,7-di-OH-flavone (chrysin) from its extracts. This flavonoid, assayed *in vitro*, had moderately high affinity for the central BZD receptors and medium to low affinity for the peripheral BZD receptors, and displayed anticonvulsant properties *in vivo*.

MATERIALS AND METHODS

Isolation of chrysin

Dried branchlets from *P. coerulea* L. were obtained in local herboristeries, and their identification was made at the Museum of Botany, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, by its Director, Dr. J. L. Amorin.

The plant material was crushed in a mechanical mill, and 120 g of the resulting powder was extracted continuously for 18 hr in a Soxhlet apparatus with 1.5 L of dichloromethane. The extract was concentrated to approximately 200 mL by distillation of the solvent and then brought to dryness in a rotary evaporator. The residue was extracted with 95% ethanol and the insoluble material discarded. The ethanolic extract was evaporated to dryness in the rotary evaporator and the residue was submitted to countercurrent distribution (CCD) in the system benzene:chloroform:methanol:water (2:1:2.3:0.7; by vol.) in a manual machine with a capacity of 10 mL in each phase. After 120 transfers the presence of ligands for the BZD receptors was investigated in the contents of several tubes along the distribution train (see Figure 1). Selected pools of active fractions were submitted to reversed-phase HPLC fractionation. Pool a, from the CCD (Fig. 1), gave rise to the chromatograms shown in Fig. 2. The major peak in E was recovered and used for identification.

Binding assays

Central BZD receptors. To determine the effects of chrysin, and other flavonoids, on the binding of tritium-labeled flunitrazepam ([^{3}H]FNZ) to the central type of BZD receptors, extensively washed crude synaptosomal membranes from bovine cerebral cortex were used. The binding of [^{3}H]FNZ (*methyl*-^{3}H; 81.8 Ci/mmol; NEN) was carried out as previously described [5, 6]. In brief, for each assay triplicate samples containing 0.2 to 0.3 mg protein were suspended in 1 mL of 25 mM Tris–HCl buffer, pH 7.3. The incubation was carried out at 4° for 60 min with 0.6 nM [^{3}H]FNZ. To study the binding saturation, a range of 0.3 to 10 nM was used. Non-specific binding was measured by performing the incubation in the presence of 3 μM FNZ or 3 μM clonazepam and represented less than 10% of the total. The assays were terminated by filtration through Whatman GF/B glass-fiber filters and three washes with 3 mL each of incubation medium. Filters were dried and counted after the addition of 5 mL

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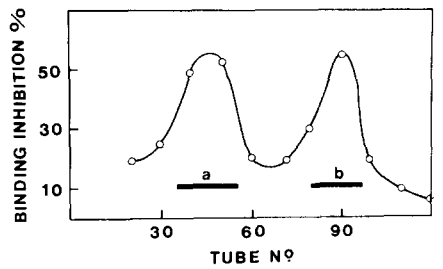

Fig. 1. Countercurrent distribution pattern obtained with the extract from 120 g of *Passiflora coerulea* submitted to 120 transfers in the system benzene:chloroform:methanol:water (2:1:2.3:0.7; by vol.). The bars indicate active components in the binding assays and the size of the pools made.

of 2,5-diphenyloxazole (PPO)-xilene as scintillation fluid.

Chrysin or the dry residues of the chromatographic fractions to be assayed were dissolved in 100 μL of ethanol and added in variable amounts to the incubation mixtures.

Peripheral BZD receptors. To examine the *in vitro* effects of chrysin, and other flavonoids, on the peripheral type of BZD receptors, [^{3}H]Ro 5-4864 binding to a crude mitochondrial fraction from rat kidneys was performed as described earlier [7]. Briefly, each assay consisted of triplicate samples containing 0.1 mg protein in 1 mL of 25 mM Tris–HCl buffer, pH 7.3, incubated for 120 min, at 4°, using a range of [^{3}H]Ro 5-4864 (76.5 Ci/mmol; NEN) between 0.2 to 13 nM. Non-specific binding was determined in the presence of 1 μM Ro 5-4864 and represented about 25% of the total binding. The assays were terminated by filtration through Whatman GF/B glass-fiber filters and three washes with 5 mL each of incubation medium. Filters were dried and counted in 8 mL of PPO-xilene.

High performance liquid chromatography (HPLC)

The instrument used consisted of two programmable pumps connected to a Rheodyne 7125 injector, a variable wavelength spectrophotometer and a solvent programmer, all from Laboratory Data Control (Riviera Beach, FL, U.S.A.). The column used was a preparative C18, 2.2 × 25 cm, 5 μm particle size, from The Separation Group (Hesperia, CA, U.S.A.).

Reference flavonoids

Pure samples of fisetin, morin, quercetin, rutin, luteolin and chalcone were donated by Drs. M. Flawiá, Instituto de Investigaciones en Ingeníeria Genética y Biología Molecular (UBA-CONICET), Buenos Aires, and G. Ferraro and V. Martino, Instituto de Química y Metabolismo del Fármaco (UBA-CONICET), Facultad de Farmacia y Bioquímica, Buenos Aires. Chrysin was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A., and its purity checked by HPLC, under conditions similar to those described for Fig. 2E.

Mass spectrometry

The spectra for the single peak in chromatogram

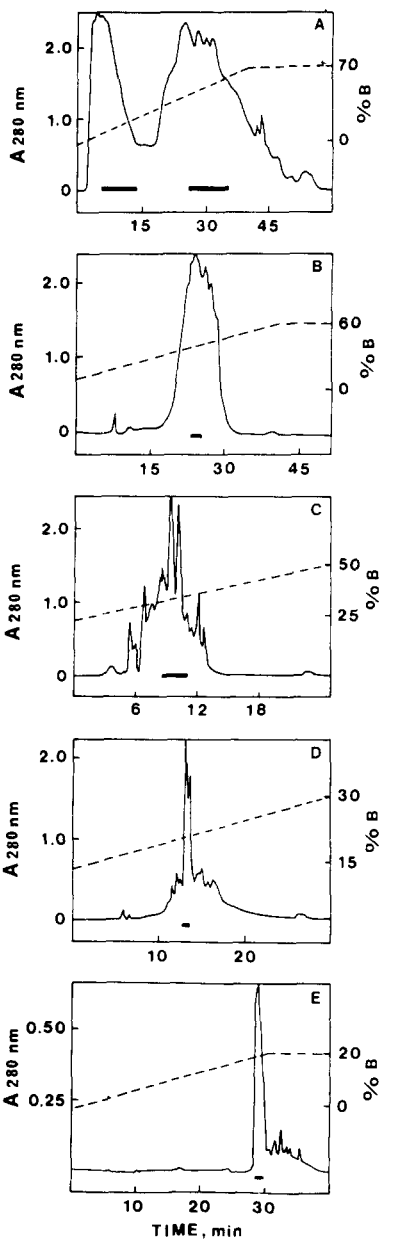

Fig. 2. Successive HPLC steps used to purify pool a (Fig. 1). All the chromatograms were obtained using a preparative C18 reversed phase column eluted with a *n*-propanol gradient in 50% methanol. Solvent B was *n*-propanol. The bars indicate pools of active components. In A the first pool (eluting between 5 and 15 min) contained BZD-like components immunologically detected; the second pool (eluting between 25 and 35 min) was submitted to further HPLC steps as shown in B, C, D and E with the gradients indicated. Each step fractionated the active pool from the previous step. The material in the main peak in E was used for mass spectrometric analysis (Fig. 3).

E of Fig. 2 was obtained with a Shimadzu QP-1000 quadrupole mass spectrometer operated in the EI mode. The ionizing voltage was 70 eV and the ionizing current 60 μA. The samples were introduced with a temperature-programmed direct insertion

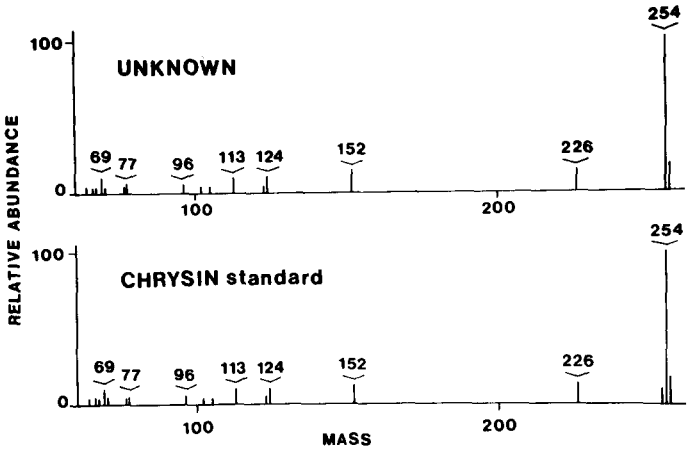

Fig. 3. Mass spectrometric identification of chrysin. Unknown: main peak in HPLC E (Fig. 2).

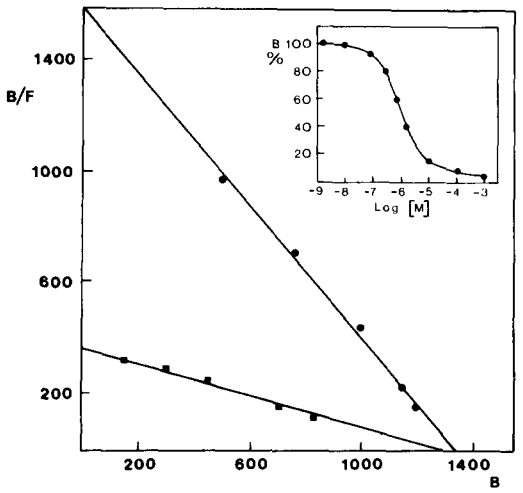

Fig. 4. Scatchard plot of [^{3}H]FNZ (81.8 Ci/mmol) binding (0.3 to 10 nM) to extensively washed crude synaptosomal membranes from bovine cerebral cortex in the absence (●) and in the presence (■) of 10 μM chrysin. B: bound [^{3}H]FNZ; F: free [^{3}H]FNZ. Both values are expressed in fmol/mg protein. Inset: representative displacement curve of [^{3}H]FNZ binding by chrysin.

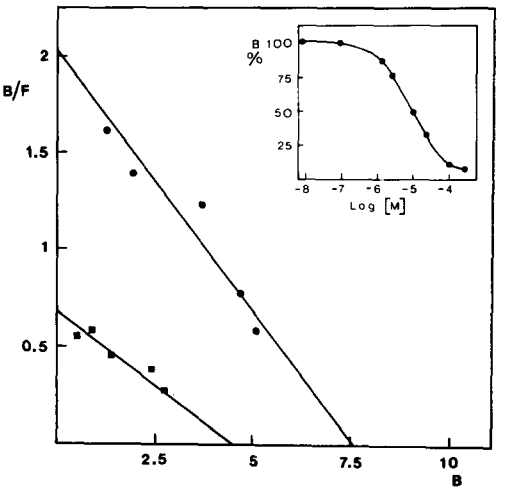

Fig. 5. Scatchard plot of [^{3}H]Ro 5-4864 (76.5 Ci/mmol) binding (0.2 to 13 nM) to a crude mitochondrial fraction from rat kidneys in the absence (●) and in the presence (■) of 10 μM chrysin. B: bound [^{3}H]Ro 5-4864; F: free [^{3}H]Ro 5-4864. Both values are expressed in fmol/mg protein. Inset: representative displacement curve of [^{3}H]Ro 5-4864 binding by chrysin.

probe. The scan rate was 1 sec/decade, and the mass range scanned was 50–500 atomic mass units. The data were analyzed and compared with a standard sample of chrysin.

Seizure testing

Swiss strain adult mice weighing between 22 and 28 g were used. Pentylenetetrazol (PTZ) was employed as a convulsant agent at 50 mg/kg, intraperitoneally. Chrysin was dissolved in dimethyl sulfoxide and injected 15 min prior to PTZ either intraperitoneally (i.p.) or by the intracerebroventricular (i.c.v.) route. Ro 15-1788, a central type BZD receptor antagonist, was dissolved in dimethylsulfoxide and injected i.p. 20 min prior to PTZ. Control mice received the solvent. All the mice were put back in the home cage right after the PTZ injection. Seizures were scored according to Zhang *et al.* [8], as follows: 0, no seizures; 1, jerks; 2, clonus no tonic extension; and 3, tonic–clonic seizure. The greatest seizures scores attained were tabulated and analyzed with the Kurskall Wallis test [9]. The latency, i.e. the time spent between the injection and the onset of the first jerk, first clonus or tonus, was recorded in seconds.

RESULTS

Isolation and identification of chrysin

The CCD of the extract of *P. coerulea* achieved a substantial purification of the materials active in the binding assay to the central BZD receptors: a major proportion of the inactive substances had very low or very high partition coefficients and could be discarded. Two groups of active substances were

Table 1. Effects of chrysin and other flavonoids (10^{-6} M) on (a) the binding of [^{3}H]FNZ at a 6×10^{-10} M concentration to extensively washed crude synaptosomal membranes from bovine cerebral cortex and (b) on [^{3}H]Ro 5-4864 binding at a 10^{-9} M concentration to crude mitochondrial fractions from rat kidney

	[^{3}H]FNZ	[^{3}H]Ro 5-4864
	(% of control)	
Chrysin	65 ± 4	80 ± 5
Fisetin	95 ± 3	87 ± 4
Chalcone	102 ± 3	71 ± 2
Quercetin	101 ± 5	93 ± 4
Rutine	97 ± 4	99 ± 6
Morin	96 ± 7	95 ± 5
Luteolin	92 ± 5	ND*

Values are the means ± SE of four independent experiments done in triplicate. Control value for [^{3}H]FNZ binding: 568 ± 25 fmol/mg protein. Control value for [^{3}H]Ro 5-4864 binding: 1.6 ± 0.2 fmol/mg protein.
* ND: not determined.

detected and pooled as indicated in Fig. 1. Pool b was fractionated further but its active components, after various steps of HPLC, could not be recovered or identified, probably because of their presence in very low amounts. Pool a was successfully purified by HPLC, as shown in Fig. 2. In chromatogram A, two groups of active substances were detected. The first one reacted with a monoclonal antibody [3], indicating the existence of BZD-like components but further efforts to identify them were not successful. The second pool in Fig. 2A was processed as shown by chromatograms B, C, D and E. The single peak in E was identified by mass spectrometry as chrysin (5,7-di-OH-flavone), as shown by the intensity pattern of the characteristic molecular ions obtained (Fig. 3). Furthermore, the retention time of the active peak in E was identical to that of authentic chrysin chromatographed under the same conditions (not shown).

Radioligand binding assays

Chrysin inhibited [^{3}H]FNZ binding to extensively washed bovine cerebral cortical membranes with a K_i of 3 μM and a Hill number of 0.91 ± 0.08 (N = 4). Scatchard analysis of saturation curves revealed a competitive interaction showing a decline in the apparent affinity without changes in the maximal number of sites (B_{max}) (Fig. 4). On the other hand, chrysin displaced [^{3}H]Ro 5-4864 binding to kidney membranes with a K_i of 13 μM and a Hill number of 0.82 ± 0.10 (N = 4) in a mixed-type competitive–non-competitive manner (Fig. 5). At a concentration of 10 μM, chrysin produced no inhibition on the binding of [^{3}H]prazosin, [^{3}H]dihydroalprenolol, [^{3}H]quinuclidinyl benzilate or [^{3}H]muscimol, respectively, to, α_1- and β-adrenoceptors, muscarinic and γ-aminobutyric acid$_A$ (GABA$_A$) receptors (N = 3, data not shown).

The effects of chrysin, and other arbitrarily selected flavonoids, at a 1×10^{-6} M concentration, on the binding of [^{3}H]FNZ and [^{3}H]Ro 5-4864 to their respective receptors, are shown in Table 1. Only chrysin displaced [^{3}H]FNZ binding. Among the other flavonoids assayed, only chalcone showed a moderate affinity for the peripheral BZD receptors with a K_i of 4 μM (N = 3).

Table 2. Effects of i.c.v. administration of chrysin (40 μg) and chrysin plus Ro 15-1788 (3 mg/kg, i.p.) on PTZ-induced seizures in mice

	N	No signs	Jerks	Forelimb clonus	Tonic–clonic
Saline	15	0	0	6	9
Chrysin	17	7	6	3	1*
Chrysin + Ro 15-1788	7	0	0	1	6

* $P < 0.02$ vs saline scores.

Pharmacological effects of chrysin

Since the i.p. injection of chrysin in mice (3–30 mg/kg) did not produce consistent results in the PTZ siezure test, we tried i.c.v. micro-injection of chrysin to explore its consequences on this test. As shown in Table 2, chrysin clearly reduced the PTZ scores. At a dose of 40 μg, chrysin showed a significant ($P < 0.02$) anticonvulsant effect mainly due to a reduction in the number of mice having tonic–clonic seizures with no clear effect on the onset-times of jerking or clonus ($P > 0.2$). This effect was abolished by the prior administration of Ro 15-1788 (3 mg/kg, i.p.) (Table 2). It is important to stress that control mice (receiving i.c.v. micro-injection of solvent) had a normal righting reflex, whereas all the chrysin-treated mice had lost it. This suggests that chrysin possesses a myorelaxant action.

DISCUSSION

Several active compounds were detected and remain to be identified in the extracts of the sedative plant *P. coerulea* L. [3], some of them of presumably BZD-like structure. Nevertheless, the monoflavonoid chrysin could be isolated, unequivocally identified, and shown to be a ligand for the BZD receptors, both central and peripheral. The central receptors bound chrysin with moderate affinity (K_i = 3 μM), and the flavonoid was a true competitor of the BZDs. The peripheral receptors showed less affinity for chrysin, and the displacement of the binding occurred by a mixed-type mechanism.

The results shown in Table 1 represent an attempt to extend these observations using a limited set of available flavonoids. It is interesting to note that chalcone, a precursor of the monoflavonoids, was as powerful a ligand towards the peripheral BZD receptors as chrysin; in fact, the affinity of chalcone was three times higher.

The pharmacological properties of chrysin could not be studied using i.p. injections since the effects were not reproducible. One possibility is that the flavonoid was rapidly destroyed after its injection, another, that it did not cross the blood–brain barrier. Alternatively, it has been demonstrated that several anticonvulsant drugs which act via potentiation of

GABAergic transmission have limited anticonvulsant effects in the PTZ test [10]. Further experiments, examining the amount of chrysin recovered in brain after its peripheral administration, are needed to clarify this point. However, using the i.c.v. route, chrysin was able to prevent the expression of tonic–clonic convulsions induced by PTZ. The blocking effect of Ro 15-1788 on the chrysin induced tonic–clonic convulsions further supports the hypothesis that chrysin acts through central BZD receptors.

The presence of BZD-like compounds in *P. coerulea* confirms our previous results [3]. Although up to now we have been unable to isolate and identify any of these natural molecules, it is important to make further efforts to establish their chemical structure and origin. Until a few years ago BZDs were considered artificial synthetic organic molecules, but evidence is accumulating that points to their widespread occurrence in nature, both in animal and vegetable sources (for a review see Ref. 11 and also Refs. 3 and 12).

Speroni and Minghetti [13] have studied the neuropharmacology of extracts of *P. incarnata* L., a closely related species to *P. coerulea* L., which is endowed with the same sedative properties. Their experiments made by administering the extracts to rats, either orally or i.p., indicated a raise in the nociceptive threshold, an increase in the sleeping time, a protection against the convulsive effects of PTZ, and a decrease in locomotor activity. No active compound could be identified by the authors, but according to our results with *P. coerulea*, it is quite probable that their extracts may contain, besides chrysin, one or more BZD-like compounds [3] which could be responsible for the effects recorded.

Amentoflavone was the first flavonoid found to be a ligand for the central BZD receptors [2]. The data in the present work incorporate chrysin as the second one.

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