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Evidence That Total Extract of *Hypericum perforatum* Affects Exploratory Behavior and Exerts Anxiolytic Effects in Rats

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VANDENBOGAERDE, A., P. ZANOLI, G. PUIA, C. TRUZZI, A. KAMUHABWA, P. DE WITTE, W. MERLEVEDE AND M. BARALDI. *Evidence that total extract of* Hypericum perforatum *affects exploratory behavior and exerts anxiolytic effects in rats.* PHARMACOL BIOCHEM BEHAV **65**(4) 627–633, 2000.—Clinical trials have extensively reported the ability of *Hypericum perforatum* extracts to exert a significant antidepressant activity. Hypericin, the main constituent of *H. perforatum* extract, is no more regarded as the active principle of the antidepressant activity of the drug. Hence, the question of which constituents are involved in the basic activity of the total extract, is still waiting for an answer. In the present study we focused our attention on the potential anxiolytic activity of *H. perforatum* total extract, and of some pure components such as protohypericin and a fraction containing hypericin and pseudohypericin. Herein we report that the total extract of *H. perforatum* increases the locomotor activity in the open field and exerts anxiolytic activity in the light–dark test, whereas the single components did not show any effect. Interestingly, the anxiolytic activity of the total extract was blocked by pretreatment of rats with the benzodiazepine antagonist Flumazenil, hence suggesting an implication of benzodiazepine receptor activation in the anxiolytic effect of *H. perforatum* extract. Electrophysiological studies, performed to gain more information on the mechanism of action, showed that hypericin reduced the GABA-activated chloride currents, while pseudohypericin did an opposite effect. Furthermore, both hypericin and pseudohypericin inhibited the activation of NMDA receptors. © 2000 Elsevier Science Inc.

Anxiolytic effect Locomotor behavior Hypericin *Hypericum* Protohypericin Pseudohypericin GABA–benzodiazepine receptor Glutamate receptor

IN the 16th century, Paracelsus recommended *Hypericum* plant extracts as medicine against general depression and related conditions (13). Meanwhile, many clinical studies have been conducted using standardized *Hypericum* extracts, with a dose of total hypericin ranging from 0.5 to 2.7 mg, which confirmed such activity. Indeed, a meta-analysis of these studies revealed that *Hypericum* extract was significantly superior to placebo, and similarly effective as standard antidepressants (18). Of importance, undesirable side effects were noted in only a small number of patients. Nowadays, it is therefore generally recognized that *Hypericum* extract is a safe and effective drug in the treatment of mild to moderately severe depressive disorders (25).

Numerous in vitro experiments and in vivo animal studies have been conducted to reveal the mechanism of action responsible for the antidepressant activity of *Hypericum* extract. First, it was suggested that *Hypericum* extracts inhibit monoamino oxidase (MAO) because of its hypericin content (27). However, later studies showed that MAO was more effectively inhibited by flavonoid aglycones present in the plant

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(2,26,29). These results are in agreement with a computer-supported model analysis comparing the molecular structures of extract components with known MAO inhibitors (15). Controversially, an ex vivo study showed no MAO inhibition in rats administered with *Hypericum* extract (2). Another possible mechanism associated with the antidepressant activity of *Hypericum* extracts includes inhibition of catechol-O-methyltransferase (COMT) by flavonoid aglycones (29). On the other hand, it was argued that the concentration of these substances is too low to be responsible for the therapeutic effect of *Hypericum* extract (29).

The effect of *Hypericum* extract and some constituents on several neurotransmitters and their receptors of the central nervous system were evaluated in vitro. It was shown that neither the extract nor hypericin or kaemferol, had any relevant effect on the tested receptors (19). However, *Hypericum* extract significantly inhibited the presynaptic reuptake of serotonine (19), and demonstrated a postsynaptic inhibition of the serotonine uptake (19,20,24). Furthermore, studies on animals that were treated with *Hypericum* extract demonstrated adaptive changes, namely a downregulation of β-receptors, a significant upregulation of postsynaptic 5-HT_2-receptors, and an increased density of 5-HT_{1A} and 5-HT_{2A} receptors (28). Recently, *Hypericum* extract has been shown to have in vitro a high affinity for $GABA_A$ and $GABA_B$ receptors (8). Moreover, the affinity of several *Hypericum* constituents (1) for the benzodiazepine binding sites that are part of the $GABA_A$ receptor was investigated in vitro. Amentoflavone showed a high affinity for this receptor binding site, while hypericin, quercitrin, luteolin, rutin, hyperoside, and I3,II8-biapigenin demonstrated a low affinity (>1 μM).

Furthermore, several animal studies have been conducted in view of identifying the active component(s). Both *Hypericum* extract and pure hypericin increased the activity of mice in a water wheel (23). It was demonstrated that the total *Hypericum* extract as well as the two fractions rich in flavonoids and hypericins induced a significant decrease of the immobility time in the Porsolt forced swimming test (4). The data obtained by Butterweck (5), administering hypericin and pseudohypericin in combination with a fraction containing procyanidins, confirm that naphthodianthrones are antidepressant constituents of *Hypericum* and suggest that the dopaminergic system is involved in their action . Recently, it has been hypothesized that hyperforin, the major lipophilic constituent of *Hypericum*, could be the active principle responsible for the antidepressant activity of the extract (7).

In the present study, the effect of *Hypericum* extracts and some pure constituents on the behavior of rats was investigated to provide better insights on the pharmacological activity of the plant extract. First, the total *Hypericum* extract, the pure naphthodianthrone derivatives (hypericin/pseudohypericin and protohypericin) were used in the open-field test to evaluate the exploratory behavior of rats.

Because the request of compounds able to treat depressive disorders combined with anxiety has increased remarkably over the past few years (17), the same substances were tested in the light–dark model of anxiety in rats. Furthermore, the specificity of the anxiolytic effect of the total extract on the GABA–benzodiazepine receptor system was challenged by using the benzodiazepine antagonist Flumazenil.

The demonstration that the total extract indeed exerts an anxiolytic effect in rats prompted us to explore further the potential mechanism of action studying its effect on the GABA–benzodiazepine receptor system and on the glutamatergic system. Thus, we have used the patch-clamp technique in the whole-cell configuration to test the modulation of GABA and NMDA currents by crude extract and by hypericin and pseudohypericin.

METHODS

Preparation of Total Extract of Hypericum perforatum

A dry extract of *Hypericum perforatum* was purchased from Indena (Milano, Italy). The amount of hypericin and pseudohypericin present in the extract was quantified using HPLC and fluorescence detection, as described (30), and was determined to be 0.11 and 0.43%, respectively, corresponding to 0.54% total hypericins (hypericin/pseudohypericin 1/4). Besides, the percentage of protoforms (protohypericin and protopseudohypericin) present in the plant extract was determined by calculating the peak difference before and after light irradiation of the extract. In the total extract, 17.4% of the total hypericins (hypericin and pseudohypericin) was present as their protoforms, which is in agreement with previously reported results (10). This means that the total extract contains 0.09% of protoforms (protohypericin and protopseudohypericin).

Preparation of Hypericin, Protohypericin, and Pseudohypericin

Hypericin and protohypericin were prepared as described previously (11). Briefly, emodin (2.5 mg), isolated from *cortex Frangulae*, was dissolved in 125 ml acetic acid, and 30 g $SnCl_2 \cdot 2H_2O$, dissolved in 75 ml HCl_{conc}, was added. After refluxing for 3 h at 120°C and cooling to room temperature, the formed emodin anthrone was filtered off and dried. Protohypericin was synthesized via oxidative dimerization by heating emodin anthrone (2 g) in a mixture of 45 ml pyridine/piperidine (10/1) in the presence of 4 g pyridine-1-oxide and 100 mg $FeSO_4$ at 100°C under nitrogen for 1 h in strict dark conditions. After synthesis, the crude protohypericin was purified by silica column chromatography (elution: ethylacetate/water 100/2.5, followed by elution of the compound with ethylacetate/acetone/water 80/20/2.5) under dark conditions to prevent photoconversion into hypericin. In a second purification step, protohypericin was chromatographed on a Sephadex LH20 column (dichloromethane/methanol/acetone 55/30/15) under the same conditions. In the case of the hypericin synthesis, protohypericin was light irradiated before the second purification step to convert protohypericin into hypericin by a photocyclization reaction (3). Hypericin was then further purified by Sephadex LH20 column chromatography. The synthesis yield for protohypericin was 20% (purity >98%, content of hypericin: 1.01%, as determined by HPLC), and in the case of hypericin 28% (purity >99%).

Pseudohypericin was prepared starting from ground herb (500 g) of *Hypericum perforatum* (Denolin, Brussels, Belgium). First, the herb was percolated with dichloromethane, ethylacetate, and finally acetone. After evaporation of the acetone extract (1200 ml) under reduced pressure, the residue (7.8 g) was dissolved in ethylacetate and purified with silica column chromatography [silica gel 60 (63–200 μm), Merck, Darmstadt, Germany] using ethylacetate, ethylacetate/water (100/2.5) and finally ethylacetate/water/acetone (100/2.5/30) as eluent. Fractions of the latter, containing high contents of pseudohypericin and hypericin as demonstrated by TLC [Alugram Sil G/UV_{254} plates (Macherey-Nagel, Düren, Germany), solvent: toluene/ethylacetate/formic acid (50/40/10)], were pooled and evaporated under reduced pressure. The

residue (534 mg) was dissolved in methanol/acetone/dichloromethane (30/15/55) and further purified with column chromatography on Sephadex LH20 (Pharmacia, Uppsala, Sweden) using methanol/acetone/dichloromethane (30/15/55) as eluent. Fractions containing pseudohypericin were pooled and dried under reduced pressure (yield: 0.03%). The compound was purified a second time with column chromatography on Sephadex LH20 under the same conditions to obtain pure pseudohypericin (purity: >99%, content of hypericin: 0.44%, as analyzed by HPLC).

All compounds were characterized with ^{1}H-NMR (Gemini 200 MHz, Varian), LSI mass spectrometry (Kratos Concept IH) and UV/VIS spectrophotometry (Hewlett-Packard, CA). The data were comparable with literature data (6,10,11).

Animals

Male Sprague–Dawley rats (Harlan–Nossan, Udine, Italy) weighing 200–220 g, were housed with free access to food and water, and manteined on a 12 L:12 D cycle, at a constant temperature of 24°C.

The experimental procedures are in compliance with the National Institutes of Health Guide for care and use of laboratory animals and with the European Communities Council Directive of 24 november 1986 (86/609/EEC).

Administration of Test Substances

Food, but not water, was withdrawn 1.5 h prior to drug administration. Test substances were freshly prepared in propyleneglycol/water (50/50) and administered orally by gavage in a final volume of 10 ml/kg body weight. The vehicle did not induce any change vs nontreated rats in both animal tests. The doses of total extract administered were 926, 1852, and 2778 mg/kg, corresponding to 5, 10, and 15 mg/kg total hypericins (hypericin/pseudohypericin 1/4) respectively. Because 17.4% of the total hypericins in the total extract was present as protoforms, a dose of 2 mg/kg protohypericin was chosen, which approximately corresponds to 10–15 mg/kg total hypericins. Tests were performed in rats, 1 h after oral administration of tested compounds (between 0900 and and 1300 h).

Locomotor Behavior

Locomotor activity was studied in rats by open-field test. Each animal was placed in the centre of a square arena (100 × 100 × 50 cm h) with a black floor. Rats were continuously filmed with a telecamera connected to a computerized system (Motion Analyzer BM800, Biomedica Mangoni, Pisa, Italy). The following parameters were recorded during the 10-min test: 1) the total pathway length during ambulatory activity, 2) number of crossings of 20 × 20 cm. squares, and 3) number of rearings. Furthermore, the system provides the route pattern for each rat.

Light–Dark Model of Anxiety

The light–dark model was used according to Crawley and Goodwin (9). A two-compartment chamber (40 × 60 × 20 cm h) was used in which a brightly illuminated area (40 × 40 cm) and a dark section (40 × 20 cm) are separated by a wall with a round hole (diameter 13 cm). At the start of the test the rats were placed in the illuminated part of the cage. The following parameters were recorded during 5 min: 1) latency time for the first crossing to the dark compartment, 2) the number of crossings between the light and the dark area, and 3) the total time spent in the illuminated part of the cage.

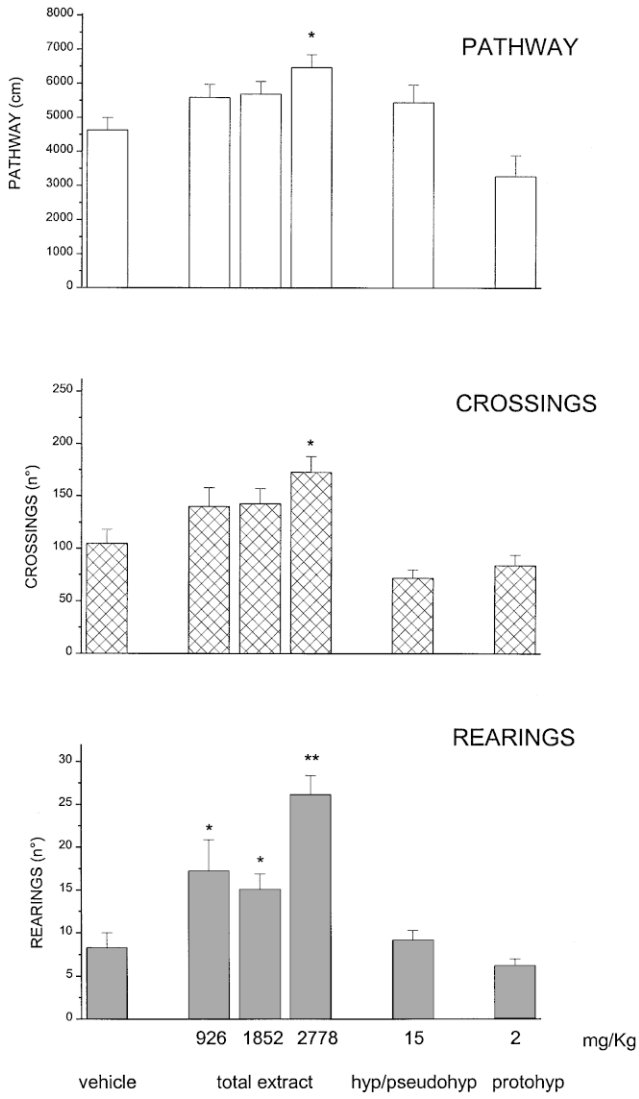

FIG. 1. Locomotor behavior of rats during the open-field test, performed 1 h after oral administration of tested compounds. Total extract was administered at different doses: 926, 1852, and 2778 mg/kg, equivalent to 5, 10, and 15 mg/kg total hypericins, respectively. The activity of total extract is compared to that of hypericin/pseudohypericin 1/4 and of protohypericin corresponding to 2778 mg/kg total extract. Values represent the mean ± SEM (n = 8–12); *$p < 0.05$, **$p < 0.01$ vs. vehicle-treated rats.

Diazepam, used as reference drug, was injected IP at the dose of 1.5 mg/kg, 20 min before the test. In the same test Flumazenil, used as BZD central receptors antagonist, was injected IP, at a dose of 3 mg/kg, 15 min after the administration of the total extract.

Statistical Analysis

Each value represents the mean ± SEM calculated from a test group of 8–22 rats. Significance of differences vs. the control group was statistically evaluated by one-way ANOVA, with the Dunnett Multiple Comparisons posttest.

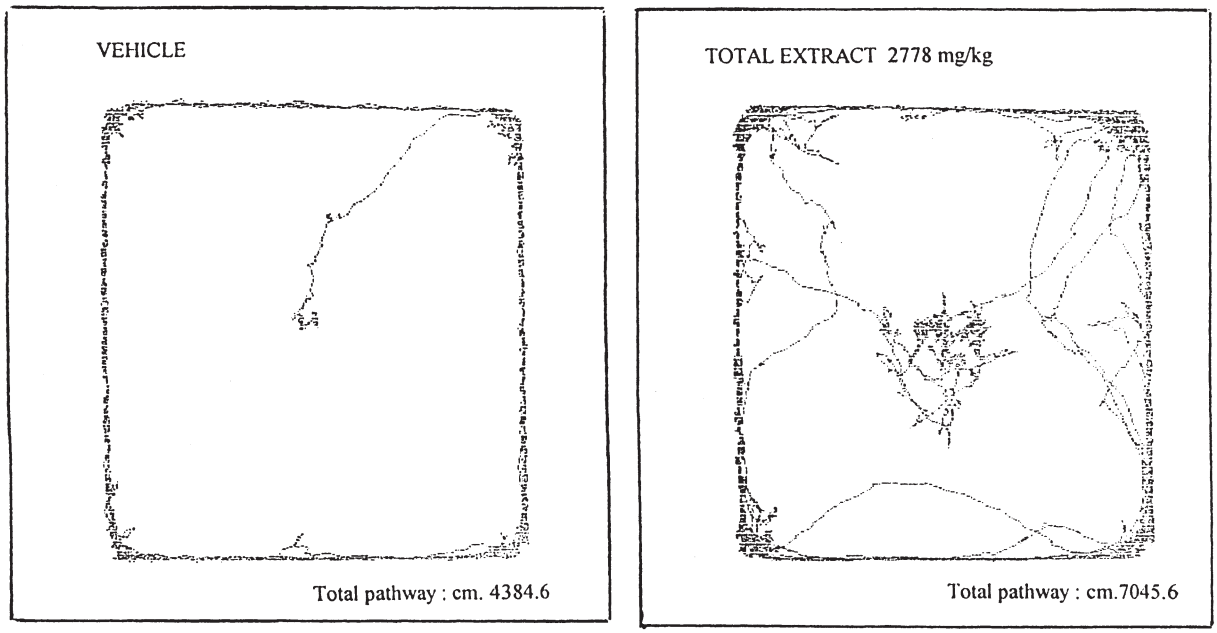

FIG. 2. Examples of pattern routes of rats treated with the higher dose of total extract (2778 mg/kg) in comparison with vehicle-treated rats.

Primary Cultures of Cerebellar Granule Cells

Primary culture of cerebellar granule neurons were prepared from 7–8-day-old Sprague–Dawley rats as previously described (12). Briefly, cells from cerebella were dispersed with trypsin (0.24 mg/ml) (Sigma, St. Louis, MO) and plated at a density of 10^6 cells/ml on 35-mm Falcon dishes coated with poly-L-lysine (10 μg/ml, Sigma). Cells were grown in basal Eagle's Medium (Irvine Scientific, Santa Ana, CA), supplemented with 10% fetal bovine serum (Hyclone Lab, UT), 2 mM glutamine, and 100 μg/ml gentamycin (Sigma), and manteined at 37°C in 5% CO_2. Cytosine arabinofuranoside (10 μM; Sigma) was added to the cultures 24 h after plating to prevent astroglia proliferation.

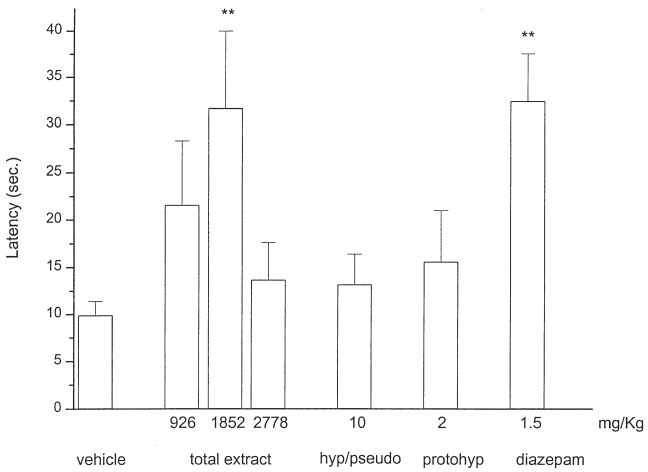

FIG. 3. Latency time (expressed in seconds) to the first crossing from the light to dark compartment evaluated in the light–dark test, 1 h after oral administration of tested compounds. Rats were treated with different doses (926, 1852, and 2778 mg/kg body weight) of total extract; the doses of hypericin/pseudohypericin 1/4 or protohypericin corresponding to 1852 mg/kg total extract were 10 and 2 mg/kg, respectively. Diazepam was used as reference drug: the test was performed 20 min after the IP injection of a dose of 1.5 mg/kg. Values represent the mean ± SEM (n = 8–22); **$p < 0.01$ vs. vehicle-treated rats.

Electrophysiological Recordings

We have used the patch-clamp technique (14) in the whole-cell configuration on single neuron after 7 days in cul-

TABLE 1

INHIBITORY EFFECT OF FLUMAZENIL ON ANXIOLYTIC ACTIVITY ELICITED BY *HYPERICUM PERFORATUM* TOTAL EXTRACT

Pretreatment (mg/kg)	Treatment (mg/kg)	Latency (s) Mean ± SEM
Vehicle	Vehicle	11.3 ± 1.7
Vehicle	Total extract 1852	31.7 ± 8.3*
Vehicle	Flumazenil 3	12.8 ± 6.3
Total extract 1852	Flumazenil 3	14.2 ± 3.0†

Pretreatments and treatments were performed respectively 60 and 15 min before the test, administering total extract per os and flumazenil IP.

*$p < 0.01$ vs. vehicle-treated rats; †$p < 0.05$ vs. total extract treated rats (ANOVA test).

ture. Electrodes were pulled from borosilicate glass (Hidelgberg, Germany) on a vertical puller (PB-7, Narishige), and had a resistence of 5–7 MOhm when filled with KCl internal solution. Currents were amplified with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA), filtered at 5 kHz and digitized at 10 kHz by using pClamp software (Axon Instruments).

Solutions and drugs. Intracellular solution contains (mM): KCl 140, $MgCl_2$ 3, EGTA 5, HEPES 5, ATP-Na 2; pH 7.3 with KOH. Cells were continuosly perfused with the external solution (mM): NaCl 145, KCl 5, $CaCl_2$ 1, HEPES 5, Glucose 5, Sucrose 20, pH 7.4 with NaOH. GABA, NMDA, and Glycine were purchased from Sigma.

Drug application. Hypericin, pseudohypericin, and the crude extract were dissolved in DMSO and diluted at the final concentration in extracellular medium (DMSO f.c. less than 0.1%). NMDA and GABA were dissolved in the extracellular solution. All drugs were applied directly by gravity through a Y-tube perfusion system (21). Drug application had a fast onset, and achieved a complete local perfusion of the recorded cell.

Data Analysis

Electrophysiological data were analysed using the software Clampex (Axon Instrument).

RESULTS

Locomotor Behavior

The data reported in Fig. 1 demonstrate that *Hypericum* extract increases the total length of pathway, the number of crossings and rearings measured during the open-field test. The effect of the total extract, when compared with that of vehicle, was statistically significant at a dose of 2778 mg/kg, containing 15 mg/kg total hypericins. Interestingly, the total extract significantly increased the number of rearings, even at the lowest dose tested. Figure 1 also shows the results obtained administering a fraction containing hypericin/pseudohypericin (1/4) and the pure protohypericin at a dose corresponding to 2778 mg/kg of the total extract (15 and 2 mg/kg, respectively). No significant difference in the different evaluated parameters was determined in comparison to vehicle-treated rats.

Examples of pattern routes, as registered by the telecamera, are reported in Fig. 2.

Anxiolytic Effect

The *Hypericum* extract increased significantly the latency time in the light–dark model of anxiety, at a dose of 1852 mg/kg, whereas the effects seen at 926 and 2778 mg/kg were not significantly different from the control group, treated with vehicle (Fig. 3). These data point out to an inverted U-shaped activity of the total extract. In Fig. 3 any change in latency time vs. vehicle-treated rats was observed for the other test substances administered at a dose corresponding to 1852 mg/kg total extract. The anxiolytic activity elicited by total extract of *Hypericum* is quite similar to that obtained with the injection of 1.5 mg/kg of diazepam. The results reported in Table 1 demonstrate that the anxiolytic effect of the total extract is blocked by the administration of BZD receptors antagonist, Flumazenil (3 mg/kg, IP).

Because in all cases only one crossing from the illuminated area to the dark part was registered, the total time spent in the illuminated area was equal to the latency time.

Electrophysiological Studies

Application of the crude extract (at a concentration that is comparable to hypericin 10 μM) alone or together with GABA or NMDA produces at the first trial an inward current followed by a complete desensitization of the cells that hampered any further analysis (data not shown). GABA-activated chloride current was decreased by the coapplication with hypericin 10 μM; the effect was reversible (Fig. 4A).

Hypericin 10 μM reduced ($-43 \pm 8\%$, $n = 13$), while the same concentration of pseudohypericin potentiated ($57 \pm 15\%$, $n = 9$), GABA-evoked Cl currents. The modulatory effects of hypericin and pseudohypericin are dose dependent (Fig. 4B).

Application of NMDA 100 μM, in the presence of Glycine 10 μM, elicits a slowly desensitizing current that is reduced by 10 μM hypericin (Fig. 5A).

NMDA-activated currents are negatively modulated either by hypericin 10 μM (-30 ± 10, $n = 7$) or by pseudohypericin 10 μM (-20 ± 8, $n = 5$) (Fig. 5B).

DISCUSSION

It is generally recognized that *Hypericum perforatum* extract is useful in the treatment of depressive disorders. There is good evidence that *Hypericum* is better than placebo in treating some depressive disorders. Moreover, *Hypericum* seems to have fewer side effects than some other antidepressants.

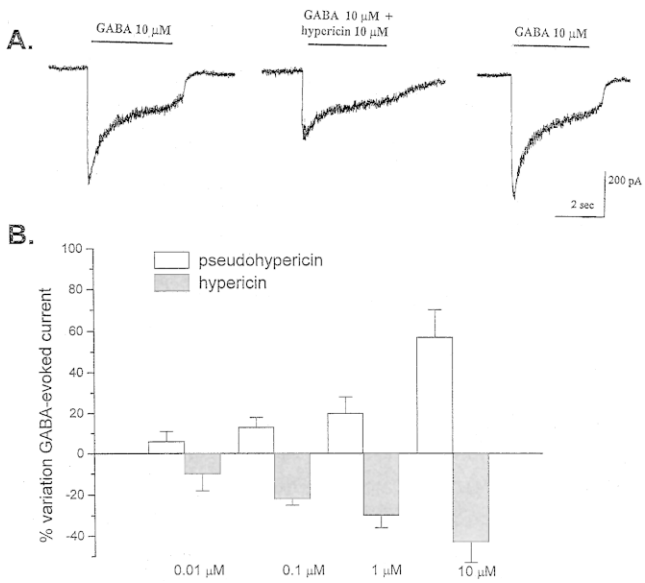

FIG. 4. Modulation of GABA-evoked current by hypericin and pseudohypericin. (A) Representative trace of Cl^- current evoked by GABA 10 μM and by GABA 10 μM + Hypericin 10 μM (straight bar = duration of the application). Holding potential (Hp) = −60 mV. (B) Histogram showing the dose response of the modulation of the GABA-elicited current by increasing doses of the two substances. Each bar is the mean ± SE of at least six cells.

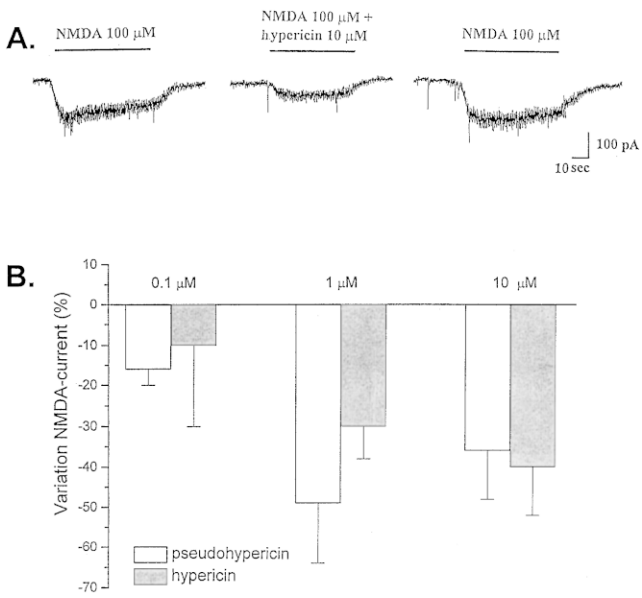

FIG. 5. Modulation of NMDA-evoked current by hypericin and pseudohypericin. (A) Recording of the current evoked by the application of 100 μM NMDA and 100 μM NMDA + Hypericine 10 μM in the presence of Glycine 10 μM (straight bar = duration of the application), Hp = −60 mV. (B) Histogram showing the % reduction of NMDA-evoked current after coapplication of increasing concentrations of hypericin or pseudohypericin. Each bar is the mean ± SE of at least six cells.

Hypericum extract contains at least 10 groups of components that may contribute to its pharmacological effect. These include naphthodianthrones, flavonoids, xanthones, phloroglucinols, and bioflavonoids (22). Despite the amount of experimental and clinical studies, the mechanism of the antidepressant effect is still under debate.

In the present study we demonstrate that the total extract of the plant affects locomotor behavior only when administered at doses much higher than those eliciting antidepressant effects, as described by Butterweck (4). The fractions containing only hypericin/pseudohypericin or protohypericin failed to alter the different parameters evaluated during the open-field test, suggesting that other components must be involved in the pharmacological activity of the plant.

Also, by the light/dark model of anxiety we demonstrated the ability of the total extract but not the single fractions to counteract anxiety in rats, submitted to an aversive stimolous (light).

The fact that the anxiolytic effect was blocked by the injection of a BZD antagonist, Flumazenil, suggests the implication of the BZD receptor system in the anxiolytic effect of *Hypericum* total extract.

Electrophysiological studies evidenced that hypericin negatively affects GABA-evoked chloride currents, but also that pseudohypericin potentiates them. The same constituents induce a reduction of NMDA mediated currents.

In light of these data, we suggest that the anxiolytic effect could be partly linked to the facilitatory activity of pseudohypericin on GABA evoked currents and to the inhibitory influence on glutamatergic transmission mediated by NMDA receptors, because it has been previously reported that an antagonism of NMDA receptor function produces an anxiolytic effect (16).

As a whole it seems likely to conclude, beside the difficulties in reconciling the role played by the single constituents, that the present demonstration of the anxiolytic effect of *Hypericum* extracts, could open up the clinical use of *Hypericum* in the treatment of depression complicated by anxiogenic components, a clinical condition where pure antidepressant or anxiolytic drugs are only modestly effective.

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