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Antagonist effect of pseudohypericin at CRF_1 receptors

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Abstract

St. John's wort (*Hypericum perforatum* L.) is widely used for the treatment of mild to moderately severe depression. However, the nature of its active principles and the exact mode of antidepressant action are still unknown. It has been suggested repeatedly in preclinical and clinical studies that the content of the acylphloroglucinol hyperforin decisively contributes to the antidepressant efficacy of St. John's wort extracts. Experimental studies in vivo also indicate that the naphthodianthrone hypericin may reduce the activity of the hypothalamic–pituitary–adrenal axis. Exacerbated hypothalamic–pituitary–adrenal activity has often been associated with depressive states in patients. Corticotropin-releasing factor (CRF) seems to be a major determinant in the regulation of the hypothalamic–pituitary–adrenal activity via activation of CRF_1 receptors. In the present study, we investigated the CRF_1 receptor antagonist activity of three main constituents of St. John's wort (hypericin, pseudohypericin and hyperforin) by measuring their effect on CRF-stimulated cAMP formation in recombinant Chinese hamster ovary (CHO) cells. As a selectivity test, the compounds were also tested against calcitonin in the same cells. Of the three compounds tested, only pseudohypericin selectively antagonised CRF (K_B 0.76 μM). Hypericin and hyperforin affected both CRF and calcitonin with similar potencies and the same type of behaviour (competitive antagonism for hypericin, noncompetitive for hyperforin). It is concluded that pseudohypericin is the only real CRF_1 receptor antagonist of the three constituents tested. In addition, evidence is provided that beside hyperforin, both pseudohypericin and hypericin are implicated in the antidepressant efficacy of St. John's wort.

Keywords: *Hypericum perforatum*; St. John's wort; Hypericin; Pseudohypericin; Hyperforin; Depression; CRF (corticotropin-releasing factor)

1. Introduction

St. John's wort (*Hypericum perforatum* L.) is a widely used phytomedicinal drug for the treatment of mild to moderate depressions. Two recent studies failed to support the efficacy of St. John's wort in moderately severe major depression (Davidson et al., 2002; Shelton et al., 2001). However, over 30 clinical trials indicate similar efficacy as low doses of tricyclic antidepressants, and better safety with regard to incidence and severity of side effects (for reviews, see Greeson et al., 2001; Schulz, 2002). Attempts to define the antidepressant principles of St. John's wort were mainly focussed on the characteristic ingredients, including the acylphloroglucinol derivative hyperforin and the naphthodianthrone hypericin (Fig. 1). Even though extracts of St. John's wort generally contain substantially larger amounts of pseudohypericin (a hydroxylated form of hypericin, see Fig. 1) compared to hypericin (Büter et al., 1998), no specific phamacological effect has ever been described for this compound. With regard to the more ubiquitous constituents of St. John's wort such as flavonoids, procyanidins, tannins, essential oil, amino acids, phenylpropanes, xanthones, organic acids, peptides, polysaccharides, etc., pharmacological data are rare or completely lacking.

Hyperforin has been shown to nonselectively inhibit the reuptake of 5-hydroxytryptamine (5-HT, serotonin), dopamine, norepinephrine, γ-aminobutyric acid (GABA) and acetylcholine into synaptosomal preparations (Müller et al., 2001; Wonnemann et al., 2001; Buchholzer et al., 2002). In addition, hyperforin reportedly interfered with the storage of monoamines in synaptic vesicles (Gobbi et al., 1999; Chatterjee et al., 2001; Roz et al., 2002). In contrast to synthetic uptake inhibitors, hyperforin failed to

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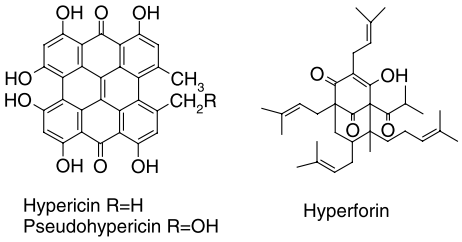

Fig. 1. Chemical structures of hypericin, pseudohypericin and hyperforin.

block these transporter proteins directly at their binding sites. It rather affected hydrogen and Na^+ channels by interaction with certain membrane structures (Singer et al., 1999; Eckert and Müller, 2001; Koch and Chatterjee, 2001; Krishtal et al., 2001). Extracts of St. John's wort as well as hyperforin also inhibited uptake of 5-HT and norepinephrine into both brain slices and cultured rat brain as well as glial cells (Kientsch et al., 2001; Neary et al., 2001). The effect of hyperforin seems, however, rather be due to cytotoxic effects than to specific inhibitory actions on transporters (Kientsch et al., 2001). Relatively scarce data are available for the mode of action of hypericin as an antidepressant compound. Based on in vivo studies, hypericin increased 5-HT concentrations in the hypothalamus after chronic treatment for 8 weeks and exhibited neuroendocrine effects in rats by decreasing plasma levels of adrenocorticotropic hormone and corticosterone after 14 days of oral treatment (Butterweck et al., 2001a, 2002). Hypericin was further shown to affect the transcription of genes involved in the regulation of the hypothalamic–pituitary–adrenal axis: mRNA levels of both corticotropin-releasing factor (CRF) and serotonin 5-HT_{1A} receptor were decreased in the hypothalamic paraventricular nucleus and in the hippocampus, respectively (Butterweck et al., 2001b). In this context, evidence has been provided that extracts of St. John's wort influence cytokine metabolism as shown either in behavioural tests with wild-type and interleukin-6 knock-out mice (Calapai et al., 2001) or by inhibiting interleukin-6 expression (Thiele et al., 1994; Fiebich et al., 2001).

A number of clinical findings suggest CRF to be a pathogenic factor in affective disorders (for a review, see Holsboer, 1999). CRF acts through CRF_1 receptors located in the pituitary gland to stimulate the release of adrenocorticotropic hormone. In turn, adrenocorticotropic hormone triggers the release of glucocorticoid stress hormones from the adrenals. Elevated CRF levels have been found in the cerebrospinal fluid of drug-free depressed patients and these levels were normalised after successful antidepressant treatment. In addition, depressed patients show a blunted adrenocorticotropic hormone response upon CRF challenge. Also, a decreased CRF receptor number has been found in the brain of depressed patients who committed suicide. Therefore, the CRF_1 receptor appears to be a major determinant in the regulation of the hypothalamic–pituitary–adrenal axis and selective CRF_1 receptor antagonists are being developed in the pharmaceutical industry as potential anxiolytics/antidepressants (Schulz et al., 1996; Zobel et al., 2000). We have formerly shown that both hypericin and pseudohypericin have some affinity for the corticotropin-releasing factor type 1 (CRF_1) receptor (Simmen et al., 2001). Preliminary functional studies, using GTP-γ-[^{35}S] binding, also indicated an antagonist property of hypericin at the CRF_1 receptor (Simmen et al., 2001).

In the present study, we investigated in more detail the functional activity of the three most typical constituents of St. John's wort, hyperforin, hypericin and pseudohypericin, at CRF_1 receptors by measuring their effect on CRF-stimulated cAMP formation in recombinant Chinese hamster ovary (CHO) cells.

2. Materials and methods

2.1. Cloning and expression of human CRF_1 receptors

Based on the published cDNA sequence of the human CRF_1 receptor (Chen et al., 1993; Genbank accession no. L23332), specific oligonucleotides for either 5′ or 3′ ends were designed. The following oligonucleotides, purchased from Microsynth (Balgach, Switzerland), were used as primers (positions within the coding sequence in parenthesis): CRF-1 ATG GGA GGG CAC CCG CAG CT (positions 1–20) and CRF-1T1 (rev) TCA GAC TGC TGT GGA CTG CTT GAT G (position 1224–1248).

Human total brain RNA was purchased from Clontech (Palo Alto, CA, USA). For the reverse transcription-polymerase chain reaction (RT-PCR), 1 μg of RNA was reverse transcribed in a final volume of 20 μl using M-MLV reverse transcriptase (Gibco-BRL) in PCR-buffer (Boehringer), 10 mM dithiothreitol, 5 μM hexanucleotides (Pharmacia), 0.2 mM of each dNTP, 40 U rRNasin (Promega) for 1 h at 37 °C. PCRs were performed in a final volume of 50 μl with 10% of the reverse transcription mixture, 10 pmol of each primer, 1 U ExpandTaq-DNA-polymerase (Boehringer) and 0.25 mM of each dNTP under the following conditions: 30 s at 55 °C, 1 min at 72 °C, 30 s at 94 °C for 38 cycles. The human CRF_1 receptor was amplified using the primer pair: CRF-1/CRF-1T1.

After filling the overhanging ends with Klenow enzyme (Boehringer), the PCR-products were separated on an agarose gel, excised and extracted using the Quiaquick kit (Quiagen). The DNA fragments were then ligated into pBluescript (Stratagene), which was linearized with *Eco*RV (Boehringer) and dephosphorylated using calf intestine phosphatase (Boehringer). Sequencing was performed using an automated sequencer (Applied Biosystems 373A). The pBluescript KSII plasmid containing the cDNA of the CRF_1 receptor was cut with *Eco*RI. The overhanging ends were filled using Klenow polymerase. The insert was then excised with *Kpn*I and ligated into the *Sal*I restriction site, which was filled with Klenow enzyme, and the *Kpn*I restriction site of the expression vector pXMT3neo7. For

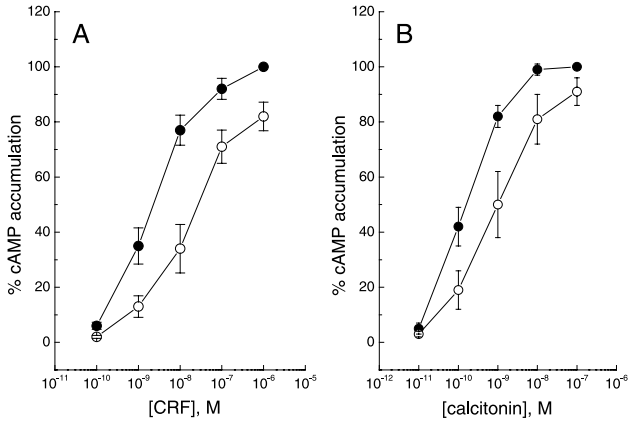

Fig. 2. Concentration–response curves of (A) CRF- and (B) calcitonin-stimulated cAMP accumulation in the absence (●) and in the presence (○) of hypericin (10 μM) in CHO-K1 cells expressing human recombinant CRF_1 receptors. Data were normalised to the effect of 1 μM CRF and 0.1 μM calcitonin in the absence of antagonist and are given as mean values ± S.E.M. of five (A) and seven (B) individual experiments.

transfections, plasmid DNA was isolated via two subsequent CsCl gradient centrifugations.

Ten micrograms of DNA (pXMT3neo7-CRF_1) were transfected into CHO-K1 cells using the protocol of Chen and Okayama (1987). The following day, cells were washed with phosphate-buffered saline (PBS) and grown for another 48 h in growth medium (DMEM, Dulbecco's Modified Eagle Medium, Gibco ref. 31885, 10% foetal calf serum (FCS), 40 mg/l L-proline and 100 nM Na-selenite). Cells were then split by serial dilutions (1:2; 1:4; 1:8 and 1:16) and maintained in selection medium (growth medium supplemented with 1 mg/ml of the antibiotic G418). Colonies of transfectants were picked and analysed functionally for the ability to stimulate cAMP formation (see below) upon incubation with the CRF-related frog peptide, sauvagine. The best 30 colonies were kept. In parallel, cells were additionally seeded in 96-well plates at a dilution that will separate single cells. Again, clones were analysed in the cAMP assay. The best clone in terms of cAMP stimulation was selected and propagated in DMEM supplemented with 10 % FCS, nonessential aminoacids (Gibco ref. 11140) and 1 mg/ml G418.

2.2. Measurements of cAMP accumulation and analysis of data

cAMP accumulation was measured in intact cells seeded in 24-well plates, using the standard [^{3}H]adenine prelabelling technique, as previously described (Schoeffter et al., 1997, 1999). The [^{3}H]cAMP/([^{3}H]cAMP+[^{3}H]ATP) cpm ratio (cAMP conversion rate) was calculated for each sample. Data were then normalised to the effect of CRF (1 μM) or calcitonin (0.1 μM), a full concentration–response curve of which was included in each experiment. Concentration–response curves were fitted to the nonlinear logistic function of the Origin 6.1 software package (OriginLab, Northampton, MA, USA). E_{max} and EC_{50} values were derived from these analyses. Results are given as means ± S.E.M. of the indicated n number of experiments, each made in duplicate. Apparent pK_B values of antagonists were calculated from the rightward shift of the CRF concentration–response curve (Furchgott, 1972), according to the formula: $pK_B = \log(\text{ratio} - 1) - \log \text{molar}[\text{antagonist}]$, wherein ratio designates the ratio (EC_{50} in the presence/EC_{50} in the absence of antagonist).

2.3. Drugs and chemicals

Hypericin, pseudohypericin and hyperforin (see Fig. 1) were gifts from Dr. Chatterjee (Dr. W. Schwabe, Karlsruhe, Germany). As verified by reverse-phase high-performance liquid chromatography, neither hypericin nor pseudohypericin showed any visible impurity measured at 590 nm. The purity of hyperforin was 88% containing 8% adhyperforin and 4% unidentified constituents as detected at 270 nm. All three compounds were dissolved in dimethylsulfoxide for antagonist assays. Human/rat CRF (hereafter referred to as CRF), sauvagine and salmon calcitonin were purchased from Bachem (Bubendorf, Switzerland). Stock solutions of these peptides (10^{-4} M) were prepared in distilled water, aliquoted, stored at −25 °C and thawed only once.

3. Results

CRF induced a maximal 33 ± 2 (n = 8)-fold stimulation of cAMP accumulation in CHO-K1 cells expressing human recombinant CRF_1 receptors (pEC_{50} 8.67 ± 0.10, corresponding to a mean EC_{50} value of 2.1 nM).

Hypericin, pseudohypericin and hyperforin were first tested at 10 μM as antagonists of CRF by measuring CRF-induced cAMP accumulation in CHO-K1 cells expressing

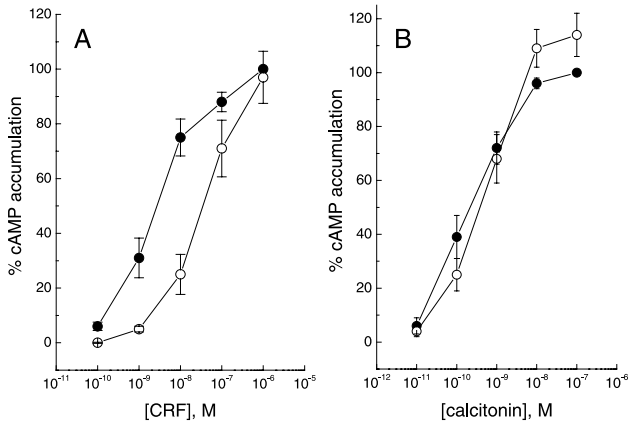

Fig. 3. Concentration–response curves of (A) CRF- and (B) calcitonin-stimulated cAMP accumulation in the absence (●) and in the presence (○) of pseudohypericin (10 μM) in CHO-K1 cells expressing human recombinant CRF_1 receptors. Data were normalised to the effect of 1 μM CRF and 0.1 μM calcitonin in the absence of antagonist and are given as mean values ± S.E.M. of five (A) and six (B) individual experiments.

human recombinant CRF_1 receptors. Hypericin (10 μM) and pseudohypericin (10 μM) induced rightward shifts of the concentration–response curve of CRF with minimal (hypericin; Fig. 2A) or no (pseudohypericin; Fig. 3A) depression of the maximal agonist effect, indicating competitive, surmountable antagonism. pK_B values were 6.03 ± 0.16 ($n = 5$) and 6.12 ± 0.09 ($n = 5$) for hypericin and pseudohypericin, respectively. By contrast, hyperforin (10 μM) caused a profound depression (by two thirds) of the maximal effect of CRF, without major shift of the agonist concentration–response curve, hinting to noncompetitive antagonism (Fig. 4A). This reducing effect of hyperforin was concentration-dependent (Fig. 4A), with an IC_{50} value of approximately 1 μM.

To assess the selectivity of their effects, hypericin, pseudohypericin and hyperforin were also tested as antagonists of calcitonin, using the same functional assay in the same cells (calcitonin receptors functionally coupled to cAMP formation are naturally present on CHO cells; George et al., 1997). Calcitonin induced a maximal 39 ± 2 ($n = 10$)-fold stimulation of cAMP accumulation in CHO-K1 cells (pEC_{50} 9.85 ± 0.11, corresponding to a mean EC_{50} value of 0.14 nM).

Hypericin (10 μM) induced a rightward shift of the concentration–response curve of calcitonin without depression of the maximal agonist effect (Fig. 2B), indicating competitive antagonism at calcitonin receptors (pK_B value 5.57 ± 0.29, $n = 7$). Pseudohypericin (10 μM) did not significantly alter the concentration–response curve of calcitonin (Fig. 3B). With hyperforin, the same pattern of inhibition was observed against calcitonin as against CRF, that is a noncompetitive, concentration-dependent reduction of the agonist maximal effect, with an IC_{50} value of approximately 1 μM (Fig. 4B).

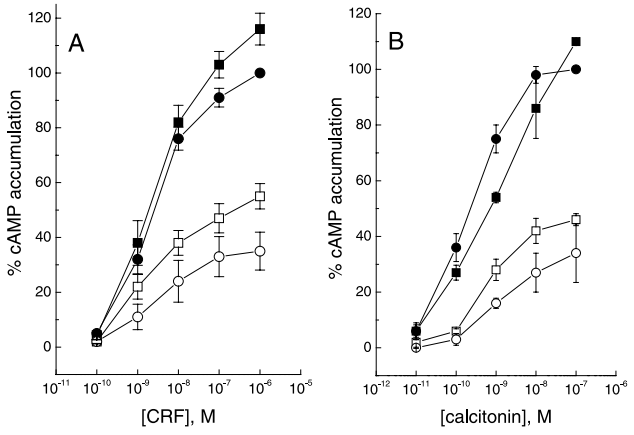

Fig. 4. Concentration–response curves of (A) CRF- and (B) calcitonin-stimulated cAMP accumulation in the absence (●) and in the presence of hyperforin 0.1 μM (■), 1 μM (□) and 10 μM (○) in CHO-K1 cells expressing human recombinant CRF_1 receptors. Data were normalised to the effect of 1 μM CRF and 0.1 μM calcitonin in the absence of antagonist and are given as mean values ± S.E.M. of four to nine (A) and two to four (B) individual experiments.

4. Discussion

The present study shows for the first time functional antagonism of CRF_1 receptors by hypericin, pseudohypericin and hyperforin, compounds which are believed to be major antidepressant constituents of St. John's wort. Increasing evidence suggests that CRF_1 receptor antagonists represent a new class of anxiolytics/antidepressants. Since the introduction of the first nonpeptide CRF_1 receptor antagonist, CP-154,526 (Schulz et al., 1996), a number of similar compounds have been described that have positive effects in animal models of anxiety and depression (for a review, see Holsboer, 2001). One such compound, R121919, displayed anxiolytic activity and inhibited stress-induced adrenocorticotropic hormone release in rats (Keck et al., 2001). Furthermore, a pilot clinical trial with R121919 in depressed patients yielded promising results (Zobel et al., 2000). In this context, the effects of hypericin, pseudohypericin and hyperforin possibly point to a novel mechanism of action of these constituents of St. John's wort.

A closer look at the effects of hypericin, pseudohypericin and hyperforin indicated however differences among the three constituents. First, hyperforin behaved differently from the other two compounds by exerting noncompetitive antagonism. It remains to be shown, whether this particular behaviour of hyperforin could be due to any interaction with ion channels or with any other membrane-related effect as has been described for this compound (Krishtal et al., 2001; Eckert and Müller, 2001; Marsh and Davies, 2002). Second, hypericin and hyperforin did not selectively antagonise CRF, since they also affected the agonist effect of calcitonin, with similar potency and the same type of behaviour (competitive for hypericin, noncompetitive for hyperforin). Calcitonin and CRF receptors belong to the same subfamily of G_S protein-coupled receptors, i.e. they share some structural properties (Segre and Goldring, 1993). Pseudohypericin was the only selective antagonist for the CRF_1 receptor, since this compound induced no significant change on the calcitonin concentration–response curve. It can therefore be concluded that pseudohypericin is the only real CRF_1 receptor antagonist among the three constituents of St. John's wort tested in the present study. Its pK_B value was estimated to be 6.12, corresponding to a K_B value of 0.76 μM.

In order to assess the relevance of the antidepressant efficacy of the compounds studied, bioavailability data have to be considered. Plasma levels of pseudohypericin and hypericin in man after chronic treatment with 600 mg of St. John's wort extract are about 25 and 60 nM, respectively (Brockmöller et al., 1997). Administration of 300 mg St. John's wort extract three times daily over 8 days resulted in plasma levels of 200–300 nM for hyperforin (Biber et al., 1998). Considering the K_B values of pseudohypericin and hypericin (0.93 μM) as well as the potency of the noncompetitive antagonism of hyperforin, none of the plasma levels determined in man would be sufficient to exert a full antagonist effect on central CRF_1 receptors. However, there

is no data available about the actual concentrations in neuronal membrane structures. Drug concentrations in subcellular structures of neurons (e.g. membranes, lysosomal vesicles) may be higher than the corresponding plasma levels as known for tricyclic antidepressants (Daniel et al., 1995). This may also be the case for pseudohypericin, hypericin and hyperforin, even though only traces of hypericin seem to penetrate in the cerebrospinal fluid of nonhuman primates (Fox et al., 2001). In any case, hypericin was shown to affect monoamine levels as well as mRNA levels of CRF and 5-HT_{1A} receptor in the hypothalamus and hippocampus indicating central pharmacological actions (Butterweck et al., 2001b; Butterweck et al., 2002). Consequently, the present data indicate antagonistic effects at CRF_1 receptors for all three tested constituents presumably relevant for the antidepressant efficacy of St. John's wort. Since hyperforin has repeatedly been suggested to be the main antidepressant component in St. John's wort extracts, the present results propose an additional mechanism of action for hypericin and particularly pseudohypericin via a reduced hypothalamic–pituitary–adrenal activity.

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