Dual Modulation of Striatal Acetylcholine Release by Hyperforin, a Constituent of St. John’s Wort

MARIE-LUISE BUCHHOLZER, CLAUDIA DVORAK, SHYAM S. CHATTERJEE, and JOCHEN KLEIN

ABSTRACT

Extracts of the medicinal plant St. John’s wort (Hypericum perforatum) are widely used for the treatment of mild to moderately severe depression. The efficacy of the extract is supported by clinical studies (reviewed by Linde et al., 1996; DiCarlo et al., 2001). Experimental studies searched for mechanisms of action known from classical synthetic antidepressants. Indeed, treatment of rodents with extracts of St. John’s wort caused changes of brain adrenergic and serotonergic receptors, and behavioral effects that were reminiscent of the effects of tricyclic antidepressants; and in vitro experiments demonstrated inhibition of amine uptake systems by these extracts (Chatterjee et al., 1998; Müller et al., 2001). Hyperforin, a lipophilic acylphloroglucinol derivative, was identified as an active constituent of St. John’s wort. Hyperforin potently inhibited the uptake of aminergic transmitters (serotonin, noradrenaline, dopamine) into synaptic nerve endings, probably by interference with mechanisms controlling the synaptic sodium concentration. Because de novo synthesis of acetylcholine (ACh) is dependent on sodium-dependent high-affinity choline uptake, we studied the effect of hyperforin on choline (Ch) uptake in vitro and on striatal ACh release in vivo using microdialysis. In rat brain synaptosomes, hyperforin inhibited high-affinity choline uptake with an IC\textsubscript{50} of 8.5 \(\mu\text{M}\), whereas low-affinity uptake was not affected. Local infusion of hyperforin (100 \(\mu\text{M}\)) via the dialysis probe caused a delayed reduction of ACh release and a concomitant increase of Ch levels. Infusion of a lower concentration of hyperforin (10 \(\mu\text{M}\)), however, increased striatal ACh release and lowered Ch levels. Systemic administration of hyperforin (1–10 mg/kg i.p.) led to therapeutic plasma levels of hyperforin and caused a significant elevation of striatal ACh release. Behavioral testing revealed a reduction of locomotor activity in mice treated with high-dose (10 mg/kg) hyperforin. We conclude that low doses of hyperforin stimulate striatal ACh release by an unknown mechanism, whereas high doses inhibit synaptic choline uptake and ACh release. The results are discussed with respect to the therapeutic use of St. John’s wort in patients with neurodegenerative disorders.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Kl 598/6-1) and the Stiftung VERUM.

ABBREVIATIONS: HACU, high-affinity choline uptake; ACh, acetylcholine; Ch, choline; LACU, low-affinity choline uptake; HC-3, hemicholinium-3; aCSF, artificial cerebrospinal fluid; HPLC, high-performance liquid chromatography; CSF, cerebrospinal fluid; KN-62, 1-[N-(2-aminoethyl)-5-methyl-3-indolyl]-3-(1-methylindol-3-yl)-maleimide methanesulfonate.
The present study presents the first data on interactions of hyperforin with the cholinergic system. We reasoned that, if hyperforin interferes with sodium-dependent uptake systems in general, it may also inhibit sodium-dependent, high-affinity choline uptake (HACU), which is coupled to acetylcholine (ACh) synthesis (Kuhar and Murrin, 1978; Tucek, 1984). The cholinergic system offers the unique opportunity to distinguish the inhibitory action of hyperforin on transmitter uptake from possible facilitatory effects on transmitter release. Whereas extracellular amine and amino acid levels would be increased by both mechanisms, they can be distinguished in the cholinergic system: inhibition of HACU would be expected to reduce ACh release, whereas a facilitatory action on transmitter release would increase ACh efflux in the microdialysis experiment.

Experimental Procedures

Materials. [3H]Choline was obtained from PerkinElmer Life Sciences (Bad Nauheim, Germany). Neostigmine and hemicholinium-3 (HC-3) were obtained from Sigma Chemical Co. (Deisenhofen, Germany), and KN-62 and Ro 31-8220 was from Calbiochem (Schwalbach, Germany). Hyperforin (sodium salt) was purified from St. John's wort as described (Chatterjee et al., 1999).

High-Affinity Choline Uptake. HACU was determined in synaptosomal (P₂) fractions obtained from Sprague-Dawley rat cortices essentially as described by Murrin and Kuhar (1976). Brain tissue was homogenized in isotonic sucrose solution containing 10 mM HEPES buffer and centrifuged at 1,000 g for 10 min. The supernatant was again centrifuged at 17,000 g for 10 min, and the resulting P₂ pellet was washed and used for HACU determinations. For this purpose, aliquots were incubated in Krebs-Ringer buffer at 37°C with 50 nM [3H]choline at 30°C for exactly 5 min. Choline uptake was stopped by addition of ice-cold buffer. After centrifugation, the pellet was washed three times with sodium-free buffer, and radioactivity associated with the pellet was determined by liquid scintillation counting. LACU was determined in parallel incubations in sodium-free buffer (sodium chloride was isotonically replaced by sucrose) or in the presence of HC-3 (1 μM) and expressed as 3H uptake (dpm/mg of protein/5 min). HACU is equivalent to the sodium-dependent and HC-3-inhibitable part of choline uptake and was calculated as the difference of total choline uptake minus LACU.

In experiments with hyperforin, the drug was dissolved in buffer immediately before the experiment and added to the incubation 15 min before [3H]choline. In experiments with prior depolarization of synaptosomes, the P₂ fraction was incubated for 15 min with buffer containing 62 mM KCl (NaCl concentration was reduced accordingly to maintain iso-osmolality). Afterward, the P₂ pellet was collected by centrifugation and resuspended in normal Krebs-Ringer buffer for [3H]choline uptake measurements. Hyperforin was added for the choline uptake period only. In some experiments, inhibitors of calcium-dependent kinases (KN-62, Ro 31-8220) were present throughout the period of depolarization and measurement. Kinase inhibitors were dissolved in dimethyl sulfoxide [final concentration 1% (v/v)], and equivalent amounts of dimethyl sulfoxide were added to control incubations.

Microdialysis. Adult Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were anesthetized with equithesin and placed in a stereotactic frame. I-shaped, concentric microdialysis probes with an exchange length of 3.5 mm (OD, 0.28 mm; ID, 0.17 mm) and a penetrable length of 3.5 mm (Paxinos and Watson, 1986). Experiments were carried out 1 or 2 days after probe implantation in freely moving animals. The probes were perfused with artificial cerebrospinal fluid (aCSF) (114 mM NaCl, 4 mM KCl, 1.2 mM CaCl₂, and 1.2 mM MgCl₂) containing 1 μM neostigmine at a perfusion rate of 2 μl/min; the in vitro recovery for ACh was 27 to 34%. For infusion experiments (see Fig. 2), hyperforin was dissolved in aCSF immediately before the experiment. For systemic application, hyperforin was dissolved in saline and injected intraperitoneally in doses of 1 and 10 mg/kg (see Fig. 3). Saline injections served as controls.

The experimental procedures used in this study met the guidelines of and were approved by the responsible governmental agency (Bezirksregierung Rheinland-Pfalz, 177-07/991-30).

Determination of Choline and Acetylcholine. Choline and acetylcholine (ACh) in dialysates were determined by microbore HPLC using a metal-free system that consisted of a low-speed pump (BAS PM80; BAS Bioanalytical Systems, Inc., West Lafayette, IN), a separation column (SepStik, 530 × 1 mm; BAS Bioanalytical Systems, Inc.), an enzyme reactor (50 × 1 mm) carrying immobilized AChE and choline oxidase, and an electrochemical detector (BAS LC-4C; Bioanalytical Systems, Inc.) with a platinum electrode operating at 0.5 V. The flow rate was 120 μl/min. Retention times for ACh and choline were 10.3 and 12.1 min, respectively. At an injection volume of 5 μl, the detection limit of this system was 10 to 20 fmol/injection. Choline and acetylcholine were quantified using external standards (standard curve). The assay was linear from 10 to 100 fmol.

Analysis and Stability of Hyperforin. For determinations of blood levels of hyperforin, blood plasma was prepared from trunk blood by centrifugation, and then mixed with 2 volumes of ethanol and centrifuged again. The supernatant was evaporated under nitrogen and taken up in eluent. Hyperforin was determined by HPLC using a Gynkotek 300C pump (Germaring, Germany), Eurosshper-100 C₄ Vertex column (125 × 4 mm; Knauer, Berlin, Germany), and a Biometra (Gottingen, Germany) EP-30 electrochemical detector operating at 1.0 V. The eluent was acetoniwater/methanol (72: 24:4) containing 25 mM perchloric acid. At a flow rate of 0.5 ml/min, the retention time of hyperforin was 8.1 min, and the detection limit was 1.0 μg/ml.

The HPLC assay was also used to determine the stability of hyperforin in aqueous solutions. When protected from light in the refrigerator, hyperforin was rather stable in aqueous solutions (aCSF; half-time of deterioration, 5 days). In contrast, when exposed to light, hyperforin decomposed rapidly (within hours). Therefore, hyperforin solutions were prepared fresh every day immediately before experimentation.

Behavioral Experiment. Spontaneous locomotor activity was measured in circular activity cages (ZW 41, diameter 24 cm; Laue-Elektronemchanik, Elmshorn, Germany). Horizontal animal movement axes were automatically recorded by means of 19 infrared photobeams. Groups of five mice (CD-1; Charles River) were preexposed to the activity cage for 2 min, and spontaneous locomotor activity was recorded for the following 2 min and calculated as total number of beam interruptions. Groups treated with hyperforin (1–10 mg/kg) received the drug by i.p. injection 15 min before exposure to the activity cage. The experiment was repeated twice, and the results (see Fig. 5) are given as average counts (beam interruptions) of three groups of mice (five mice per group).

Results

Effects of Hyperforin on High-Affinity Uptake of Choline (HACU). In the presence of hyperforin (10 μM), the total uptake of [3H]choline into the synaptosomal pellet (P₂ fraction from rat cortex) was significantly inhibited (−32%; p < 0.05). To distinguish high- and low-affinity choline uptake, we made use of two characteristics of HACU, namely, its sodium dependence and its high sensitivity toward HC-3 (Kuhar and Murrin, 1978; Tucek, 1984). When HACU and LACU were distinguished by omission of sodium, we found that the hyperforin-induced reduction of choline uptake was...
solely due to an inhibition of HACU, which was inhibited by 68% (*p < 0.01; Fig. 1A). The sodium-independent uptake representing the LACU system was unaffected by hyperforin (Fig. 1A). Essentially the same results were obtained when HC-3 (1 μM) was used to distinguish high- and low-affinity uptake: in the presence of hyperforin (10 μM): total choline uptake was inhibited by 31% (*p < 0.05), and HACU was reduced by 54% (*p < 0.01), whereas LACU was, again, unchanged (not illustrated). The concentration-response relationship of the inhibitory effect of hyperforin revealed an IC50 of 8.5 μM (r² = 0.98; Fig. 1B).

We conducted additional experiments to characterize the effect of hyperforin on HACU more closely. We found that preincubation of the synaptosomes under depolarizing conditions (62 mM KCl) caused a stimulation of HACU activity by 66% (*p < 0.01), corroborating previous findings by Murrin and Kuhar (1976). Interestingly, the postdepolarization stimulation was not affected in the presence of two inhibitors of calcium-dependent kinases, KN-62 (1 μM; an inhibitor of calcium/calmodulin kinase II) and Ro 31-8220 (1 μM; a protein kinase C inhibitor), but was efficiently inhibited (by 76%) in the presence of hyperforin (10 μM). Thus, hyperforin inhibited both basal and depolarization-induced HACU activity by a mechanism independent of the major synaptic, calcium-dependent protein kinases (data not shown).

**Extracellular ACh Concentration in the Striatum of Freely Moving Rats.** The results of the microdialysis experiments are summarized in Figs. 2 and 3. When hyperforin was infused into the rat striatum in a high concentration (100 μM), extracellular choline was found to increase steadily during infusion (Fig. 2A). Concomitantly, the recovery of ACh, which was initially increased, dropped to 50% of basal values, indicating an inhibition of ACh synthesis. In contrast,
infusion of hyperforin at a lower concentration (10 μM; Fig. 2B) induced a release of ACh reflected in a higher ACh efflux while extracellular choline levels decreased.

When hyperforin was given systemically by i.p. injection, striatal ACh release increased to 150% of control levels (1 mg/kg; Fig. 3B) or doubled (10 mg/kg; Fig. 3A), whereas choline levels dropped. Injection of saline did not affect striatal ACh or choline levels.

**Plasma Levels of Hyperforin.** After systemic administration of hyperforin (10 mg/kg i.p.), we determined plasma levels of the drug in the low micromolar range (Fig. 4). No hyperforin could be detected in CSF sampled from the cisterna magna (not illustrated). When a lower dose of hyperforin was administered (1 mg/kg), we failed to detect drug in either plasma or CSF (not shown).

**Behavioral Experiment.** Locomotor activity in mice decreased significantly 15 to 60 min past administration of 10 mg/kg hyperforin (Fig. 5A). A lower dose of hyperforin (1 mg/kg; Fig. 5B) was ineffective.

**Discussion**

**Inhibitory Effects of Hyperforin on the Cholinergic System.** This is the first study on possible effects of hyperforin on (central) cholinergic systems. Our in vitro experiments in synaptosomes (Fig. 1) demonstrated that hyperforin is an inhibitor of sodium-dependent HACU, whereas sodium-independent LACU was not affected. This result is compatible with previous data obtained with noncholinergic transmitters in which uptake is inhibited by hyperforin in a
nonselective manner by interference with intracellular sodium homeostasis (Singer et al., 1999; Wonnemann et al., 2000). Choline and its high-affinity uptake system must now be added to the list of sodium-dependent targets possibly involved in the effects of hyperforin.

Recent work indicated that hyperforin may also influence calmodulin-dependent mechanisms (Fisunov et al., 2000). Because HACU was previously found to be activated via calcium- or calmodulin-dependent processes (Chatterjee and Bhatnagar, 1990; Yamada et al., 1991), we tested the possibility that HACU activation may be sensitive to inhibition of calcium/calmodulin-dependent kinase II or protein kinase C. In our hands, however, two inhibitors of these kinases (KN-62 and Ro 31-8220, respectively) did not affect HACU activity that was stimulated by prior depolarization. In contrast, hyperforin inhibited depolarization-induced HACU activity to an extent similar to that of unstimulated HACU in synaptosomes. Thus, hyperforin’s mechanism of action apparently does not involve direct inhibition of the major calcium-dependent kinases in the synaptic endings. We cannot exclude, however, the idea that hyperforin interacts with other calcium/calmodulin-dependent processes (e.g., activation of P-type calcium channels; Fisunov et al., 2000) to affect depolarization-induced HACU activation. The possible relationship of these actions to the effect of hyperforin on sodium levels is presently unknown.

The sodium-dependent HACU is coupled to ACh synthesis and release by mass action (Tucek, 1984), and inhibition of HACU, e.g., by HC-3, has been reported to reduce extracellular ACh concentrations and to increase choline levels in rat striatum in vivo (Ikarashi et al., 1997). We report here that hyperforin, infused at a concentration of 100 M, has identical effects to HC-3: ACh release is reduced (albeit in a delayed manner), whereas the extracellular choline level is increased (Fig. 2A). Although this effect of hyperforin directly reflects the in vitro results on HACU inhibition, it cannot easily be discussed in quantitative terms because the local concentrations of hyperforin that are attained during infusion are unknown. From previous experience, we estimate extracellular hyperforin concentrations in the brain at approximately 5 to 10% of the infusion concentration, so that an average concentration of 5 to 10 M hyperforin may be reached in the brain tissue surrounding the probe. This concentration is similar to the IC50 value for HACU inhibition determined in vitro (8.5 M; Fig. 1B).

Stimulatory Effects of Hyperforin on the Cholinergic System. When we infused hyperforin at 100 M into rat striatum, we observed an initial increase of ACh efflux possibly due to a facilitatory effect of the drug on neurotransmitter release (see the introduction). At lower concentrations (10 M), we observed only an increase of the ACh efflux (Fig. 2B). This was accompanied by a decrease of choline efflux, which again confirmed the inverse relationship between extracellular ACh and choline concentrations in rat striatum (Ikarashi et al., 1997). After systemic administration (1 and 10 mg/kg of hyperforin given intraperitoneally), striatal ACh efflux increased, whereas choline efflux decreased; the higher dose was more effective (Fig. 3). Thus, the ACh-releasing effect of hyperforin was the more prominent effect after systemic dosing, whereas inhibition of ACh release was only observed during intrastratal infusion of a high hyperforin concentration.

The mechanism of action of the stimulatory effect of hyperforin remains unknown at present, but several recent observations are relevant. Hyperforin was found to increase the synaptosomal calcium concentration, to lower synaptosomal pH, and to directly stimulate the release of 5-HT, glutamate, and α-aminobutyric acid from synaptosomes (Gobbi et al., 1999; Chatterjee et al., 2001). In synaptic vesicles, hyperforin interfered with the pH gradient, an action that may underlie its property to release neurotransmitters (Chatterjee et al., 2001). Finally, hyperforin was reported to modulate several ionic conductances in cerebellar Purkinje cells including a P-type calcium channel that is known to be involved in neurotransmitter release (Fisunov et al., 2000). Additional studies are required to decide whether interactions with pH or calcium homeostasis are responsible for the stimulatory actions of hyperforin on ACh release.

Therapeutic Implications. For the detection of hyperforin in body fluids, we developed a novel HPLC procedure with electrochemical detection that proved to be of comparable sensitivity to previously described procedures using UV detection (Chi and Franklin, 1999; Orth et al., 1999). With this method, we measured plasma levels of 3 to 5 M, 30 to 120 min after i.p. application of 10 mg/kg hyperforin (Fig. 4). We were unable to detect hyperforin in CSF, a failure we ascribe to the high lipophilicity of the drug (cf. Hilgert et al., 1999). Unfortunately, hyperforin plasma levels after injection of 1 mg/kg were below the limit of detection. However, Biber et al. (1998) demonstrated linear pharmacokinetics of hyperforin in humans treated with therapeutic doses of St. John’s wort extract; a single therapeutic dose of St. John’s wort extract gave rise to plasma hyperforin levels of 0.28 M on average (Biber et al., 1998). If we assume linear pharmacokinetics in the present study, we can estimate that the low dose of hyperforin (1 mg/kg) likely produced plasma levels of 0.3 to 0.5 M in the rat, which is close to the therapeutic plasma level of hyperforin in humans. Consequently, the predominant effect of hyperforin in patients most likely would be a facilitatory effect on striatal ACh release.

What could be the consequences of hyperforin’s stimulatory effect on brain ACh release? In the striatum, such an effect may be expected to cause parkinsonism, and, indeed, high-dose hyperforin (10 mg/kg) caused a significant decrease of spontaneous locomotor activity in our study (Fig. 5A). However, the lower dose of hyperforin (1 mg/kg) was ineffective in this respect (Fig. 5B). Thus, motor effects of hyperforin in experimental animals were only observed at a dose that caused plasma levels surpassing those measured in humans under therapeutic conditions. Nevertheless, a severe overdosage of St. John’s wort extracts may have effects on motility and should be avoided, particularly in patients with disorders of the motor systems (e.g., Parkinson’s disease). On the other hand, if the facilitatory action of hyperforin on ACh release is also present in other brain areas such as hippocampus or cortex, it may explain beneficial effects of hyperforin in learning and memory tests (e.g., scopolamine-induced amnesia; Klusa et al., 2001) and may form a basis for the use of hyperforin (and St. John’s wort) in cognitive disorders (Chatterjee et al., 1999). This possibility is currently under investigation in our laboratory.
References


Address correspondence to: Dr. Jochen Klein, Department of Pharmacology, Johannes Gutenberg University of Mainz, Obere Zahlbacher Str. 67, D-55101 Mainz, Germany. E-mail: jklein@ama.ttuhsc.edu