Inhibition of synaptosomal 5-[^{3}H]hydroxytryptamine uptake by endogenous factor(s) in human blood

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Received 23 March 1984

The inhibition of 5-[^{3}H]hydroxytryptamine uptake into rat forebrain synaptosomes by human plasma extracts was studied. Highly potent, small (M_r < 10000), and heat stable factor(s) were found to inhibit 5-[^{3}H]hydroxytryptamine uptake specifically, reversibly and in a non-competitive manner. The possible role of these factor(s) as endogenous modulators of serotonergic activity is discussed.

5-Hydroxytryptamine uptake *Plasma factor* *Synaptosome* *Imipramine* *Endogenous modulator*

1. INTRODUCTION

Serotonin (5-hydroxytryptamine; 5HT) is accumulated into the presynaptic nerve terminal by a specific high affinity transport process [1]. The postulated role of this transport system is to terminate the synaptic action of 5HT and to regulate its concentration in the synaptic cleft [2]. Antidepressant drugs, such as imipramine, inhibit the uptake of 5HT into nerve terminals, a process which has been considered to be of major importance for their antidepressant effects. Imipramine has been shown in both brain preparations [3,4] and platelets [5] to inhibit serotonin uptake specifically by binding to a site which is structurally and functionally associated with the 5HT transporter (so-called [^{3}H]imipramine binding site). Nevertheless, it is not clear whether these sites are identical to the 5HT recognition site, as some pharmacological studies have suggested, or are allosteric sites which are functionally coupled to the 5HT transporter [6–8]. Clinical studies in severely depressed patients have demonstrated alterations in the density of [^{3}H]imipramine binding sites and in the ability of platelets to accumulate [^{3}H]5HT [9–11]. One possible explanation for these changes is the differential presence of humoral endogenous modulators of the transport system. Thus, if the [^{3}H]imipramine binding site serves to allosterically modulate (inhibit or enhance) serotonin transport, the presence of endogenous effectors of these sites is plausible. We here report the presence and preliminary characterization of substance(s) in blood that inhibit synaptosomal [^{3}H]5HT uptake and also inhibit [^{3}H]imipramine binding.

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2. MATERIALS AND METHODS

2.1. *Preparation of crude synaptosomes*

Adult male Osborne-Mendel rats (180–250 g) were killed by decapitation and their forebrains (whole brain minus cerebellum) were pooled and homogenized in 20 vols of 0.32 M sucrose. All procedures were carried out on ice at 0–4°C. The homogenate was centrifuged (1000 × g, 10 min) and the supernatant was further centrifuged at 17000 × g for 30 min. The resulting supernatant was decanted and the pellet (P_2) was resuspended in 20 vols of 0.32 M sucrose.

2.2. *Measurement of neurotransmitter uptake*

The uptake of several neurotransmitters into the crude synaptosomal preparation was assayed using

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a modification of the methods in [1,2]. Briefly, 0.2 ml crude synaptosomes were preincubated for 10 min at 37°C with 0.7 ml of either Krebs-Ringer buffer (NaCl, 120 mM; KCl, 4.8 mM; $CaCl_2$, 2.3 mM, $MgSO_4$, 1.2 mM; dextrose, 2 mg/ml; containing EDTA, 0.06 mg/ml; Pargyline, 10 μM; ascorbic acid, 0.2 mg/ml, K_2HPO_4, 15.6 mM, pH 7.4) or varying volumes of the blood extract prepared in the same buffer in a total assay volume of 1.0 ml. The reaction was started by the addition of 0.1 ml aliquots of radiolabelled neurotransmitter at a final concentration of approx. 40 nM (spec. act. [^{3}H]5HT, 23.4 Ci/mmol; [^{3}H]dopamine, 30.4 Ci/mmol; [^{3}H]norepinephrine, 43.9 Ci/mmol and [^{3}H]GABA, 38.0 Ci/mmol; all from NEN, Boston, MA). After a 2 min incubation at 37°C the reaction was stopped by dilution with 4 ml ice-cold Krebs-Ringer buffer and by rapid filtration under reduced pressure, using Whatman GF/C filters (0.45 μm pore size). The filters were further rinsed 3 times with 4 ml ice-cold buffer and the radioactivity on the filters counted in a Beckman LS 9000 scintillation counter. Net uptake was calculated by subtracting parallel assay tubes incubated at 0°C.

2.3. *Preparation of blood extracts*

Blood was obtained from medication-free volunteers by venipuncture and collected in tubes containing 16 mM citrate buffer and 1 mM EDTA. Cellular elements were separated by low-speed centrifugation (600 × *g*, 10 min) and the plasma separated by further centrifugation at 12000 × *g* for 20 min. Plasma was subsequently diluted 1:4 with Krebs-Ringer buffer and boiled for 10 min at 90°C. After cooling, the denatured proteins were separated by centrifugation at 20000 × *g* for 20 min and the supernatant fluid was collected.

3. RESULTS

The effects of diluted and boiled human plasma on [^{3}H]5HT uptake into rat forebrain synaptosomes were studied. In this synaptosomal preparation, [^{3}H]5HT uptake was linear with incubation time up to 3 min and plateaued at approx. 10 min (not shown). The data presented here represent 2 min incubations and therefore correspond to the initial rate of uptake.

As demonstrated in fig.1, addition of a very small aliquot of the plasma extract induced a marked inhibition of [^{3}H]5HT uptake. Under the assay conditions described, as little as 25 μl of plasma extract inhibited the uptake of [^{3}H]5HT by 50%, whereas about 200 μl completely inhibited the uptake. Very similar dose-response curves were also obtained using untreated plasma, serum or boiled serum (not shown).

Kinetic analysis of [^{3}H]5HT uptake into forebrain synaptosomes and the effect of various plasma concentrations are shown in fig.2. Using [^{3}H]5HT concentrations ranging from 4–400 nM, a single saturable high affinity uptake site was found. This transport process has an apparent K_m of 60 nM and a V_{max} of 5 pmol/mg protein per min. Fig.2 demonstrates that the inhibitory effect of boiled plasma was non-competitive in nature (e.g., there is no apparent change in the affinity of [^{3}H]5HT to its transporter, but the V_{max} of transport is markedly reduced). Thus the inhibitory factor(s) in plasma do not directly compete with [^{3}H]5HT for the transport recognition site, but rather interact with the transport system

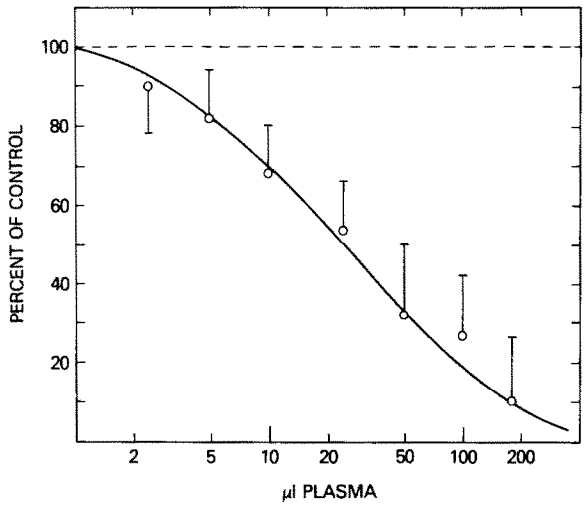

Fig.1. The effect(s) of human plasma extract on [^{3}H]5HT uptake in rat brain synaptosomes. Uptake of [^{3}H]5HT in the absence and presence of various plasma concentrations was carried out at 37°C for 2 min at 40 nM [^{3}H]5HT as described in the text. Values are the mean ± SE of at least 23 separate experiments. The control value for net [^{3}H]5HT uptake was 2.08 ± 0.07 pmol·mg protein^{-1}·min^{-1}.

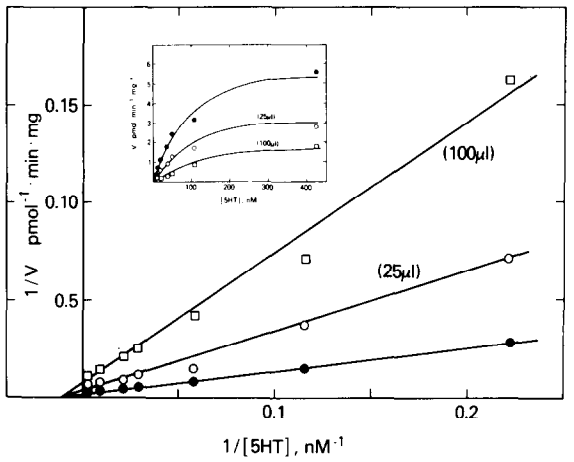

Fig.2. Kinetic analysis of the effect of various plasma concentrations on [^{3}H]5HT uptake into forebrain synaptosomes. The uptake of various [^{3}H]5HT concentrations was conducted for 2 min at 37°C and 0.4 mg synaptosomal protein/ml. Non-specific uptake was measured at 0°C and corresponds to less than 10% of total uptake. (●) Control, (○) 25 μl plasma, (□) 100 μl plasma. Values are from a representative experiment repeated 3 times with similar results.

through other mechanisms. Since the presence of endogenous inhibitors of $(Na^+ + K^+)$-ATPase in human blood has been demonstrated [12] and since these inhibitors would be expected to non-specifically inhibit any $(Na^+ + K^+)$-ATPase-dependent transport system we examined whether the present uptake inhibition was specific for [^{3}H]5HT.

The effect of the plasma extract on the transport of other neurotransmitters into synaptosomes was studied, and the extract volumes resulting in 50% inhibition of uptake (IC_{50}) were calculated and are shown in table 1.

Significantly greater amounts of plasma extract were necessary to inhibit the specific transport of [^{3}H]dopamine, [^{3}H]norepinephrine or [^{3}H]GABA into synaptosomes. The plasma extract volume required to inhibit 50% of [^{3}H]dopamine or [^{3}H]norepinephrine uptake, is approx. 10 times greater than that for inhibiting [^{3}H]5HT uptake whereas approx. 6–7 times more plasma is necessary to produce a similar inhibition of [^{3}H]GABA uptake. These data indicate that the plasma factor(s) are more selective for the blockade of [^{3}H]5HT uptake, and that they may be

Table 1

Effect of plasma extract on the uptake of different neurotransmitters

Neurotransmitter	n	IC_{50} (μl)
[^{3}H]5HT	17	28 ± 4
[^{3}H]Dopamine	6	340 ± 86
[^{3}H]Norepinephrine	6	367 ± 81
[^{3}H]GABA	8	192 ± 23

Specific uptake of the neurotransmitters was carried out as described. IC_{50} values representing the volume of plasma (in μl) which caused 50% inhibition of ^{3}H-labeled neurotransmitter uptake (each at 40 nM), were calculated from dose-response curves using at least 7 concentrations of plasma. Values represent the mean ± SE of the indicated number of separate experiments

different from the endogenous $(Na^+ + K^+)$-ATPase inhibitors.

Further characterization of the effect of plasma extract on [^{3}H]5HT uptake has revealed that the inhibition is fully reversible (table 2). Synap-

Table 2

Reversibility of plasma extract-induced inhibition of synaptosomal [^{3}H]5HT uptake

	[^{3}H]5HT uptake (pmol·mg protein^{-1}·min^{-1})	Plasma (μl)	% Control
Control	2.78 ± 0.38	–	
		25	45.5 ± 0.4
		100	27.0 ± 2.4
Pretreated with plasma	2.55 ± 0.17	–	
		25	49.5 ± 0.4
		100	26.5 ± 2.0

Crude synaptosomes (4 ml) were incubated with 1 ml of either Krebs-Ringer buffer or plasma extract for 10 min at 37°C. Subsequently both membrane preparations were washed twice by centrifugation at 17000 × g for 20 min, resuspended in 5 ml of 0.32 M sucrose, and assayed for [^{3}H]5HT uptake (at 40 nM) as described. Data represent means ± SE of 3 experiments. Membranes pre-treated with plasma extract sufficient to inhibit ⩾80% of specific [^{3}H]5HT uptake had the same basal uptake rate after washing as control membranes. Subsequent inhibition by plasma extract was also similar in pretreated and control membranes

tosomes incubated with plasma extract at a concentration sufficient to inhibit [^{3}H]5HT uptake completely showed no significant difference in [^{3}H]5HT uptake after washing and resuspension in the original buffer. Furthermore, no difference was found in the ability of previously incubated synaptosomes to be inhibited by newly added plasma extract (table 2). This finding rules out the possibility that the inhibition of [^{3}H]5HT uptake is due to a non-specific detergent-like effect of the plasma extract that may have physically disrupted the integrity of the synaptosomal membrane.

4. DISCUSSION

This study reports the presence of factor(s) in human plasma, which inhibit the transport of [^{3}H]5HT into rat forebrain synaptosomes and presents a preliminary characterization of this effect. Since rather crude plasma preparations were used, it is at present not known whether a single factor or multiple substances are responsible for the observed effects. Nevertheless, that the observed inhibition was not due to several known candidates was ruled out. One possible candidate for an endogenous inhibitor of [^{3}H]5HT uptake is 5HT itself. 5HT is stored in rather high concentrations in platelets [13] and is also found free in plasma. Nevertheless, the human plasma concentrations reported for 5HT are rather low, approx. 70 nM [14], whereas the IC_{50} for 5HT on [^{3}H]5HT uptake using our assay conditions was found to be approx. 200 nM (not shown). Since the IC_{50} of plasma on [^{3}H]5HT uptake was shown to be about 25 μl (table 1, fig.1), the plasma concentration of 5HT would need be approx. 8 μM to elicit the same effect. Furthermore, we have shown (fig.2) that the inhibitory effect of the plasma factor(s) is non-competitive in nature, whereas 5HT inhibits [^{3}H]5HT uptake competitively (not shown). Another candidate for an endogenous inhibitor of [^{3}H]5HT uptake is the endogenous ouabain- and digitalis-like inhibitor of ($Na^+ + K^+$)-ATPase, which has also been shown to be present in plasma [12]. This circulating ATPase inhibitor has been associated with renal sodium excretion [15] and with essential hypertension [16]. Recently such inhibitors have been shown to inhibit [^{3}H]5HT uptake into human blood platelets [17] and [^{3}H]norepinephrine uptake into canine saphenous vein [18]. However, this substance(s) appears to be a rather non-specific inhibitor of ($Na^+ + K^+$)-ATPase, and therefore would be expected to uncouple many transport systems. Consequently, it is likely that the plasma factor(s) reported here are not endogenous ouabain-like substances, since inhibition was rather specific for [^{3}H]5HT uptake, as opposed to the uptake of other neurotransmitters, that are similarly dependent on the activity of ($Na^+ + K^+$)-ATPase. Furthermore, the endogenous ($Na^+ + K^+$)-ATPase inhibitor is reported to be several orders of magnitude less potent than the [^{3}H]5HT uptake inhibitor reported here. For example, at least 10 vol. of plasma extract were necessary to produce 50% inhibition of [^{3}H]5HT transport into platelets by the endogenous ($Na^+ + K^+$)-ATPase inhibitor [17], whereas only 0.025 vols of plasma extract results in an equivalent inhibition of synaptosomal [^{3}H]5HT uptake by the factor(s) reported here. Hence, it appears unlikely that the factor(s) reported here are similar to the previously reported ATPase inhibitor(s).

Preliminary purification and characterization of the plasma factor(s) indicate that it is heat stable, and partially resistant to proteolytic cleavage. Using ultrafiltration and dialysis, the inhibitory activity (not shown) has been shown to have a small M_r (<10000).

At present, the possible physiological significance of the inhibitory factor(s) is unknown. However, since preliminary experiments suggest a parallel inhibition of [^{3}H]5HT uptake and [^{3}H]imipramine binding to synaptosomal membranes, this material could represent an endogenous imipramine-like substance, and could have a role in regulating serotonergic activity.

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