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Enhancing effect of zinc on astroglial and cerebral endothelial histamine uptake

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Abstract

We have studied the effect of zinc ion on the uptake of histamine (HA) into cultured astroglial and cerebral endothelial cells and established that Zn^{2+} enhances the uptake of the amine dose-dependently and in remarkable extents by increasing the V_{max} to about 3-fold (from 3.25 ± 0.42 to 8.50 ± 0.97 pmol/mg protein/min in astroglial cells) without altering the K_M (0.20 ± 0.03 μM) significantly. The stimulatory effect of zinc ion showed strong sensitivity for VUF 8407, an inhibitory compound of astroglial and cerebral endothelial uptake of HA. In the presence of 20 μM VUF 8407 the zinc-enhanced uptake was reduced by about 50% in both cell types. Binding measurements revealed increased capacities of the zinc-exposed HA binding (B_{max}= 0.41 ± 0.05 increased to 1.21 ± 0.16 pmol/mg protein in astroglial membranes and B_{max} = 0.25 ± 0.03 enhanced to 1.05 ± 0.12 pmol/mg protein in cerebral endothelial membranes) but statistically unchanged affinity of the ligand for HA carrier (K_D values calculated as 35.2 ± 3.4 nM and 45.1 ± 3.8 nM for astroglial bindings; whereas 25 ± 2.1 nM and 30 ± 2.6 nM for cerebral endothelial bindings of the amine). The compound VUF 8407 reduced the B_{max} of zinc-exposed HA binding of astroglial membranes but did not modify the K_D of the zinc-exposed membrane significantly. The *ex vivo* experiments confirmed our in vitro findings; an i.c.v. dose of 0.4 μmol/kg $ZnSO_4$, 24 hr after the injection, enhanced the uptake of [^{3}H]HA into dissociated hypothalamic and cerebellar cells to about 2- and 3-fold, respectively. Present data clearly showed that zinc exposures enhance the astroglial and the cerebral endothelial uptake of HA *in vitro* and it might be considered that zinc produces similar effects *in vivo*. Free zinc may participate in the regulation of the extraneuronal HA concentration and this metal ion (endogenous or exogenous) might be favored in the removal of the amine from the interstitial space especially in conditions with relatively high HA.

Keywords: Histamine uptake; Zinc; Cultured astroglial cells; Cerebral endothelial cells

1. Introduction

A recognition of HA as a neurotransmitter can be traced back to 1980 [1]. Now it is well known that histaminergic nerve fibers (with many varicosities) are distributed in several brain regions and form synaptic contacts with other neurones and varicosities approximate not only to neurones but also to glial cells and capillaries [2–5]. HA is synthesised from L-histidine by a specific enzyme histidine decarboxylase (EC 4.1.1.22), localized in synaptosomes or in synaptic vesicles and released by depolarization stimuli both *in vivo* and *in vitro* [6–9]. As no high-affinity and selective neuronal reuptake system for HA has been demonstrated [10–13], inactivation of neuronal HA has been supposed to proceed by its direct enzymatic transformation to N-methylimidazole acetic acid through N-methylation by histamine-N-methyltransferase (EC 2.1.1.8) and oxidative deamination by monoamine oxidase B and aldehyde dehydrogenase [14–16]. The cellular localisation of histamine-N-methyltransferase, the main metabolic enzyme of neuronal HA, however, could not be visualized in the brain by an immunohistochemical study, using polyclonal antibody raised

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Abbreviations: HA, histamine; FCS, fetal calf serum; MEM, minimal essential medium; and NEM, N-ethylmaleimide.

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against the enzyme purified from rat kidney [17], therefore the localization of HA inactivation is still uncertain.

The last ten years in our laboratories have been devoted to study the uptake of HA into non-neuronal (astroglial and cerebral endothelial) cells as possible main and/or alternative routes of HA inactivation (clearance) in the brain [18]. Our studies have shown that HA uptake by astroglial and cerebral endothelial cells represents a carrier-mediated, energy- and sodium-dependent high-affinity mechanism, which is definitely involved in the inactivation and clearance of neuronal HA. A unique characteristic of this uptake is its activation by mercuric salts added in distinct (25–200 μM) concentrations to the incubation medium [19]. This enhanced uptake of HA showed similar characteristics to those described for basic uptake (e.g. high sensitivity for energy and sodium deprivation and specific uptake blockers). Mercury, however, as in the group of IIB metals Cd^{2+} prevails several toxic effects, including severe damages of neurons, which excludes its *in vivo* application.

As the group of IIB metal cations share many chemical properties, we observed in a preliminary study that zinc ion also produced enhancements in HA uptakes by cultured astroglial and cerebral endothelial cells [20]. Zinc is the most widely used of the IIB metals and unlike others, it is virtually non-toxic. It has traditionally been identified in biology as a trace element, detectable in several biological systems, but the concentration of zinc in the brain is actually quite high. The typical concentration of zinc in the gray matter (about 0.l5–0.20 mM) is l0-l00 times higher than that of classical neurotransmitters such as acetylcholine and the monoamines. There are three rather separate pools of zinc in the CNS: vesicular, free, and protein-bound zinc. In neuronal vesicles, the concentration of zinc is about 0.1 mM and it could reach 0.3 mM or even higher concentrations in the local microenvironment during neuronal release [21]. This appears sufficient to induce many of neurobiological effects without being toxic.

This article presents new findings concerning the enhancing effects of zinc on astroglial and cerebral endothelial uptake of HA and discusses its possible importance in HA physiology.

2. Materials and methods

2.1. Materials

[2,5-^{3}H]-Histamine dihydrochloride (1.67 TBq per mmol) was purchased from Amersham International. Histaminergic agents were generously provided by SKF (Smith Kline and French Laboratories), except for thioperamide (Cookson Chemicals). FCS was obtained from Life Technologies, the 0.1% collagenase-dispase enzyme mixture from Boehringer; MEM, tripolidine, NEM Amphotericin-B, and Dulbecco's modified Eagle's MEM from Sigma. The compound VUF 8407 was synthesized in the Division of Medical Chemistry, Center for Drug Research, Vrije Universiteit [22] and generously provided by Prof. Timmerman for these studies. Zinc chloride and zinc sulphate were of the highest analytical grade and were obtained from Reanal Finechemical. All other chemicals were purchased from commercial sources and were of the highest purity available.

2.2. Cell preparation

2.2.1. Primary cultures

Primary cultures of astroglial cells from the forebrain of postnatal (P_0-P_6) Wistar CFY Long Evans rats were made as previously described [19]. Briefly; after removal of the meninges and blood vessels, forebrain tissue of newborn rats was chopped and the pieces were incubated in 0.05% w/v trypsin (in a phosphate buffered saline) for 10 min. The enzyme solution was aspirated and the pre-digested tissue was triturated in Eagle's MEM containing 10% v/v FCS. Single cells were separated from non-dissociated tissue fragments by sieving through a nylon mesh (45-μm pore diameter). The cells were counted and seeded onto Petri dishes (100-mm diameter) coated with poly-L-lysine or onto 24 well plates, at cell densities of 10^5 cell/well. The cells were grown in MEM supplemented with 10% FCS; glutamine (4 mM) and gentamycin (40 μg/mL). After plating, in the first two days, fungizone (Amphotericin-B: 0.5 μg/mL) was added to the medium. Cells were grown at 100% humidity, 5% CO_2, 95% air at 37° and the media changed twice a week. The cell composition of the cultures was checked by immunocytochemistry: astroglial cells with GFAP, oligodendrocytes with MBP and microglia with anti-Fc-receptor antibody or with 4B isolectin. Cultures at 19–21 days old, composed of predominantly astroglial cells (were 95–98% positive for GFAP immunostaining and contained no neurons) were used for experiments. Under the conditions described, microglia could not be identified and oligodendrocytes appeared only incidentally (<2%). Astroglial cultures did not contain endothelial cells which was verified by negative F VIII staining.

Cultivation of endothelial cells from the isolated cerebral microvessels of 2-week-old rats were carried out as previously [19,23]. Briefly, minced cortical tissue was first incubated with 0.1% collagenase-dispase enzyme mixture for 1.5 hr, then the microvessels were collected by a centrifugation through 25% w/v bovine serum albumin in the medium. The washed pellet was further digested in collagenase-dispase solution for an additional 1.5 hr. Endothelial cells were separated by a centrifugation step on a 35% w/v continuous Percoll gradient. The collected and washed cells were seeded onto collagen-coated plastic dishes. The cultures were maintained in a Dulbecco's modified Eagle's medium supplemented with antibiotics, FCS (20% v/v), glutamine (2 mM), and N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (10 mM). Spindle shaped, tightly attached cerebral endothelial cells in cultures (grew to con-

fluency and formed a monolayer at the 14th day) were used for the experiments. Cells gave positive immuno-histochemical staining with anti-Factor VIII antibody (that is a general marker for endothelial cells) and bound the galactose specific BSL I B4 isolectin, considered to be a marker for rodent cerebral endothelium.

2.2.2. Dissociated brain cells

For *in vivo* experiments a fraction of dissociated hypothalamic and/or cerebellar cells of rats was prepared as described previously [24]. Briefly; brains were removed, cleared from meninges, the brain parts, cerebellum, and hypothalamus were separated, sliced, and dissociated mechanically by using a glass homogenizer. The dissociated cells were passed through a nylon mesh (pore size 50 μm) and centrifuged in a refrigerated centrifuge (600 *g* for 10 min). The pellet was resuspended in a Krebs buffer (pH 7.4) containing 134 mM NaCl, 4.7 mM KCl, 4.5 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 11.5 mM glucose (previously saturated with 95% air and 5% CO_2) and aliquots (30–50 μg protein) of the suspensions were taken for the determination of HA uptake.

2.3. Membrane preparation

For membrane preparations, cultured astroglial and cerebral endothelial cells were collected from the dishes, washed with 1–2 mL of cold PBS by centrifugation (2000 *g* for 10 min at 4°), re-suspended (at least twice) and finally the cells were re-suspended for obtaining membrane in a "binding medium" containing 1.2 mM $CaCl_2$ and 1.2 mM $MgCl_2$ in 50 mM sodium phosphate buffer (pH 7.4) before being stored in a frozen state at −20°. Plasma membranes were obtained from the frozen cells after repeated thawings and freezings (at least four times) and by centrifugation (2000 *g* for 10 min at 4°) and re-centrifugation of the supernatant (50,000 *g* for 30 min at 4°). The resulting pellet (membranes) was suspended in a "binding medium" (0.5–1.0 mg protein/mL) and kept at −20° until used.

2.4. [^{3}H]HA uptake

[^{3}H]HA uptake measurements were carried out as previously described [19]. Briefly; after removing the growth medium from the cultures and washing with 1–2 mL Krebs buffered salt medium containing 134.5 mM NaCl, 4.7 mM KCl, 4.5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 11.5 mM glucose (previously saturated with 95% air and 5% CO_2) the cells were carefully scraped into the above medium, centrifuged (600 *g* for 10 min) and then taken up into the incubation medium (prewarmed Krebs buffered salt medium; pH 7.4) and aliquots (50–100 μL) of the cell suspension (30–80 μg protein), were pre-incubated in the medium (total volume 480 μL), at 37° for 15 min. The assay was started by the addition of 20 μL of [^{3}H]HA (0.1–0.2 μCi) containing unlabelled amine (See in the text; routinely: 0.04 μM) and the cells, in tubes plugged with rubber stoppers, were incubated further at 37° for 3–15 min. The incubation was terminated by adding 1 mL of ice-cold Krebs buffered salt medium and rapid removal of the medium by centrifugation. After several (at least three) washings, cells were de-proteinized by adding 400 μL of trichloroacetic acid to the pellets, left to stand overnight, centrifuged (2000 *g* for 10 min) and aliquots were taken from the supernatants for scintillation counting. The protein content was determined by the method of Lowry *et al.* [25]. Compounds tested, were pre-incubated with the cells for 15 min before the assay. Assays, carried out under similar experimental conditions but at 4° were used as blanks and subtracted from the experimental values obtained at 37°. Blank values were from 10–20% of the experimental values and they were done in parallel with the experiments, usually carried out in duplicates and replicated at least three times with different cultures.

2.5. [^{3}H]HA binding assay

[^{3}H]HA binding to the astroglial HA carrier was measured in plasma membrane preparations as described [19]. Aliquots of the membrane suspension (30–60 μg protein) were incubated at 37° for 30 min in duplicate with 0.01–0.04 μM [^{3}H]HA (0.2–0.5 μCi) in 500 μL of binding medium containing 1.2 mM Ca^{2+}, 1.2 mM Mg^{2+} and a receptor antagonist cocktail, (tripolidine, cimetidine, and thioperamide; 40 μM each) in a 50 mM sodium phosphate buffer (pH 7.4). The reaction was terminated by the addition of 1 mL of cold buffer and centrifugation at 2000 *g* for 20 min at 4°. The supernatant was aspirated and the pellet resuspended in 2 mL of ice-cold buffer and centrifugation at 20,000 *g* for 10 min. Finally, 0.4 mL of 10% trichloroacetic acid was added to the pellet and allowed to stand overnight. Radioactivity was measured from an aliquot (350–400 μL) of the supernatant obtained after a centrifugation at 3000 *g* for 10 min. The protein content of the pellet was determined simultaneously by using the method of Lowry *et al.* [25]. Specific binding was defined as the difference between the binding of [^{3}H]HA in the presence and absence of 40 μM unlabeled HA. The effect of the zinc ion was tested using concentrations of zinc sulphate ranging between 50–100 μM.

2.6. Calculation

2.6.1. Calculations of free zinc and HA concentrations

Calculations were carried out by standard equilibrium calculation methods, based upon themodynamic constants in the Critical Stability Constants [26].

2.6.2. Statistical Analysis

Data were presented as the mean ± SEM of 3–5 individual experiments with at least 2–2 parallel aliquots prepared from individual cultures. Significant differences between groups

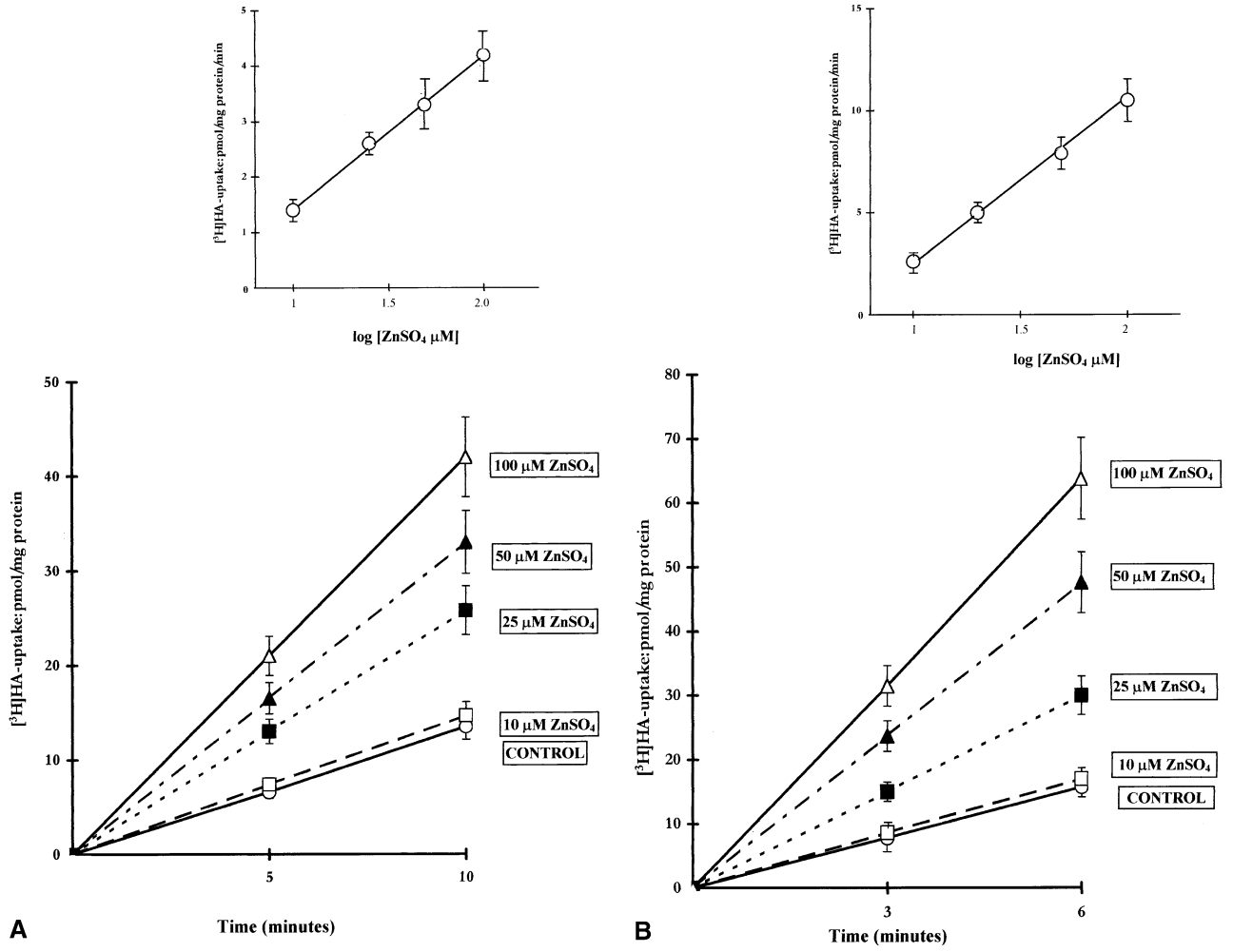

Fig. 1. Effect of zinc ion on [^{3}H]HA uptake by cultured astroglial (A) and cerebral endothelial cells (B). Uptake measurements were carried out with cultured cells and 0.04 μM [^{3}H]HA in a buffered Krebs medium after 15-min pre-incubations followed by 5–10 min incubations in the absence and the presence of various (10–100 μM) concentrations of zinc sulphate as described in Methods. In these batches of cultures, the mean value of the control rate was 1.32 $\pm$ 0.12 pmol/mg protein/min for astroglial cells (A) and 2.6 $\pm$ 18 pmol/mg protein/min for cerebral endothelial cells (B) respective to 0.04 μM HA. Data points represent mean values of four individual experiments with duplicates (n = 8). SEM is indicated with vertical bars. In the range of 25–100 μM, the zinc-enhanced uptake differed from control significantly ($P < 0.001$). Inset: Concentration dependency of zinc-enhanced [^{3}H]HA uptake: uptake rates vs. log [$ZnSO_4$].

were determined by analysis of variance (one-way ANOVA) followed by Dunnett's *t*-test.

The kinetic parameters of the uptake (K_M and V_{max}) were determined by using the Eadie–Hofstee equation; while the binding parameters, the binding capacity (B_{max}) and the dissociation constant (K_D) were determined by the Scatchard analysis. The untransformed data were analyzed by using linear and/or non-linear least-square curve fitting procedures [27].

3. Results

3.1. Effects of zinc on cultured astroglial and cerebral endothelial cells

Similarly to our preliminary data [20], zinc displayed a dose-dependent enhancing effect in concentrations of 10–100 μM on [^{3}H]HA uptake by cultured astroglial cells (Fig. 1A) and cerebral endothelial cells (Fig. 1B). Zn^{2+} produced similar effects on both cell types but with somewhat higher efficiency on the later cells.

To characterize the effect of zinc ion, we followed the kinetics of control and zinc-enhanced HA uptake within a wide range of HA concentrations (0.02–10.00 μM). Within this range, the Eadie–Hofstee analysis of the data revealed two components for HA uptake by cultured astroglial cells (inset Fig. 2A and B); but we concentrated for the high-affinity range (0.02–0.40 μM) in this work and estimated the kinetic parameters of the high-affinity uptake by linear regression of the plots (Fig. 2 panel). With 100 μM Zn^{2+}, an approximate 3-fold increase in the V_{max} was observed without a significant change in the K_M. More precisely, the V_{max} increased from 3.25 $\pm$ 0.42 pmol/mg protein/min

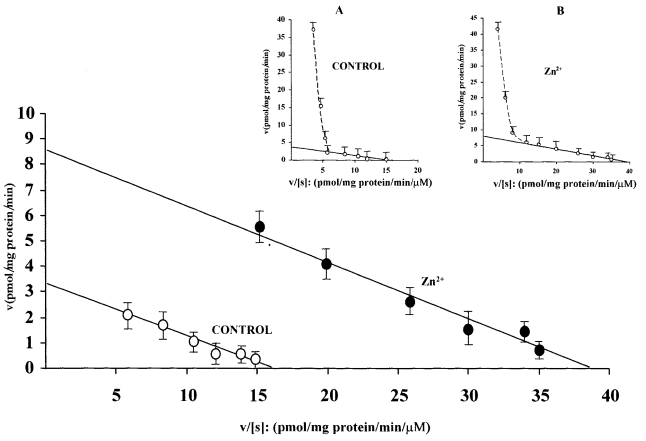

Fig. 2. Kinetic characteristics of the zinc effect on astroglial HA uptake. Inset A and B: [^{3}H]HA uptake (in the concentration range of 0.02–10.00 μM) was determined in the absence and the presence of 100 μM zinc sulphate, after a 15-min pre-incubation followed by 5-min incubation, at 37° as described in Methods. The uptake rates (v) (expressed in pmoles/mg protein/min) were plotted against v/S according to the Eadie–Hofstee equation. Panel: Kinetic parameters, K_M and V_{max} values were calculated by linear regression of the Eadie–Hofstee plots of the high affinity uptake. Data points represent mean values of five individual experiments with duplicates ($n = 10$) for control and three individual measurements with duplicates ($n = 6$) for zinc.

(range: 2.7–4.1 pmol/mg protein/min) ($n = 10$) to 8.50 ± 0.97 pmol/mg protein/min (range: 7.6-l0.8 pmol/mg protein/min) ($n = 6$) and this difference was highly significant ($P < 0.001$). The values obtained for K_M were 0.20 ± 0.02 μM (range: 0.15–0.25 μM) and 0.25 ± 0.018 μM (range: 0.19–0.35 μM). The variations in K_M values were insignificant ($P < 0.2$).

In order to quantitate the population of HA in its various protonated and Zn^{2+}-complexed forms, (to establish whether the free zinc ion can bind to [^{3}H]HA directly under conditions described in Section 2), calculations, based on association constants, were carried out.

The successive formation constants of the various species are as follows: $\log K_{1_{H(HA)^+}} = 9.81$, $\log K_{2_{H_2(HA)^{2+}}} = 6.07$, $\log K_{1_{Zn(HA)^{2+}}} = 5.17$, $\log K_{2_{Zn(HA)_2^{2+}}} = 4.56$, where the constants in log K units refer to the formation of the species shown in the respective subscript. Taking 100 μM total Zn^{2+} and 0.05 μM total HA concentrations, the concentrations of free Zn^{2+} HA and Zn-HA complexes were calculated, and are listed as a function of pH near the physiological region, in Table 1.

Table 1
Concentrations as function of pH

PH	[HA]	[H(HA)$^+$]	[H$_2$(HA)$^{2+}$]	[Zn(HA)$^{2+}$]	[Zn(HA)2^{2+}]
7.2	1.10E − 10*	4.50E − 08	3.33E − 09	1.60E − 09	6.41E − 15
7.3	1.39E − 10	4.52E − 08	2.66E − 09	2.01E − 09	1.02E − 14
7.4	1.75E − 10	4.52E − 08	2.11E − 09	2.51E − 09	1.60E − 14
7.5	2.20E − 10	4.50E − 08	1.67E − 09	3.11E − 09	2.49E − 14
7.6	2.74E − 10	4.46E − 08	1.32E − 09	3.82E − 09	3.82E − 14

* See text for abbreviations and total concentrations.

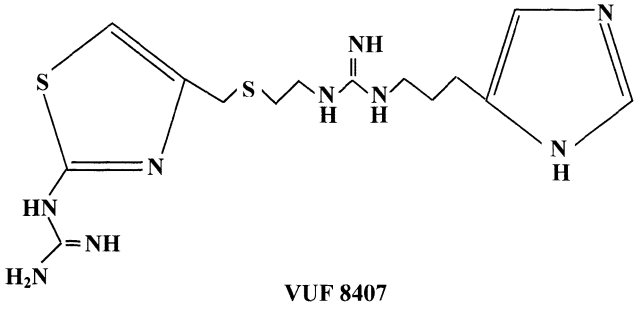

Fig. 3. Chemical structure of the compound VUF 8407: Guanidine imidazole thiazole tripicrate.

The concentration values clearly indicate that 95% of the HA is free of Zn^{2+} at pH 7.4, despite the fact that the total Zn^{2+} concentration exceeds that of HA, by a factor of 2000. Calculations in a wider pH range show that the zinc-complexed HA reaches its highest % concentration at pH 8.8, with a value of slightly above 20%. This fact can be interpreted in terms of the proton- and zinc-binding constants of HA, where H^+ and Zn^{2+} are Lewis acids, competing for the electron pair-donating nitrogen(s) of HA, a bidentate Lewis base. The stability constants indicate that HA prefers proton over zinc by nearly five orders of magnitude (compare log K_1, for $H(HA)^+$ and $Zn(HA)^{2+}$ in the first association step), and to a lesser extent, in other forms as well. All these indicate that HA does not chelate Zn^{2+} under conditions described.

To test the characteristics of basic and the zinc-enhanced uptake of HA, we investigated the effect of VUF 8407 (Fig. 3), a structure analog of impromidine, the control substance, which has been described as a potent agonist of the H_2, subclass and antagonist of the H_3 subclass of HA receptors [22], revealing remarkable inhibitory effect on the astroglial HA uptake [18,24]. VUF 8407 showed potent inhibitory effect on the basic uptake of HA in both cell types (I_{50} values detected as 20 μM for astroglial and 15 μM for cerebral endothelial uptake of HA); and could reverse the zinc-induced enhancement in [^{3}H]HA uptake by inhibiting the total uptake by about 50% (Fig. 4AB; Table 2).

The effect of zinc on [^{3}H]HA-binding was assessed by measuring total and non-specific binding to the HA carrier of cell membranes in the absence and the presence of zinc sulphate (100 μM). Analysis of the data indicated that zinc treatment enhanced specific binding of [^{3}H]HA (determined in the presence of HA receptor antagonists) but did not affect the non-specific binding (determined in the presence of 40 μM HA) (Fig. 5A and B Inset).

The Scatchard analysis revealed a considerable increased density of binding sites (B_{max}) and a statistically unchanged apparent affinity ($1/K_D$) in the presence of 100 μM zinc ion. In astroglial membranes, the K_D ranged from 27 to 46 nM with a mean value of 35.2 ± 3.4 ($n = 6$), whereas the B_{max} ranged from 0.32 to 0.52 pmol/mg protein with a mean value of 0.41 ± 0.05 ($n = 6$). In the zinc-treated membranes

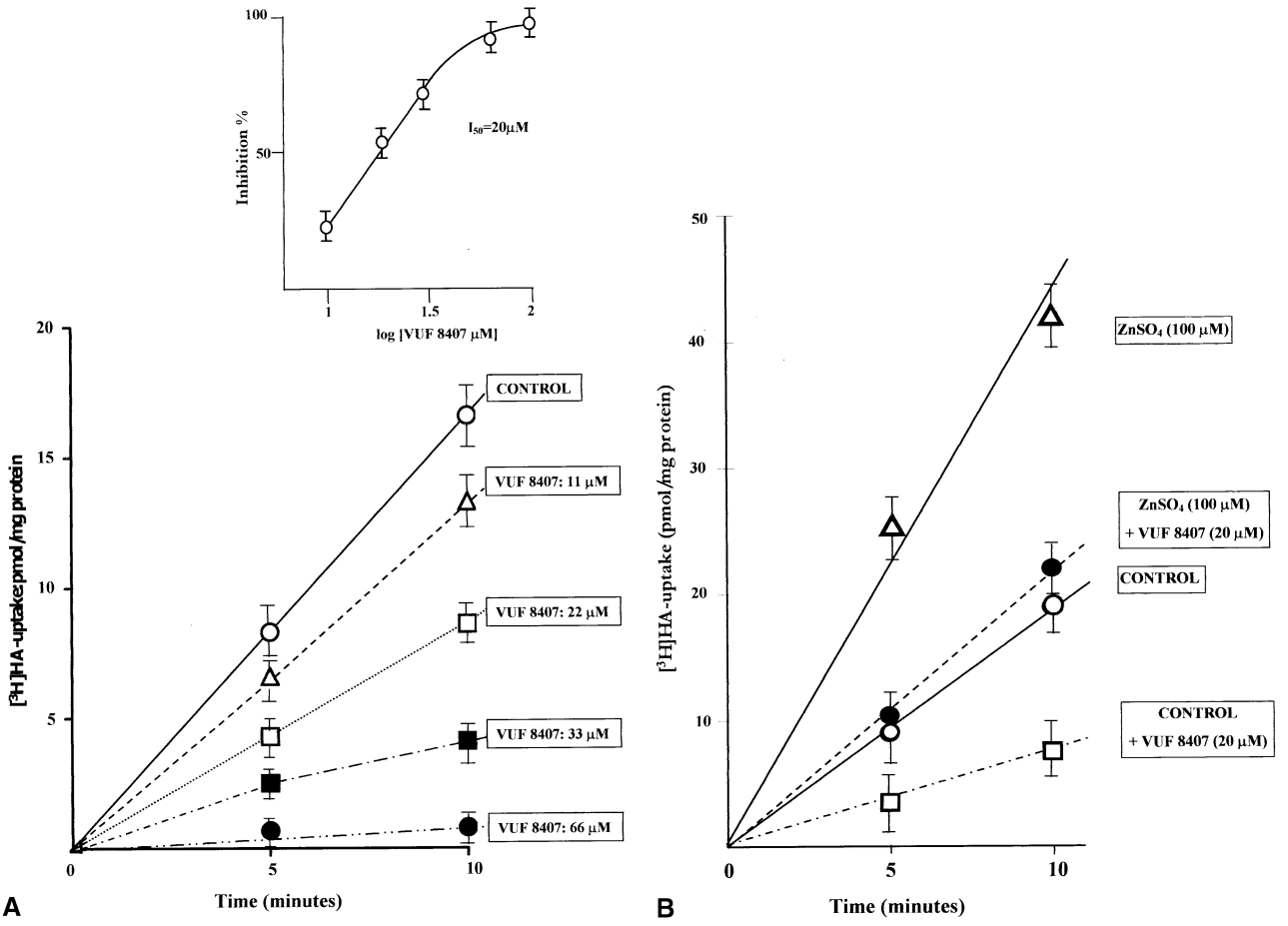

Fig. 4. Effect of VUF 8407 on basic (A) and zinc-enhanced [^{3}H]HA uptake (B) by astroglial cells. Uptake experiments were carried out on cultured cerebral endothelial cells with 0.04 μM [^{3}H]HA in the absence and the presence of 100 μM zinc sulfate after 15 min pre-incubations followed by 5–10 min incubations at 37° with or without 20 μM VUF 8407 as described in Methods. Inset: Inhibition curve of VUF 8407 on astroglial [^{3}H]HA uptake under control conditions. Respective to these batches of cultures, the control rate was 1.68 ± 0.14 pmol/mg protein/min. Data points of the panel represent mean values of three individual experiments with duplicates (n = 6). SEM are indicated with vertical bars. Differences between control and zinc-induced uptake and between VUF 8407-treated, and non-treated zinc-exposed uptake were highly significant ($P < 0.001$).

the apparent K_D and B_{max} ranged within the same magnitude, yielding a mean value of 45.1 ± 3.8 nM (n = 6) for K_D (range: 32–55 nM) and 1.21 ± 0.16 for B_{max} (range: 0.98–1.35 pmol/mg protein). In conformity with uptake kinetics, the difference between control and zinc-treated B_{max} was highly significant ($P < 0.001$); whereas the variations in the K_D were statistically insignificant ($P < 0.2$).

Table 2
Effect of VUF 8407 on basic and zinc-enhanced HA uptake by cultured cerebral endothelial cells

Experimental condition	v: pmol/mg protein/min			
	Basic intake		Zinc-enhanced uptake	
		Inhibition %		Inhibition %
None	2.60 ± 0.30[a]	—	7.90 ± 0.68	—
VUF 8407 (20 μM)	1.15 ± 0.12[b]	56	4.10 ± 0.35[c]	48

Uptake experiments were carried out on cultured cerebral endothelial cells with 0.04 μM [^{3}H]HA in the absence and presence of 50 μM zinc sulphate after a 15-min pre-incubation followed by 3–6 min incubation at 37°C with or without 20 μM VUF 8407.

[a] Mean ± SE.

[b,c] Inhibitory effects, compared to basic and zinc-enhanced uptake rates, were highly significant ($P < 0.001$).

The analysis of the zinc effect on [^{3}H]HA binding revealed specificity for VUF 8407 to decrease [^{3}H]HA binding, (B_{max}) without significant modification of the affinity for uptake carrier ($1/K_D$); resulting in an apparent K_D = 54 ± 5.2 nM (n = 6) (range: 47–65 nM) and a B_{max} = 0.65 ± 0.072 pmol/mg protein (range: 0.46–0.72 pmol/mg protein) (Fig. 5A). The decrease in B_{max} compared to the zinc enhanced control was statistically significant ($P < 0.01$) whereas the variations detected in K_D values of VUF 8407 treated or non-treated zinc-exposed membranes were statistically insignificant ($P < 0.2$). The possibility that VUF 8407 just competes with HA for complex formation with Zn^{2+}, can be ruled out because HA does not chelate Zn^{2+} under these conditions; moreover it showed potent,

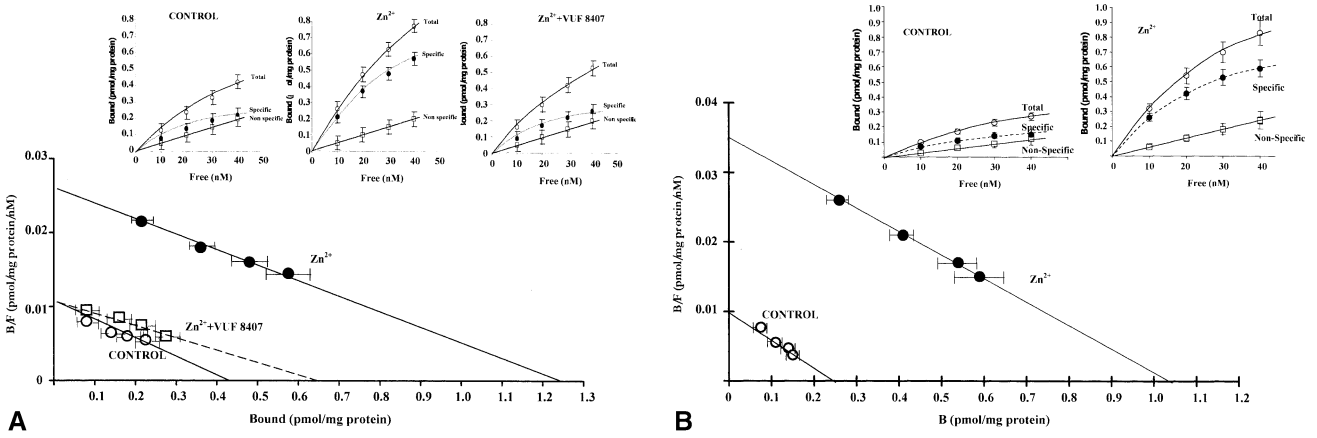

Fig. 5. (A) Effect of zinc ion on the [^{3}H]HA binding in astroglial plasma membranes and the effect of VUF 8407 on the zinc-enhanced [^{3}H]HA binding. Inset: Saturation curves of HA binding in equilibrium (30 min incubation at 37°); total (○), non-specific (□) and specific (●) binding of [^{3}H]HA under control condition and in the presence of zinc sulfate (100 μM) or zinc sulphate (100 μM) + VUF 8407 (20 μM). Binding-measurements, carried out as described in Methods, represent three experiments with duplicates (mean ± SEM). Lines were fitted by linear and non-linear regressions. Panel: Scatchard plots of the above data (SEM are indicated with error bars in x axis): Kinetics of control and zinc-enhanced [^{3}H]HA binding to uptake carrier in the absence and the presence of VUF 8407. Lines were fitted by linear regression and had correlation coefficients of 0.90–0.95. (B) Effect of zinc ion on [^{3}H]HA binding in cerebral endothelial plasma membranes.Inset: Saturation curves of HA binding in equilibrium (30-min incubations at 37°); total (○), non-specific (□), and specific (●) binding of [^{3}H]HA under control condition and in the presence of zinc sulfate (100 μM). Data represent three individual measurements with duplicates, in membranes from one batch of cerebral endothelial cells (mean values ± SEM). Lines were fitted by linear and non-linear regressions. Panel: Scatchard plot of the above data (SEM are indicated with error bars in x axis): Kinetics of [^{3}H]HA binding to the uptake carrier in the absence and the presence of zinc sulfate (l00 μM). Lines were fitted by linear regressions and had a correlation coefficient of 0.90.

closely equal inhibiting potency on both basic and zinc-induced HA uptake.

The analysis of [^{3}H]HA binding in cerebral endothelial cell membranes (Fig. 5B) revealed similar effect, namely, increased apparent B_{max} (from 0.25 ± 0.03 pmol/mg protein increased to 1.05 ± 0.12 pmol/mg protein; and this increase was statistically significant $P < 0.01$) and no change in the apparent K_D (K_D values calculated as 25 ± 2.1 nM for control and 30 ± 2.6 nM for zinc-enhanced bindings did not differ statistically; $P < 0.2$). The apparent K_D and B_{max} values ranged within the same magnitude and the data of different cell types, were within the same range. Binding capacities of the different batches of cultures varied in some extents.

3.2. Effect of zinc on HA uptake ex vivo

The uptake of [^{3}H]HA into dissociated cells of hypothalamic and cerebellar fractions of the brain was measured 24 h after injecting of 0.4 μmol/kg i.c.v. dose of zinc sulphate to male rats under ether anesthesia (Table 3). In zinc-treated animals, [^{3}H]HA, taken up into the dissociated hypothalamic cells, under conditions described, increased to 200% above the controls, whereas in the cerebellum, this increase went up to 292%, suggesting considerable effectiveness of zinc ions on HA uptake in both brain parts.

4. Discussion

Present studies provide an evidence that distinct amounts of zinc ion acting directly on astroglial and cerebral endothelial cell membranes can stimulate HA uptake into these cells. Zinc ion produced marked enhancement in the uptake rates by increasing the apparent V_{max} without affecting the ligand affinity (apparent K_M). In the range of 0.02–0.40 μM, kinetics confirmed carrier-mediated transport of HA both in the absence and presence of zinc ion. The mechanism by which zinc ion affects HA uptake is uncertain but it cannot be a simple aspecific aggregation of the precipitated protein

Table 3
[^{3}H]HA uptake ex vivo

	[^{3}H]HA uptake (pmol/mg protein/min)	
	Hypothalamus	Cerebellum
Control	2.0 ± 0.28[a]	1.1 ± 0.16
Zinc sulphate[a]	4.2 ± 0.45[b]	3.1 ± 0.35[c]

0.04 μmol $ZnSO_4$ (in 10 μL, physiological saline) was injected into 100-g male rates ($n = 5$) i.c.v. under ether anesthesia, and the animals were killed 24 hr after the injection. (The animals were cared and treated according to the principles presented in the "Guidelines for the use of Animals in Nueroscience Research"). Controls ($n = 5$) received 10 μL physiological saline i.c.v. and they were killed at the same time. The rats were given only water for the next 24 hr, whereupon they were killed. After decapitation, the brain was immediately removed, the hypothalamus and the cerebellum were separated and dissected, and the cells from the brain fractions dissociated mechanically (as described in Methods), and were used for the *ex vivo* uptake measurements. [^{3}H]HA uptake measurements were carried out in buffered Krebs medium (500 μL) with 0.04 μM tritiated amine and 50-μL aliquots (30–50 μg of protein) of dissociated cell fractions, after 15-min preincubation followed by 5–10-min incubation, at 37°C as described.

[a] Mean ± SE.

[b,c] The effects of zinc, compared to control, was highly significant ($P < 0.001$; $n = 6$).

because Zn^{2+} did not increase the aspecific binding of HA (see insets of Fig. 5A and B). Moreover, we consider the possibility of a binding site for Zn^{2+} on HA carrier.

We considered the possibility that it might be a Zn^{2+}-HA complex that modulates the operation of the carrier rather than the free zinc but our calculations concerning the free concentrations of HA and zinc, ruled out that HA would chelate zinc under conditions described and a Zn^{2+}-HA complex might be responsible for the increased capacity (V_{max}) of HA uptake. One could, however, expect an enhanced Zn^{2+}-HA complex formation at high pH where the hydrogen ion concentration drops and becomes concomitantly less competitive. The lack of enhanced zinc-HA complex formation even at higher pH is due to the hydroxide-complex formation of Zn^{2+}, which dramatically decreases the viability of Zn^{2+} in the competition for the binding nitrogen(s) of HA.

Because of the effectiveness of mercuric ion by enhancing astroglial and cerebral endothelial HA uptake by interacting with the carrier thiols [19], we considered that zinc might create similar bindings with carrier thiols. The relative insensitivity of the zinc-enhanced HA uptake for the sulfhydryl agent NEM (showing only a slight decrease in the ligand affinity and no change in the maximal velocity of the zinc-induced HA uptake after NEM treatments), however, questioned such interactions between zinc and the carrier thiols. In spite of it, this possibility is not excluded; the relative insensitivity for NEM may simply reflect a steric hindrance of the access of the compound to the critical SH group. Anyway, it is noteworthy that the effect of the zinc ion on HA binding sites was much more specific than that of mercuric ion, since zinc ion did not affect the non-specific binding of the amine (see insets of Fig. 5A and B).

VUF 8407, the structure analog of the previously described potent inhibitor of the astroglial HA uptake, impromidine [18,23] showed inhibiting potency on HA uptake. Its effectiveness in inhibiting both basic and zinc-enhanced uptake of the amine by astroglial and cerebral endothelial cells, was suggestive of common recognition sites for the amine and the compound in both cell membranes (carriers). VUF 8407 showed somewhat (~10 times) less inhibiting activity on HA uptake than that of impromidine [18,24], but closely equal stimulating/inhibiting activities on the subclasses of HA receptors [22]. Thus, both compounds may belong to a new class of substances that reveal remarkable affinities for both the uptake carrier and the subclasses (H_2 and H_3) of the receptors of HA. Such compounds are expected to increase the activity and level of HA in the extraneuronal space and might be useful (much more than compounds bearing only H_3 antagonistic activity) for the therapy of narcolepsy and dementia [28]. Yet, the recognition sites on HA receptors do not appear to be involved in HA uptake, because H_2 -receptor agonists, including dimaprit, or H_3 -receptor antagonists, including thioperamide, did not generally influence the uptake of HA (data not shown).

As the [^{3}H]HA binding to the uptake carrier is an integral part of the [^{3}H]HA uptake, we focused our interpretation on both uptake and binding measurements in the presence of zinc ion. Zinc sulfate that had been identified as a stimulator of [^{3}H]HA uptake was effective in enhancing the capacity (B_{max}) of the binding sites for HA in both astroglial and cerebral endothelial membranes without affecting its affinity (K_D) for the ligand. The analysis of the enhancing effect of zinc revealed specificity for HA carrier in both cell types. The compound VUF 8407 was found to decrease the enhanced capacity of HA binding in astroglial membranes such as the basic and zinc-enhanced uptake of HA in cultured astroglial cells (20 μM VUF 8407 produced an about 50% reduction in the enhanced capacity of binding which was identical with its IC_{50} value calculated for the astroglial uptake of HA).

If we extrapolate our findings from in vitro studies to the physiological situation *in vivo*, this might lead to a speculation, namely that zinc has a regulatory role in HA inactivation.

The following questions might, however, arise: 1) Is this enhancing effect of zinc operative in situ? 2) Is zinc in valuable concentrations within or nearby HA-ergic neurones? 3) Is this enhancing effect of zinc operative under pathological conditions and would it be helpful?

Under normal conditions, HA uptake processes, at least the high-affinity HA uptake into astroglial cells [29] appeared to be operative in the brain. Current *ex vivo* data were insufficient to distinguish between neuronal and non-neuronal uptake of the amine or to give the complete character of the uptake, but it clearly showed the effectiveness of zinc ion on HA uptake by dissociated brain cells.

The presence of zinc within soluble matrix of secretory vesicles raised the possibility that zinc is released into the extracellular space during exocytosis [30, 31]. Glutamatergic nerve terminals of the hippocampal mossy fibers and the nerve endings of the neurones in the cortex have been demonstrated to contain zinc in high concentrations and that zinc is co-released with glutamate from the presynaptic nerve terminal [21]. Moreover, the released metal ion may modulate both the uptake and the release of the amino acid by astroglial cells [32] and the activity of the post-synaptic NMDA-receptor [33]. Thus, zinc ion might be an important endogenous modulator of the post-synaptic response to the co-released transmitter glutamate.

HA-immunoreactive fibers and nerve terminals originating from the tuberomammilary nucleus, have been observed in almost all major regions of the brain including different hypothalamic nuclei (most numerous), the cerebral cortex and the basal parts of the hippocampus [2,34]. It is not yet known, whether HA-ergic neurones contain soluble zinc, which might be released (probably with HA) from the neurones and could accelerate the removal of the amine from the extracellular space. However, we might consider that endogenous zinc, can reach astroglial cells nearby HA-ergic neurones and might modulate HA-ergic neurotrans-

mission under normal and pathological conditions. Moreover, the exogenous zinc that had been demonstrated to affect the neuronal activity by modulating GABA-ergic [35] and the NMDA-activated glutamatergic neurotransmission [36], might affect the activity of HA-erg neurones. Zinc ion might be expected to enhance HA uptake into astroglial and cerebral endothelial cells and facilitate the removal of the amine from the interstitial space serving a temporary protection from a long lasting (non-desirable) effect of the amine via its own receptors. In experimental models of cerebral ischemia, marked enhancements in the extracellular HA levels, accompanied with increased vascular permeability and oedema formation have been estimated [37,38]. These non-desirable effects appeared to attribute to the elevated extracellular level of HA [38,39]. It is apparent that the concentration of free zinc would be critical in the regulation of extracellular HA level especially if it is extremely high, such as in the ischemic brain [37].

Large amounts of zinc (200–600 μM), and a long exposure, might result, however, in a widespread glial and neuronal injury in mature cells especially in the presence of high concentrations of sodium [40]. But this is not the case we assumed zinc (endogenous or exogenous) to modulate extracellular HA levels and the HA responses via various subclasses of HA receptors.

In conclusion, we showed that zinc enhances astroglial and cerebral endothelial high-affinity uptake of HA *in vitro* and we might consider that it affects also *in vivo*. Free endogenous or free exogenous zinc might be favored to facilitate HA removal from the interstitial space to maintain or to re-store the HA homeostasis, especially in the ischemic brain.

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