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In vitro Effect of *Ginkgo biloba* Extract (EGb 761) on the Activity of Presynaptic Cholinergic Nerve Terminals in Rat Hippocampus

Key Words
Ginkgo biloba
High-affinity choline uptake
[^{3}H]Hemicholinium-3
Nootropic drugs
Arachidonic acid

Abstract
The effects of *Ginkgo biloba* extract (EGb) applied in vitro to hippocampal synaptosomes from young Wistar rats on the specific binding of [^{3}H]hemicholinium-3 ([^{3}H]HCh-3), high-affinity choline uptake (HACU) and activity of Na^+,K^+-ATPase were examined. EGb at a concentration of 100 μg/ml markedly elevated the specific binding of [^{3}H]HCh-3 (to 306%) and moderately elevated HACU values (to 115%). Scatchard analysis revealed an increase in the B_{max} for [^{3}H]HCh-3 binding, Lineweaver-Burk analysis an increase in the V_{max} for choline uptake. No marked changes in the activity of the sodium pump were discovered. EGb was not able to influence the specific 'second messenger' effect of arachidonic acid.

Introduction

The Na^+-dependent, high-affinity choline uptake (HACU) system is perhaps located selectively on presynaptic cholinergic nerve terminals and subserves acetylcholine synthesis as one of the rate-limiting steps [1]. Measurement of the HACU values in vitro on hippocampal synaptosomes can be used as a marker of the activity of septohippocampal cholinergic nerve terminals in vivo [2]. Several authors have reported significant alterations in animal hippocampal HACU after intensive memory testing. It seems that short-term working memory tasks result in a long-lasting increase [3, 4], whereas long-term reference memory tasks result in an acute increase [4] followed by a secondary decrease and long-lasting inhibition [4–6]. In vivo administration of many cholinergic as well as noncholinergic drugs changes hippocampal HACU levels. For example, the cholinergic antagonist scopolamine increases acetylcholine release and HACU values but causes a decrease in acetylcholine levels and subsequent amnesia [2]. On the other hand, the cholinergic agonist oxotremorine reduces HACU levels [2]. Anesthetics, hypnotics or sedatives have generally been found to depress [2, 7] and some convulsants to stimulate [2, 7, 8] hippocampal HACU.

Nootropics, a new class of psychotropic drugs, are characterized by a positive regulatory effect on impairment of noetic functions (e.g., disorders in vigilance, orientation, comprehension, cognitive performance, memory) and are used in the treatment of senile dementias including Alzheimer disease. The class of nootropics involves drugs of various chemical structures and of diverse mechanisms of action [for review, see 9]. It seems very likely that the central cholinergic nervous system plays a crucial role in the memory and learning processes [10] and that the deterioration of cognitive functions in Alzheimer disease is directly related to the degeneration of basal forebrain cholinergic neurons [11]. Consequently,

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nootropic drugs are expected to activate the function of central cholinergic neurons. However, commonly used nootropics are characterized by a different effect on the hippocampal HACU levels, i.e. a marker of the activity of presynaptic septohippocampal cholinergic nerve terminals. A significant increase (e.g., 3,4-diaminopyridine, pramiracetam, oxiracetam, pyritinol, ginsenoside Rb_1 [12–14]), no change (e.g., piracetam, aniracetam, 4-aminopyridine [12, 13]) and even a decrease of HACU levels after in vivo administration (centrophenoxine, tacrine [12, 15]) have been reported. Cognition-enhancing properties of nootropic drugs with a direct effect on presynaptic cholinergic terminals (i.e. changes after in vivo as well as in vitro administration) can consist in an increased availability of acetylcholine. No simple relation between the efficiency of cholinergic nootropics and HACU levels has been found.

In our laboratory, we have tested *Ginkgo biloba* extract (EGb), a standardized extract of the leaves of a subtropical tree, containing specific flavonoidglycosides and terpenoides. We have reported that the new nootropic drug EGb significantly increases HACU levels estimated by means of [^{3}H]choline in hippocampal synaptosomes of 24-month-old Wistar rats after long-term administration (50 mg/kg/day for 30 days in drinking water, experiment in vivo) and after direct application of drug to synaptosomes (15–30 μg/ml, i.e. 50–100 μg/mg protein, experiment in vitro) [15]. Taylor [16] has described an increase of muscarinic receptor population in the hippocamus of 24-month-old Fisher 344 rats after chronic administration of EGb (100 mg/kg/day for 28 days) in drinking water. And finally, Rapin et al. [17] have reported an increase of acetylcholine release in the hippocampus of 4-month-old rats after acute administration of EGb (100 mg/kg i.p.) and in the frontal cortex, hippocampus and corpus striatum after chronic treatment (100 mg/kg/day p.o. for 21 days). Although the beneficial EGb effect is due to the combination of its various protective, curative and modulating properties against the pathological process [18], the positive effect on the cholinergic nervous system could contribute in part to its therapeutic action.

The availability of [^{3}H]hemicholinium-3 ([^{3}H]HCh-3), a potent, selective and competitive inhibitor of HACU, has permitted the development of a ligand-binding technique for the detection of high-affinity choline carriers. Parallel estimation of HACU by means of [^{3}H]choline and [^{3}H]HCh-3, as well as the analysis of the choline uptake kinetics, facilitate the determination of whether the changes are influenced by a change in the transport velocity or by a change in the number of carriers. Our previous results [19, 20] have indicated that, during normal aging, Alzheimer disease and during oxidative impairment in vitro a diminished transport of choline rather than a decrease in the number of carriers is likely to occur in the human and rat brain tissue.

The aim of this study is to obtain more detailed information about the effect of EGb in vitro on the activity of presynaptic cholinergic nerve terminals in rat hippocampus and to contribute to a better elucidation of its nootropic effectivity.

Materials and Methods

Animals

Experiments were carried out on 40 male Wistar rats aged 3–4 months of the Konárovice breed housed under controlled conditions and kept on a standard pellet diet. Animals were decapitated, the hippocampi dissected on an ice-cooled plate, weighed and immediately used for the preparation of synaptosomal fraction.

Methods

Preparation of Synaptosomes. The hippocampi were homogenized in 10 volumes of 0.32 *M* sucrose, the homogenates were centrifuged at 1,000 *g* for 10 min and the supernatant fluids at 20,000 *g* for 20 min. The resulting pellets were resuspended in the same volume of 0.32 *M* sucrose and used for estimation of the [^{3}H]choline uptake, [^{3}H]HCh-3 binding and activity of sodium pump. Proteins were measured by the method of Bradford [21] with bovine serum albumin as a standard.

[^{3}H]Choline Uptake. Synaptosomal suspension was added to Krebs-Ringer-HEPES-glucose buffer (128 m*M* NaCl, 5 m*M* KCl, 2.7 m*M* $CaCl_2$, 1.2 m*M* $MgSO_4$, 5 m*M* glucose and 10 m*M* HEPES, pH = 7.4) and incubated for 4 min at 37°C with 10 n*M* [^{3}H]choline ([methyl-^{3}H]choline chloride, NEN). The incubation was terminated by rapid cooling and filtration under vacuum (Whatman BF/B filters). HACU was defined by its sensitivity to hemicholinium-3 (Sigma) and was calculated as the difference between the uptake in samples incubated without and with 1 μ*M* hemicholinium-3. In kinetic experiments the concentrations of choline varied from 0.01 to 1 μ*M*.

[^{3}H]HCh-3 Binding. Synaptosomal suspension was added to glycylglycine buffer (50 m*M* glycylglycine, 200 m*M* NaCl, pH = 7.8) and incubated for 30 min at 24°C with 20 n*M* [^{3}H]HCh-3 ([methyl-^{3}H]hemicholinium-3, diacetate salt, NEN). Parallel incubations in the presence of 10 μ*M* hemicholinium-3 were used to define the nonspecific binding. The reaction was terminated by filtration under vacuum on Whatman GF/B filters, presoaked in 0.15% (v/v) polyethylenimine [22]. Scatchard analysis was performed with increasing concentrations of [^{3}H]HCh-3 from 1 to 50 n*M*.

Activity of Na^+,K^+-ATPase. Synaptosomal suspension was added to the buffer (100 m*M* NaCl, 20 m*M* KCl, 5 m*M* $MgCl_2$, 20 m*M* TRIS, pH = 7.5) and incubated for 30 min at 37°C with 5 m*M* ATP (Sigma). Parallel incubation in the presence of 0.1 m*M* ouabain (Sigma) was used to measure the difference between total and Mg^{2+} ATPase activities. ATP hydrolysis was quantified according to Rousser et al. [23].

Table 1. Effect of EGb on the specific binding of [^{3}H]HCh-3

Experiment	Dose of EGb		n	Spec. binding of [^{3}H]HCh-3 fmol/mg prot.
	µg/ml	µg/mg prot.		
1	0	0	12	204.0 ± 71.6
	5	11.8	3	219.3 ± 111.1
	10	23.6	3	142.6 ± 19.7
	25	59.1	3	180.6 ± 53.6
ANOVA				$p = 0.5940$
2	0	0	6	154.6 ± 46.4
	50	118.2	6	232.0 ± 63.4*
	75	177.3	6	333.3 ± 119.0*
	100	236.4	6	473.5 ± 95.2***
ANOVA				$p < 0.001$

Statistical significance (t test) was calculated with respect to control samples (* $p < 0.05$; *** $p < 0.001$).

Experiment 1 was performed on mixed synaptosomes of 11 3-month-old rats, concentration of proteins equaled 0.423 mg/ml and concentration of [^{3}H]HCh-3 28.1 n*M*. EGb was first preincubated with synaptosomes for 10 min and then incubated with [^{3}H]HCh-3 for 30 min.

Experiment 2 was performed on mixed synaptosomes of 11 3-month-old rats, concentration of proteins equaled 0.423 mg/ml and concentration of [^{3}H]HCh-3 28.6 n*M*. EGb was first preincubated with synaptosomes for 10 min and then incubated with [^{3}H]HCh-3 for 30 min.

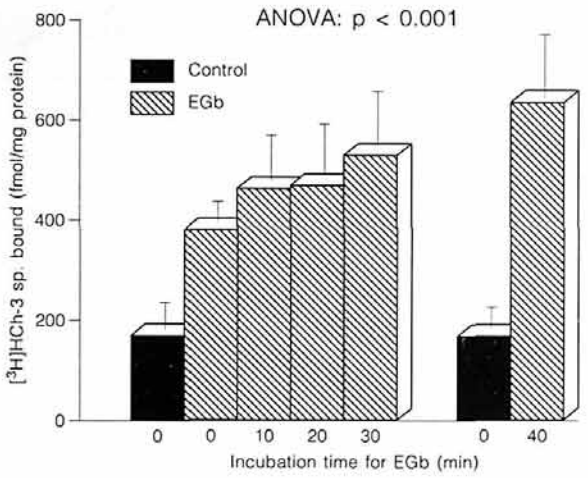

Fig. 1. Time dependency of the specific binding of [^{3}H]HCh-3 in the presence of 100 µg/ml of EGb. The experiment was performed on mixed synaptosomes of 11 3-month-old rats, concentration of proteins equaled 0.423 mg/ml, concentration of [^{3}H]HCh-3 21.3 n*M*. EGb was added at different time intervals to the synaptosomes incubated with [^{3}H]HCh-3 for 30 min (left bars) or preincubated with the synaptosomes for 10 min and then incubated with [^{3}H]HCh-3 for 30 min (right bars). Control samples of left bars were incubated for 30 min and of right bars for 40 min totally.

In vitro Test. Solutions of EGb (extrait de *Ginkgo biloba* – EGb 761, Beaufour Ipsen Industrie) in corresponding buffers and of AA (arachidonic acid, sodium salt, Sigma) in ethyl alcohol were prepared shortly before the experiments.

Data Analysis. Lineweaver-Burk and Scatchard plots were analyzed using least-squares linear regression analysis employing the BMDP 6D program. To determine statistical significance, ANOVA and Student's t test (BMDP 7D program) were applied [24]. Data in tables are presented as the means ± SD.

Results

Table 1 demonstrates the effect of different doses of EGb on the specific binding of [^{3}H]HCh-3. 50–100 µg/ml of EGb significantly increased binding of [^{3}H]HCh-3 from 150 to 306%. The nonspecific binding measured in the presence of 10 µ*M* hemicholinium-3 (less than 46%) was unchanged. The administration of EGb induced an immediate and time-dependent effect (fig. 1). Scatchard analysis revealed that the increase is due to a change in B_{max} rather than in K_D (fig. 2).

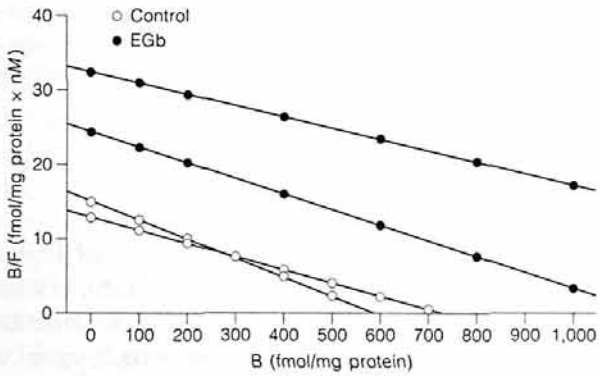

Fig. 2. Scatchard analysis of data on specific binding of [^{3}H]HCh-3 influenced by 50 µg/ml of EGb. The experiment was performed twice in duplicate on mixed synaptosomes of 11 3-month-old rats, concentration of proteins equaled 0.423 mg/ml. EGb was incubated with synaptosomes and [^{3}H]HCh-3 for 30 min. Control: B_{max} = 659.4 ± 65.9 fmol/mg prot., K_D = 48.6 ± 8.5 n*M*. EGb: B_{max} = 1,631.0 ± 473.1 fmol/mg prot., K_D = 56.6 ± 8.6 n*M*.

Table 2. Effect of EGb on the high-affinity choline transport

Dose of EGb		n	HACU
µg/ml	µg/mg prot.		fmol/4 min/mg prot.
0	0	6	334.5±38.9
5	13.3	3	363.4±56.9
10	26.6	3	325.2±58.9
25	66.5	3	354.4±27.2
50	133.0	3	361.8±23.7
75	199.5	3	315.3±2.8
100	266.0	3	383.9±20.6*
ANOVA			p = 0.3155

Statistical significance (t test) was calculated with respect to control samples (* $p < 0.05$).

The experiment was performed on mixed synaptosomes of 5 3.5-month-old rats, concentration of proteins equaled 0.376 mg/ml and concentration of [^{3}H]choline 12.6 n*M*. EGb was preincubated with synaptosomes for 5 min and then incubated with [^{3}H]choline for 4 min.

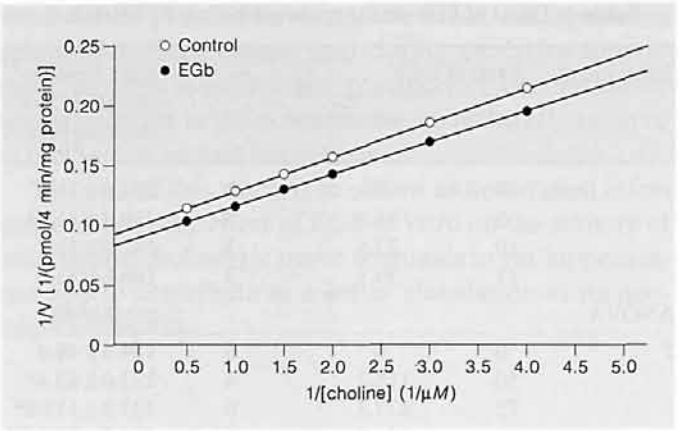

Fig. 3. Lineweaver-Burk analysis of high-affinity choline uptake influenced by 100 µg/ml of EGb. The experiment was performed in duplicate on mixed synaptosomes of 3 4-month-old rats, concentration of proteins equaled 0.348 mg/ml. EGb was first preincubated with synaptosomes for 5 min and then incubated with [^{3}H]choline for 4 min. Control: V_{max} = 10.18 pmol/4 min/mg prot., K_M = 0.30 µ*M*. EGb: V_{max} = 11.35 pmol/4 min/mg prot., K_M = 0.30 µ*M*.

Table 3. Effect of 100 µg/ml of EGb on the activities of ATPases

Group	n	Na^+, K^+-ATPase µmol P_i/h × mg prot.	Mg^{2+}-ATPase µmol P_i/h × mg prot.
Control	6	8.3±4.6	6.7±1.8
EGb	6	9.5±5.7	7.5±4.7
ANOVA		p = 0.7068	p = 0.6799

The experiment was performed on mixed synaptosomes of 11 4-month-old rats, concentration of proteins equaled 0.342 mg/ml. EGb was preincubated with synaptosomes for 5 min and then incubated with ATP for 30 min.

Table 4. Effect of 100 µg/ml of EGb on the specific binding of [^{3}H]HCh-3 and HACU transport influenced by AA

Group	n	[^{3}H]HCh-3 fmol/mg prot.	HACU fmol/4 min/mg prot.
Control	6	153.6±12.9	321.0±26.1
EGb	6	304.9±35.6***	356.3±46.2
AA	6	210.8±31.3**	193.9±43.8***
EGb + AA	6	393.3±36.2***	174.3±33.5***
ANOVA		p < 0.001	p < 0.001
t test (AA × EGb + AA)		p < 0.001	p = 0.4044
t test (EGb × EGb + AA)		p = 0.0016	p < 0.001

Statistical significance (t test) was calculated with respect to the control samples (** $p < 0.01$; *** $p < 0.001$).

The experiment with [^{3}H]HCh-3 was performed on mixed synaptosomes of 11 3-month-old rats, concentration of proteins equaled 0.423 mg/ml and concentration of [^{3}H]HCh-3 24.9 n*M*. EGb and/or 1 m*M* AA were preincubated with synaptosomes for 10 min and then incubated with [^{3}H]HCh-3 for 30 min. All samples contained 9% of ethyl alcohol.

Experiment with [^{3}H]choline was performed on mixed synaptosomes of 5 3-month-old rats, concentration of proteins equaled 0.382 mg/ml and concentration of [^{3}H]choline 9.0 n*M*. EGb and/or 100 µm AA were preincubated with synaptosomes for 5 min and then incubated with [^{3}H]choline for 4 min. All samples contained 0.9% of ethyl alcohol.

Table 2 shows the effect of different doses of EGb on HACU values. 100 µg/ml of EGb increased HACU values to 115%. Values of low-affinity choline uptake measured in the presence of 1 µ*M* hemicholinium-3 were unchanged. Lineweaver-Burk analysis revealed that the increased transport was due to a change in V_{max} but not in K_M (fig. 3).

No marked changes in the activities of Na^+,K^+-ATPase and Mg^{2+}-ATPase of EGb compared to control samples were measured (table 3).

Table 4 demonstrates the effect of EGb on the specific binding of [^{3}H]HCh-3 and HACU values influenced by

AA. 1 m*M* AA significantly increased the specific binding of [^{3}H]HCh-3 to 137%, whereas 100 μ*M* AA decreased HACU values to 60%. The effects of 100 μg/ml of EGb and AA on the specific binding of [^{3}H]HCh-3 were additive. Choline transport values reduced by AA were not influenced by EGb.

Discussion

This study confirms our previous results [15] as well as results of other authors [16, 17] that EGb influences the cholinergic neurotransmitter system by activating presynaptic nerve terminals. This effect is not restricted to cholinergic neurons but results are more marked and less controversial here than in the case of other neurotransmitter systems [for review, see 25]. Therefore EGb can be counted among the nootropic drugs that stimulate the release of acetylcholine and also concomitantly the HACU values [for review, see [14]. Pharmacological strategies based on the cholinergic theory want to increase the availability of acetylcholine in the brain tissue of aged or Alzheimer disease patients. A comparison of nootropics that stimulate an acetylcholine release as well as choline uptake (e.g., ginsenoside Rb_1 [14], physostigmine [25, 26]) with those that reduce directly both values (cholinesterase inhibitors tacrine and methoxytacrine [15, 26, 28, 29] is surprising. However, results indicate that for the synthesis of acetylcholine, the utilization of intracellular choline perhaps from intracellular choline esters is increased by tacrine and methoxytacrine [28]. It is evident that testing of new cholinergic nootropic drugs only by means of HACU estimation could be doubtful and misleading. Nevertheless, the majority of cholinergic nootropics are expected to stimulate HACU levels with regard to the decreased velocity of choline transport during normal and pathological aging [19, 20].

Increase in HACU levels can be accounted for either by an increase in the number of carriers or in the turnover rate of each carrier. The rises in V_{max} and B_{max} indicate that EGb can enhance the number of choline carriers. This confirms results of several groups that drugs which stimulate acetylcholine release also concomitantly increase HACU levels as well as the specific binding of [^{3}H]HCh-3 [for review, see 14]. However, a marked increase in the maximal number of [^{3}H]HCh-3 binding sites accompanied only by a mild rise in the maximal velocity of transport indicates that EGb either evokes also the binding of a number of [^{3}H]HCh-3 molecules to one carrier or mobilizes the occult pool of carriers that are not totally available for choline transport. The membrane-stabilizing actions of EGb can influence only the extracellular part of carrier characterized by a specific binding of [^{3}H]HCh-3 and perhaps the part of membrane phospholipid domain [30]. Therefore, the mobilization of the occult carriers especially from a putative cytoplasmatic pool [31] appears to be improbable.

It is interesting to note that EGb applied in vitro to rat synaptosomes inhibits the uptake of biogenic amines [32]. The EGb-stimulated rise in the number of carriers is evidently specific for cholinergic neurons.

It is well known that the Na^+-dependent high-affinity transport is directly linked to the function of Na^+,K^+-ATPase. It has been published that flavonoids could inhibit the activity of the sodium pump as well as of many other membrane enzymes [33]. However, our results (table 3) indicate no direct effect of EGb.

It has been suggested that AA acts as a second messenger in the regulation of HACU transport [34, 35]. The effect of AA on the membrane proteins and lipids has not yet been elucidated in detail. Free AA readily intercalates into the membrane and produces significant changes in its fluidity, increases the peroxidation of lipids and the concentration of intracellular Na^+, induces synaptosomal swelling and disruption of synaptosomal integrity. In the case of cholinergic neurons, AA unmasks previously occult carriers and increases [^{3}H]HCh-3 binding in vitro [35]. However, the disruption of electrochemical gradient and transmembrane electric potential required for the Na^+-dependent transport brings about a decrease in HACU values [34]. Our results indicate that EGb applied in vitro is not able to influence the specific 'second messenger' effect of AA (table 4). It seems that the influence of EGb is largely restricted to the extracellular domain in contrast to AA. However, EGb treatment in vivo (100 mg/kg/day for 14 days, p.o.) had an effect on an electroconvulsive shock-induced accumulation of free fatty acids in rat brain and reduced the pool of free fatty acids in the hippocampus [36]. Therefore, EGb applied in vivo affects lipid metabolism and a lipid-derived second messenger release indirectly.

In conclusion, in addition to the anti-ischemic, antiedema, antihypoxic, radical-scavenging and metabolic effects of EGb, its marked influence on the presynaptic cholinergic terminals probably contributes to its nootropic action. Effective EGb concentrations applied in vitro are about 100 μg/ml (200–300 μg/mg protein, i.e. approximately 20–30 mg/g brain tissue. These levels could probably be achieved in the brain tissue after long-term EGb administration. In vivo application of EGb to old rats

(50 mg/kg/day for 30 days) significantly increases HACU values [15]. However, doses commonly used in human patients with disturbances of CNS functions [for review, see 37] are slightly lower (120–360 mg/day, i.e. approximately 2–6 mg/kg/day).

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