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I. KALVINSH, ET AL. [2006] MED HYPOTHESES RES 3: 803-812.

HYPOTHETICAL GAMMA-BUTYROBETAINE ESTERASE-DEPENDENT SIGNAL TRANSDUCTION SYSTEM: POSSIBLE LINK TO MILDRONATE ACTION

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REVIEW ◇ HYPOTHESIS

ABSTRACT. THE IDEA ABOUT EXISTENCE OF an electron transfer system outside the nervous system arises from simple consideration that if the signal transfer from nerve fibres to organs is of electric nature, a recurrent flow of electrons from effector cells to nervous system should exist in order to form a closed electric circuit. Several lines of scientific considerations all indicate that the gamma-butyrobetaine and gamma-butyrobetaine esters could perform this function. The gamma-butyrobetaine esterase activity, a necessary prerequisite for the existence of such a system, was detected in rat blood. Our recent results suggest that action of the anti-ischemic drug mildronate could be mediated, in part, by the stimulation of the gamma-butyrobetaine and gamma-butyrobetaine ester pathway and the production of the nitric oxide as a secondary messenger.

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• MEDICAL HYPOTHESES AND RESEARCH • THE JOURNAL FOR INNOVATIVE IDEAS IN BIOMEDICAL RESEARCH •

1. PREREQUISITES FOR EXISTENCE OF GAMMA-BUTYROBETAIENESTERASE-DEPENDENT ELECTRON TRANSFER SYSTEM

The idea about existence of an electron transfer system outside the nerve system arises from simple consideration that if the signal transfer from nerve fibres to organs is of electric nature, a recurrent flow of electrons from effector cells to nervous system should exist in order to form a closed electric circuit. The hypothetical signal transducer should have following features: (i) It should be capable to accept and to and to donate electrons. Most ionogenic compounds can be involved in this process. (ii) To our opinion such a compound should be sought among analogues of acetylcholine. In higher organisms acetylcholine-dependent signal transfer system based on electron transfer is localized in nervous system. In lower multicellular organisms the nervous system does not exist, however cells of these organisms are able to transfer signals. Probably some acetylcholine-like molecule could serve as signal transmitter during early phase of evolution. (iii) Somatic cells are much more numerous in organisms than neurons, thus the concentration of hypothetical "somatic' transmitter in the organism should exceed that of acetylcholine. (iv) The concentration of hypothetical neurotransmitter in biological fluids should correlate with intensity of irritation; it should rapidly increase in response to stressor and decrease when the stress factor is removed. This is possible when all the cells in an organism can synthesize this signalling molecule. Moreover all the cells should possess receptors to this signalling molecule. With no doubt, this compound is known already, although its signalling function is not revealed yet. (v) The CNS should be able to analyse signals transferred by this transmitter from the periphery, but the transmitter itself should be stopped by the blood-brain barrier. Thus, presumably it should be a positively charged molecule.

After thorough analysis of the literature data taking into consideration the above arguments we came up to conclusion that esters of gamma-butyrobetaine (GBB) are the most suitable candidate for the role of "somatic" transmitter, a hypothetical GBB-esterase enzyme system should enable electron transfer. The presumable reaction is given in FIG. 1, reaction of electron transfer in the acetylcholine–choline system is also given for comparison. The idea about existence of such a system is suggested by facts of an increase of the GBB concentration in stressed animals [1] and the cholinergic activity of GBB esters [2].

2. DESIGN OF MILDRONATE: DUALISM IN ITS MECHANISMS OF ACTION

Although described long time ago gamma-butyrobetaine esters and related compounds remain out of the attention focus of the researchers. Numerous studies performed by E. Hosein and co-workers in sixties and seventies indicate multiple functions of these compounds [2,4-6]. Our attention to this class of compounds was triggered by successful design, synthesis and introduction of mildronate, a structural analogue of the gamma-butyrobetaine, and revelation of its benevolent influence on cell proliferation and metabolism of ischemised myocardium [7-9] and brain [10]. Pharmacological effects of mildronate can be explained in part by its capability to block carnitine biosynthesis and to prevent accumulation of cardiotoxic intermediate products of fatty acid catabolism [7-11].

However not all pharmacological effects of mildronate can be explained by inhibition of carnitine biosynthesis. First of all, to achieve this effect the drug should be administered for several days, however numerous observations indicate that mildronate elicits several fast effects related to vasorelaxation [8,12]. It was reported that bolus administration of mildronate increased the animal survival after experimental myocardium infarction and improved the bioenergetic parameters of ischemic myocardium in rats [12]. Bolus intravenous injection of mildronate increased the blood flow in aorta arch and decreased the peripheral resistance in blood vessels of anesthetized cats. In dogs it increased blood flow in carotid, mesentery and femoral arteries. In

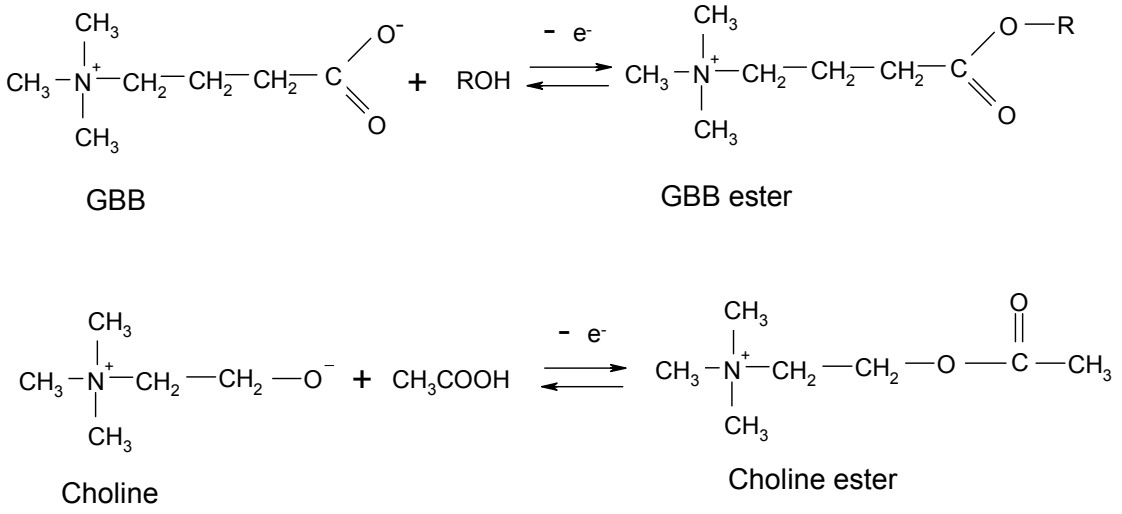

FIGURE 1. REACTIONS OF ELECTRON TRANSFER IN GBB – GBB ESTER AND CHOLINE – ACETYLCHOLINE SYSTEMS.

isolated vessels of rabbit ears it reduced the spasms induced by epinephrine. It also prevented the evolution of heart insufficiency symptoms in cats caused by stenosing of lung artery (M. Veveris, personal communication). In the clinical study by Enina et al. [13] a transient decrease of arterial blood pressure was detected in 30 minutes after the drug administration, several parameters of the cerebral circulation changed in 1 hour after the bolus intravenous injection of Mildronate. In clinical conditions single administration of Mildronate normalized the cerebrovascular reactivity for 60–90 minutes [14]. Mildronate appeared to be able to increase the beta-adrenoreactivity of smooth muscles in aortic rings in *ex vivo* system [15]. Similarly, Mildronate produced negative chronotropic and inotropic effects when perfused in high concentrations through atrial and ventricular preparations of dogs [16]. Mildronate administration favours wound and ulcer healing [17-20], however carnitine biosynthesis and transport block should rather inhibit the cell proliferation [11]. Mildronate interferes with membrane receptor and secondary messenger activity [21], in few hours it triggers DNA replication, repair and methylation [8,22,23]. It is capable to trigger the RNA-polymerase activity in isolated neurons *in vitro* [24]. Increase of the pre-mRNA synthesis was observed in rat liver, spleen, heart and intestine during 6 hours after the drug administration [25]. Decrease of the ADP-ribosylation of loosely-bound chromatin non-histone proteins can be also observed soon after the drug administration [26]. None of the above effects could be explained by inhibition of the carnitine biosynthesis.

In order to explain all biological effects of mildronate, including its "rapid" effects, Kalvinsh proposed the hypothesis about the signal-transducing effects of GBB esters as presented above. In order to confirm the existence of the GBB esterase-dependent electron transfer system, we have tried to elucidate existence of the GBB esterase enzymatic activity in mammal tissues.

3. EVIDENCE FOR EXISTENCE OF GAMMA-BUTYROBETAINE ESTERASE ENZYMATIC ACTIVITY

The phenylated derivative of gamma-butyrobetaine ethyl ester (PhGBB-OEt) was synthesised in order to follow the gamma-butyrobetaine ester hydrolysis by means of HPLC.

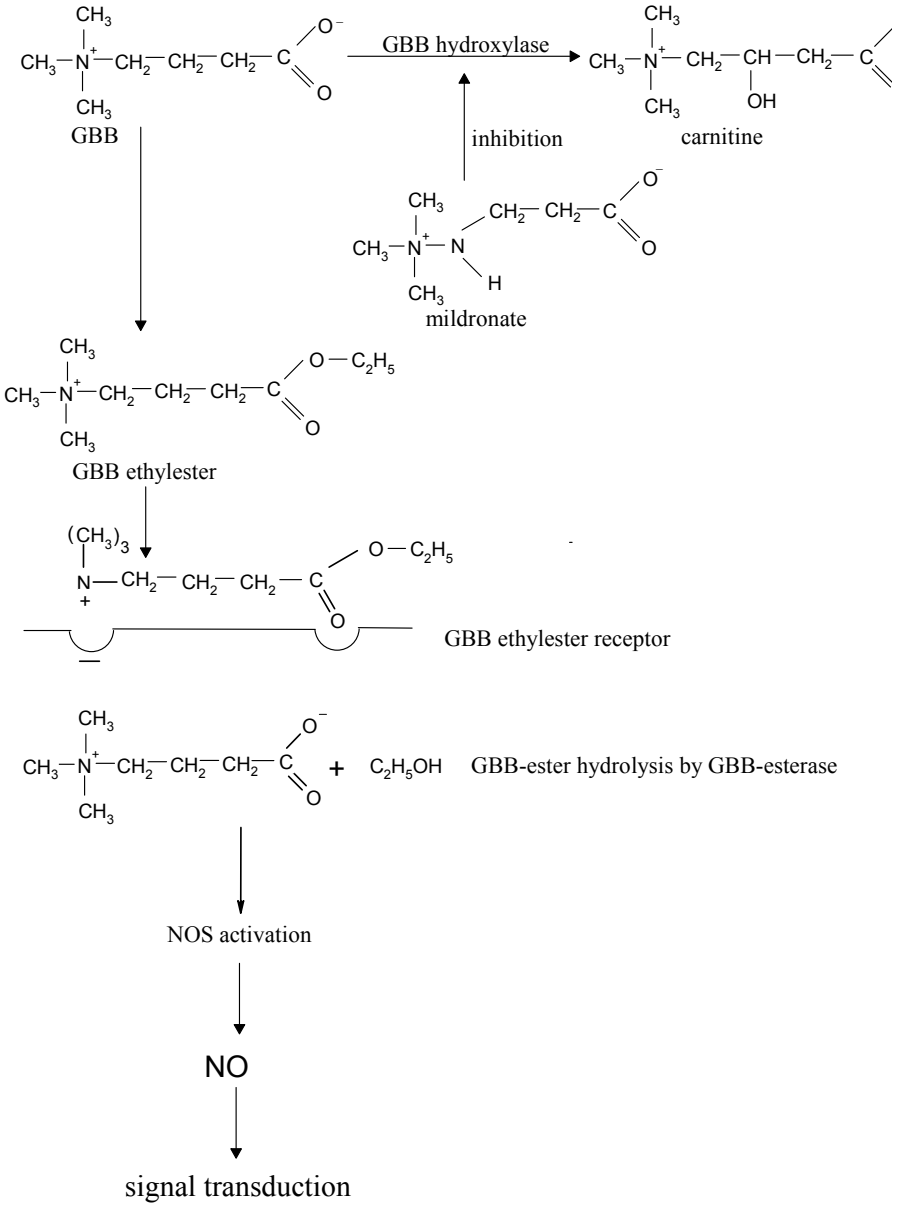

FIGURE 2. HYPOTHETICAL MECHANISM OF ACTION OF MILDRONATE BASED ON GBB-ESTERASE ACTIVITY AND NO PRODUCTION.

The substance was stable in water solutions; however its rapid decay and conversion of the gamma-butyrobetaine ester to gamma-butyrobetaine was observed when the substance was incubated in mixture with blood plasma or serum. This clearly indicated an enzymatic hydrolysis of the gamma-butyrobetaine esters by an enzyme or enzymes of blood plasma. It was not evident if the hydrolysis was performed by a specific activity ("gamma-butyrobetainesterase") or gamma-butyrobetaine esters could serve as substrates for acetylcholine esterase. In order to clarify this point the incubation was performed in presence of acetylcholine in quantity equimolar to that of the phenylated gamma-butyrobetaine ester (3×10^{-4} M). Presence of the "good" substrate for

acetylcholinesterase did not decrease the rate of the gamma-butyrobetaine ester hydrolysis; these results gave evidence in favour of hypothesis that some enzyme other than acetylcholineesterase possesses the gamma-butyrobetaine esterase activity. Experiments with addition to the incubation mixture of neostigmine, a specific inhibitor of acetylcholine esterase, confirmed the above conclusion, neostigmine did not interfere with kinetics of the gamma-butyrobetaine ester hydrolysis. The rate of the gamma-butyrobetaine ester hydrolysis was not reduced by the substrate for an analogue of the acetylcholinesterase — pseudocholinesterase or butyrylcholine esterase (E.C.3.1.1.8). However dibucaine, a specific inhibitor of the butyrylcholinesterase drastically decreased the rate of the reaction. It was hypothesised that the gamma-butyrobetaine ester hydrolysis was performed by the butyrylcholinesterase. To test this hypothesis we have performed incubation of the gamma-butyrobetaine ethyl ester with purified butyrylcholinesterase and acetylcholinestrase. However none of the purified enzymes performed hydrolysis of the gamma-butyrobetaine esters. We interpreted these results as first experimental evidence for existence of gamma-butyrobetaine esterase activity in mammals [27].

Formation of the GBB esters is the minor pathway of the GBB metabolism, it is mainly converted to carnitine, fatty acid transporter to mitochondria. Interestingly, although brain brain cells use glucose as the sole source of energy, carnitine is synthesized in brain cells, the GBB hydroxylase gen gene is expressed in brain [28]. This indicates a probably different function of carnitine in brain tissue, probably linked to the signal-transferring function of the GBB esters. It

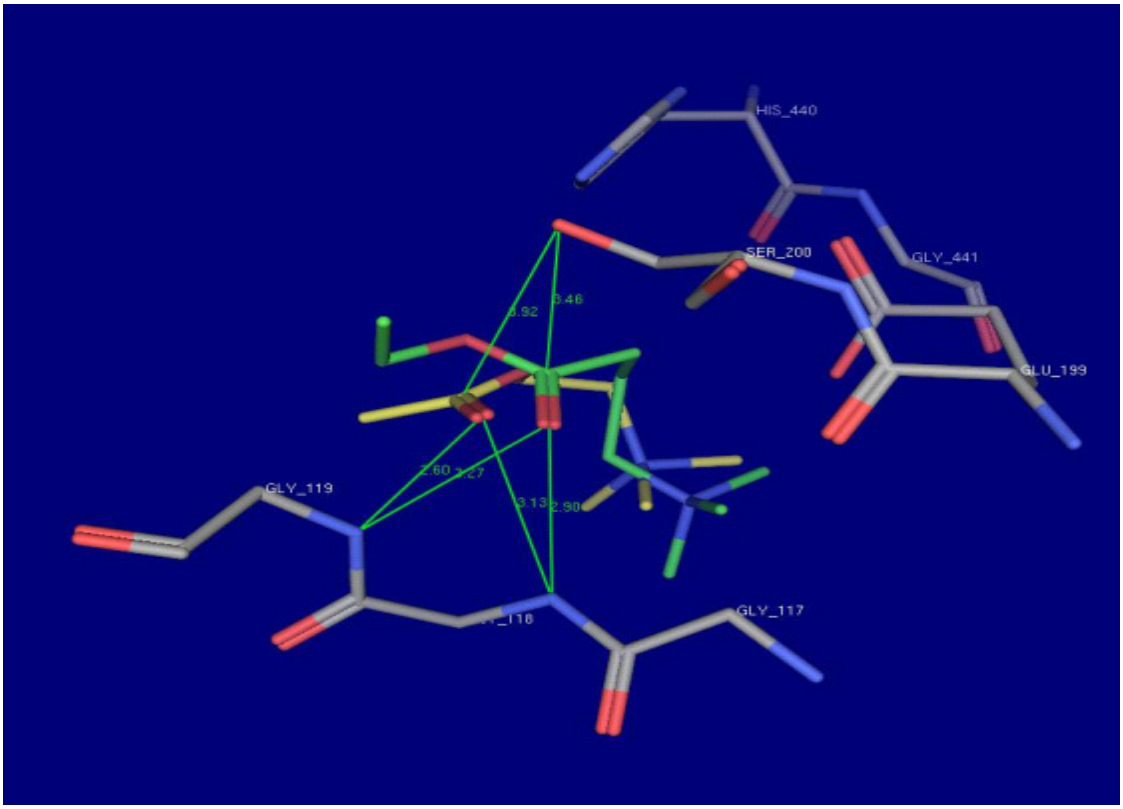

FIGURE 3. DOCKING RESULTS OF GBB ETHYL ESTER (YELLOW) AND ACETYLCHOLINE (GREEN) INTO THE ACTIVE CENTER OF NATIVE ACETYLCHOLINESTERASE FROM *TORPEDO CALIFORNICA*. Details are given in ref. [10].

was reported, that besides being the precursor of carnitine GBB can undergo etherification in mammal brain tissues [5].

The proposed hypothetical "fast" mechanism of mildronate action involving the GBB esterase-dependent signal transfer could consist of following steps: (i) Mildronate administration shifts the equilibrium between GBB hydroxylation to carnitine and GBB esterification towards the GBB esters. Trace amounts of the GBB esters are physiologically active, besides other organs the process is performed also in brain, thus the effect should be rather fast. (ii) The GBB ester binds its specific receptor; the GBB esterase acting like acetylcholineesterase performs hydrolysis of the ester. (iii) GBB ester hydrolysis triggers the signal transduction. Secondary messengers can be involved in the process (FIG. 2).

Existence of the GBB esterase cannot exclude action of the GBB esters via acetylcholine receptors, as the substance can bind them. Recent *in vitro* data obtained by Dambrova et al. [29] have shown that GBB methyl ester is potent agonists for *m*-type of acetylcholine receptors, GBB affinity to these receptors is much lower. A computer model of the molecular interactions between the GBB ethyl and methyl esters and the active centre of acetylcholine esterase performed by A. Gutcaits indicates that acetylcholine and GBB ethyl ester have the same binding modes (FIG.3). This is a clear indication of a possibility for hydrolysis of GBB esters by this enzyme.

4. NITRIC OXIDE AS POSSIBLE SECONDARY MESSENGER IN GBB ESTERASE-DEPENDENT SIGNAL TRANSFER PATHWAYS

Concerning the following transducers of the GBB esterase signalling pathway the nitric oxide appeared to be the most probable, as it was reported that mildronate and gamma-butyrobetaine (GBB) composition abolished the vasoconstriction produced by nitric oxide synthase (NOS) inhibitors [30]. We hypothesized that Mildronate might act also via a nitric oxide-dependent mechanism. In a preliminary study [31] we tried to reveal possible effects of mildronate on NO concentration in rat organs. Changes in the NO content in different rat tissues (brain cortex, cerebellum, liver, heart, kidneys) were evaluated after administration of mildronate by electron paramagnetic resonance method (EPR). It was revealed that mildronate triggered a slight but reproducible wave-like increase of NO level in the brain cortex and cerebellum 30 minutes after the drug administration. Administering in the same time the NOS inhibitor Nω-nitro L-arginine, caused a pronounced decrease of the NO concentration that indicates the necessity of NOS activation to produce the mildronate effect. This was the first indication on a putative NO-dependent mechanism of the drug action. Interestingly, the effect was pronounced in brain, where "ununderstandable" carnitine biosythesis takes place, moreover the time course of the effect resembled that for vascular effects of Mildronate described by Enina et al. [13]. In our following studies the NO-producing effects of Mildronate were studied in comparison to gamma-butyrobetaine and GBB esters. We observed an induced transient increases in nitric oxide (NO) concentrations in rat blood and myocardium, produced by mildronate, GBB and GBB methyl ester [32]. The latter produced similar effect to GBB and Mildronate in 100 times lower concentrations. Intraperitoneal administration of the GBB and mildronate composition induced a short-termed increase of the NO concentration in brain cortex, detectable only 15 after the injection. Similar effect was produced by the GBB methyl ester administered in thousand times lesser concentration. The ester increased NO concentration also in the cerebellum. Also the ethyl ester triggers increase of NO synthesis in brain cortex and cerebellum [10]. *In vitro*, these compounds neither modified the activities of purified neuronal and endothelial recombinant nitric oxide synthases (NOSs), nor were able to interact with their active sites. GBB induced vasodilatation at high concentrations only (EC_{50} = 5×10^{-5} M), mildronate alone displayed no vasodilating effect, however enhanced the GBB vasodilating activity. GBB methyl and ethyl esters

were found more potent vasodilators (EC_{50} = 2.5 × 10^{-6} M). Pre-treatment of aortic rings with NOS inhibitor N^{ω}-nitro-L-arginine methyl ester abolished vasodilating effects of the compounds [32]. The above results provide evidences that GBB methyl and ethyl esters are potent NO- and endothelium-dependent vasodilators. While mildronate alone elicits no activity, it sharply potentiates the activity of GBB in endothelium- and NOS-dependent responses. These data suggest that fast anti-ischemic effects of mildronate may be in part related to the stimulation of formation of NO by the endothelium. As none of the studied compounds could modify the NOS activity *in vitro*, we think that our results suggest that some receptor-mediated mechanisms are involved in the activation of the formation of NO in blood vessels. Both still hypothetic GBB esterase-dependent receptors (GBB esterase is not hypothetic any more!) and acetylcholine receptors are possible candidates for this role. Cholinomimetic activity of GBB esters and compounds of similar structure has been described long time ago [5]. Mildronate ethyl ester EDIHYP is even considered to be a synthetic analogue of acetylcholine [33]. Ability of the GBB methyl ester to bind *m*-type of acetylcholine receptors also supports this possibility [29]. The described above synergistic effects of mildronate on GBB activity may involve the esterification of GBB, as GBB esters trigger their vasorelaxing effects at much lower concentrations than GBB itself. These results suggest us that fast anti-ischemic action of mildronate could be mediated, at least in part, by stimulation of NO production in the vascular endothelium through a modification of the GBB/GBB esters pools. This stimulation of NO formation might be rationalized in the following ways (FIG. 2): (i) administration of mildronate inhibits GBB hydroxylation and increases the intracellular pool of GBB; (ii) a part of GBB is released from cells, and, after esterification, forms GBB esters; (iii) GBB esters, via specific GBB-esterase receptor as shown on FIG. 2 or acetylcholine receptors on endothelial cells could activate eNOS. Our data provide evidence for most steps of this hypothetical mechanism, the increase of GBB esterification after mildronate administration remaining the missing link. Interestingly, also the carnitine has been found to produce endothelium-dependent vasorelaxation in aortic rings [34], an activity that might be mediated by esterification, as carnitine esters also possess cholinergic activity [5].

5. IMMUNOMODULATING ACTION OF MILDRONATE – ADDITIONAL EVIDENCE FOR EXISTENCE OF GBB ESTERASE-DEPENDENT SIGNAL TRANSFER SYSTEM

Evidence for interference of mildronate with stress signals in the organism can be deduced also from immunomodulating activities of mildronate. The drug is an active interferon inducer in mice when administrated simultaneously with antigen and shows a protective effect against influenza virus when used according to therapeutic and preventive schedules. We suppose that the drug acts as a strong stressing signal dissipating from the site of vaccine injection [35]. This property of mildronate enabled to propose its use as immunoadjuvant, especially during vaccination against influenza [36,37]. It was also reported that mildronate enhances the immune response in fasting animals [38] and in patients after surgery [39]. Interestingly, GBB and carnitine also increase efficiency of vaccination [40].

Taken together, our recent data on GBB-esterase activity of blood serum and on mechanisms of the "fast" mildronate effects provide more evidence for the existence of the GBB ester-dependent signal transfer pathway. Purification and characterization of the GBB esterase from mammalian tissues is underway. We have worked out an assay that enables spectrophotometric or colorimetric determination of the reaction products. For this purpose, gamma-butyrobetaine naphtyl ester was synthesized in the Latvian Institute of Organic Synthesis by the P. Trapencieris team. Hydrolysis of this ester should lead to release of 1-naphthol; this causes increase of the optical absorbance at 322 nm. On other hand, the same compound was applicable for determination of the activity in gels, as 1-naphthol

forms insoluble complex with Fast Blue RR dye. Analogous reactions of 1-naphtylacetate are successfully used for determination of acetylcholine esterase activity. At the initial stage of the work, it was important to test applicability and specificity of the gamma-butyrobetaine naphtyl ester. This was performed using inhibitor analysis. Native or partially fractionated rat blood serum manifested ability to hydrolyze the gamma-butyrobetaine naphtyl ester; the reaction was followed by monitoring absorbance at 322 nm. Acetylcholine (at 0.1 mM) did not produce any effect on the reaction rate, on the contrary phenylated gamma butyrobetaine ethyl ester in the same concentration significantly decreased the reaction rate, so did also gamma butyrobetaine ethyl and methyl esters. This indicated that the enzyme which hydrolyzes the gamma-butyrobetaine naphtyl esters is inhibited by GBB-esters, thus our assay appeared to reveal *bona fide* the gamma-butyrylesterase activity. The assay was applied to detect GBB-esterase activity in fractions of rat blood serum proteins obtained by various methods. Active fractions obtained by different purification methods were subjected to gel electrophoresis in native or denaturing conditions. Gels were incubated with gamma-butyrobetaine naphtyl ester and stained with Fast Blue RR dye in order to reveal bands possessing the enzyme activity. Incubation with 1-naphthylacetate was used as control to compare with acetylcholine esterases. Three distinct bands of 150, 70 and 35 kDa were detected by the gamma-butyrobetaine naphtyl ester assay; the bands co-localized neither with purified butyrylcholine esterase, nor with polypeptides revealed by 1-naphthylacetate assay (acetylcholine esterase). Further analysis of the polypeptides will enable us to characterize the enzyme.

ACKNOWLEDGEMENTS

This work as supported in part by Taiwan-Latvian-Lithuanian project "Purification and Characterisation of the gamma-Butyrobetaine Esterase".

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RECEIVED ON 10-25-2005.
ACCEPTED ON 5-8-2006.