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Enzymes cooperating in the hydrolyzing process of benzoylcholines: subcellular and inter-tissue comparison

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Within benzoylcholine biotransformation, butyrylcholinesterase (3.1.1.8, BuChE) appears to be the key enzyme in the hydrolyzing process. Except for BuChE in the process of benzoylcholine hydrolysis, carboxylesterase (3.1.1.1, CE) could play a role in the splitting of the ester bond. The aim of this work was to clarify the interaction between BuChE and CE in the hydrolyzing process of a homologic row of benzolycholines on subcellular and inter-tissue level. Two fractions, microsomes and cytosol of rabbit lung and liver were investigated. Participation of the enzyme activities was determined on the base of kinetic inhibitory studies, using eserine as cholinesterase inhibitor. Despite the fact that in all studied fractions of both organs BuChE and CE were confirmed, only in lung microsomes exclusive BuChE activity in benzoylcholine hydrolyzing process was observed, without substrate specifity. In the other fractions studied interaction of both enzymes were recorded, whereas the benzoylcholine structure played an important role. It seems that, the portion of CE depends predominantly on substrate structure and elevates with bulk of alcoholic part of benzoylcholines. Despite the same enzyme equipment in all tissue fractions studied, the affinity of hydrolyzing enzymes interestingly differs. This might be as a result of distinct subcellular pattern of CE activity localization in lung and liver.

1. Introduction

Many drugs contain an ester bond, which is very sensitive to esterases attack. It is a very complicated process depending on many biological, toxicological and biochemical aspects. It depends on the overall substrate structure and concentration, as well as species, tissue and subcellular localization, if one or more esterases participate in the hydrolyzing process. While the action of the majority of drugs is terminated on this basis (local anesthetics, antidepressants ... etc.), there are some releasing products, which can also contribute to the pharmacological effects (bambuterol, irinotecan ... etc.).

Butyrylcholinesterase (EC 3.1.1.8, BuChE) has been in a focus of many scientists for a long time. While this enzyme occures in various tissues (brain, liver, lung, kidney, heart, blood, adipose tissue) its natural substrate has not been found and so the exact role of BuChE remains unexplained. In general, it is supposed that BuChE is synthesized in liver and transported to other tissues, but some authors assume its synthesis also in some other tissues (Lucic et al. 2005). Whereas a lot is known about BuChE gene, sequence of amino acids, formation of molecular forms, active site and etc., there are several contradictions about BuChE subcellular distribution, as well as the unusual behaviour of BuChE molecular forms in rabbit tissues (Rush et al. 1980; Jbilo et al. 1994). The most significant changes have been observed in the liver microsomal fraction on the inter-species level (Pauliková et al. 2006). Moreover BuChE individually varies in the rabbit livers mainly (unpublished results). Although some observed discrepancies of BuChE activity could be caused here by very low content of BuChE, since the enzyme expression was surprisingly low in comparison to carboxylesterases (Jbilo et al. 1994). Not of neglectable importance are genetic variations, which lead to lower or higher BuChE activity. BuChE is probably not necessery for life, since individuals with no BuChE activity do not suffer from any kind of disease relating to its lack (Li et al. 2006). Even though, this statement could turn premature in the course of future investigations. From the toxicological point of view the participation of BuChE activity in metabolism of several xenobiotics, especially organophosphates is important.

CE is an enzyme widely distributed in animal cells and is, together with BuChE, involved in the hydrolyzing process. However a lot of isoenzymatic forms of CE occuring on not only interspecies, but even on intertissue levels were described.

Unexpected response of BuChE activity in the rabbit liver has previously been the reason for the elucidation and characterization of BuChE as well as the participating enzymes in the hydrolyzing process on the subcellular and inter-tissue level using substrates of the benzoylcholine row (Pauliková et al. 2006). These compounds are N-alkylderivates of benzoylcholines, with following general formula: *N*-(2-benzoyloxyetyl)alkyldimetylammonium bromides, where alkyl is 1–14 carbons long (abbreviated BCHn, where n is the number of carbons in the alkyl side

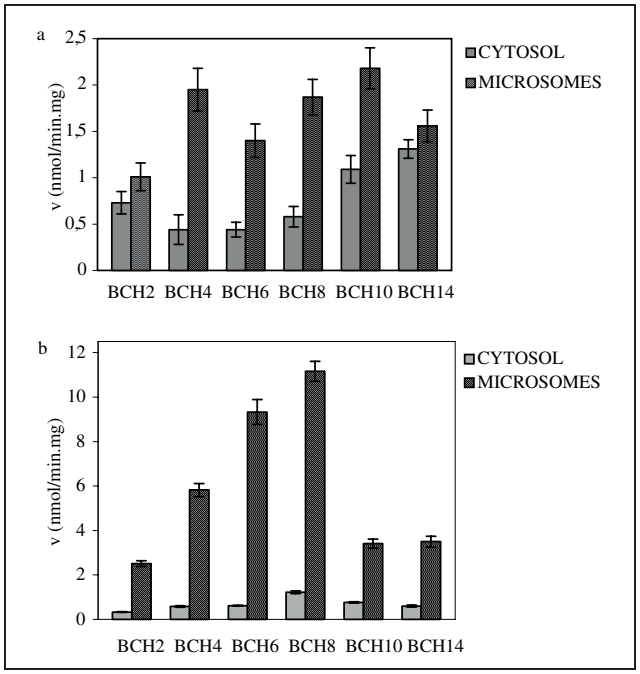

Fig. 1: The overall hydrolysis rate (v) of benzoylcholines with different alkyl side chain length in cytosol and microsomes of rabbit lung (a) and liver (b), when 1 mmol/l substrate concentration was used. Values are mean ± SEM from 6 separate experiments

chain) (Csiba et al. 1986). They are quaternary ammonium salts with antimicrobial activity.

Two fractions of lung and liver were investigated: microsomes and cytosol.

2. Investigations and results

Before the experiments with benzoylcholines, both tissue fractions were tested for the presence of BuChE and CE activity. BuChE activity was confirmed with aliphatic substrate – S-butyrylthiocholine. Carboxylesterase activity was measured with *p*-nitrophenylacetate. Results are showen in the Table.

Figure 1a displays the whole hydrolyzing activity in cytosolic and microsomal fractions of rabbit lung in relation to the lenght of alkyl side chain in benzoylcholines, when a 2 mmol/l substrate concentration was used. The course of the hydrolyzing activity of benzoylcholines with shorter alkyl side chain from BCH2 up to BCH8 appears to be mutualy reverse in cytosolic and microsomal fractions in both studied tissues. In general, the hydrolyzing process is more intensive in microsomal fractions of liver and lung than in the cytosol (Fig. 1a, b).

Since the affinity of BuChE to benzoylcholines differs depending on the lenght of their alkyl side chain, two substrates were chosen for further kinetic experiments. Fig. 2 is a review of the kinetic study of two designated substrates with short and middle length chain, BCH2 and BCH8 respectively, measured in cytosol and microsomes of rabbit lung and liver. In lung cytosol the saturation curve assigns two peaks (Fig. 2a), and so does not seem to follow the typical Michaelis-Menten kinetics. In the other studied tissue fractions this effect was not observed (Fig. 2b, c, d). Further studies provided evidence of participation of two enzymes in the hydrolysis of benzoylcholines in all studied fractions except for the lung microsomes.

The cooperation of two enzymes in the hydrolyzing process was confirmed by the inhibitory kinetic studies with the cholinesterase inhibitor eserine. Low eserine concentration enabled to distinct between eserine-sensitive - true BuChE and non-sensitive – remaining hydrolyzing activity. In cytosol of lung and liver relatively low inhibition was recorded, when BCH8 was used as substrate (Fig. 3b, d). When BCH2 was the substrate, in cytosol full inhibition was attained in lung and approximately 50% of re-

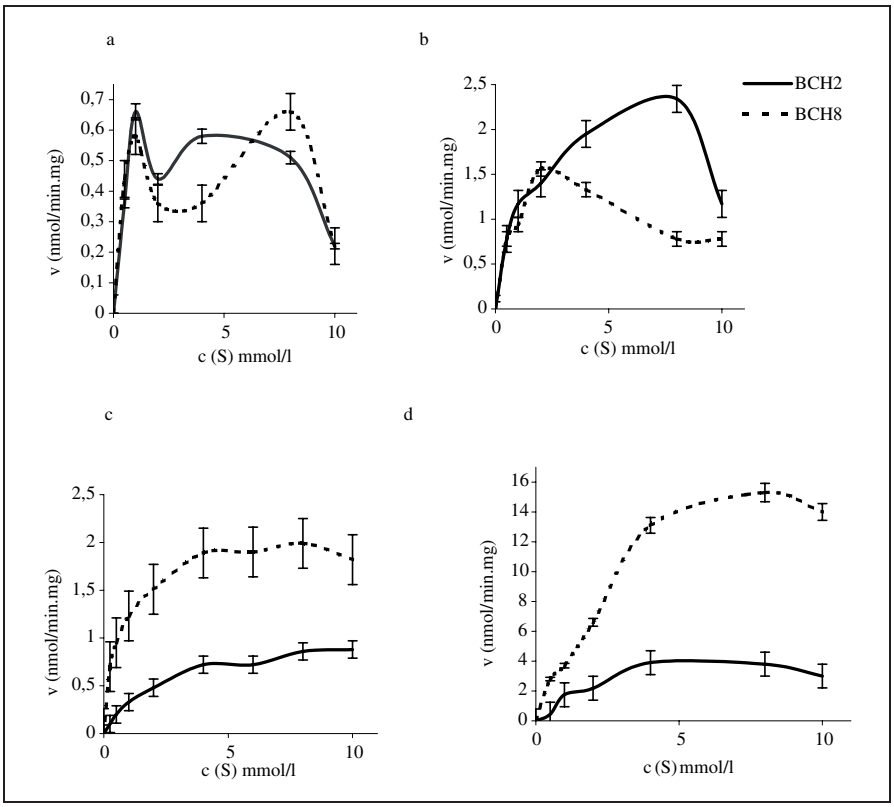

Fig. 2:
Kinetic study of BCH2 and BCH8 hydrolysis in rabbit lung (cytosol (a), microsomes (b)) and liver (cytosol (c), microsomes (d)). The substrate (S) concentration ranges from 0.5 to 10 mmol/l. Values are mean ± SEM from 6 separate experiments

Table: Butryrylcholinesterase and carboxylesterase activity in cytosolic and microsomal fractions of rabbit lung and liver

Activity	Lung		Liver	
	Cytosol	Microsomes	Cytosol	Microsomes
BuChE (μmol/min · mg)	1.26 ± 0.062	1.46 ± 0.030	5.32 ± 0.112	7.37 ± 0.097
CA (μmol/min · mg)	34.46 ± 4.223	88.13 ± 5.852	125.19 ± 10.936	107.38 ± 8.293

Values are mean ± SEM from 6 separate experiments

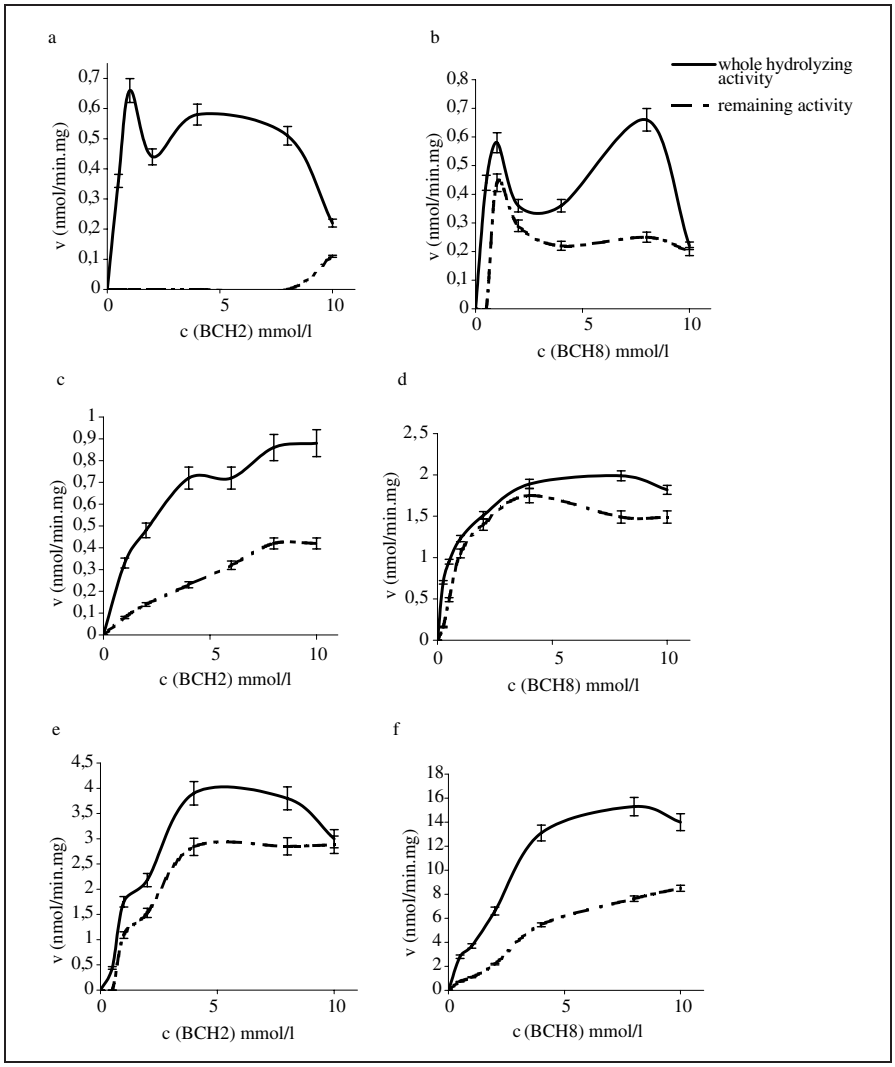

Fig. 3:
0.1 mmol/l eserine was used in the inhibition kinetic study of BCH2 and BCH8 hydrolysis in subcellular fractions of rabbit lung and liver. The substrate concentration ranged from 0.5 to 10 mmol/l. For in lung microsomes full inhibition of hydrolyzing activity was achieved, the responsible enzyme appears to be BuChE and only the results in cytosol (a, b) are displayed. Neither in liver cytosol (c, d), nor in microsomes (e, f), full BuChE inhibition was attained. Values are mean ± SEM from 6 separate experiments

maining activity was observed in liver (Fig. 3a, c). The inhibitory pattern of BCH2 and BCH8 in liver microsomes turned out to be reverse to that in liver cytosol (Fig. 3e, f).

3. Discussion

Despite at first sight the hydrolyzing process is metabolically simple and not energetically challenging, in reality it is a complex of various factors and reactions which relate to each other. On one hand there are substrates, their structure and concentration, on the other, there are enzymes and their izoenzymes, as well as individual conditions of this reactions. Due to this it is inevitable to investigate the metabolic processes on subcellular, inter-tissue and inter-species levels, since izoenzymes are likely to occur. In addition, the hydrolyzing reaction is often catalysed by more then one typical enzyme. From this reason the aim of this paper was not only a general study of BuChE and the participating enzymes, but individual differences of subcellular fractions on inter-tissue level in the hydrolyzing process using different substrates.

Two fractions of rabbit lung and liver were chosen for elucidation of enzymes responsible for the hydrolysis of benzoylcholines. In previous studies, BuChE was confirmed to be the enzyme responsible for benzoylcholines hydrolysis, whereas there was no evidence about an another enzyme, cooperating with BuChE (Pauliková et al. 2006).

When S-butyrylthiocholine was used as substrate, BuChE activity was in liver microsomal fraction significantly elevated compared to cytosol. No activity changes were recorded between microsomes and cytosol in the lung. However, BuChE activity in the liver was severalfold higher than in the lung. The CE activity in the liver also exceeded the CE activity in the lung. But whereas in the lung the CE microsomal activity reached more than double the values of those in cytosol, in liver the activity of CE was approximately equal (Table). Interestingly, in the

rat lung CE activity was mainly found in the cytosolic fraction (Gaustad et al. 1991).

For the kinetic study benzoylcholines were used due to previous BuChE activity studies. A reverse tendency of benzoylcholines hydrolyzing activity, depending on the alkyl side chain, was observed in cytosol and microsomal fractions only in the lung. The most appropriate substrates for hydrolyzing enzymes in lung cytosol are long-lenght substrates in opposite to microsomes, where the convenient substrates appear to be the middle-lenght benzoylcholines. In both subcellular fractions of rabbit liver middle-lenght benzoylcholines show the highest hydrolyzing activity. Within interspecies distinctions in rat microsomes the hydrolyzing velocity reaches its maximal values in short-lenght alkyl side chain substrates, which gradually decreases towards middle-lenght chain substrates. Exactly the opposite course of hydrolyzing activity was observed in rabbit liver, where hydrolyzing activity increased from short to middle-lenght side chain benzoylcholines (Olaszová et al. 1998, Pauliková et al. 2006). Although the lung microsomal BuChE activity copies that in the liver, the total hydrolyzing velocity is significantly lower.

Except BuChE carboxylesterase could be the enyzme, playing a role in the process of benzoylcholine hydrolysis, but never before participation of CE was considered. In view of this, BuChE inhibitory kinetic studies were carried out using eserine. Deliberately two substrates with different affinity to BuChE were selected to elucidate the participation of mentioned hydrolyzing enzymes in rabbit lung and liver on subcellular level.

Exclusive responsibility of BuChE for studied benzoylcholines hydrolysis was affirmed in the lung microsomes and in lung cytosol explicitly for BCH2, while the middle-length alkyl side chain substrate BCH8 required a cooperating CE. This is probably caused by the bulk of the alcoholic part of benzoylcholines, that suit rather carboxylesterase than BuChE. Similar results were observed in the rat lung. In contrast to rabbit microsomes small portion of CE activity, however without benzoylcholine specifity, was recorded in rat lung microsomes (unpublished results). In cytosol and microsomes of rabbit liver the BuChE activity in hydrolyzing process was supported by CA in both the benzoylcholines. Participation of BuChE activity decreased with elongation of the alkyl side chain on ammonia atom. Similar results were observed in rat liver microsomes (Pauliková et al. 2004).

Based on this facts, it can be concluded, that the majority of the specific changes of benzoylcholine hydrolysis have been limited to the microsomal fractions. No too significant differences brought the cytosolic fraction on interspecies and inter-tissue level. Both of enzymes BuChE and CE cooperated with each other, whereas the portion of CE elevated when the alkyl part of benzoylcholines was growing.

4. Experimental

4.1. Chemicals

Except of eserine salicylate (Kulich, Czech Republic) all chemicals: bw284c51, 5,5′-dithiobis(2-nitrobenzoic) acid, butyrylthiocholine iodide and *p*-nitrophenylacetate were from Sigma (Slovakia).

4.2. Animals

Six New Zealand white male rabbits, six month old, of approximately 2,5 kg were used in the experiment. The experiment was approved by the statement No. SK CH 29004. Rabbits were single-housed in the cages with food and water available *ad libitum*. 24 h before decapitation, the animals were starving.

4.3. Preparation of subcellular fractions

After decapitation, lung and liver were immediately perfunded, rinsed in saline and stored at −20 °C until analysis. Tissues were homogenized in a medium consisting of: 0.25 mol/l saccharose solution, adjusted by 0.15 mmol/l Tris-HCl buffer to pH 7.4. Liver and lung subcellular fractions (microsomes and cytosol) were obtained by differential centrifugation of 10–20% crude organ homogenate according to Cinti (1972). Protein content in fractions was determined by the Lowry method (Lowry et al. 1951).

4.4. Enzyme activity

4.4.1. Determination of BuChE activity according to Ellman

The BuChE activity was determined by a modified Ellman method (Ellman et al. 1961). The activity of the sister enzyme acetylcholinesterase (3.1.1.7, AChE) was inhibited by 20 min preincubation of the tissue homogenate with a specific AChE inhibitor (bw284c51, 0,02 mmol/l) at 37 °C. After the following 30 min incubation of the homogenate in 0,1 mol/l phosphate buffer, pH 7,4 with 0,5 mmol/l 5,5′-dithiobis(2-nitrobenzoic) acid at 37 °C, the substrate – 1mmol/l butyrylthiocholine was added. The absorbance was read at 436 nm for 5 min in 60 s intervals. The exclusive participation of BuChE and AChE in butyrylthiocholine brakedown was prooved by 5 min preincubation of the tissue homogenate with common AChE and BuChE inhibitor – eserine (0.1 mmol/l).

4.4.2. Determination of CE activity

The carboxylesterase activity was determined with the substrate *p*-nitrophenylacetate (Clement and Erhardt 2000). The absorbance of *p*-nitrophenol was measured at 400 nm and room temperature. The 1.5 ml sample cuvette contained 50 µl of sample, 0.3 ml of 0.55 mmol/l *p*-nitrophenylacetate in methanol and 1.15 ml of 100 mmol/l potassium phosphate buffer, pH 7.4.

4.4.3. Kinetic study

4.4.3.1. Substrates

The own kinetic study of BuChE in cytosolic and microsomal fractions of rabbit lung and liver was carried out with substrates of benzoylcholine type. These compounds are *N*-alkylderivates of benzoylcholines, with following general formula: *N*-(2-benzoyloxyetyl)alkyldimetylammonium bromides, where alkyl is 1–14 carbons long.

4.4.3.2. Incubation

The enzyme reaction was performed under aerobic conditions, at 37 °C for 15 min (in inhibitory studies the enzyme was preincubated for 5 min with eserine in final concentration 0.1 mmol/l). Incubation mixtures contained in a total volume of 4 ml 0.15 mol/l potassium phosphate buffer, pH 7.4 with 0.57 mmol/l $MgCl_2$, substrate in different concentration ranging according to experiment and microsomal or cytosolic fractions. The reaction was initiated by addition of the substrate to the mixture and finished by lowering the pH value to 2.5 with 1 mol/l HCl.

4.4.3.3. Extraction and HPLC analysis

After the incubation, *p*-iodobenzoic acid in CH_3OH was added to all incubation mixtures as internal standard. Incubation mixtures were extracted 3 times into $CHCl_3$, using 10 ml. Extracts were combined, filtered and vacuum dried. Dried samples were re-dissolved in CH_3OH and analyzed by the means of HPLC (Helia et al. 1995). Enzyme activity was expressed in nmol of benzoic acid formed per minute, calculated per 1 mg of protein. Obtained results were subjected to a statistical analysis using the Student t-test.

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