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HYDROLYSIS OF ESTER- AND AMIDE-TYPE DRUGS BY THE PURIFIED ISOENZYMES OF NONSPECIFIC CARBOXYLESTERASE FROM RAT LIVER

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Abstract—Five purified carboxylesterases from rat liver microsomes show a differing capacity for the hydrolysis of ester- and amide-type drugs. The two closely related enzymes that are responsible for the microsomal hydrolysis of palmitoyl-CoA and long chain monoacylglycerides exhibit the highest propanidid-and aspirin-cleaving rates. The predominant nonspecific esterase of microsomes is responsible for the hydrolysis of procaine, clofibrate, isoarecaidine esters, butanilicaine, octanoylamide, and possibly butyryl thiocholine. Finally, the palmitoyl carnitine-cleaving esterase splits phenacetin and acetanilide. The purified nonspecific esterase with the lowest isoelectric point is not involved in the metabolism of the drugs mentioned.

It has often been reported that the nonspecific carboxylesterases of liver are involved in the detoxication of ester- and amide-type drugs (reviews [1–3]). During the last few years it became evident that the liver of mammals contains a variety of carboxylesterases with differing substrate specificity. Multiple esterases have been isolated and characterized from pig liver [4, 5], rabbit liver [6, 7] and rat liver [8, 9]. Thus, the question arose, which of the individual esterases was responsible for the hydrolysis of a certain drug. We have now investigated this problem *in vitro* using a variety of drugs that are known to be metabolized hydrolytically in the liver [2]. In this study we used five microsomal carboxylesterases/amidases from rat liver that have been purified in our laboratory [8]. These enzymes have preliminarily been classified as carboxylesterases (EC 3.1.1.1) and subclassified according to their isoelectric points (e.g. esterase pI 6.0). Recently, we could show that the esterase pI 5.6 corresponded to microsomal palmitoyl-carnitine-hydrolase (EC 3.1.1.28), and that the esterases pI 6.2 and 6.4 represented the microsomal palmitoyl-CoA hydrolase (EC 3.1.2.2) [10]. However, all of the five enzymes used here have high activities with small xenobiotic esters [8, 10] and are enzymes of the serine hydrolase type (R. Mentlein and E. Heymann, unpublished). Thus, it seems justified to speak of isoenzymes of nonspecific esterase EC 3.1.1.1 according to the present official nomenclature. The nomenclature problem has recently been discussed [10].

MATERIALS AND METHODS

Enzymes and reagents

The five carboxylesterases were isolated from rat liver microsomes as previously described [9]. Protein was estimated with a modified biuret procedure [11].

Propanidid, aspirin and paraoxon (diethyl 4-nitrophenyl phosphoric acid triester) were gifts from Bayer (Leverkusen, FRG). Butanilicaine (Hostacain) and procaine were gifts from Hoechst AG (Frankfurt, FRG). The isoarecaidine esters were gifts from Dr. G. Lambrecht (Frankfurt, FRG). Acetanilide, bis(4-nitrophenyl)phosphate and physostigmin (eserin sulfate) were purchased from Merck (Darmstadt, FRG). Serva (Heidelberg, FRG) supplied acetylcholine iodide, butyrylcholine iodide, acetylthiocholine iodide and clofibrate. Phenacetin was from Fluka (Buchs, Switzerland).

Enzyme assays

The hydrolysis of acetyl choline, butyryl choline, propanidid (Fig. 1/I) and clofibrate (Fig. 1/III) was followed by autotitration with 40 mM NaOH at a constant pH of 8.0 or 7.4 without addition of buffer. The hydrolysis of the isoarecaidine esters (Fig. 1/IV) was autotitrated at varying pH-endpoints.

The release of aniline derivatives from anilides was recorded spectrophotometrically in the presence of 50 mM Tris–HCl buffers using the following substrates/recording wavelengths: Acetanilide/286 nm [12]; butanilicaine/285 nm [12]. The release of 4-amino-benzoic acid from procaine (Fig. 1/II) was recorded at 316 nm [13] in the presence of 50 mM Tris–HCl, pH 8.6; a $\Delta\varepsilon$ of 5000 $l \times mol^{-1} \times cm^{-1}$ was used for the calculation. The standard 4-nitrophenyl acetate assay was performed spectrophotometrically at pH 8.0 [9]. The hydrolysis of aspirin (acetyl salicylic acid) and of phenacetin (Fig. 1/VI) was assayed in 50 mM Tris–HCl buffers by fluorometric determination of the released salicylic acid (λ_{ex} = 325 nm; λ_{em} = 408 nm) or 4-ethoxy-aniline (λ_{ex} = 305 nm; λ_{em} = 372 nm) [12] using an Aminco–Bowman fluorometer with two monochromators. The fluorometric assays were standardized with 1 μM solutions of the reaction products.

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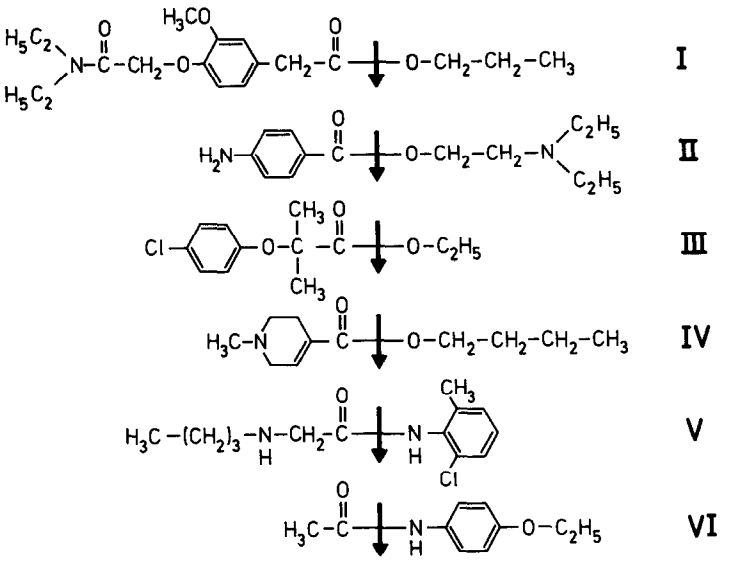

Fig. 1. Ester- and amide-type drugs. I, Propanidid. II, Procaine. III, Clofibrate. IV, Isoarecaidine butylester. V, Butanilicaine (Hostacain). VI, Phenacetin. The arrows indicate the bonds cleaved by enzymatic hydrolysis.

The hydrolysis of thiocholine esters [14] was assayed in 50 mM Tris–HCl buffer, pH 7.4, by spectrophotometric determination (412 nm) of free thiol groups after reacting with 0.3 mM 5,5′-dithiobis-(2-nitrobenzoic acid).

The hydrolysis of octanoyl amide was assayed photometrically using a determination of free NH_3 [15] after incubation of enzymes and substrate in 80 mM phosphate buffer, pH 7.4, containing 1 mM taurocholate at 37° for 2 hr. The assay was standardized with ammonium sulfate.

Kinetic constants

The K_m-values and maximal velocities (V) were calculated as described by Wilkinson [16]. Only those ranges of the substrate concentration that gave linear Lineweaver–Burk plots were used for the calculation. Specific activities (v) and maximal velocities (V) are given in units (U) per mg of purified hydrolase. One unit corresponds to the hydrolysis of 1 μmol of substrate in 1 min under the conditions described.

RESULTS AND DISCUSSION

Hydrolysis of propanidid

The narcosis with propanidid is terminated by the hydrolysis of the carboxylester bond. This reaction occurs more rapidly in the liver than in the blood [1]. The anesthetic action of propanidid can be prolonged *in vivo* by application of bis(4-nitrophenyl) phosphate [17]. Since all of the carboxylesterases used in the present study are sensitive to inhibition by this organophosphate [8, 18] it was likely that at least one of these esterases was involved in the metabolism of propanidid.

The data of Table 1 show that both esterase pI 6.2 and the closely related esterase pI 6.4 have a high propanidid-cleaving activity. Both enzymes are long-chain palmitoyl CoA and monoglyceride-hydrolyzing carboxylesterases [10]. Esterase pI 6.2 probably is responsible for most of the propanidid-cleaving capacity of rat liver, as is indicated by its high specific activity and low apparent K_m-value (Table 1).

A propanidid-cleaving enzyme from rat liver has been described before [19]. This purified carboxylesterase was also active with aspirin and phenyl acetate, but the specific activities with these three substrates were about 20-fold lower as compared to esterase pI 6.2. The kinetic data [19] suggest that the former enzyme preparation comprised one or both of our highly purified esterases pI 6.2 and 6.4. However, it has been proposed that the former enzyme was an "arylesterase" because it could not be inhibited by paraoxon or diisopropyl fluorophosphate in a simple qualitative experiment [19]. We oppose this opinion because the esterases pI 6.2 and 6.4 clearly are of the serine-esterase type (B-esterases) [8, 18], although their reaction with these organophosphates is comparatively slow [18].

Hydrolysis of aspirin

Although it is not clear whether the liver plays a major role in the metabolism of aspirin [20], liver enzymes capable of cleaving aspirin have been described [19, 21]. The aspirinase of guinea-pig liver has been identified as a microsomal carboxylesterase of the serine-esterase type [21]. Thus, it is not surprising that two of our purified carboxylesterases, namely the same enzymes that are active with procaine and palmitoyl CoA (see above), have a relatively high aspirinase activity. The other purified esterases—except esterase pI 5.2—also hydrolyze aspirin (Table 1).

Hydrolysis of procaine

The well-characterized "nonspecific" liver carboxylesterases isolated from many species usually cleave procaine [1]. Of rat liver, esterase E1 [22] and an

Table 1. Hydrolysis of ester-type drugs by the purified carboxylesterases

Substrate (Formulas see Fig. 1)	pH/ Temperature	Constant*	Substr. conc. (mM)	Data obtained with esterase pI† 5.6	6.0	6.2	6.4
Propanidid (I)	8.0/30°	v	2	0.0	0.0	13.6	3.7
		K_m	0.2–2			0.37	
		V				16.1	
Procaine (II)	8.6/37°	v	0.25	0.078	0.225	0.00	0.00
		K_m	0.005–0.5		0.070		
		V			0.285		
Clofibrate (III)	8.0/37°	v	2	0.0	14.7	2.8	0.5
		K_m	0.1–2		0.24		
		V			16.4		
Aspirin	8.0/37°	v	1	0.51	0.37	2.37	4.78
		K_m	0.05–1.5				0.95
		V					9.3
Isoarecaidine Butylester (IV)	8.6/30°	v	1	1.13	3.14‡	0.10	0.35
		K_m	0.05–1	0.050	0.018		
		V		1.2	3.2		
Isoarecaidine Propylester	8.6/30°	v	1	0.48	2.30‡		
		K_m	0.05–1	0.42	0.014		
		V		0.68	2.33		
Butyryl Thiocholine	7.4/37°	v	20	0.00	0.12	0.00	0.00
		K_m	2–80		40		
		V			0.37		

* V and v in U/mg, K_m in mM.
† Esterase pI 5.2 shows no activity with any of these esters.
‡ Substrate activation above 1mM (Fig. 2).

isocarboxazid metabolizing esterase [23] have been reported to hydrolyze procaine. Here we report additional kinetic data for esterase pI 6.0 (Table 1) that is identical with esterase E1 [8]. Esterase pI 5.6 also has some activity with procaine. In contrast, the purified esterases pI 5.2, 6.2 and 6.4 do not cleave this analgesic.

The activities shown in Table 1 have been measured at pH 8.6. It is remarkable that at pH 7.0 the esterases exhibit only about 2% of these activities.

Hydrolysis of clofibrate

Administration of clofibrate induces long-chain acyl-CoA hydrolases in rat liver [24, 25]. Therefore, we expected that this drug would be hydrolyzed by the corresponding enzymes, i.e. esterases pI 6.2 and 6.4 [10]. Surprisingly, these esterases have low clofibrate-cleaving activity as compared to esterase pI 6.0 (Table 1), the dominant nonspecific esterase of rat liver [8]. The purified esterases with lower isoelectric points were not active with this drug. Clofibrate is also hydrolyzed by purified esterases from human liver and intestine [26].

Hydrolysis of isoarecaidine esters

Isoarecaidine esters reduce blood pressure in cats and rats [27]. It has been postulated that the esters might act as prodrugs and are activated by enzymatic hydrolysis [27]. Four of our purified esterases are capable of cleaving esters of this type; esterase pI 6.0 has the highest activity (Table 1). It is remarkable that the apparent K_m-values are in the order of 10 μM. These are the lowest K_m-values found so far with this enzyme and xenobiotic substrates [2, 10, 22]. Substrate concentrations above 1 mM seem to have an extra activating effect upon esterase pI 6.0 (Fig. 2). The reason for this anomalous kinetic is not clear. Possibly, a second binding site exists, as has been discussed for pig liver esterase [5].

The hydrolysis of isoarecaidine esters by the purified rat liver esterases is extremely sensitive to pH in the range of pH 7–9. At pH 7.0 the esters are almost stable to enzymatic hydrolysis. At pH values above 9 these esters are hydrolyzed spontaneously at high rates.

Hydrolysis of choline esters

Butyryl choline or butyryl thiocholine is hydrolyzed by a number of purified liver carboxylesterases from various species [1, 5, 6]. Of the hydrolases investigated here, only the esterase pI 6.0 has a low butyryl thiocholine-cleaving capacity. Physostigmin (0.1 mM), a specific inhibitor of cholinesterases, inhibits the hydrolysis of butyryl thiocholine by 27%, whereas the 4-nitrophenyl acetate activity of esterase pI 6.0 decreases by only 13% under the same conditions. Therefore, it is possible that our purified esterase may be contaminated with a low amount of a more specific cholinesterase. On the other hand, the high apparent K_m-value (40 mM) for the hydrolysis of butyryl thiocholine by esterase pI 6.0 may indicate that this is a minor activity inherent to this esterase. Acetyl thiocholine (photometric assay), acetyl choline and butyryl choline (titrimetric assay) were not measurably cleaved by any of the five carboxylesterases.

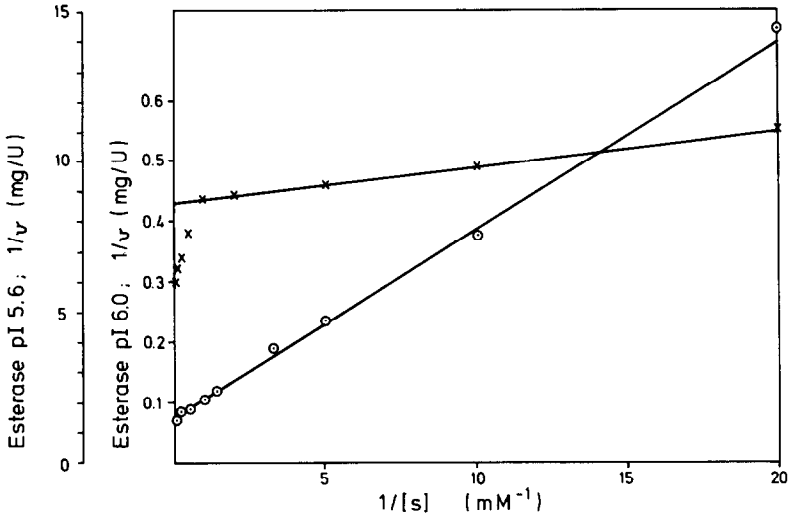

Fig. 2. Hydrolysis of isoarecaidine propylester by esterases pI 5.6 and 6.0. Lineweaver–Burk plot. Assay at pH 8.6, 30°. (×), esterase pI 6.0 (○), esterase pI 5.6.

Hydrolysis of amides

The amide-type drugs used in this study are preferably cleaved by the esterases pI 5.6 and 6.0 (Table 2). Esterase pI 5.6 that represents most of the acetanilide-cleaving capacity of rat liver [2] has the relatively highest activity with the aromatic amides acetanilide and phenacetin. In contrast, the anilide-type local anesthetic butanilicaine is preferred by esterase pI 6.0. Since this enzyme also represents the highest octanoyl-amide-cleaving activity (Table 2) it seems that the amidase activity of these hydrolases depends on the chain length and lipophilicity of the acyl moiety rather than on the chemical differences between aliphatic and aromatic amides.

At pH 7.0, 37°, the butanilicaine-cleaving activity of esterase pI 6.0 amounts to only one third (6.6 U/mg with 5 mM substrate) of the corresponding activity at pH 8.6 (Table 2). Using a titration curve of butanilicaine we obtained a pK-value of 7.4 for the secondary amino group in the acyl moiety of this substrate. This means that butanilicaine is almost uncharged at pH 8.6, whereas more than two-thirds of this substrate are positively charged at pH 7.

A number of purified carboxylesterases have been reported to cleave butanilicaine [2]. Arndt and Krisch [22] reported a K_m of 0.25 mM and V of 5.0 U/mg for the hydrolysis by purified rat liver esterase E1 at pH 8.6, 30°.

Deacetylation is a minor pathway in the metabolism of phenacetin [1, 28]. However, the deacetylation product phenetidin has a considerable toxicity. There is evidence that the mutagenicity of phenacetin depends on the deacetylation rate in liver [29]. In an *in vivo* study it could be demonstrated that the esterase inhibitor bis(4-nitrophenyl)phosphate reduced phenacetin hydrolysis and, as a consequence, phenacetin-induced methemoglobinemia in rats [30].

Esterase pI 5.6 is responsible for most of the phenacetin-cleaving activity of rat liver. This can be concluded from the data of Table 2 in connection with the preparative yield of this enzyme [8] and the specific activity of rat liver homogenates (0.16 mU/mg protein at pH 8.6, 37°). It may be important that this esterase can specifically be inhibited by bis(4-cyanophenyl)phosphate *in vivo* [18].

The hydrolysis of octanoyl amide as described in

Table 2. Hydrolysis of amide-type drugs by the purified carboxylesterases

Substrate (Formulas in Fig. 1)	pH/ Temperature	Constant*	Substr. conc. (mM)	Data obtained with esterase pI 5.2	5.6	6.0	6.2	6.4
Butanilicaine (V)	8.6/37°	v	5	0.0	3.0	20.4	0.0	0.0
		K_m	0.1–10		3.4	0.97		
		V			5.1	24.4		
Phenacetin (VI)	8.6/37°	v	5	0.00	0.060	0.004	0.000	0.00
		K_m	1–7.5		2.8			
		V			0.094			
Acetanilide	8.6/37°	v	10	0.55	14.0	0.00	0.00	0.00
		K_m	0.5–15		15.0			
		V			35.0			
Octanoylamide	7.4/37°	v	1	0.0	0.2	1.5	0.5	0.2

* V and v in U/mg, K_m in mM.

Table 2 is the first example of an aliphatic amide that is hydrolyzed by a purified liver carboxylesterase. Simple amides of fatty acids exhibit narcotic action [31]. Both this action and the rate of hydrolysis in the body increase with the length of the paraffin chain [31]. Octanoyl amide and other aliphatic amides also induce the porphyrin biosynthesis [32]. These amides were reported to be hydrolyzed by a bis(4-nitrophenyl)phosphate-sensitive enzyme in chicken liver [32].

CONCLUSIONS

All liver carboxylesterases used in this study have a high specific activity with simple aromatic esters, e.g. the histochemical substrate 1-naphthyl acetate [10]. In contrast, these esterases exhibit a marked specificity if assayed with various ester- or amide-type drugs. Although the limited number of drugs used here does not allow a detailed description of the nature of the active sites of these enzymes, some general conclusions may be drawn, if earlier results on the specificity of these esterases [10, 22] are taken into account. Esterase pI 5.2 that does not significantly hydrolyze any of the drugs assayed here is excluded from the further discussion.

Two features of the substrate structure appear to influence the activity of the individual esterases: the overall length of the acyl moiety, and the presence of charged groups. Although all good substrates of the esterases are lipophilic to a certain extent, we do not see a clear correlation between lipophilicity and esterase activity.

The influence that charged groups exert on the esterase activity is most obvious with esterase pI 6.0, but can also be seen with esterase pI 5.6. Two of the drugs cleaved by these enzymes have amino groups with pK-values above 7 in their acyl moiety, namely butanilicaine (pK of the secondary amino group = 7.4) and the isoarecaidine esters (pK of the *N*-methyl piperidino group ≈ 9–10). In both cases the hydrolase activity was much higher at pH 8.6 than at pH 7.0. The isoarecaidine esters that occur exclusively in their positively charged form at pH 7.0 are not cleaved at all at this pH. These results are in contrast to the broad pH-optima observed for the esterases with totally uncharged substrates, e.g. the esterases cleave phenyl butyrate at pH 7.0 and 8.6 at about the same rates [10]. Therefore, we assume that substrates with cationic acyl moieties are enzymatically hydrolyzed only in their deprotonized form. (The diethylamido group of propanidid and the 4-aminobenzoyl residue of procaine have low pK-values and are uncharged at neutral pH.)

A charged group in the alcohol moiety of the substrate also seems to reduce the activity of esterase pI 6.0, as can be concluded from the pH-dependency of the hydrolysis of procaine (pK of the diethylamino group = 9.0). Such a charge in the alcohol residue has less influence on the other esterases: esterase pI 5.6 is active with palmitoyl carnitine and lysophospholipids [10], and the esterases pI 6.2 and 6.4 cleave palmitoyl CoA [10] and aspirin.

We calculated the molecular dimensions of a variety of esterase substrates, including the drugs used here, using the standard atomic distances. Considering these data, it appears that the esterases pI 6.2 and 6.4 prefer substrates with a relatively long acyl residue, e.g. palmitoyl (2.0 nm), lauryl (1.5 nm), or the acyl moiety of propanidid (1.5 nm). Substrates with medium sized acyl residues, such as octanoyl (1.0 nm) or the acyl moieties of procaine (0.7 nm), clofibrate (0.95 nm), isoarecaidine (0.75 nm), and butanilicaine (0.9 nm), are optimally hydrolyzed by esterase pI 6.0. Esterase pH 5.6 exhibits the relatively highest activities with substrates that have a short acyl residue (<0.5 nm) [10, 33], provided that the alcohol or amide moieties have a certain lipophilicity.

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