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# CHOLINESTERASES OF HEART MUSCLE

## CHARACTERIZATION OF MULTIPLE ENZYMES USING KINETICS OF IRREVERSIBLE ORGANOPHOSPHORUS INHIBITION

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**Abstract**—Cholinesterases of porcine left ventricular heart muscle were characterized with respect to substrate specificity and inhibition kinetics with organophosphorus inhibitors *N,N'*-di-isopropylphosphorodiamidic fluoride (Mipafox™), di-isopropylphosphorofluoridate (DFP), and diethyl *p*-nitrophenyl phosphate (Paraoxon). Total myocardial choline ester hydrolysing activity (234 nmol/min/g wet wt with 1.5 mM acetylthiocholine, ASCh; 216 nmol/min/g with 30 mM butyrylthiocholine, BSCh) was irreversibly and covalently inhibited by a wide range of inhibitor concentrations and, using weighted least-squares non-linear curve fitting, residual activities as determined with four different substrates in each case were fitted to a sum of up to four exponential functions. Quality of curve fitting as assessed by the sum of squares reached its optimum on the basis of a three component model, thus, indicating the presence of three different enzymes taking part in choline ester hydrolysis. Final classification of heart muscle cholinesterases was obtained according to both substrate hydrolysis patterns with ASCh, BSCh, acetyl-β-methylthiocholine and propionylthiocholine, and second-order rate constants for the reaction with organophosphorus inhibitors Mipafox, DFP, and Paraoxon. One choline ester-hydrolysing enzyme was identified as acetylcholinesterase (EC 3.1.1.7), and one as butyrylcholinesterase (EC 3.1.1.8). The third enzyme with relative resistance to organophosphorus inhibition was classified as atypical cholinesterase.

About 70 years after Otto Loewi first documented the role of the "vagus substance" (acetylcholine, ACh‖) for neuro-humoral nerve impulse transduction in the isolated frog heart [1], it is now generally accepted that ACh released from vagal nerve endings in mammalian heart muscle has to be removed rapidly from neuroeffector junctions by both diffusion and hydrolysis of the transmitter substance to reestablish postsynaptic responsiveness [2–4].

The impact of diffusion followed by extracellular washout for rapid ACh removal has been elucidated in a number of studies using isolated heart preparations [2, 3, 5]. In contrast, the role of ACh hydrolysis for cholinergic transmission in heart muscle has been investigated only indirectly by measuring ACh effects in the presence of substances with inhibitory action on heart muscle cholinesterases [6, 7].

Studies on heart muscle cholinesterases have been concerned mainly with histochemical investigations using so-called "specific" substrates or inhibitors of either AChE (EC 3.1.1.7) or BChE (EC 3.1.1.8) [7, 8]. On the basis of histochemical studies, cholinergic innervation of mammalian ventricles had been disputed for a long time [10]. However, evidence for cholinergic innervation of right and left ventricular heart muscle has been reviewed extensively [4, 9, 10].

Earlier results from this and other laboratories on cholinesterase isoenzymes of serum, liver and the central nervous system revealed that neither substrates nor inhibitors can be regarded as absolutely specific for either AChE or BChE [11–14]. However, differentiation and characterization of cholinesterase isoenzymes may be achieved by combining patterns of substrate hydrolysis and second-order inhibition rate constants for the reaction with organophosphorus compounds [13, 14].

In the present paper, irreversible covalent inhibition by anticholinesterase agents *N,N'*-di-isopropylphosphorodiamidic fluoride (Mipafox™), diethyl *p*-nitrophenyl phosphate (Paraoxon), and DFP is used to differentiate choline ester-hydrolysing enzymes of heart muscle. With analysis of inhibition data by least-squares non-linear curve fitting, three enzymes can be differentiated and characterized according to their substrate specificity. One esterase is identified as AChE, one as BChE. For an additional enzyme which is not inhibited by the organophosphorus compounds Mipafox, Paraoxon, and DFP at up to millimolar concentrations, preliminary classification as atypical cholinesterase is suggested.

### MATERIALS AND METHODS

*Materials.* The following inhibitors, substrates and

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‖ Abbreviations: AβMSCh, acetyl-β-methylthiocholine; ACh, acetylcholine; AChE, acetylcholinesterase; ASCh, acetylthiocholine; BSCh, butyrylthiocholine; BChE, butyrylcholinesterase; DFP, di-isopropylphosphorofluoridate; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); PSCh, propionylthiocholine; SoS, sum of squares; w.w., wet wt.

reagents were used: Mipafox from Bayer AG (Leverkusen, Germany); DFP from Fluka AG (Buchs, Switzerland); Paraoxon (E 600), DTNB, ASCh, AβMSCh, PSCh and BSCh from Serva Feinbiochemica (Heidelberg, Germany). All other reagents were of analytical grade and purchased from local suppliers.

*Tissue preparation.* Heart muscle samples of about 5 g w.w. were obtained from the left ventricular free wall of healthy Landrace pigs (about 50 kg) of either sex from a local slaughterhouse. Hearts were excised after thoracotomy, immediately and thoroughly rinsed off with 0.9% (w/v) saline to remove blood and stored in ice-cold cardioplegic solution containing 0.18 M KCl, 10 mM Tris–HCl pH 7.4. All following preparation steps were carried out in a cold room (4°). Heart muscle samples were freed from endocardial and epicardial tissue, minced with scissors and homogenized in 6 mL/g w.w. cold phosphate buffer (0.2 M $NaH_2PO_4/Na_2HPO_4$, 50 mM NaCl, 1 mM Na-EDTA, pH 7.0) using an Ultraturrax (IKA-Labortechnik, Staufen, Germany) homogenizer (10 sec at maximal setting). Crude homogenates were adjusted to pH 7.0 using 1 M Tris–base. The final homogenization was performed by one down-pass with a loosely fitting rotating Teflon pistil in a Potter–Elvejhem homogenizer.

*Cholinesterase assay.* Cholinesterase activities were determined spectrophotometrically using the time-dependent increase in the concentration of thionitrobenzoic anion formed with DTNB (Ellman's reagent) after thiocholine ester hydrolysis [13–15]. Heart muscle homogenate (50 μL) containing 7.14 mg of left ventricular w.w. was preincubated with 50 μL 0.9% (w/v) NaCl for 5 min at 25°. The enzymatic reaction was started by adding 500 μL substrate solution (final concentration either 1.5 mM ASCh, 2.4 mM AβMSCh, 1.5 mM PSCh or 30 mM BSCh). After 60 min incubation (25°) the reaction was stopped by adding 500 μL of ice-cold perchloric acid (0.33 M) followed by centrifugation (3 min, 14,000 rpm; Eppendorf centrifuge 5415). Clear supernatant (550 μL) was transferred to a freshly prepared mixture of 500 μL $Na_2HPO_4$ (0.5 M) and 50 μL DTNB solution (10 mM in 0.1 M phosphate buffer, pH 7.0). Absorbance at 412 nm was determined in a spectrophotometer (Zeiss PM4). A blank receiving 50 μL of heart muscle homogenate after perchloric acid stop was run under identical conditions.

*Cholinesterase inhibition.* Heart muscle homogenate (50 μL) containing 7.14 mg of left ventricular w.w. was preincubated with 50 μL of organophosphate solution (60 min, 25°). Final buffer concentrations were 0.1 M phosphate buffer pH 7.0, 25 mM NaCl, and 0.5 mM Na-EDTA. Inhibitor stock solutions (Mipafox, Paraoxon or DFP, 0.1 M in ethanol) were stored at 4° and diluted with 0.9% (w/v) NaCl immediately before inhibition experiments to yield 50 different inhibitor concentrations ranging from 0.1 nM to 5 mM. Organophosphate inhibition was stopped by adding 500 μL of substrate solution and residual enzyme activities were determined as described above. Inhibition experiments were repeated with inhibition time reduced 4-fold (to 15 min) and inhibitor concentrations increased 4-fold.

*Analysis of cholinesterase inhibition kinetics.* Organophosphate inhibition data of heart muscle cholinester-hydrolysing activities were analysed using weighted least-squares non-linear curve fitting according to the Marquardt–Levenberg algorithm (SIGMAPLOT™, 4.0; Sigma-Chemie GmbH, München, Germany). Briefly, data on residual activities $A$ determined with ASCh, AβMSCh, PSCh and BSCh as substrates after inhibition by Mipafox, Paraoxon or DFP (inhibition time $t$ at 25°) and corresponding inhibitor concentrations $c$ were fitted to a sum of exponentials:

$$A = \sum_{i=1}^{m} A_0^i \times e^{-k_2^i \times t \times c} \qquad (1)$$

for $m = 1$, $m = 2$, $m = 3$, and $m = 4$ (single component, two component, three component, or four component model, respectively) with $A_0$ being the uninhibited activity at $t = 0$ and $k_2$ being the second-order rate constant ($M^{-1}\,min^{-1}$). The iterative process was started with initial parameter estimates for $A_0^i$ and $k_2^i$ graphically obtained from exponential inhibition curves. The terms $\Sigma A_0^i = 100$ and $k_2^i > 0$ were used as constraints. The reciprocals of variances $s_i^2$ of residual activities $A^i$ were used as weight values. After convergence of the fitting process, the set of parameters $A_0^i$ and $k_2^i$ that caused Eqn (1) to best fit the data was determined and the minimized SoS of the differences between equation and actual data values was indicated as a parameter or curve fitting quality. For all individual inhibition experiments parameter dependencies in the case of a four component model suggested that the model applied was too complex for the data set.

*Statistical analysis.* Data are given as means ± SEM.

## RESULTS

Total choline ester hydrolysing activity of porcine left ventricle (N = 5) with ASCh as substrate was found to be 234 ± 6 nmol/min/g w.w. ASCh hydrolysis was irreversibly and covalently inhibited using the organophosphorus anticholinesterase agent Mipafox (60 min; 25°) at a wide range of inhibitor concentrations (Fig. 1). As evidenced in Fig. 1, about 30% of the total ASCh hydrolysing, activity was inhibited by Mipafox concentrations of less than 5 μM. In contrast, an activity component of about 15% of the total remained active even after Mipafox inhibition with concentrations of more than 1 mM.

As has been confirmed with purified cholinesterases, organophosphate inhibition in the presence of excess inhibitor follows first-order kinetics [13, 14]. Thus, the non-sigmoidal shape of the Mipafox inhibition curve depicted in Fig. 1 strongly indicated enzymatic heterogeneity of left ventricular total ASCh-hydrolysing activity. Using weighted least-squares non-linear curve fitting a sum of $m$ exponentials comprising parameters for both ASCh hydrolysing activities $A_0^i$ (nmol/min/g w.w.) and bimolecular Mipafox inhibition rate constants $k_2^i$

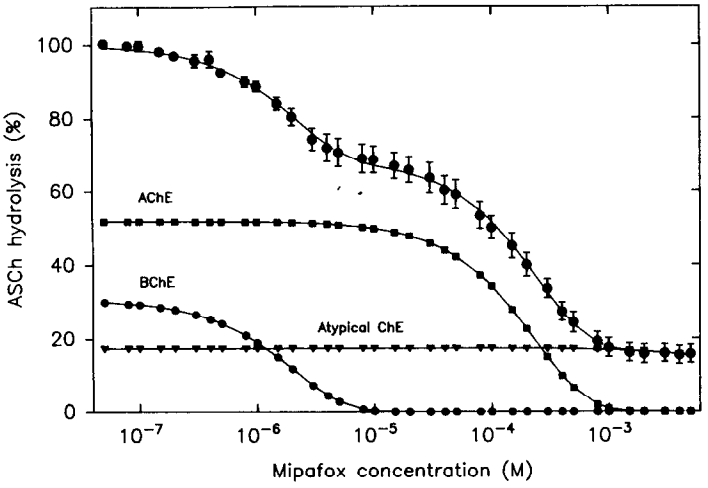


Fig. 1. Mipafox inhibition of ASCh hydrolysis in porcine heart muscle (60 min; 25°). Means ± SEM of experiments on N = 5 individual hearts. The inhibition curve superimposed upon experimental points was obtained by non-linear least-squares curve fitting on the basis of a three component model (cf. Materials and Methods). Residual activities of activity components atypical ChE (▼), AChE (■) and BChE (●) were calculated for each inhibitor concentration from respective Mipafox inhibition rate constants $k_2$ and uninhibited activities $A_0$.

($M^{-1}\,min^{-1}$) was fitted to Mipafox inhibition data. On the basis of either two ($m = 2$) or three ($m = 3$) activity components hydrolysing ASCh and being inhibited by Mipafox, parameter estimates for $A_0^i$ and $k_2^i$ were derived from paired data of residual enzyme activities and corresponding inhibitor concentrations (cf. Materials and Methods).

For residual ASCh hydrolysing activities (means ± SEM; N = 5) after Mipafox inhibition shown in Fig. 1, the quality of curve fitting as measured by the SoS of the differences between equation values and actual data values was significantly ($P < 0.01$) better on the basis of a three component model (SoS 1.57) as compared to a two component model (SoS 6.38). As shown in Fig. 1, the curve obtained from the least-squares procedure (three component model) is in excellent agreement with the experimental points. For each of the five individual Mipafox inhibition experiments better curve fitting was obtained based on a three component model (SoS 6.45 ± 1.98; range: 1.53–11.18) as compared to a two component model (SoS 44.80 ± 9.77; range: 17.40–75.71).

Bimolecular Mipafox inhibition rate constants $k_2^i$ as well as ASCh hydrolysing activities $A_0^i$ of corresponding uninhibited activity components obtained by the non-linear curve fitting process for $m = 3$ (three component model) are listed in Table 1. Activity components were listed according to their inhibition rate constants for the reaction with Mipafox and identified by roman numbers. Inhibition experiments were repeated routinely with inhibitor concentrations increased 4-fold and time of inhibition reduced 4-fold to 15 min. Since rate constants of organophosphate inhibition $k_2^i$ and ASCh hydrolysing activity components $A_0^i$ obtained by weighted least-squares curve fitting showed no significant differences, a major impact of inhibitor saturation kinetics (mainly concentrations of enzyme–inhibitor complexes) on bimolecular inhibition rate constants determined for cholinesterase isoenzymes could be excluded.

The rate constant of component I (ASCh hydrolysis 40 nmol/min/g w.w.) was close to zero indicating relative resistance to Mipafox inhibition. The bulk of total ASCh hydrolysing activity (52%) was associated with activity component II characterized by a bimolecular rate constant of $6.6 \times 10^1\ M^{-1}\,min^{-1}$ for Mipafox inhibition. Activity component III was inhibited about 130 times faster than was component II.

In Fig. 2 inhibition of left ventricular ASCh hydrolysing activity by organophosphorus compounds DFP and Paraoxon is compared. The curve best fitting to experimental points is included for both Paraoxon and DFP inhibition. The non-sigmoidal shape of the inhibition curves again indicates enzymatic heterogeneity. A slow reacting activity component could be identified with both inhibitors. Paraoxon inhibition in the intermediate range of inhibitor concentrations resulted in a more uniform decline of ASCh hydrolysis than inhibition with both DFP (Fig. 2) and Mipafox (Fig. 1). Analysis of DFP inhibition kinetics (Fig. 2) by least-squares curve fitting revealed ASCh hydrolysing activity components I, II and III in an identical activity range as documented for Mipafox inhibition (component I: 34 nmol/min/g; component II: 115 nmol/min/g; component III: 85 nmol/min/g). Corresponding bimolecular rate constants for DFP inhibition are listed in Table 1. With least-squares non-linear curve fitting applied to Paraoxon inhibition of left ventricular ASCh hydrolysing activity (Fig. 2) the best fit was obtained on the basis of a two component model (30 nmol/min/g and 204 nmol/min/g, respectively). The component of 204 nmol/

Table 1. Cholinesterases of porcine left ventricular heart muscle: substrate specificities and rate constants for organophosphorus inhibition

| Activity component | Substrate hydrolysis (nmol/min/g) | | | | Inhibition rate constants, $k_2$ ($M^{-1} min^{-1}$) | | |
|---|---|---|---|---|---|---|---|
| | ASCh | AβMSCh | PSCh | BSCh | Mipafox | Paraoxon | DFP |
| I Atypical cholinesterase | 36 ± 4 | 31 ± 4 | 18 ± 1 | 77 ± 13 | $3.5 (\pm 0.8) \times 10^{-1}$ | $1.5 (\pm 0.3) \times 10^{0}$ | $5.8 (\pm 1.1) \times 10^{-1}$ |
| II AChE | 119 ± 4 | 96 ± 6 | 71 ± 3 | 0 | $6.8 (\pm 2.3) \times 10^{1}$ | $3.6 (\pm 0.6) \times 10^{4}$* | $7.4 (\pm 0.1) \times 10^{3}$ |
| III BChE | 79 ± 7 | 32 ± 1 | 80 ± 3 | 139 ± 9 | $7.3 (\pm 0.8) \times 10^{3}$ | $2.9 (\pm 0.7) \times 10^{4}$* | $3.7 (\pm 1.0) \times 10^{5}$ |

Organophosphate inhibition of choline ester hydrolysis was performed at 25° (60 min). Data on cholinester hydrolysis are mean values ± SEM of N = 8 experiments. Bimolecular inhibition rate constants are mean values ± SEM obtained by analysis of inhibition kinetics (cf. Materials and Methods) of N = 4 experiments performed with the respective inhibitor and four different substrates. Mipafox inhibition of ASCh hydrolysis was analysed in a total of N = 5 individual hearts.

* Paraoxon inhibition rate constants listed for AChE and BChE show a slight difference in mean values because AChE, in contrast to BChE, cannot be detected in Paraoxon inhibition experiments of BSCh hydrolysis.

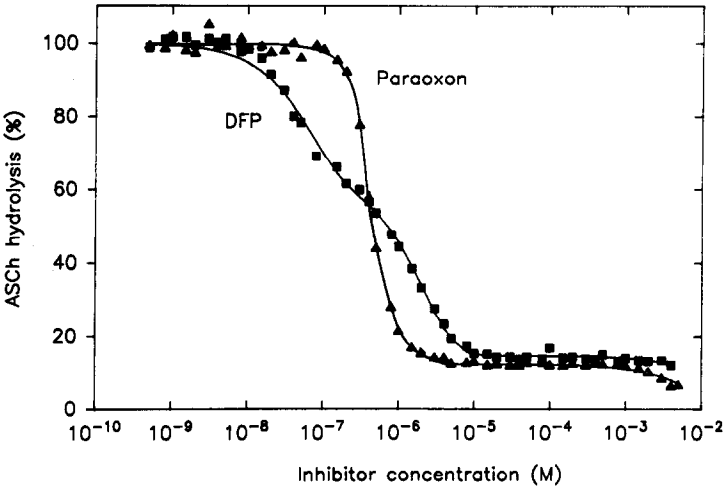


Fig. 2. Paraoxon (▲) and DFP (■) inhibition (60 min; 25°) of ASCh hydrolysis in an individual porcine left ventricular heart muscle. The inhibition curve was calculated by non-linear least-squares curve fitting on the basis of a three component model (see Materials and Methods).

min/g was equivalent to the sum of activity components II and III as listed in Table 1 and showed a Paraoxon inhibition rate constant of $k_2 = 3.0 \times 10^4 M^{-1} min^{-1}$. ASCh hydrolysing activity component I was only slowly inhibited by paraoxon (cf. Table 1).

Bimolecular inhibition rate constants for the reaction with organophosphorus compounds can be easily used to identify and characterize individual activity components. Since the rate constant of a specific activity component for the reaction with a certain organophosphorus inhibitor is independent of the substrate used to test its residual activity [13], comparison of residual hydrolytic activities towards different choline esters after organophosphorus inhibition can help to establish substrate specificity of choline ester hydrolysing activity components. An example is given in Fig. 3. After inhibition of choline ester hydrolysing activity in left ventricular porcine myocardium by DFP, residual activity was determined with both ASCh and AβMSCh. In a semi-logarithmic plot of log activity versus inhibitor concentration residual activity shows a linear decay with increasing inhibitor concentrations if only one single component is active. With DFP inhibition this could be observed for DFP concentrations of more than 15 μM (Fig. 3). The slowest reacting activity component with both substrates (ASCh and AβMSCh) was identified as activity component I using bimolecular rate constants of DFP inhibition (ASCh experiment: $6.9 \times 10^{-1} M^{-1} min^{-1}$; AβMSCh experiment: $7.1 \times 10^{-1} M^{-1} min^{-1}$). Uninhibited

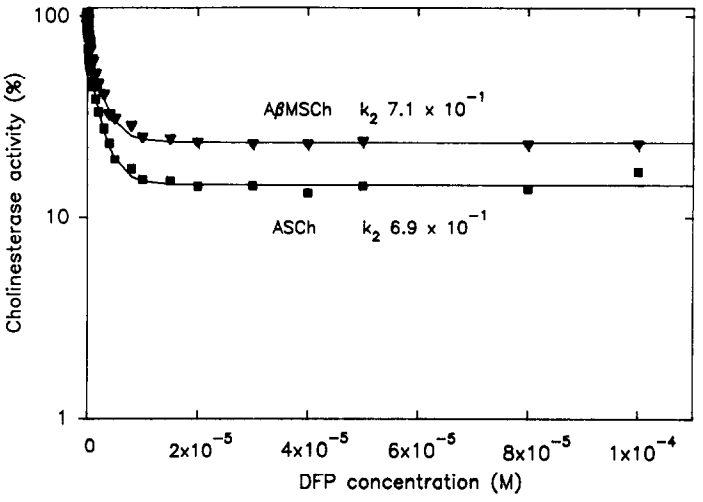


Fig. 3. DFP inhibition (60 min; 25°) of heart muscle cholinesterase activities from an individual pig heart. Residual activity determined with either ASCh (■; uninhibited activity 236 nmol/min/g), or AβMSCh (▼; uninhibited activity 187 nmol/min/g). Inhibition curves and DFP inhibition rate constants $k_2$ were calculated by non-linear least-squares curve fitting on the basis of a three component model (cf. Materials and Methods).

activity of component I was obtained by extrapolation to zero inhibitor concentration: 34 nmol/min/g with ASCh, and 44 nmol/min/g with AβMSCh. Identical experiments were performed using PSCh and BSCh as substrate. When BSCh was used as a substrate, inhibition rate constant $k_2$ of activity component II could not be detected with any of the inhibitors Paraoxon, DFP and Mipafox.

In Table 1, substrate specificity and organophosphate inhibition rate constants of left ventricular choline ester hydrolysing activity components are summarized. The major ASCh hydrolysing activity component II did not take part in BSCh hydrolysis and because of its substrate specificity can be classified as AChE. Activity component III hydrolysed BSCh faster than ASCh. Its rate constants for both Mipafox and DFP inhibition were about two orders of magnitude higher than those of AChE. Therefore, choline ester hydrolysing activity component III can be classified as BChE. As compared to AChE and BChE, choline ester hydrolysing activity component I exhibited extremely low reactivity towards all three organophosphates up to millimolar inhibitor concentrations. The best substrates for this enzyme were BSCh and AβMSCh. According to both inhibition kinetics and the pattern of substrate hydrolysis, activity component I may be referred to as atypical cholinesterase.

## DISCUSSION

According to a generally accepted definition, cholinesterases catalyse the hydrolysis of choline esters at a higher rate than that of other esters and can be inhibited by low concentrations ($\leqq 10\ \mu M$) of organophosphorus compounds [16]. Cholinesterase isoenzymes of different tissues and organs including serum, liver, and the central nervous system have been characterized using so-called specific substrates and inhibitors [11–14]. AChE located predominantly in erythrocytes and nerve tissue plays a central role in synaptic transmission. Isoenzymes of BChE, on the other hand, have been characterized in serum and liver and are thought to preferentially hydrolyse choline esters propionylcholine and butyrylcholine. Their physiological significance until now has not been known.

In the present paper, we were able to differentiate three choline ester hydrolysing enzymes of porcine left ventricular heart muscle on the basis of inhibition kinetics with the organophosphorus compounds Mipafox, DFP and Paraoxon. According to classic criteria, choline ester hydrolysing activity components II and III are classified as AChE and BChE, respectively (cf. Table 1).

As evidenced in Table 1, AChE does not hydrolyse the substrate BSCh. In this respect, BSCh can be referred to as substrate which is specific for non-AChE cholinesterases of heart muscle. All other substrates listed in Table 1 are non-specific, the best substrates for AChE being ASCh and AβMSCh.

Analysis of cholinesterase inhibition kinetics can be applied to crude tissue homogenates without prior purification of enzyme proteins, and isoenzyme patterns can be established without solubilization and separation techniques. However, it should be taken into account that ventricular myocardium consists of up to 8% capillaries on a volume percent basis [17]. In our experiments, a major contribution of blood cholinesterases to total myocardial choline ester hydrolysing activity of heart muscle could be ruled out. With low BSCh hydrolysing activity of porcine serum (185 ± 11 nmol/min/mL blood; N = 10) and no BSCh hydrolysis of porcine erythrocyte AChE, possible contributions of blood cholinesterases to myocardial choline ester hydrolysis

are limited to a maximum of about 7% of total. Moreover, bimolecular inhibition rate constants ($M^{-1}$ $min^{-1}$) of porcine serum cholinesterase for the reaction with Paraoxon ($k_2 = 1.8 \times 10^5$) and Mipafox ($k_2 = 2.1 \times 10^4$) differ from those of cholinesterase isoenzymes in heart muscle (cf. Table 1).

The presence of parasympathetic fibers in mammalian ventricles has been well established [8, 18]. However, the question of whether or not AChE and BChE activities in the myocardium on a cellular level are limited to nerve elements remains to be decided. Histochemical investigations, partly based on the questionable specificity of cholinesterase inhibitors, have demonstrated activities of both AChE and BChE in the atrioventricular node, the bundle of His and its two main branches, neurons of cardiac ganglia, postganglionic fibers, and atrial as well as ventricular muscle fibers [8]. In contrast, relatively few data are available on the subcellular distribution of myocardial cholinesterase isoenzymes. Nyquist-Battie *et al.* [19] using velocity sedimentation on sucrose gradients differentiated three major molecular forms of cholinesterases from rat heart atria and ventricles (two globular, one asymmetric). Gonzalez *et al.* [20] solubilized both globular and asymmetric AChE forms from human right atrial auricles without detailed characterization of enzyme proteins.

The main effects of the transmitter substance ACh on heart muscle which are mediated by binding to muscarinic $M_{2\alpha}$ receptors [21] can be summarized as negative chronotropic (sinus node), dromotropic (atrioventricular node and possibly His-Purkinje fibers) and inotropic (atrial and ventricular contractility). Cholinergic effector substances in heart muscle (ACh and structural analogues like AβMCh, metacholine) increase the ventricular fibrillation threshold [22] and, independent of their influence on mechanical performance and coronary perfusion, exhibit primary metabolic regulatory effects on creatine kinase enzyme kinetics and fluxes of high energy phosphates in the myocardium [23].

The rate of ACh hydrolysis has been demonstrated to be an important determinant of the chronotropic response to repetitive vagal stimulation and of the phase dependency of this response [24]. Effects of vagal stimulation on heart rate and myocardial contractility are potentiated in the presence of cholinesterase inhibitors [6, 7]. Brown *et al.* [6] suggested that endogenous ACh is released spontaneously even in the absence of neuronal activity or depolarization–secretion coupling. Results presented in this paper emphasize the significance of myocardial cholinesterases for the hydrolytic removal of the cholinergic transmitter substance ACh in heart muscle under physiological and pathophysiological conditions.

Our results also pertain to toxicological considerations. In the case of an organophosphate intoxication *in vivo*, with irreversible dose- and time-dependent inhibition of both central nervous system AChE and serum BSCh resulting in so-called "endogenous ACh intoxication" [25], atypical cholinesterase which is relatively resistant to organophosphate inhibition would diminish continuously heart muscle ACh concentrations and thus support endogenous detoxification of organophosphorus compounds [26]. However, until now, nothing has been known about the physiological substrate(s) of myocardial atypical cholinesterase. Preliminary results from our laboratory indicate that atypical cholinesterase does not hydrolyse phenyl valerate and cannot therefore be classified as carboxylesterase (EC 3.1.1.1).

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