study once again demonstrate an important role of carboxylesterase in the detoxification of organophosphate anticholinesterases and the influence that this detoxification mechanism has on animal variation and the measured response, especially at sublethal doses.

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# Identification of the novel rat liver IBMX-insensitive phosphodiesterase as a non-specific phosphodiesterase capable of hydrolysing cCMP

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Cyclic nucleotide phosphodiesterases (PDE*) may be classified into five isoenzyme families. These families may be characterized by different kinetic properties and sensitivity to physiological and pharmacological effectors. All known members of these families are selective for purine cyclic 3′:5′-nucleotides, $Mg^{2+}$-dependent, and are inhibited by IBMX [1, 2]. Lavan *et al.* [3] have reported that rat liver contains a PDE that is not inhibited by IBMX and does not require $Mg^{2+}$ for activity. These properties made it distinct from any of the members of the five isoenzyme families, therefore it was suggested to be a novel activity. However, the properties of the rat liver IBMX-insensitive PDE are remarkably similar to those of a cCMP-PDE previously purified from pig liver [4–7]. In addition to insensitivity to IBMX and $Mg^{2+}$, both activities elute early from DEAE, have a size of 33 kDa on gel filtration and do not bind to Affigel blue. The pig liver cCMP-PDE will hydrolyse both cyclic 3′:5′-nucleotides and cyclic 2′:3′-nucleotides [5] and is inhibited by AMP and $P_i$ [6, 7]. In this manuscript we report that the rat liver IBMX-insensitive PDE will also hydrolyse all these substrates and

* Abbreviations: EGTA, ethyleneglycol-bis(β-aminoethyl ether)*N*,*N*,*N*′,*N*′-tetraacetic acid; PMSF, phenylmethylsulphonyl fluoride; IBMX, 3-isobutyl-1-methylxanthine; PDE, phosphodiesterase; $P_i$, inorganic phosphate; cAMP, adenosine 3′:5′-cyclic monophosphate; cGMP, guanosine 3′:5′-cyclic monophosphate; cCMP, cytidine 3′:5′-cyclic monophosphate.

is also inhibited by AMP and $P_i$. It is proposed that the rat liver IBMX-insensitive PDE is very similar, if not identical to, the previously described cCMP-PDE from pig liver.

*Materials and Methods*

A high-speed supernatant was prepared from perfused rat livers and chromatographed on a Mono-Q column as described by Lavan *et al.* [3]. Buffer A contained 50 mM Tris–HCl, 5 mM benzamidine, 0.1 mM EGTA, 0.1 mM dithiothreitol, 50 μM PMSF (pH 7.5). For the experiments shown in Fig. 1 a linear gradient of 25–175 mM NaCl in Buffer A was used, the flow rate was 0.5 mL/min and 0.5 mL fractions were collected. Gel filtration was performed on a Superose-12 column equilibrated in Buffer

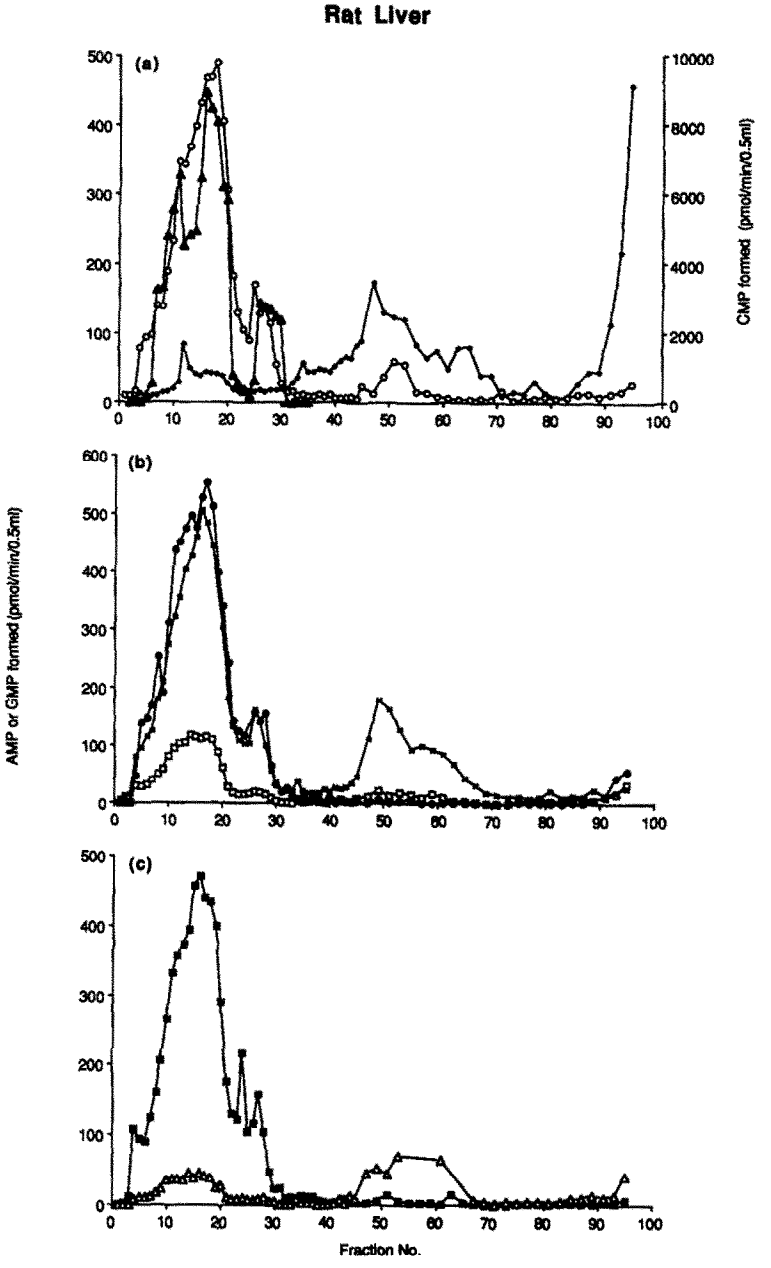


Fig. 1. Chromatography of rat liver high speed supernatant on Mono-Q. A high speed supernatant was prepared and chromatographed as described in Materials and Methods. Unless stated all assays were in the presence of 5 mM $MgCl_2$. (a) The fractions were assayed for PDE activity using the following substrates: (○) 1 μM [$^3$H]cAMP; (◆) 1 μM [$^3$H]cGMP; (▲) 1 mM cCMP (for clarity only the first 35 fractions are shown; no activity was observed in fractions 35–95). (b) The fractions were assayed for PDE activity using 1 μM [$^3$H]cAMP as substrate with the following additions: (●) 100 μM IBMX; (×) 10 units calmodulin/10 mM $Ca^{2+}$; (□) 1 mM cCMP. (c) The fractions were assayed for PDE activity using 1 μM [$^3$H]cAMP as substrate (■) with the addition of 1 mM AMP and (△) in the absence of $MgCl_2$.

A containing 0.5 M NaCl. PDE assays using [$^3$H]cAMP and [$^3$H]cGMP were as described [8]; hydrolysis of cCMP was assayed by the release of $P_i$ [9] following treatment with 5′-nucleotidase [10], results using cAMP showed that both assays gave quantitatively similar results. Other non-radioactive cyclic nucleotides were assayed by separating the product on TLC [6]. The concentrations of cyclic nucleotides used are shown in the figure legends, amounts of PDE were used so that the assays were linear. Cyclic nucleotides and other biochemicals were obtained from the Sigma Chemical Co. (Poole, U.K.), radiochemicals were from Amersham International (Amersham, U.K.).

*Results and Discussion*

When, using the conditions of Lavan *et al.* [3], a high-speed supernatant from rat liver was chromatographed on a Mono Q column and assayed using 1 μM cAMP or 1 μM cGMP as a substrate, a profile similar to that of these authors was obtained (see figure 3 of Lavan *et al.* [3]). The first activity to elute was the IBMX-insensitive PDE, this was followed by a $Ca^{2+}$/CaM-activated PDE and then by a cGMP-stimulated PDE and two peaks of a cAMP-specific PDE (results not shown). Subsequent chromatography used a different gradient so that the IBMX-insensitive PDE could be studied in more detail (see Fig. 1).

On this gradient two peaks of PDE are obtained: the first corresponds to the IBMX-insensitive PDE, and the second to the $Ca^{2+}$/CaM-activated PDE (the cGMP-stimulated PDE elutes at the very end of this gradient). The first peak has greater activity with 1 μM cAMP compared to 1 μM cGMP as substrate (Fig. 1a) and its activity is unaffected by the addition of calmodulin or IBMX (Fig. 1b) or the removal of $Mg^{2+}$ (Fig. 1c). These properties are identical to those reported by Lavan *et al.* [3]. The complete lack of effect of these agents strongly suggests that this peak does not contain a member of one of the five purine cyclic 3′:5′-nucleotide isoenzyme families. When the column fractions were assayed with cCMP as substrate a peak of activity which co-eluted with the first peak of cAMP hydroysis was obtained (Fig. 1a). The fact that cCMP inhibits the hydrolysis of cAMP (Fig. 1b) indicates that both cyclic nucleotides are being hydrolysed by the same enzyme. In addition, the first peak was inhibited by the addition of AMP (Fig. 1c) which is a reported inhibitor of the pig liver cCMP-PDE [5]. The shoulder on the first peak may be a proteolytic product as when frozen and thawed supernatant was used the proportion of the shoulder increased. No kinetic differences between the shoulder and the main peak have been observed.

The evidence above indicates that the first peak contains only one PDE activity. This was confirmed when the peak was pooled, concentrated and subjected to gel filtration and a single peak of PDE activity was obtained. This had a size of 25 kDa which, considering the differing chromatographic conditions, is similar to that obtained for the rat liver IBMX-insensitive PDE and the pig liver cCMP-PDE (see Table 1). The pooled peak was analysed kinetically and for selectivity towards substrates and inhibitors (Table 1). The results show that the rat liver IBMX-insensitive PDE has very similar properties to the pig liver cCMP-PDE [4–7]. In particular it may be noted that cCMP was a competitive inhibitor of cAMP hydrolysis (Fig. 2) indicating that both cyclic nucleotides are being hydrolysed by the same PDE. In addition, the enzyme showed no absolute selectivity and all purine and pyrimidine cyclic 3′:5′-nucleotides and cyclic 2′:3′-nucleotides were hydrolysed. The rank order of hydrolysis was identical to that reported for the pig liver cCMP-PDE [6]. The enzyme was also inhibited (> 95%) by 1 mM AMP and 1 mM $P_i$; none of the members of the five purine cyclic nucleotide isoenzyme families is affected by AMP and $P_i$ at these concentrations. In agreement with the results of Lavan *et al.* [3], the enzyme is not inhibited by the commonly used PDE inhibitors milrinone, zaprinast, rolipram and Ro 20-1724 [1–3] at 100 μM.

In conclusion, a PDE has been isolated from rat liver that has identical properties to the IBMX-insensitive PDE reported by Lavan *et al.* [3] and described by these authors as novel. However, as the properties of the rat liver IBMX-insensitive PDE are very similar, and in many cases, identical to a PDE isolated from pig liver that has been termed cCMP-PDE [4–7], we propose that they are the same enzyme. A PDE capable of hydrolysing cCMP ("cCMP-specific PDE") has been previously purified from rat liver [11]. A number of properties distinguish this enzyme from that reported here. In particular the cCMP-specific PDE has a very low rate of hydrolysis of other cyclic nucleotides (< 1% of the rate with cCMP). The physiological role of the cCMP/IBMX-insensitive PDE is

Table 1. Comparison of the properties of rat liver IBMX-insensitive PDE with pig liver cCMP-PDE

| Tissue | IBMX-insensitive PDE | cCMP-PDE | |
|---|---|---|---|
| Reference | Rat liver [3] | Pig liver [4–7] | Rat liver [This paper] |
| Molecular weight | 33 kDa | 33 kDa | 25 kDa |
| $K_m$ (μM) | | | |
| cAMP | 25 | 25 | 47 |
| cGMP | 237 | ND | >500 |
| cCMP | ND | 182 | >100 |
| $K_i$ (μM) | | | |
| cAMP* | ND | 21 | ND |
| cCMP† | ND | 242 | 452 |
| Rate of hydrolysis (at 2 mM) | | | |
| cyclic 3′:5′nucleotide | ND | cCMP>cAMP>cGMP | cCMP>cAMP>cGMP |
| cyclic 2′:3′nucleotide | ND | cCMP>cGMP>cAMP | cCMP>cGMP>cAMP |
| $Mg^{2+}$, IBMX | NE | NE | NE |

Experimental details are given in Materials and Methods and in the legend to Fig. 2.
* Determined with cCMP as substrate.
† Determined with cAMP as substrate.
ND, not determined; NE, no effect on activity.

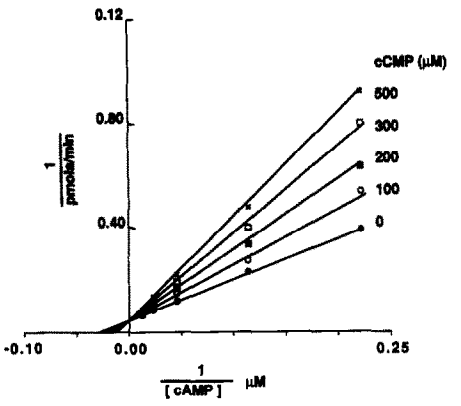


Fig. 2. Competitive inhibition of cAMP hydrolysis by cCMP of the IBMX-insensitive PDE. The first peak of activity (as described in Fig. 1) was pooled and concentrated by dialysis against solid sucrose, the PDE activity of this fraction was completely unaffected by IBMX. The enzyme was assayed as described in Materials and Methods using radiolabelled cAMP (4–90 μM) in the presence of the indicated concentrations of cCMP. The data were fitted by non-linear regression analysis to a series of equations representing different types of inhibition. The best fit to the data, measured as the smallest sum of square residuals, was to the equation for competitive inhibition. The $K_m$ and $K_i$ values obtained are given in Table 1.

unknown, although it has been suggested that it may be a major cAMP-hydrolysing enzyme in liver [3, 12]. However, its lack of substrate selectivity and the fact that it is inhibited by common metabolites such as AMP, ADP, GMP and GDP indicate that this may not be the case.

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