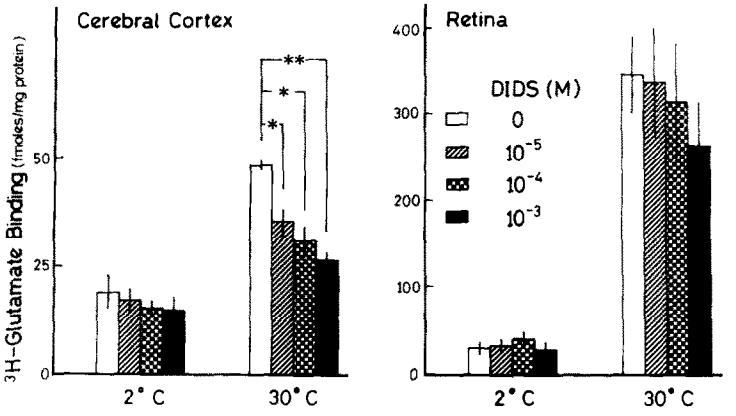

Fig. 3. Effect of DIDS on [^{3}H]Glu binding. Each membranous preparation was incubated with 10 nM [^{3}H]Glu in 50 mM Tris-acetate buffer (pH 7.4) at 2° or 30° for 60 min in the presence of various concentrations of DIDS. Each value represents the mean ± SE obtained from four to six independent experiments. Key: *$P < 0.05$ and **$P < 0.01$, compared with each control value.

In summary, it appears that the retinal Glu receptors may be lightly linked to the anion transport carriers, which is distinctly different from the coupling in the cerebral cortex. Further biochemical and pharmacological characterizations of the retinal Glu receptors are now under way in our laboratory.

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Department of Pharmacology and † Department of Biology Setsunan University Hirakata, Osaka 573-01, *Japan*

YUKIO YONEDA*
KIYOKAZU OGITA
HIROMICHI NAKAMUTA
YOSHIE FUKUDA
MASAO KOIDA
YASUNAO OGAWA†

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* Address correspondence to: Dr. Yukio Yoneda, Department of Pharmacology, Faculty of Pharmaceutical Sciences, Setsunan University, 45-1, Nagaotoge-cho, Hirakata, Osaka 573-01, Japan.

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Effect of hypolipidemic drugs on the metabolism of lauric acid and dimethylaminoazobenzene by rat liver microsomes

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Hepatic responses to clofibrate and other hypolipidemic drugs have been studied for many years [1, 2]. Aside from frank toxicity and carcinogenicity, prominent effects include hepatomegaly, associated with both hyperplasia and hypertrophy of hepatocytes, and proliferation of peroxisomes and endoplasmic reticulum [1, 2]. Enzymic activity associated with these organelles is often, but not always, induced. Liver enlargement occurs after clofibrate feeding.

After withdrawal of the drug, liver weight returns to normal within 1 week although increased numbers of peroxisomes may persist for somewhat longer [3, 4]. Administration of clofibrate to rodents is followed by a modest increase in total hepatic cytochrome P-450 [5–7], a 5- to 10-fold increase in laurate hydroxylation [6, 8, 9] and induction of epoxide hydrolase activity [10, 11]. Stabilization by clofibrate of cytochrome P-450 levels in primary cultures of rat hepatocytes has also been reported [12]. The structurally related hypolipidemic agent, nafenopin, causes marked hepatomegaly and profound proliferation of the hepatic endoplasmic reticulum, although it does not appreciably induce total hepatic levels of cytochrome P-450. In fact, clofibrate and nafenopin, although they elicit major hepatic responses, do not induce major changes in most cytochrome P-450-catalyzed oxidative pathways [13–17], unlike many other hepatotrophic chemicals [18]. Long-term (18–25 months) feeding of nafenopin leads to hepatocellular carcinomas in rodents [19, 20]. The drug, however, exhibits no promoting properties and, in fact, suppresses early histological and histochemical responses during experimental induction of tumors with acetylaminofluorine [21]. The plasticizer, di(2-ethylhexyl)phthalate (DEHP), is hypolipidemic [22], causes peroxisomal proliferation [23], and is hepatocarcinogenic [24]. A recent study showed that DEHP induces cytochrome P-450-dependent hydroxylation of lauric acid in primary rat hepatocyte cultures [25].

Dimethylaminoazobenzene (DAB) is metabolized in the liver to ring-hydroxylated, N-hydroxylated, N-demethylated and azoreduced products [26–30]. It was observed recently in this laboratory that treatment of rats with clofibrate markedly induces microsomal DAB azoreductase activity but suppresses N-demethylation, an oxidative pathway [9]. Nafenopin treatment, on the other hand, suppresses DAB azoreduction by 90% but leaves oxidative pathways relatively unaffected [9]. Other inducing agents, phenobarbital, β-naphthoflavone, methylcholanthrene, isosafrol, pregnenolone-16-α-carbonitrile, and triacetyloleandomycin, do not appreciably affect DAB azoreduction ([9] and unpublished observations), suggesting that a unique form of cytochrome P-450 catalyzes the reaction. Gibson and his colleagues [31] have reported that clofibrate and several other hypolipidemic agents induce a specific form of cytochrome P-450 (cytochrome P-452) that catalyzes laurate hydroxylation. In view of these findings, the possibility was considered that hypolipidemic chemicals were selective inducers of DAB azoreductase and, if so, could be valuable tools for the study of this activity. Therefore, an investigation was conducted on the effects of a number of hypolipidemic agents and structurally related compounds on laurate hydroxylation and both reductive and oxidative pathways of DAB metabolism.

Materials and methods

Animal treatment. The drugs (see Table 1) were purchased from the Sigma Chemical Co. (B, D, E, F) and the Aldrich Chemical Co. (M), or were gifts from Ayerst Laboratories (A), Sterling-Winthrop Research Laboratories (C), Ciba-Geigy (G), Wyeth Laboratories (H, I, J, K) and Merrill Dow Research Institute (L). A and M were injected i.p. and the others were administered by gastric tube as aqueous suspensions with a few drops of Tween 80, to male Wistar rats (200–250 g), 200 mg/kg/day for 7 days. Ciprofibrate (C) was given in a dose of 20 mg/kg/day since it is considerably more potent than clofibrate in inducing peroxisome proliferation and hypolipidemia. Controls were given vehicle alone by the corresponding route of administration. One day after the last dose [2 days after nafenopin (G)], livers were homogenized in 0.01 M Tris buffer, pH 7.4, containing 0.15 M KCl, and washed microsomes were prepared by differential centrifugation and stored at −70°.

Enzyme assays. Microsomal laurate hydroxylation and oxidative as well as reductive metabolism of DAB were measured as previously described [9, 30]. Protein content of microsomes was measured by the method of Lowry *et al.* [32], using bovine serum albumin as standard.

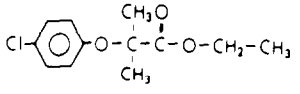
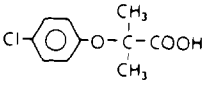
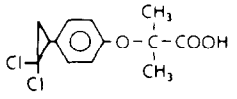
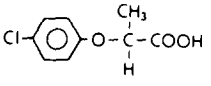
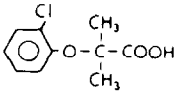
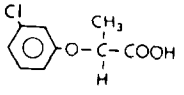
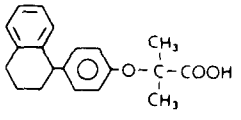
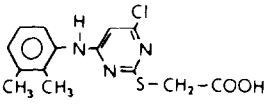
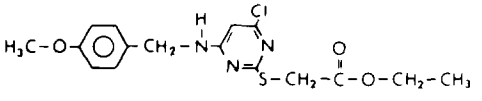
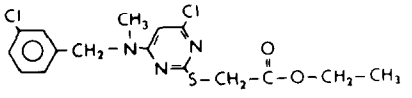
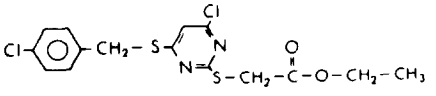
Results and discussion

The drugs studied are listed in Table 1. They include clofibrate (A), the deesterified derivative, clofibric acid (B) and several structural analogs (C–F), including nafenopin (G). Ciprofibrate has approximately ten times the hypolipidemic potency of clofibrate [33]. Wy-14643 (H) has potent hypolipidemic properties and causes hepatomegaly, peroxisomal proliferation and hepatocellular carcinoma [34, 35]. Several of its structural analogs (I–K) are devoid of hypolipidemic effects (R. Fenichel, Wyeth Laboratories, personal communication, and ref. 36). Probucol (L), although it does not cause hepatomegaly, lowers plasma cholesterol but not triglycerides [37]. The relative liver weight was increased 40–60% by all drugs except I and L where there was no significant increase (data not shown). Each compound in this structurally diverse group induced laurate hydroxylation 2- to 8-fold irrespective of effect on liver weight or hypolipidemic capacities (Table 1). Their effects on metabolism of DAB were quite varied. Clofibrate induced and nafenopin suppressed DAB azoreduction, confirming previous work in this laboratory [9]. Clofibric acid also induced DAB azoreduction. This was expected since clofibrate is readily deesterified to clofibrate acid in the body [38]. Ciprofibrate depressed azoreduction of DAB, as was seen with nafenopin. The other three clofibrate analogs (D–F) had relatively little effect on DAB azoreductase although laurate hydroxylation was induced markedly. A 2-fold induction of laurate hydroxylation and a slight induction of DAB azoreduction were seen after treatment with DEHP.

Of the four Wyeth compounds, treatment with Wy-14643 (H), the only one with hypolipidemic activity, markedly induced laurate hydroxylation but suppressed azoreductase activity. The subtle structural changes which remove hypolipidemic properties did not alter ability to induce laurate hydroxylase which was seen for each of these four compounds. Interestingly, treatment with the non-hypolipidemic compounds also induced DAB azoreduction. These findings, however, do not permit conclusions regarding hypolipidemic properties and induction of DAB azoreduction by this group of compounds.

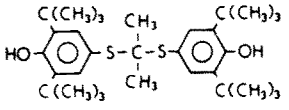
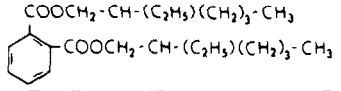
As previously seen for clofibrate and nafenopin [9], effects on the oxidative pathways, ring-hydroxylation and N-demethylation, were varied. Little induction was seen with any compound and responses of each oxidative pathway were independent of each other and of the azoreduction pathway. Administration of compounds A–H and L led to considerable suppression of N-demethylation, whereas little or no effect on ring-hydroxylation was apparent. This is consistent with our previous report that the several pathways of DAB metabolism are catalyzed to some extent by different forms of cytochrome P-450. It is also clear that DAB azoreductase activity is not restricted to cytochrome P-452 since all of the drugs induced laurate hydroxylation, a marker for cytochrome P-452, but only selective ones induced azoreduction. Similarly, Lake *et al.* [39] found that a group of hypolipidemic agents markedly induces laurate hydroxylation in cultured rat hepatocytes, whereas there is no consistent effect on the deethylation of 7-ethoxycoumarin. The results imply that there is no correlation between the hypolidemic property of a drug and its ability to induce either laurate hydroxylation or DAB azoreduction. This is consistent with a recent study [40] which showed that hypolipidemic action of clofibrate does not depend on cytochrome P-450-dependent laurate hydroxylation. Furthermore, there appeared to be no correlation between peroxisome proliferation and induction of laurate hydroxylase or DAB azoreduction. For example,

Table 1. Effects of hypolipidemic and structurally-related compounds on the metabolism of lauric acid and DAB by rat liver microsomes

	Compounds*	Enzymic activity (percent of controls†)			
		Laurate Hydroxylation	DAB metabolism		
			Azo reduction	N-demethylation‡	Ring-hydroxylation‡
A		579	344	33	115
B		645	205	68	123
C		492	38	33	115
D		556	116	30	89
E		719	179	26	94
F		716	86	26	93
G		626	33	75	112
H		783	75	15	68
I		408	159	115	168
J		435	235	101	139
K		267	205	116	126

Continued on next page

Table 1. *Continued*

Compounds*	Enzymic activity (percent of controls†)			
	Laurate Hydroxylation	DAB metabolism		
		Azo reduction	N-demethylation‡	Ring-hydroxylation‡
L 	758	68	28	74
M 	176	133		

* A: clofibrate; B: clofibric acid; C: ciprofibrate; D: 2-(*p*-chlorophenoxy) propionic acid; E: 2-(*o*-chlorophenoxy)2-methylpropionic acid; F: 2-(*m*-chlorophenoxy) propionic acid; G: nafenopin; H: WY-14643; I: WY-14807; J: WY-15672; K: WY-14730; L: Probucol; and M: di(ethylhexyl)phthalate.

† Enzymic activity was calculated as nmoles of metabolites formed per mg of protein per minute. Control activity ranged from 1.4 to 2.6 nmoles/mg protein/min. The values are means of three trials and the standard errors did not deviate from the mean by more than 10%

‡ N-Demethylation and ring-hydroxylation of DAB are the rates of formation of 4-aminoazobenzene and 4'-hydroxy-dimethylaminoazobenzene respectively.

treatment with WY-15672 (J) induced both enzyme activities, although it was reported not to have hypolipidemic or peroxisome proliferating properties [36].

In summary, a study has been performed on the effects of a number of hypolipidemic drugs, and compounds of similar structure, on laurate hydroxylation and oxidative and reductive metabolism of DAB by rat liver microsomes. Although clofibrate has been shown to induce markedly both laurate hydroxylation [8, 9] and DAB azoreduction [9], no parallel was seen between these two activities after treatment with other drugs. It is concluded that the forms of cytochrome P-450 which catalyze these two reactions are probably distinct.

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Department of Molecular Pharmacology
Albert Einstein College of Medicine
Bronx, NY 10461, U.S.A.

HAIDER RAZA
WALTER G. LEVINE*

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* To whom all communications should be addressed.

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Inhibition of cardiac phosphodiesterases by amiloride and its *N*-chlorobenzyl analogues

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Amiloride (3,5-diamino-6-chloro-*N*-(diaminomethylene)-pyrazinecarboxamide) is widely employed as an anti-kaliuretic agent, and has recently gained importance as an inhibitor of numerous plasmalemmal Na^+ transport systems. These include epithelial Na^+ channels, the Na^+/H^+ exchange (for review see ref. 1), and the Na^+/Ca^{2+} exchange [2–4]. The diuretic effect of the drug has been ascribed to the inhibition of one or more of these systems. Other pharmacological effects of amiloride such as the increase in cardiac inotropism [4–6], the antagonism toward the toxic effects of digitalis both *in vivo* [7] and *in vitro* [4, 6], and the vascular smooth muscle relaxing effect [8], have been tentatively ascribed to inhibition of the Na^+/Ca^{2+} exchange system [4, 6, 8].

Amiloride has also been observed to inhibit other enzymatic systems, including the Na^+/K^+ ATPase [9], protein-kinases [10–13], and adenylate cyclase [14]. These effects are not strictly related to the action of the drug on kidney and heart.

The amiloride molecule can be modified to obtain derivatives that show more selective inhibitory effects on the different Na^+ transport or exchange systems. In particular, the 5-amino nitrogen substituted analogues are potent inhibitors of Na^+/H^+ exchange while compounds bearing substituents on terminal nitrogen of the guanidino group block the Na^+/Ca^{2+} exchange. Of these, benzamil, chloro-, dichloro-, and dimethyl-benzyl derivatives of amiloride are the most potent agents [15–17].

In the present paper we describe the inhibition of partially purified cardiac bovine soluble phosphodiesterases by amiloride, *o*-chlorobenzyl amiloride and 3′,4′-dichlorobenzyl amiloride.

Materials and methods

Amiloride and 3′,4′-dichlorobenzyl amiloride were kindly supplied by Merck-Sharp and Dohme (Roma, Italy, and Rahway, NJ, respectively); *o*-chlorobenzyl amiloride was prepared according to Cragoe *et al.* [18–19].

Preparation of phosphodiesterase. Soluble cAMP and cGMP phosphodiesterases were partially purified from bovine heart by the procedure of Butcher and Sutherland as modified by Carpenedo *et al.* [20] to the stage of DEAE-cellulose column. The 0.2 M KCl fraction used as the source of phosphodiesterase showed good hydrolyzing activity against both cAMP and cGMP, contained calmodulin, and required micromolar concentrations of exogenous Ca^{2+} for full activity. A Lineweaver–Burk plot of the enzyme preparation was downward curvilinear. A low K_m of 3 μM and a high K_m of 40 μM for cAMP and a low K_m of 5 μM and a high K_m of 30 μM for cGMP were obtained by extrapolation. When cAMP phosphodiesterase activity was assayed in the presence of increasing concentrations of cGMP, its activity was completely blocked by concentrations of cGMP that were only slightly higher than the K_m value of the enzyme for cAMP, but when cGMP phosphodiesterase was assayed in the presence of increasing concentrations of cAMP, amounts 80 times higher than the K_m of the enzyme for cGMP had to be added to obtain a 50% inhibition of the hydrolyzing activity.

Assay of phosphodiesterase activity. Phosphodiesterase activity was assayed at 37° for 10 min using the two step procedure of Thompson *et al.* [21]. Tritiated cyclic nucleotides were converted to [^{3}H] labelled 5′-nucleotides in a 0.4 ml reaction mixture containing 40 mM Tris·HCl, pH 8, 5 mM $MgCl_2$, 20 μM $CaCl_2$, 12 U calmodulin (bovine brain, Sigma) and a proper amount of enzyme. The reaction was stopped by freezing the sample in liquid nitrogen (30 sec) and then thawing it in boiling water (90 sec). The 5′-nucleotides were then converted to [^{3}H] nucleosides with 0.3 U of 5′-nucleotidase (Crotalus atrox, grade II, Sigma). The radioactive nucleosides were separated from labelled cyclic nucleotides using Dowex 1 × 2 anion-exchange resin in two volumes of ethanol–water 1:1. When assaying for Mg^{2+}-dependent phosphodiesterase activity, $CaCl_2$ and calmodulin were omitted and 1 mM EGTA pH 8 was added.

Amiloride and the *N*-chlorobenzyl analogues were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in incubation medium never exceeded 1%. This amount did not affect phosphodiesterase activity.

Protein assay. Protein content was determined according to Lowry *et al.* [22] by using bovine serum albumin as standard.

Results and discussion

IC_{50} values calculated from the concentration–response curves of the inhibition of cardiac soluble Ca^{2+}-calmodulin activated phosphodiesterase by amiloride and the *N*-chlorobenzyl analogues (Table 1) show that amiloride was a weak inhibitor of the enzyme when both cAMP and cGMP were employed as substrates (IC_{50}s in the millimolar range).