Table 1. Effect of oral tobacco administration on hepatic esterases/amidases in rat

Esterase/amidase	Control	Enzyme activity (units/mg protein) Treated 1 day	7 days	25 days
ASA esterase I	230 ± 11	205 ± 12	305 ± 18*	351 ± 17*
ASA esterase II	198 ± 9	179 ± 7	245 ± 6*	270 ± 18*
Procaine esterase	3.58 ± 0.12	4.02 ± 0.22	3.25 ± 0.21	3.72 ± 0.11
NPA esterase	7152 ± 221	8053 ± 526	7632 ± 331	6857 ± 412
Acetanilid *N*-deacetylase	52.1 ± 1.8	58.3 ± 2.4	104 ± 4*	126 ± 6.8*
Butyrylcholine esterase	15.5 ± 1.2	14.8 ± 1.0	13.2 ± 0.91	17.2 ± 0.8

Values are means ± S.E. with separate homogenates of liver obtained from 8–10 rats. One unit of esterase/amidase activity is expressed as the amount of enzyme causing disappearance or appearance of 0.1 nmole of substrate or product respectively under the experimental conditions described.
* $P < 0.01$.

to alter the activities of esterases/amidases necessary for the metabolism of ASA, procaine, NPA, acetanilid and butyrylcholine in kidney and brain throughout the course of study. Similar findings have been reported previously, where phenobarbital [17, 18] and DDT [17] induced hepatic carboxylesterases/amidases but did not modify the enzyme activity of extrahepatic tissues. It seems that extrahepatic enzymes are probably non-inducible in nature.

Oral administration of nicotine (10 mg/kg, twice a day), equivalent to that present in 250 mg tobacco, did not elicit any change in the rate of hydrolytic metabolism of the xenobiotics by hepatic or extrahepatic esterases/amidases. It suggests that nicotine probably does not share the inductive effect of tobacco on hepatic esterases/amidases necessary for the metabolism of aspirin and acetanilid.

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Clonidine effect on hepatic cGMP levels *in vivo* could be mediated by α_1-adrenoceptors

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Clonidine has been used for some years as an α_2-adrenergic agonist. Recent work from our laboratory has shown that, at low doses, clonidine administration produces a significant decrease in cerebellar cGMP levels due to its action on α_2-adrenoceptors [1].

In parallel with the study of clonidine action in the

cerebellum, we investigated the effect of clonidine on mouse hepatic cGMP levels. We studied its action in the liver for two main reasons. Firstly, clonidine has been demonstrated to cause hyperglycemia both in animals [2] and humans [3] and, secondly, because cGMP could be involved in the regulation of carbohydrate metabolism [4].

In order to characterize the action of clonidine in the liver, the effect of a number of adrenergic agonists and antagonists on hepatic cGMP levels was also investigated.

The results obtained in the present study indicate that unlike the action of clonidine in the cerebellum, in the liver clonidine increases cGMP levels via α_1-adrenergic mechanisms.

Materials and methods

Male Swiss OF1 mice (22 g) were allowed free access to food and water. Drugs were freshly prepared and administrated i.p. in a volume of 250 μl. The following drugs were injected in saline: clonidine (Boehringer Ingelheim GmbH), piperoxan (Rhône Poulenc), phenoxybenzamine (Smith, Kline and French Lab.), phentolamine and guanethidine (Ciba-Geigy), amphetamine and phenylephrine (Sigma). Yohimbine (Boyer) was dissolved in 0.1 N HCl, adjusted to pH 5–6 with 0.1 N NaOH and made up to the required volume with saline. Prazosin (Pfizer GmbH) was dissolved in hot water. Control mice were injected with saline only.

The mice were killed by rapid microwave exposure (2 sec, Litton model 70–91, 2450 MHz). All animals were killed between 10:00 and 11:30 a.m.

All procedures for extraction and determination of cGMP were essentially as previously described in detail [1]. In brief, after dissection, each liver was homogenized in 5 ml of 10% trichloroacetic acid (TCA). After centrifugation and filtration of supernatants, aliquots of 750 μl were extracted with 2 × 7.5 ml of water-saturated diethyl ether. Dry residues were taken up in 350 μl of 0.2 N Tris buffer, pH 7.5, containing 10 mM EDTA. If necessary, the pH of the solutions were adjusted to 7.5 with 0.1 N NaOH and 100-μl aliquots were used for cGMP determinations.

Protein was determined on the TCA pellets after solubilization in 1 N NaOH [5].

All results represent the mean of at least 10 experiments ± S.E.M. The significance of differences was determined by Student's "*t*"-test; $P < 0.05$ was considered as the limit of statistically significant differences.

Results

Action of α-adrenergic agonists. Both clonidine and phenylephrine increased liver cGMP concentrations. This rise was dose-dependent for agonists concentrations up to 2 mg/kg and remained practically constant for higher concentrations (Fig. 1). The maximal increase (+ 129%) occurred 10 min after injection in the case of clonidine, but only after 30 min for phenylephrine (+ 128%, Fig. 2). Methoxamine also increased hepatic cGMP levels (+ 49%, Table 1).

When clonidine (3 mg/kg) was injected 20 min after phenylephrine (5 mg/kg), we observed a significant increase (+ 98%) in cGMP levels. This increase was of the same magnitude as that obtained when clonidine or phenylephrine were injected separately (Table 1). There is no additive effect of these two drugs.

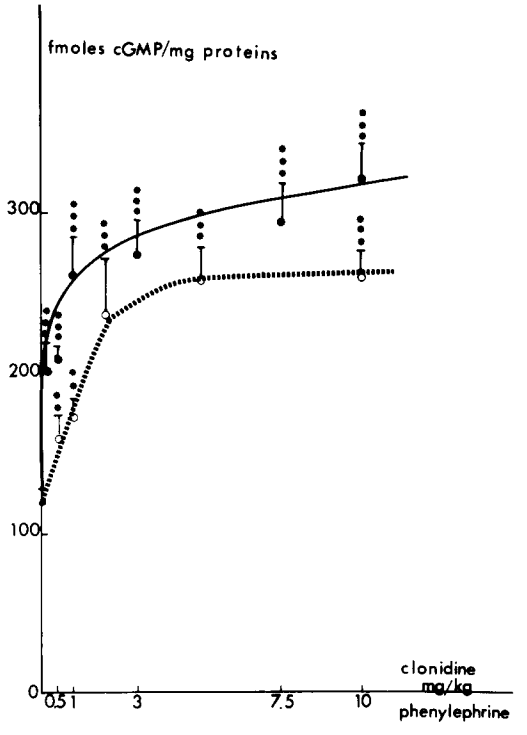

Fig. 1. Effect of clonidine and phenylephrine on mouse hepatic cGMP levels. Each point represents the mean ± S.E.M. for groups of at least five animals: dose–response curve for clonidine (●——●) and for phenylephrine (○- - -○). Ordinate: fmoles cGMP/mg protein; abscissa: quantity of clonidine or phenylephrine injected i.p. (mg/kg). $^{**}P < 0.01$, $^{***}P < 0.001$—relative to control values.

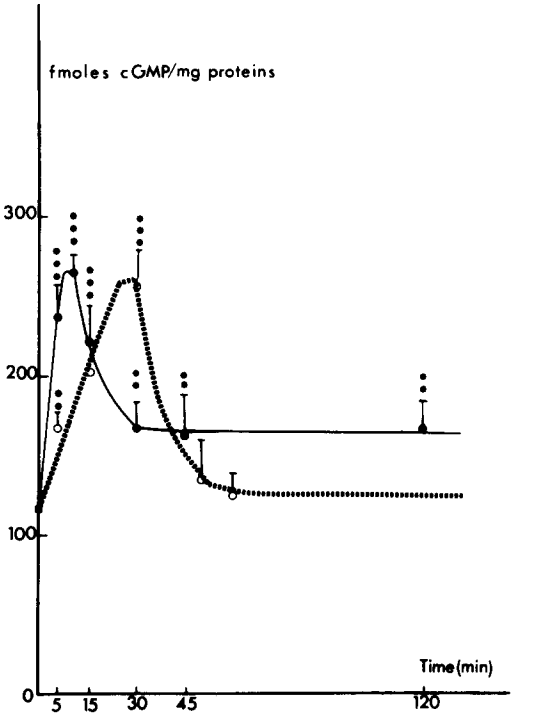

Fig. 2. Variation in mouse liver cGMP levels with time after a single dose of clonidine (3 mg/kg) (●——●) or of phenylephrine (5 mg/kg) (○- - -○). Each point represents the mean ± S.E.M. for groups of at least six animals. Ordinate: fmoles/mg protein; abscissa: time (min). $^{**}P < 0.01$, $^{***}P < 0.001$—relative to control values.

Table 1. Action of clonidine and adrenergic agonists and antagonists on mouse hepatic cGMP content

	Cyclic GMP (fmoles/mg protein)
Controls	113.30 ± 2.73
Clonidine (3 mg/kg:10 min)	259.98 ± 7.32*
Piperoxane (7 mg/kg:30 min)	113.83 ± 4.60 (n.s.)
Piperoxane + clonidine	237.23 ± 11.35*
Yohimbine (7 mg/kg:30 min)	116.39 ± 9.66 (n.s.)
Yohimbine + clonidine	226.47 ± 7.92*
Phenoxybenzamine (10 mg/kg:30 min)	115.95 ± 8.45 (n.s.)
Phenoxybenzamine + clonidine	182.81 ± 9.15†‡
Phentolamine (5 mg/kg:30 min)	95.09 ± 5.90 (n.s.)
Phentolamine + clonidine	267.99 ± 7.53*
Prazosin (5 mg/kg:30 min)	98.45 ± 9.42 (n.s.)
Prazosin + clonidine	144.94 ± 8.10 (n.s.)‡
Phenylephrine (5 mg/kg:30 min)	258.65 ± 11.00*
Phenylephrine + clonidine	224.85 ± 7.60*
Methoxamine (5 mg/kg:30 min)	169.03 ± 5.71*
Guanethidine (5 mg/kg:30 min)	137.72 ± 8.40 (n.s.)
Guanethidine + clonidine	259.48 ± 28.68*

Mice were killed by microwave irradiation at the appropriate time after injection. The numbers in parentheses represent the dose of drug and time of injection. For all compounds, a dose–response curve and a time course were carried out. The dose and time noted here correspond to the maximal effect observed if any. When two compounds were injected, clonidine was administered 20 min after the first drug and mice were killed 10 min later. Values are the average of at least 10 determinations ± S.E.M.

* $P < 0.001$ relative to control values.

† $P < 0.01$ relative to control values.

‡ $P < 0.0001$ relative to the clonidine-treated animals' values.

n.s. = not significant.

Action of α-adrenergic antagonists. The α-adrenergic antagonists, phenoxybenzamine, phentolamine, piperoxan, yohimbine and prazosin had no significant effect on hepatic cGMP content under our experimental conditions for all concentrations tested and at any time (Table 1).

The α_2-selective antagonists, piperoxan and yohimbine, and the non-selective α_1-adrenergic antagonist, phentolamine, did not inhibit the clonidine-induced increase in cGMP levels. On the other hand, the clonidine effect was completely blocked by prior injection of the α_1-selective antagonist, prazosin (Table 1), since the level of cGMP observed after injection of both compounds together was not significantly different from that of controls. Similarly, phenoxybenzamine partially antagonized clonidine action, since the increase in cGMP content (+ 61%) when phenoxybenzamine and clonidine were administered together was significantly less evident than the increase observed with clonidine alone (+ 129%).

Action of guanethidine. Guanethidine did not exhibit any effect on hepatic cGMP concentrations when injected alone, and did not inhibit the clonidine action (Table 1).

Discussion

In recent years, clonidine has been shown to act on both α_1- and α_2-adrenoreceptors, irrespective of their anatomical (pre- or postsynaptic) localization [6–8]. The present experiments indicate that the effects of clonidine on hepatic cGMP are due to activation of α_1-adrenoreceptors. Firstly, phenylephrine, a preferential α_1-agonist, has the same effect as clonidine (differences in the time courses of phenylephrine and clonidine could reflect differences in the relative hydrosolubility and liposolubility of the two compounds). Secondly, phenoxybenzamine and prazosin, preferential α_1-antagonists, reduced or abolished the effects of clonidine, whereas piperoxan and yohimbine, preferential α_2-antagonists, caused no significant inhibition of clonidine action.

Since pretreatment with guanethidine did not alter the effect of clonidine on hepatic cGMP, an indirectly mediated effect or an effect depending on the sympathetic system (such as a central action) must be ruled out. In addition, phenylephrine, which does not easily cross the blood–brain barrier, was also effective in increasing the hepatic cGMP content; therefore the comparable action of clonidine must be peripheral.

In the cerebellum, clonidine, in a dose range from 0.1 to 10 mg/kg, was shown to act on α_2-adrenoreceptors and produced an important decrease in cGMP content [1–9]. In contrast, α_1-adrenergic agonists, such as phenylephrine, caused an increase in the concentration of cerebellar cGMP [1].

As observed by Pointer *et al.* [10], in isolated hepatocytes, we failed to provoke a decrease in cGMP levels with any of the tested compounds in the present study.

These results are in agreement with the report of Wood *et al.* [11] that in rat liver plasma membranes, α-adrenergic receptors are predominantly of the α_1-subtype. In order to confirm them and to avoid all indirect effects due to the complexity of our *in vivo* system, we are going to study this effect of clonidine on liver cGMP content *in vitro* with isolated hepatocytes.

All these observations are consistent with the hypothesis that clonidine action on hepatic cGMP content is associated with stimulation of α_1-receptors present in liver membranes. More studies are necessary to show if other α_1-agonists such as cirazoline or α_2-agonists like azepexole can or can not induce the same response and to establish a clear correlation between cGMP and carbohydrate metabolism in this organ. Moreover it would be interesting to know if this increase is, like Exton proposed [12], secondary to a rise in intracellular Ca^{2+}. These studies are presently under investigation in our laboratory.

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Evidence for binding of certain acidic drugs to α_1-acid glycoprotein

Human serum albumin (HSA) and α_1-acid glycoprotein (α_1-AGP) are the major circulating plasma proteins involved in drug binding. Acidic (anionic) drugs ionized at plasma pH, are assumed to be bound preferentially to HSA in plasma. The binding forces involve both hydrophobic and electrostatic interactions [1]. In contrast, α_1-AGP is the main plasma protein for the binding of basic (cationic) drugs [2]. In this case, the nature of the binding forces is not clearly established, though recent studies show that electrostatic interactions seem to be poorly involved: Lemaire and Tillement [3] showed that the different hydrophobic characteristics of some pindolol derivatives led to important differences in their binding. Then hydrophobic interactions between α_1-AGP and drugs are strongly to be expected. If this hypothesis is true, anionic drugs may also interact with α_1-AGP by hydrophobic bonds. So, the aim of this study is to investigate the qualitative and quantitative aspects of plasma binding of anionic drugs regarding their possible interactions with α_1-AGP. Two types of anionic drugs were used depending on the presence or absence of a carboxylic group. We attempted to find out whether acidic and basic drugs are bound to distinct or common sites on α_1-AGP.

Materials and methods. The binding of the drugs was measured by equilibrium dialysis at pH 7.4 in 0.066 M phosphate buffer ($\mu = 0.284$) at 37° for 4 hr using a Dianorm apparatus according to an experimental scheme previously described [4]. Drug solutions were prepared by isotopic dilution of a constant amount of [^{14}C]warfarin (49 Ci/mole, Amersham), [^{14}C]acenocoumarol (7 Ci/mole, Ciba-Geigy), [^{14}C]phenylbutazone (28 Ci/mole, Ciba-Geigy), [^{14}C]benoxaprofen (2.5 Ci/mole, Eli-Lilly), [^{14}C]indomethacin (13.8 Ci/mole, Merck Sharp & Dohme), [^{14}C]itanoxone (1.8 Ci/mole, Pierre Fabre), [^{14}C]salicylic acid (47 Ci/mole, Amersham), [^{14}C]clofibric acid (20 Ci/mole, CEA), [^{14}C]fenofibric acid (5.8 Ci/mole, Fournier) and [^{14}C]valproic acid (0.5 Ci/mole, CEA) with increasing amounts of unlabelled drugs. All labelled drugs had a chemical purity greater than 99%. α_1-AGP (Behringwerke) (99% pure) was dissolved in phosphate buffer at a concentration of 22.5 μM. Estimation of the binding parameters was performed by fitting the data to a theoretical relationship for drug–protein binding derived from the mass action law:

$$\frac{B}{R} = \frac{nKF}{1 + KF} \quad (1)$$

where B and F are the bound- and free-drug concentrations respectively, n and K the number of binding sites and the association constant, and R the total protein concentration. The data were analyzed by an iterative non-linear regression program using a Gauss–Newton algorithm [4]. Data obtained with an inhibitor were treated as follows: bound (B) vs free (F) ligand plots obtained without and with different amounts of inhibitor were analyzed altogether assuming either n or K values depended on the inhibitor concentration. The correct model was then chosen according to the best fit.

Some theoretical calculations were made to evaluate the respective concentrations of drug bound to HSA and α_1-AGP in plasma using therapeutic plasma levels. So the general equation used was:

$$B_i = K_i n_i RF \quad (2)$$

applied to each drug–protein binding of the ith class. Then

Table 1. Binding characteristics of some anionic drug–α_1-AGP interactions

Drug	% bound*	n	K (10^{-3} M^{-1})
Warfarin	88	1.09 ± 0.03	212 ± 38
Acenocoumarol	85	1.08 ± 0.02	201 ± 35
Phenylbutazone	26	0.71 ± 0.08	35 ± 9

*At a drug to α_1-AGP molar ratio of 0.04.