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In vitro and in vivo α-blocking activity of thymoxamine and its two metabolites

M. H. CREUZET*, G. PRAT, A. MALEK†, F. FAURAN, J. ROQUEBERT†

Sarget Research Department, Avenue J. F. Kennedy, 33701 Merignac, and † Laboratory of Pharmacology, University of Bordeaux II, Rue Leyteire 33000, Bordeaux, France.

The α-adrenoceptor potency of thymoxamine and its two metabolites deacetylthymoxamine and demethyldeacetylthymoxamine were determined on the contraction of rat vas deferens induced by noradrenaline, the blood pressure increase induced by noradrenaline given i.v. to dogs and the contraction of the nictitating membrane induced by electrical stimulation in cats. In vivo the three drugs were administered at $6{\cdot}35 \times 10^{-6}$ mol kg^{-1} intravenously. Deacetylthymoxamine presented nearly the same α-blocking activity as the parent drug. This was ascribed in vivo to the rapid deacetylation of thymoxamine. Demethyldeacetylthymoxamine was less active. In vitro its pA_2 was $6{\cdot}20 \pm 0{\cdot}09$ compared with $6{\cdot}75 \pm 0{\cdot}20$ for thymoxamine and $6{\cdot}57 \pm 0{\cdot}13$ for deacetylthymoxamine. In vivo, it was inactive in dog and less active than the other two drugs soon after its administration in the cat. The oral LD 50 values in the mouse for the three drugs were respectively 0·81, 0·71 and 1·14 mmol kg^{-1} for thymoxamine, deacetylthymoxamine and demethyldeacetylthymoxamine.

We have already identified two metabolites of thymoxamine as conjugated forms in the urine of rats (Feniou et al 1980): deacetylthymoxamine (4-dimethylaminoethoxy-5-isopropyl-2-methylphenol) and demethyldeacetylthymoxamine (5-isopropyl-2-methyl-4-methylaminoethoxyphenol). Unchanged thymoxamine was never identified. From the sequence of appearance of the two metabolites in the urine and the comparative in vitro assays of deacetylation and demethylation, it was concluded that the circulating form of the drug was its deacetylated metabolite.

Credner & Graebner (1967) showed that deacetylthymoxamine had the same α-adrenolytic activity as thymoxamine in the cat on blood pressure increase induced by adrenaline. Furthermore they determined the LD 50 of the two metabolites in the mouse after subcutaneous injection and they found similar results. Later Arbab et al (1973) determined the pA_2 of deacetylthymoxamine against noradrenaline on muscle strips from human colon and ileum and the saphenous vein. They found deacetylthymoxamine acted as a competitive antagonist on colon and ileum but as a non-competitive antagonist on the saphenous vein. The pA_2 values for deacetylthymoxamine varied between 6·1 to 6·51 compared with 6·72–6·96 for thymoxamine (Coupar & Turner 1970). The α-adrenolytic activity of *N*-demethyldeacetylthymoxamine has never been described. We have compared the in vitro and in vivo α-blocking activity and oral toxicity of thymoxamine and its two metabolites.

* Correspondence.

MATERIALS AND METHODS

In vitro measurement of potency of antagonism at α-adrenoceptors

The vas deferens of Wistar male rats (230 ± 20 g) were placed under a preload of 0·5 g in a 20 ml organ-bath containing Krebs–Henseleit solutions at 37 °C and bubbled with 5% CO_2 in oxygen. Contractions were recorded with a isotonic Racia N° 1 transducer attached to a recorder (Electronic Polyrecorder Model E.P.R. 104). After a 30 min rest period, the cumulative dose-response curves were determined with noradrenaline (NA), at 15 min intervals each curve requiring 3 to 5 min. The incubation medium was changed twice at the end of each determination. After the contractile response induced by the agonist had been established, the tissues were exposed to varying concentrations of thymoxamine, deacetyl and *N*-demethyldeacetylthymoxamine for 5 min. Three other dose-response curves were determined for the agonist, the first curve in the presence of antagonist, and the two other curves after changing incubation medium. Five isolated organs were used for each antagonist dose. The results were expressed as a percentage of maximal contraction.

The classical pA_2 affinity parameters for competitive antagonism were determined according to van Rossum (1963).

In vivo measurement of potency of antagonism at α-adrenoceptors

The antagonism of intravenous NA on blood pressure of dogs was assessed using 8 mongrel dogs (6–20 kg) of either sex anaesthetized with sodium pentobarbitone (25 mg kg^{-1}). The trachea was intubated. The blood pressure was recorded with a Bell & Howell transducer from the left common carotid artery, the arterial pulse pressure was also used to trigger a heart ratemeter. Blood pressure and heart rate were recorded on a Beckman R 411 chart recorder. Before an experiment, the vagus nerves were sectioned in the neck. The brachial part of the cephalic vein was canulated for drug administration. The experiment was not begun before blood pressure and heart rate had been stabilized. The responses to intravenous NA were recorded. Doses of 0·1, 0·25, 0·5 and $1 \cdot 10^{-6}$ g kg^{-1} were injected with an interval of 2 min between doses. 8 min after the last injection, the four doses of NA were repeated. Then thymoxamine or its metabolites were injected and again the four doses of NA were repeated 4, 30, 60 min after drug administration. This was at $6 \cdot 35 \times 10^{-6}$ mol kg^{-1} (2 mg kg^{-1}) for thymoxamine hydrochloride, 1·73 mg kg^{-1} for deacetylthymoxamine hydrochloride and 1·64 mg kg^{-1} *N*-demethyldeacetylthymoxamine hydrochloride) as solutions in 0·9% NaCl (saline) at a concentration of 5 mg ml^{-1}. The pH of the solutions were adjusted to neutral with sodium bicarbonate.

Antagonism of the response of the nictitating membrane to sympathetic nerve stimulation was adapted from Westfall et al (1969) and Langer & Pinto (1976). Cats of 1·8 to 2·5 kg and of either sex were anaesthetized with sodium pentobarbitone (30 mg kg^{-1}) and the response of nictitating membrane was recorded by means of a microdisplacement myograph transducer (F 1000 Narcobiosystems). The resting tension was adjusted to 8 g. Blood pressure was recorded from the left femoral artery and injections made into the right femoral vein. For stimulations, the postganglionic fibres of the cervical sympathetic chain were exposed and bipolar platinum electrodes were fitted in and covered with warm liquid paraffin. The sympathetic chain was cut. Rectangular impulses (1 ms) of supramaximal voltage (6–8 V) were supplied by a stimulator HSE. Stimulation frequencies varying from 0·2 to 25 Hz were applied every 2 min and stopped as soon as the response reached a plateau (25 to 40 s). Two control frequency-response curves were obtained for each preparation. The frequency-response curves were then recorded 4, 60 and 120 min after drug administration. The conditions of administration and the doses used were the same as in the dog experiment except that the concentration of drug was of 2 mg kg^{-1}.

Mice toxicity

Solutions of test compounds in a 6% suspension of gum arabic were given orally as single doses to groups of each of 5 male and 5 female CD1 Charles River mice. The oral dose producing 50% lethality (LD 50) was determined, after an observation period of 15 days, according to Litchfield & Wilcoxon (1949).

Materials

Thymoxamine hydrochloride was provided by Dedieu Laboratories. Deacetylthymoxamine and demethyldeacetylthymoxamine hydrochlorides were synthesized as described by Buzas et al (1959) and Gödecke (1972) respectively. All chemicals were submitted to ^1H n.m.r. and m.s. For the in vitro assay, (−)-noradrenaline bitartrate was purchased from Serva. For the two in vivo assays we used (−)-noradrenaline (+)-bitartrate as solution of 200 mg noradrenaline bitartrate monohydrated, 200 mg sodium metabisulphite, 800 mg NaCl, bidistilled water to 100 mg (vials of 4 ml for intravenous injection, Winthrop). Sodium pentobarbitone was Nembutal (Abbott).

Statistical treatment

The significance of regression lines and of difference between the drugs were determined for the in vitro assay by Student's *t*-test. For the in vivo assays, Quality of Regression was determined by the Snedecor's F-test (Lellouch & Lazar 1974). Values of $P < 0 \cdot 05$ were considered significant.

RESULTS

Thymoxamine, deacetylthymoxamine and *N*-demethydeacetylthymoxamine produce a dose-dependent inhibition of NA-induced contraction of the rat vas deferens. The three substances bring about an almost parallel displacement of the dose-response curves towards the right, without depression of maximal amplitude. This suggests competitive antagonism. On the other hand, maxima were enhanced in the presence of the drug and its metabolites. This phenomenon has already been described for phentolamine (Barnett et al 1968: Van Nueten et al 1977), yohimbine, piperoxane, tolazoline and phentolamine (Jurkiewicz & Jurkiewicz 1976). The pA_2 values are 6·75 ± 0·20 for thymoxamine, 6·57

± 0·13 for deacetylthymoxamine and 6·20 ± 0·09 for *N*-demethyldeacetylthymoxamine. The effects of these three substances are easily reversed by replacement of perfusion liquid. Thymoxamine and its deacetyle derivative have practically the same activity (non-significant difference), *N*-demethyldeacetylthymoxamine is significantly less active ($P < 0{\cdot}05$). A supplementary criterion for validation of the competitive antagonism hypothesis is the existence of a linear relationship between the log (dose ratio −1) for NA and the log of the molar concentration of antagonist (Arunlakshana & Schild 1959). The regression slopes are, however, very low: 0·50 for thymoxamine, 0·60 for deacetylthymoxamine and 0·89 for *N*-demethyldeacetylthymoxamine. These deviations from theoretical values may be explained by interference with neuronal uptake process (Furchgott 1972). If the experiments are repeated in the presence of cocaine (3×10^{-6} M) the values for the slope of the regression curve are 0·92 for thymoxamine, 0·95 for deacetylthymoxamine and 0·94 for *N*-demethyldeacetylthymoxamine which are not significantly different from 1. It thus appears that the three substances have characteristic properties of competitive inhibitors of α-adrenoceptors.

Preliminary studies of the effect of thymoxamine administered to dogs at doses varying from 0·15 and 4 mg kg^{-1} on blood pressure increase induced by a single dose of noradrenaline showed that 2 mg kg^{-1} of thymoxamine induced an almost maximal effect. This adrenolytic effect remained stable for at least 10 min. For two in vivo adrenolytic experiments we therefore chose the dose of 2 mg kg^{-1} for thymoxamine and equimolecular corresponding doses for the two metabolites. NA administered intravenously to dogs at increasing doses from $0{\cdot}1 \times 10^{-6}$ g kg^{-1} produced blood pressure increases which were linearly related to dose (Fig. 1). 4 to 10 min after drug administration the dose-response curves remain linear but with thymoxamine and deacetylthymoxamine the two straight lines shifted to the right and their slopes decreased whereas with demethyldeacetylthymoxamine the line remained close to the control. For example the increase of blood pressure induced by the 10^{-6} g kg^{-1} of noradrenaline was decreased from 75·7 ± 10·31 to 42·4 ± 13·07 mm Hg 10 min after thymoxamine and from 67·7 ± 8·51 to 34·0 ± 15·41 mm Hg 10 min after deacetylthymoxamine. 10 min after demethyldeacetylthymoxamine the increase of blood pressure was still 70·4 ± 6·54 mm Hg. 30 and 60 min after thymoxamine and deacetylthymoxamine their curves shifted towards the control line without reaching it and the slopes of the dose-response lines increased but remained

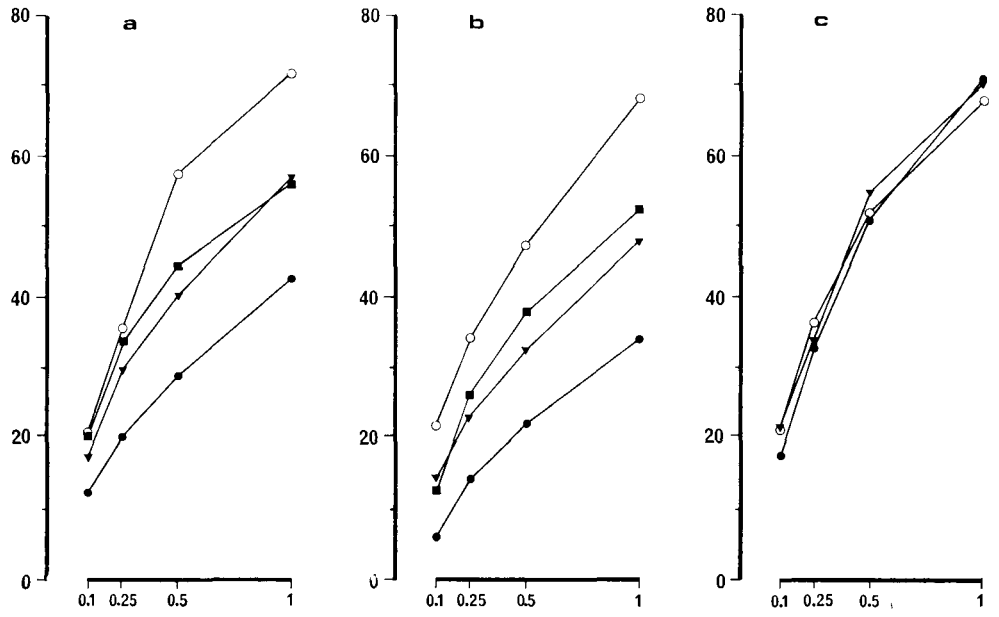

FIG. 1. Dose-response curves for blood pressure increase induced by noradrenaline in dogs: before (○—○) and 4–10 min (●—●), 30–36 min (▼—▼), 60–66 min (■—■) after i.v. administration of (a) 2 mg kg^{-1} thymoxamine HCl, (b) 1·73 mg kg^{-1} deacetylthymoxamine HCl, (c) 1·64 mg kg^{-1} demethyldeacetylthymoxamine HCl, the three doses corresponding to $6.35.10^{-6}$ mol kg^{-1}. The results are the mean of 5 assays for drug curves and 10 assays for control curves. Ordinate: blood pressure increase in mm Hg. Abscissa: noradrenaline dose in 10^{-6} g kg^{-1}, i.v.

lower than the slope of the control lines. These results are similar to those obtained by Birmingham et al (1967) in the analysis of the antagonism of intravenous noradrenaline by thymoxamine on the blood pressure of the conscious cat.

In the cat, stimulation of the postganglionic fibres produced contractions of the nictitating membrane; the percentages of contraction versus maximal contraction were linearly related to the log of the stimulation frequency (Fig. 2). 4 to 20 min after drug administration the frequency-response lines for the three drugs were shifted to the right and their slopes same proportion, so that after 2 h the demethyldeacetylthymoxamine was equivalent to the other two products.

Mice toxicities of the three drugs administered by oral route are reported in Table 1. When expressed in mmol kg^{-1} the LD 50 of the thymoxamine and of the deacetylthymoxamine are close. The demethyldeacetylthymoxamine was less toxic.

DISCUSSION

Thymoxamine and its *O*-deacetylated derivative both in vitro and in vivo have similar α-adrenolytic

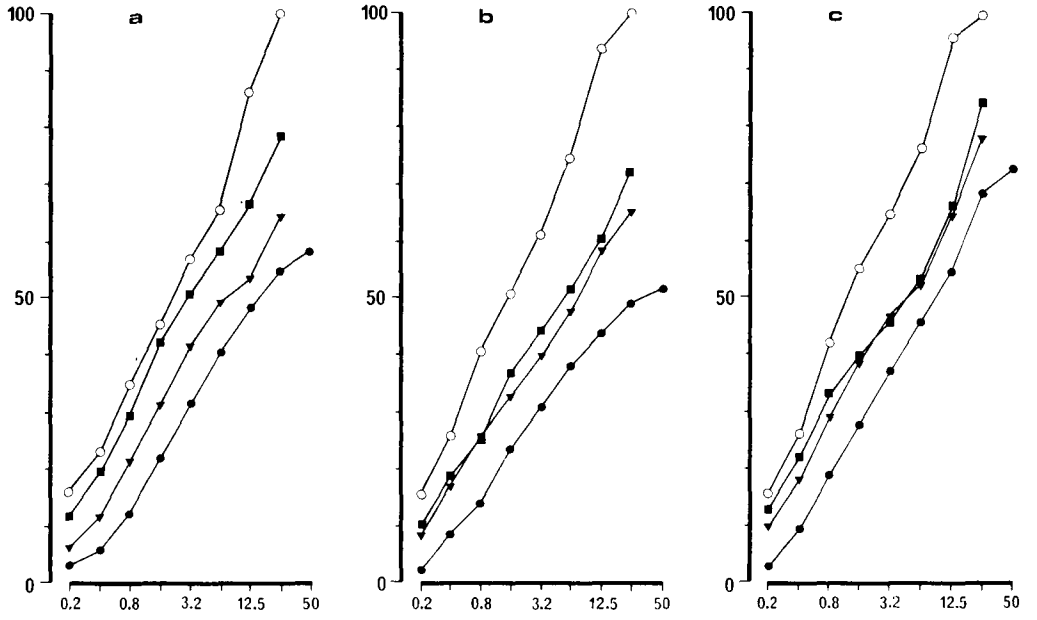

FIG. 2. Frequency-response curves for contraction of nictitating membrane induced by stimulation of postganglionic fibres in the cat: before (○—○) and 4–20 min (●—●), 60–76 min (▼—▼), 120–136 min (■—■) after i.v. administration of (a) 2 mg kg^{-1} thymoxamine hydrochloride, (b) 1·73 mg kg^{-1} deacetylthymoxamine hydrochloride, (c) 1·64 mg kg^{-1} demethyldeacetylthymoxamine hydrochloride, the three doses corresponding to $6{\cdot}35.10^{-6}$ mol kg^{-1}. The results are the mean of 4 assays for drug curves and 8 assays for control curves. Ordinate-percentage of contraction versus maximal contraction. Abscissa-frequency of stimulation, expressed in Hz, on a log scale.

were decreased. These modifications were more pronounced for thymoxamine and its deacetyl compound than for demethyldeacetylthymoxamine. For example at the maximal frequency of stimulation the contraction for the nictitating membrane was 20 min after drug administration, equal to 54·2 ± 6·74% (thymoxamine), 48·3 ± 6·43% (deacetylthymoxamine) and 67·6 ± 8·00% (demethyldeacetylthymoxamine) of the maximal contraction. We have verified that the drug effect remained stable for at least 20 min. One and 2 h after administration of the three drugs the activity decreased but not in the activity. We have previously shown that thymoxamine is rapidly deacetylated by rat plasma esterases (Feniou et al 1980) and found it is true also for dog plasma esterases. The almost identical results obtained in the two in vivo experiments can then be explained by the fact that as early as the first measurement (4 min after drug administration) thymoxamine is already transformed into deacetylthymoxamine. Hence the active form of thymoxamine in vivo is its deacetylated form.

Demethyldeacetylthymoxamine, formed in vivo by microsomal hepatic demethylation of deacetylthym-

Table 1. Toxicity (LD50) of thymoxamine and its metabolites in mice calculated according to Litchfield & Wilcoxon (1949).

Drug	LD50 (mg kg^{-1})	LD50 (mmol kg^{-1})
Thymoxamine HCl	255 (227–286)	0·81 (0·72–0·91)
Deacetyl-thymoxamine HCl	195 (160–237)	0·71 (0·59–0·87)
Demethyldeacetyl-thymoxamine HCl	295 (266–327)	1·14 (1·03–1·26)

oxamine, presents an α-adrenolytic activity differing from that of the two other drugs. In vitro its activity was significantly lower than that of the other two. In vivo the two experiments lead to different conclusions; in the dog the demethyldeacetylthymoxamine was inactive at dose of 1·64 mg kg^{-1} whereas in the cat it was active, although less so than the other two drugs, for a short time. This apparent contradiction may be explained as the two experiments involved two different models. In the dog we studied the activity towards exogenous NA whereas in the cat we measured the activity towards endogenous NA released by electrical stimulation. In the dog, the amount of NA injected was far greater than the physiological concentration so that we recorded mainly effects involving postsynaptic receptors. In the cat, the regulatory mechanisms took over so we recorded a total effect involving pre and postsynaptic receptors. Drew (1976) has demonstrated that thymoxamine is much more potent at the postsynaptic than at presynaptic receptor.

The in vitro activities of the three drugs, in conditions without metabolism, is in accordance with the general facts known about the α-adrenoceptor structure. The most important molecular fragment for interaction with the α-receptor is the amine moiety. In the dynamic receptor hypothesis of Bloom & Goldman (1966) the α-blocking tertiary amines bind coulombically to the terminal phosphate oxygen of ATP preventing the approach of an agonist amine to this centre. In *N*-demethyldeacetylthymoxamine, the secondary amine induced a smaller steric hindrance than the bulky cationic head present in thymoxamine and deacetylthymoxamine. On the other hand the substituents on the aromatic moiety may be varied without large modifications in α-blocking activity.

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