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SEMICARBAZIDE-SENSITIVE AMINE OXIDASE (SSAO) OF THE RAT AORTA

INTERACTIONS WITH SOME NATURALLY OCCURRING AMINES AND THEIR STRUCTURAL ANALOGUES

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Abstract—The influence of a number of naturally occurring amines and their structural analogues has been examined on the metabolism of radiolabelled benzylamine (BZ) by the membrane bound semicarbazide-sensitive amine oxidase (SSAO) of the rat aorta. Only primary monoamines were effective in reducing the deamination of BZ. In the phenylethylamine series, addition of hydroxyl groups to the benzene ring decreased their potency as inhibitors while addition of a hydroxyl group at the β position increased the inhibitory potency. Stereoselectivity of action was shown with octopamine, the L-isomer being the more active form. Kinetic analysis of these interactions showed predominantly competitive inhibition and kynuramine had the lowest K_i of 5.4 μM. The aliphatic monoamines, isoamylamine and isobutylamine both competed with BZ. 5-Hydroxytryptamine (5-HT) was the only amine that inhibited non-competitively. Direct evidence for metabolism by SSAO of some of the competing amines such as isoamylamine, phenylethylamine, tyramine and tryptamine was obtained by fluorimetric or radiochemical assays. The inhibitors clorgyline and (E)-2-(3′,4′-dimethoxyphenyl)-3-fluoroallylamine (MDL 72145) were used to characterise the amine oxidase activity responsible for the deamination. Octopamine and phenylethanolamine (PeOH) were not SSAO substrates and inhibited BZ metabolism in the fluorimetric assay. It is possible that the activity of SSAO is controlled by octopamine released from sympathetic nerve endings or 5-HT released from platelets.

Amine oxidase enzyme activities sensitive to inhibition by semicarbazide and resistant, *in vitro*, to the acetylenic inhibitors of monoamine oxidase (MAO; EC 1.4.3.4), form a heterogeneous group of enzyme activities termed semicarbazide-sensitive amine oxidases (SSAO; EC 1.4.3.6) (see Ref. 1 for review). SSAO enzyme activities are widely distributed through the tissues of many species, particularly in the blood vessels [2]. In the rat, one such SSAO activity has been shown to reside in the plasma membrane [3] of vascular smooth muscle cells [4]. Like many of the SSAO enzymes, this activity has a high affinity for the synthetic amine, benzylamine [5], whilst its physiological substrate has yet to be determined. Direct evidence has been obtained that this SSAO enzyme in homogenates of rat aorta is capable of metabolising the amines kynuramine [6], phenylethylamine [7], dopamine [8], tyramine [9], tryptamine [10] and most recently methylamine [11]. However, no detailed study of the relative affinities of related amines for the enzyme has been undertaken with this tissue.

The presence of SSAO in the blood vessel wall and its location in the plasma membrane of smooth muscle cells could be taken as supporting a possible role for the enzyme inactivating circulating amines without the need for an uptake process [12]. Recent evidence has also shown that SSAO, *in situ*, in the intact mesenteric arterial bed of the rat, is capable of metabolising amines present in fluid perfusing these vessels [13]. Despite this, direct evidence for a scavenging role of this SSAO activity is lacking and the inability to potentiate the pressor response to tyramine in mesenteric arteries, when SSAO had been selectively inhibited, casts doubt on this suggestion. Indeed, it has been speculated that the products of oxidative deamination may be more important than the amines that are metabolised with respect to the function of this membrane bound activity [13]. In particular the generation of the reactive molecule, hydrogen peroxide, at the surface of the cell could be of importance in modulating signals controlling the activity of this contractile tissue.

In addition to a search for naturally occurring enzyme substrates, it is also important to determine whether or not any physiological regulators of enzyme activity exist. One way in which the enzyme's activity might be controlled is by a compound with a high affinity for the enzyme's active site but which is not itself metabolised. We have looked at a number of structurally related amines to see if they reduced the metabolism of radiolabelled benzylamine by aortic tissue homogenates and determined both type and potency of inhibition of the most effective compounds. Where possible direct evidence as to whether these compounds are enzyme substrates or regulators has been obtained by a fluorimetric assay. Throughout these studies care has been taken to identify the type of amine oxidase responsible for the metabolism measured by the use of selective inhibitors and/or selective substrates. This tissue contains both SSAO and MAO-A but little or no MAO-B [9].

MATERIALS AND METHODS

Materials

Substrates for the radiochemical amine oxidase assays were [7-^{14}C]benzylamine hydrochloride and [G-^{3}H]tyramine hydrochloride from Dupont U.K. Ltd (Stevenage, Herts, U.K.), and 5-hydroxy [G-^{3}H]tryptamine creatinine sulphate and [2,5,6-^{3}H]dopamine from Amersham International (Amersham, U.K.). The following unlabelled amines were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.): *p*-tyramine hydrochloride, 5-hydroxytryptamine creatinine sulphate, dopamine hydrochloride, the hydrochlorides of tryptamine, DL-*p*-octopamine, L-phenylephrine and DL-synephrine, DL-α-(methylaminomethyl) benzyl alcohol (halostachine) and DL-phenylethanolamine (both as the free base), L-noradrenaline bitartrate, L-adrenaline bitartrate, kynuramine dihydrobromide, cadaverine dihydrochloride, histamine dihydrochloride, putrescine dihydrochloride, spermidine trihydrochloride and spermine tetrahydrochloride. *m*-Tyramine hydrochloride was purchased from Research Biochemicals Inc. (Natik, MA). Benzylamine, isoamylamine, isobutylamine and phenylethylamine were obtained as the free base (from Sigma) and crystallised as the hydrochlorides. The isomers of octopamine, L-*p*-octopamine hydrochloride, D-*p*-octopamine hydrochloride and DL-*m*-octopamine bitartrate were gifts from Prof. T. L. Sourkes.

Clorgyline hydrochloride was a gift from May and Baker Ltd (Dagenham, Essex, U.K.) and [E]-2-(3′,4′-dimethoxyphenyl)-3-fluoroallylamine (MDL 72145) was a gift from Merrell Dow Research Institute (Strasbourg, France). The following chemicals were used in the fluorimetric assays; horseradish peroxidase (type II) and 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid; HVA) both obtained from Sigma. Catechol and L-cysteine hydrochloride were purchased from BDH (Poole, Dorset, U.K.) and recrystallised from benzene and ethanol, respectively. The hydrogen peroxide (100 vol.) was obtained from Fisons Scientific Apparatus (Loughborough, Leics, U.K.) and standardised by titration with potassium permanganate. The sodium salicylate and Brij 35 were from Hopkin and Williams (Chadwell Heath, Essex, U.K.). All other reagents were of analytical grade where possible. Male Wistar rats (200–400 g) were supplied by A. J. Tuck and Son (Rayleigh, Essex, U.K.).

Methods

Rats were killed by stunning followed by decapitation. The aorta was removed, rinsed in physiological saline to clear any adhering blood and freed of surrounding fat. The tissues were frozen (−20°) until assayed. Some rats were treated with 1 mg/kg of MDL 72145 (dissolved in distilled water) by intraperitoneal injection 1 hr before death. This compound is a potent and irreversible inhibitor of SSAO [14] and used in this way has been shown to inhibit SSAO selectively, in this tissue, without affecting MAO-A [15]. Injections of distilled water alone have no effect on the enzyme activities.

Homogenates of aortas were made on the day of the assay. The tissue was chopped finely with scissors and homogenised 1 in 20 w/v in 1 mM potassium phosphate buffer (pH 7.8) using a mechanical homogeniser (Polytron Mark 5, Kinematica) with three bursts of 5 sec each at speed number 10. The resulting homogenates were spun at 600 *g* (MSE Chillspin) for 10 min to separate unbroken cells and nuclei and the supernatant was used in the assay. Some homogenates were preincubated with clorgyline before the assay to inactive MAO-A. The preincubation was carried out at 37° for 30 min with 500 μM clorgyline.

Radiochemical method. A modification of the method of Callingham and Laverty [16] was used to study the metabolism of benzylamine (BZ), 5-hydroxytryptamine (5-HT), tyramine and dopamine. In this assay, a water saturated mixture of ethyl acetate and toluene was used to extract the radiolabelled products of metabolism from the unchanged amine. The specific activities of aortic homogenates from control and MDL 72145 treated rats for all four amines were measured. In addition, the effect of preincubating the homogenates with 500 μM clorgyline on the metabolism of the amines was studied. The concentrations of amines used were 10 μM BZ, 500 μM 5-HT, 100 μM tyramine and 500 μM dopamine. These were chosen as they were in excess of the K_m and K_i values calculated in the studies described below. Incubation times and homogenate concentrations were chosen for each amine such that the reaction was linear with time and not more than 15% of the available substrate was metabolised during the incubation period. The partition of the labelled products between the organic and aqueous phases was determined by a double extraction method [17] for each of the amines. The protein content of each homogenate was measured by the Lowry method [18] and the specific activities calculated in nmoles of product formed per hr per mg protein.

In order to determine which type of monoamine oxidase was responsible for the amine metabolism that was sensitive to inhibition by clorgyline, aortic homogenates were preincubated in the absence and presence of a range of clorgyline concentrations from 0.1 nM to 500 μM at 37° for 30 min. The resulting homogenates were then assayed with all four amines and the metabolism measured was expressed as a percentage of the control which had been preincubated in the absence of the inhibitor.

Having shown that the metabolism of 10 μM BZ was catalysed by SSAO alone, we examined the ability of a series of unlabelled amines to interfere with the metabolism of BZ. The homogenate (25 μl), unlabelled amine (25 μl) and 10 μM radiolabelled BZ (50 μl) were all incubated together and the metabolism of BZ measured and compared with the metabolism seen when 25 μl of water were added instead of the unlabelled amine. Again, the specific activities were calculated in nmoles per hr per mg protein and comparison between control and in the presence of unlabelled amine made using a paired *t*-test. The unlabelled amines were tested first at 100 μM and those that did not give significant reduction in BZ metabolism at this concentration were examined at 200, 500 μM and 1 mM. The amines were also added to the mixture after incubation but before the extraction process and the effect of their presence on the

efficiency of extraction of the metabolites of BZ was examined. No effect was seen with any of the amines on the efficiency of the extraction process.

The kinetics of the interaction of many of the amines that did interfere with BZ metabolism were investigated. The metabolism of 1, 2.5, 5, 7.5, 10 and 20 μM radioactive BZ by homogenates of aorta was measured. Preliminary experiments with homogenate concentrations of 1 in 200 w/v and with an incubation time of 5 min showed that the reaction was linear with time and that less than 15% of the available substrate was metabolised at all concentrations. Similar substrate velocity curves were also measured in the presence of two or three different concentrations of cold amine and the kinetics of the interaction analysed on homogenates from three rats for each amine. The results from these kinetic studies were plotted by a computer producing Hanes–Woolf plots using the weighted linear regression method of Wilkinson [19] to obtain apparent K_m and V_{max} values. Most of the amines examined in this way showed a competitive interaction.

Fluorimetric assay. This method was used to determine whether those amines, which significantly inhibited BZ metabolism in the radiochemical assay described above, were metabolised by SSAO. The method was based on that of Guilbault *et al.* [20] and a semi-automated assay similar to that used by Sharman and Cooper [21] was employed. Homogenates from control rats and from MDL-treated rats and homogenates preincubated for 30 min with 500 μM clorgyline were used. All homogenates were shaken with manganese dioxide (20 mg/ml) to oxidise any substances present in the homogenate, with an oxidation reduction potential less than that of homovanillic acid, that would interfere with the assay. This treatment did not affect the activity of either MAO-A or SSAO as measured by the metabolism of 5-HT and BZ, respectively, in the radiochemical assay. Each tissue sample tested consisted of two aortas homogenised 1 in 20 w/v as previously described. A mixture containing homogenate, HVA (0.5 mg/ml), horseradish peroxidase (0.2 mg/ml) and potassium phosphate buffer (0.1 M, pH 7.8) was made (solution A). In the assay, each tube contained 190 μl of solution A plus 10 μl of the amine under investigation (final concentration 100 μM). The tubes were incubated for 20 min at 37° and then placed on ice followed by the addition of 3 ml of a solution consisting of 0.01 M tris (hydroxymethyl)-methylamine (Tris) containing diaminoethane tetraacetic acid disodium salt (EDTA: 0.2 mg/ml), cysteine HCl (0.2 mg/ml) and catechol (0.2 mg/ml) (solution B). This mixture was freshly prepared for each assay and was added to stop the reaction and to stabilise the fluorescence. Blank assays were made by incubating solution A in the absence of the amine. In addition, the amines were all tested for fluorescence with solution A in the absence of homogenate and only tryptamine gave a slight positive reaction. The values obtained for tryptamine were corrected for this. The fluorescence (activation wavelength 313 nm; emission wavelength 430 nm) of the final mixture was measured automatically in a Locarte filter fluorimeter fitted with a flow-through cuvette and fed from an automatic sampler (Carlo Erba SO3) by means of a Gilson Minipuls 2 pump. The wash solution for the automatic sampler was 0.01 M Tris containing 10 mg/l. Brij 35.

The assay was standardised by measuring the fluorescence of a 250 ng/ml solution of sodium salicylate in 0.01 M Tris at the beginning and end of each batch of samples. This fluorescence remained constant throughout the series of experiments. A standard curve for hydrogen peroxide was constructed in each assay by the addition of known amounts of peroxide to solution A (which had been incubated in the absence of the amine). After 2 min at room temperature, 3 ml of solution B was added and the fluorescence read. The metabolism of each amine was measured on the three groups of homogenates referred to above with five pairs of rats providing the tissue for each group. The protein content of each homogenate was measured and the results expressed in nmoles of peroxide formed per hr per mg protein. The MDL and clorgyline treated groups were compared with the control group by Student's *t*-test.

Octopamine, phenylethanolamine (PeOH) and spermine all inhibited BZ metabolism in the radiochemical assay but were shown not to be metabolised by SSAO in the fluorimetric assay. These amines were therefore tested for their ability to inhibit BZ metabolism as measured by the fluorimetric assay. Control homogenates were used for the studies with PeOH and spermine and clorgyline treated homogenates for octopamine. The incubation mixture consisted of 180 μl of solution A, 10 μl of BZ (25 or 50 μM) and 10 μl of octopamine or PeOH (at 25, 50, 100 or 200 μM) or spermine (at 100, 250, 500 μM or 1 mM). The resulting metabolism of BZ was measured in nmoles of peroxide formed per hr per mg protein and the results plotted on a Dixon plot.

RESULTS

Table 1 summarises the results obtained from experiments designed to identify the amine oxidase activities responsible for the deamination of radiolabelled BZ, 5-HT, tyramine and dopamine in homogenates of rat aortas. The specific activities illustrated have been corrected for the efficiency of extraction of the labelled products. The partition of the metabolites of dopamine into the organic phase was less than that of the other amines. BZ was metabolised by an activity resistant to inhibition by clorgyline but sensitive to inhibition by MDL 72145. 5-HT metabolism was completely inhibited by clorgyline but was not significantly affected by MDL 72145 treatment of the rats. At these concentrations, BZ and 5-HT were SSAO and MAO substrates, respectively. Tyramine and dopamine metabolism was inhibited by both inhibitors indicating that these amines were substrates for both types of activity. Figure 1 shows the clorgyline inhibition plots for the metabolism of these four amines and, where inhibition by clorgyline was seen, this reached its maximum at 0.1 μM clorgyline. No further inhibition occurred at higher clorgyline concentrations. This confirms that MAO-A was responsible for that part of the metabolism that was sensitive to inhibition by clorgyline [22].

Table 1. Specific activities of some amines metabolised by rat aortic homogenates measured by a radiochemical assay method

Amine	Control	Clorg treated	MDL treated	Extraction efficiency (%)
Benzylamine (10 μM, N = 5)	73.5 ± 2.6	87.5 ± 7.7	2.4 ± 0.5‡	100.6 ± 0.9
5-HT (500 μM, N = 10)	97.0 ± 10.6	1.7 ± 0.3‡	83.7 ± 8.7	77.0 ± 6.0
Tyramine (100 μM, N = 5)	95.6 ± 4.0	28.5 ± 4.5‡	49.9 ± 8.3‡	93.0 ± 1.5
Dopamine (500 μM, N = 6)	64.8 ± 7.5	13.3 ± 1.6†	39.6 ± 12.9*	49.5 ± 6.0

The specific activities for the metabolism of the four amines were measured using a radiochemical method and extracting the radiolabelled products into organic solvent. The values given are expressed as nmoles of product formed per hr per mg protein in the aortic homogenates and have been corrected for the extraction efficiency. The three groups of homogenates used were those from control rats (control), homogenates that were preincubated for 30 min at 37° with 500 μM clorgyline (Clorg treated) and those from rats pretreated with 1 mg/kg MDL 72145 by intraperitoneal injection 1 hr before death (MDL treated). In each case N refers to the number of animals in each group and for each amine the Clorg and MDL group have been compared with the control group by Student's *t*-test (* $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$). The efficiency of extraction of the radiolabelled products was determined for each amine by a double extraction procedure.

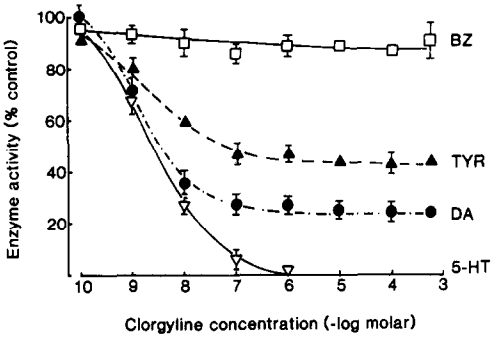

Fig. 1. Inhibition by clorgyline of amine oxidase activities in rat aortic homogenates. Substrates used were benzylamine (BZ, 10 μM), tyramine (TYR, 100 μM), dopamine (DA, 500 μM) and 5-HT (500 μM). The homogenates were preincubated for 30 min at 37° prior to assay with the radiolabelled amines. Each point is the mean activity from four aortic homogenates expressed as a percentage of the activity in the absence of the inhibitor. The standard error of the ratio is shown where larger than the symbol.

When experiments to measure the deamination of radiolabelled BZ in the presence of other amines were undertaken, primary monoamines were effective inhibitors (see Table 2). Of all the other amines tested only spermine had an effect at 100 μM. Secondary monoamines (adrenaline, phenylephrine, synephrine and halostachine), diamines (putrescine, cadaverine and histamine) and the polyamine spermidine were tested at higher concentrations to see if they interfered with BZ metabolism. Only histamine gave significant inhibition at 200 μM and the results obtained at 1 mM are listed in Table 2. Several of these amines were studied to determine the kinetics of their inhibition of BZ metabolism as described in the methods. Most of them showed a competitive interaction giving a parallel shift of the Hanes–Woolf plot and the results obtained with PeOH are shown in Fig. 2a as an example where a parallel shift in the kinetic plots can be seen. 5-HT was the one exception in that the interaction was mainly non-competitive. The Hanes–Woolf plot for this amine is shown in Fig. 2b. The K_i values for those amines tested in this way are also listed in Table 2. The kinetic parameters for BZ measured in these experiments were: K_m 2.45 ± 0.1 μM and V_{max} 122.6 ± 6 nmol/hr/mg protein.

Kynuramine was the most potent amine tested in this way. Of the amines in the phenylethylamine series, the addition of a hydroxyl group to the benzene ring reduced the affinity of the amine slightly. The addition of a second hydroxyl group caused a more substantial reduction in affinity (e.g. phenylethylamine > tyramine > dopamine and PeOH > octopamine > noradrenaline). The compounds used to determine in which position the hydroxyl group had the greatest effect gave equivocal results since *m*-tyramine was a better inhibitor than *p*-tyramine and *p*-octopamine was a better inhibitor than *m*-octopamine. β-Hydroxylation, in this series of structures, increased the affinity of the amine (PeOH > phenylethylamine and octopamine > tyramine). The optical isomers of octopamine showed that the L-isomer was the most active. The aliphatic amine, isoamylamine, inhibited BZ metabolism competitively showing that the presence of an aromatic ring was not essential for recognition by the active site of the SSAO.

Some of the amines, which gave significant inhibition, were examined by the fluorimetric method to determine whether they were metabolised by SSAO. The results are presented in Fig. 3, which gives the specific activities found for each amine in control, clorgyline and MDL 72145 treated aortic homogenates. The amines were studied at 100 μM and

Table 2. The effect of some amines on the metabolism of benzylamine by homogenates of rat aorta

Amine	% inhibition (100 μM amine vs 10 μM ^{14}C BZ)	K_i (μM)	Amine	% Inhibition (1 mM amine vs 10 μM ^{14}C BZ)
Kynuramine	100	5.4 ± 0.5	Histamine	45.0 (K_i 520 ± 90 μM)
DL-PeOH	74.2	8.6 ± 0.1		
L-*p*-Octopamine	72.8	—	DL-Halostachine	23.7
Phenylethylamine	69.3	16.5 ± 2.0	L-Phenylephrine	21.2
DL-*p*-Octopamine	62.9	12.3 ± 0.4	DL-Synephrine	15.9
Isoamylamine	62.9	44.7 ± 8.0	Cadaverine	5.7
m-Tyramine	53.5	—	Spermidine	3.1
L-*m*-Octopamine	49.9	—	Putrescine	2.0
Tryptamine	45.2	28.0 ± 4.3	DL-Adrenaline	1.6
p-Tyramine	43.0	77.1 ± 10.9		
5-HT	37.8	216.8 ± 10.6		
Isobutylamine	32.4	—		
Spermine	25.1	153.0 ± 37.8		
Dopamine	24.4	184.0 ± 19.7		
L-Noradrenaline	15.1	192.8 ± 26.2		
D-*p*-Octopamine	5.9	—		

The amines were used unlabelled together with radiolabelled benzylamine (BZ, 10 μM) to see if they interfered with BZ metabolism by rat aortic homogenates. For the first group of amines the percentage inhibition with 100 μM of the amine is given (mean inhibition; N = 4 or 5). The inhibition was significant for all the amines in the first column (except D-*p*-octopamine) when the specific activities obtained in the presence of the amine were compared with those found with no amine present using a paired *t*-test ($P < 0.05$). The kinetics of the interaction of many of these amines was studied and the K_i values given are the mean values ± SE from three rats where two or three concentrations of the amine were used to inhibit a range of BZ concentrations (see Materials and Methods). The inhibition seen was predominantly competitive for all the amines with the exception of 5-HT which showed a non-competitive interaction. The second group of amines did not show significant inhibition of BZ metabolism when included at 100 μM. Only histamine inhibited significantly at 200 μM, DL-halostachine, L-phenylephrine and DL-synephrine inhibited significantly at 500 μM ($P < 0.05$) and none of the other amines gave significant inhibition even at 1 mM. The percentage inhibition seen at 1 mM for all these amines is given in the table. The kinetics of the interaction of this second group of amine structures was not studied.

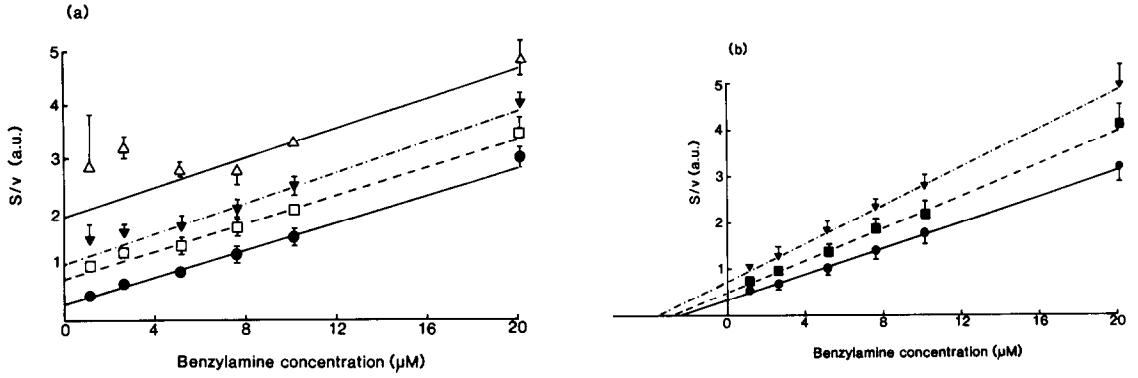

Fig. 2. The kinetics of the interaction between unlabelled phenylethanolamine (PeOH) and 5-HT with the metabolism of radiolabelled BZ by homogenates of rat aorta. Homogenates (1 in 200 w/v) were incubated with BZ concentrations ranging from 1 to 20 μM at 37° for 5 min in the presence or absence of either 5-HT or PeOH. The results have been calculated in nmoles of product formed per hr per mg protein and plotted by computer on a Hanes–Woolf plot by weighted linear regression [19]. (a) The regression lines in the absence of PeOH (closed circles), in the presence of 10 μM PeOH (open squares), 20 μM PeOH (closed triangles) and 40 μM PeOH (open triangles) are shown. Each point represents the mean value from three aortic homogenates ± SE where larger than the symbol. The kinetic parameters in the absence of PeOH are K_m 2.28 ± 0.46 (μM) and V_{max} 140 ± 8 (nmol/hr/mg protein). (b) The regression lines in the absence of 5-HT (circles), in the presence of 50 μM 5-HT (squares) and 100 μM 5-HT (triangles) are shown. Each point is the mean value from three homogenates ± SE where larger than the symbol. The kinetic parameters in the absence of 5-HT are K_m 2.28 ± 0.23 (μM) and V_{max} 118 ± 3.9 (nmol/hr/mg protein).

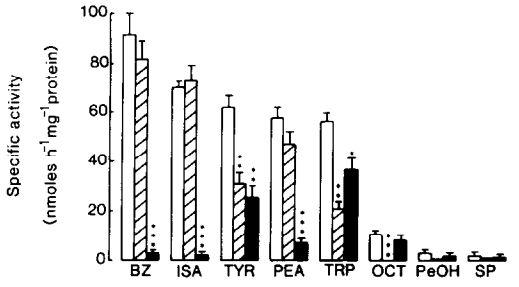

Fig. 3. The metabolism of some amines by rat aortic homogenates measured by a fluorimetric method. The assay method used detects the fluorescence produced when hydrogen peroxide is acted upon by peroxidase in the presence of homovanillic acid. The metabolism of each amine at a concentration of 100 μM is given in nmoles of peroxide formed per hr per mg protein in the homogenate. For all the amines the metabolism has been studied in three different types of homogenate: homogenates from control rats (open columns); control rat homogenates that have been preincubated with 500 μM clorgyline at 37° for 30 min prior to incubation with the amines (hatched columns) and homogenates from rats treated with 1 mg/kg MDL 72145 by intraperitoneal injection 1 hr before death (solid columns). The values given for each amine in all three groups are the mean ± SE (N = 5 for each group). The clorgyline and MDL treated groups have been compared with the control group using a Student's t-test and the levels of significance are indicated (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). The amines are benzylamine (BZ), isoamylamine (ISA), tyramine (TYR), phenylethylamine (PEA), tryptamine (TRP), octopamine (OCT), phenylethanolamine (PeOH) and spermine (SP).

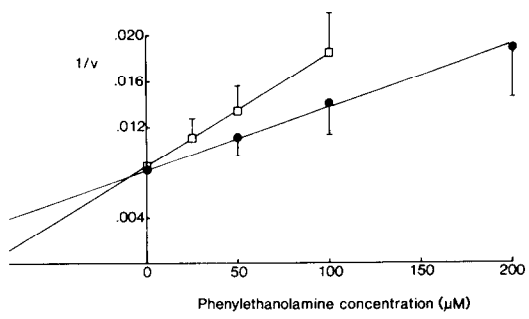

Fig. 4. The kinetics of the interaction between phenylethanolamine (PeOH) and the metabolism of benzylamine (BZ) in rat aortic homogenates measured by the fluorimetric assay method. Benzylamine metabolism has been studied at 25 μM (open squares) and 50 μM (closed circles) and the reciprocal of the velocity (v, nmoles of peroxide formed per hr per mg protein) has been plotted against the PeOH concentration. Each point represents the mean ± SE from three aortic homogenates. The K_i value for PeOH derived from this plot is 9 μM.

BZ was examined for comparison. The metabolism that was resistant to inhibition by clorgyline (of BZ, isoamylamine, and phenylethylamine) was, in each case, almost completely inhibited by MDL 72145 showing that these amines were SSAO substrates. Octopamine, whose metabolism was resistant to MDL 72145 and completely inhibited by clorgyline, was, therefore, a substrate only for MAO at this concentration. BZ had the highest specific activity and the results obtained by this method are similar to those obtained by the radiochemical method. PeOH and spermine showed almost no metabolism using this assay and the metabolism of a very small amount of PeOH was prevented by clorgyline. Tyramine and tryptamine were metabolised by activities which were inhibited significantly by both inhibitors.

PeOH, octopamine and spermine were investigated for their ability to inhibit BZ metabolism as measured by the fluorimetric method. PeOH and octopamine both decreased the amount of BZ metabolism detected and the data from the PeOH experiments have been used to draw the Dixon Plot shown in Fig. 4. The data from octopamine gave a similar pattern when plotted in the same way giving a K_i of 21 μM. These K_i values are in close agreement with those obtained by the radiochemical assay. No inhibition of BZ metabolism by spermine could be detected by this method even at a concentration of 1 mM. At this concentration spermine metabolism was still not detectable (triplicate observations made in two separate experiments with aortic homogenates from two pairs of rats).

DISCUSSION

Clorgyline and MDL 72145 have been used to inhibit selectively MAO and SSAO, respectively, in the rat aorta. BZ has been used as a selective substrate for SSAO as its deamination was completely inhibited by MDL 72145 and totally resistant to inhibition by clorgyline. By contrast, 5-HT deamination was completely inhibited by 0.1 μM clorgyline but was resistant to inhibition by MDL 72145 indicating that MAO-A was responsible. MDL 72145 is also a potent inhibitor of MAO-B in many tissues [23] but the rat aorta would seem to contain very little MAO-B from the results of these studies. For example, with both tyramine and dopamine, the clorgyline plots showed maximum inhibition at 0.1 μM clorgyline indicating that MAO-A catalysed this clorgyline-sensitive portion of the deamination. The demonstration that an amine competitively inhibits the metabolism of radiolabelled BZ and that it is itself deaminated by an activity that is resistant to inhibition by clorgyline but sensitive to inhibition by MDL 72145 would be strong evidence that this amine is a substrate for SSAO. Using these criteria, it was possible to investigate the substrate specificity of SSAO in rat aorta without the need to purify the enzyme and so risk altering its properties in the purification procedure.

Twenty-one amines have been examined for an interaction with SSAO by investigating their ability to interfere with BZ metabolism. The concentration of BZ chosen for these studies was 10 μM. From the kinetic analysis of BZ metabolism, this substrate concentration should enable any amine competing with BZ for the active site of SSAO to be readily detected. The results suggest certain rules for amines with the phenylethylamine structure to describe the affinity for this SSAO activity. Kapeller-Adler [24] and Blashcko [25] both proposed that this group of

enzymes (SSAO) only metabolised primary amines. In the present study, the results show substitution of the amino-nitrogen in this series greatly reduced the ability of the amine to inhibit BZ metabolism. Addition of a hydroxyl group to the benzene ring reduced the affinity slightly, although the position in which this hydroxyl group caused the greatest decrease differed between the isomers of tyramine and octopamine. Possibly, a β-hydroxyl group influences the distribution of charge over the benzene ring and alters the effect of a hydroxyl group in the *para* or *meta* position on the amine's affinity for the enzyme. Adding the second hydroxyl group to form a catecholamine substantially reduces the affinity. The presence of a β-hydroxyl group increases the amine's affinity for the BZ metabolising activity. These structural requirements for recognition at the active site of SSAO (which is suggested from the fact that these amines showed competitive inhibition of BZ metabolism) were also seen in the membrane bound SSAO enzyme found in the brown adipose tissue of the rat [26] suggesting that these two SSAO enzyme activities may have very similar properties.

The studies with the isomers of octopamine demonstrate that the L-isomer is the active form of octopamine. This finding is similar to those of Giachetti and Shore [27] who showed that the L-isomers of octopamine, noradrenaline and adrenaline were more rapidly metabolised by monoamine oxidase from a variety of sources. It appears that, like MAO, SSAO is able to distinguish between optical isomers.

Kynuramine was the most active amine tested in the competition studies having the lowest K_i. There is direct evidence that this amine, which may be formed naturally as a by product of tryptophan metabolism in many tissues [28], is a substrate for both MAO and SSAO in the rat aorta [6]. The amines which are tryptophan derivatives, tryptamine and 5-HT, both interacted with SSAO to reduce BZ metabolism. 5-HT was the only amine tested that gave a mixed but mainly non-competitive interaction with SSAO. This type of interaction has been reported previously between SSAO and 5-HT in the cat uterine artery [29] as well as in the brown adipose tissue of the rat (M. A. Barrand, personal communication). The evidence from the inhibition studies with clorgyline and MDL 72145 confirm that 5-HT is a substrate for MAO-A in the rat aorta. Indeed, relatively few tissues have amine oxidising activities that metabolise 5-HT, which are resistant to inhibition by clorgyline. An exception to this general rule is the dental pulp of the ox and pig where membrane bound SSAO enzymes capable of deaminating 5-HT have been found [30]. It is possible that 5-HT acts as an allosteric regulator for SSAO in vascular smooth muscle. The local release of 5-HT from platelets activated following endothelial damage would expose SSAO to high concentrations of this inhibitory amine, which could modify the function of this enzyme.

The aliphatic amines, isoamylamine and isobutylamine, both interacted strongly with SSAO. These findings support the work of Precious *et al.* [11] who showed that methylamine is a substrate for the enzyme in this tissue with a K_m of 100 μM and showed that an aromatic group is not essential for an amine to be recognised by the active site of SSAO. The relatively low affinity of histamine for this SSAO activity and the lack of effect of the diamines putrescine and cadaverine demonstrate that the vascular SSAO activity differs from the tissue bound SSAO enzymes of the diamine oxidase (DAO) group. DAO enzymes show a much higher affinity for diamines, including histamine, than they do for BZ and other monoamines [31]. Similarly, the lack of inhibition of BZ metabolism seen with the polyamine spermidine indicates that this activity does not interact with this type of amine structure unlike the SSAO enzyme present in the plasma of ruminants [32]. The apparent interaction of spermine with BZ metabolism as measured by the radiochemical method was not confirmed by the fluorimetric method where 1 mM spermine was neither metabolised nor did it interfere with BZ metabolism. A possible explanation for this anomaly is that spermine forms a complex with the metabolites of BZ holding them in the aqueous phase and so preventing their detection in the radiochemical method. It would seem that these complexes may require time as well as incubation at 37° to form since the addition of spermine just prior to the extraction process in the radiochemical assay did not affect the efficiency of extraction of the metabolites of BZ. This explanation is probable since spermine shows a tendency to bind to naturally occurring compounds such as nucleic acids.

The demonstration of competitive inhibition of radiolabelled BZ metabolism by an unlabelled amine suggests an interaction at the active site of the SSAO activity but does not differentiate between an amine which is metabolised by this enzyme or one which binds without being metabolised. Direct demonstration of metabolism of the amine in question by aortic homogenates is necessary to give this information.

The fluorimetric detection of hydrogen peroxide using homovanillic acid and peroxidase provided a sensitive method for detecting metabolism of some of these amines without the need to have them radiolabelled. The specific activities found with BZ by this method were similar to those found with the radiochemical method. At a concentration of 100 μM, BZ might show some substrate inhibition of SSAO [9] but nevertheless, BZ is the most actively metabolised of all the amines tested. Isoamylamine is also rapidly metabolised by SSAO and fulfils the criteria of showing competitive inhibition of BZ metabolism, being deaminated itself and its deamination being sensitive to inhibition by MDL 72145 but resistant to clorgyline. Phenylethylamine behaved in a similar manner, which confirms the findings of Fuentes & Neff [7] that this amine is a substrate for SSAO in the rat aorta.

Tyramine metabolism was shown by the radiochemical and fluorimetric methods, to be due to both MAO-A and SSAO. Tryptamine falls into the same category and the results here confirm the findings of Lyles and Taneja [10], who demonstrated metabolism of tryptamine by both SSAO and MAO in rat aorta.

The β-hydroxylated amines, octopamine and PeOH, are not substrates for SSAO in this tissue at

100 μM. Both these amines are good inhibitors of BZ metabolism as measured by both assays. The K_i values obtained by the two methods are in close agreement. It would seem, therefore, that the addition of a β-hydroxyl group to a phenylethylamine derivative increases the affinity of the amine for the active site of SSAO but the amine is no longer metabolised. This finding may have important consequences with regard to the physiological regulators of this enzyme activity. Both octopamine and noradrenaline are present in sympathetic nerve endings [33] and octopamine has been shown to be released along with noradrenaline on sympathetic stimulation [34]. Liberation of this amine in small amounts, but in close proximity to the enzyme activity, could alter the production of hydrogen peroxide by SSAO and, therefore, the oxidation–reduction conditions at the smooth muscle cell surface. These changes may affect the response of this tissue to hormones and locally acting agents. Noradrenaline is a weaker inhibitor of BZ metabolism but unfortunately we have no direct evidence as to whether it is metabolised by or inhibits the SSAO activity. This amine is not suitable for study by the fluorimetric assay as it has a lower redox potential than homovanillic acid and so is oxidised by the peroxidase catalysed reaction in preference to HVA [35]. The efficiency of extraction of the products of radiolabelled noradrenaline, using a solvent system such as the one employed here, is low, making the radiochemical assay insensitive and unreliable [36].

A similar problem exists for both dopamine and 5-HT which means they are not suitable for use in the fluorimetric assay. However, both can be used in the radiochemical assay and metabolism of dopamine by SSAO was detected by the use of the selective inhibitors despite the lower efficiency of extraction of dopamine metabolites when compared with the other labelled amines. It would be useful to have a method that could confirm whether or not 5-HT is an allosteric regulator of this enzyme.

In conclusion, these studies have characterised further the amine structures which interact with the SSAO activity of the rat aorta and provided some information as to their relative affinities for this enzyme. A number of amines which are present in the diet or are formed in the intestine by the action of microbial amino acid decarboxylases [37] such as phenylethylamine, tyramine, isoamylamine and tryptamine, are substrates of SSAO. BZ remains the amine which is most effectively metabolised by SSAO in the rat aorta. Naturally occurring regulators of this activity, such as octopamine and possibly 5-HT and noradrenaline, have been uncovered. The enzyme shows stereoselectivity in recognising the L-form of octopamine and it is possible that sympathetic nerve activity and the release of octopamine could be the physiological regulation system reducing the activity of this enzyme and the products it generates. It may well be that these products, and the regulation of their production, are more important to the smooth muscle cell than the deamination and inactivation of circulating amines which may function merely to fuel the enzyme for the generation of reactive products such as hydrogen peroxide.

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