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Allosteric modulation of semicarbazide-sensitive amine oxidase activities *in vitro* by imidazoline receptor ligands

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1 Evidence indicates that imidazoline I_2 binding sites (I_2BSs) are present on monoamine oxidase (MAO) and on soluble (plasma) semicarbazide-sensitive amine oxidase enzymes. The binding site on MAO has been described as a modulatory site, although no effects on activity are thought to have been observed as a result of ligands binding to these sites.

2 We examined the effects *in vitro* of several imidazoline binding site ligands on activities of bovine plasma amine oxidase (BPAO) and porcine kidney diamine oxidase (PKDAO) in a spectrophotometric protocol.

3 While both enzymes were inhibited at high concentrations of all ligands, clonidine, cirazoline and oxymetazoline were seen, at lower concentrations, to increase activity of BPAO *versus* benzylamine, but not of PKDAO *versus* putrescine. This effect was substrate dependent, with mixed or biphasic inhibition of spermidine, methylamine, *p*-tyramine and *β*-phenylethylamine oxidation observed at cirazoline concentrations that increased benzylamine oxidation.

4 With benzylamine as substrate, clonidine decreased K_M (EC_{50} 8.82 μM, E_{max} 75.1% of control) and increased V_{max} (EC_{50} 164.6 μM, E_{max} 154.1% of control). Cirazoline decreased V_{max} (EC_{50} 2.15 μM, E_{max} 91.4% of control), then decreased K_M (EC_{50} 5.63 μM, E_{max} 42.6% of control) and increased V_{max} (EC_{50} 49.0 μM, E_{max} 114.4% of decreased V_{max} value).

5 Data for clonidine fitted a mathematical model for two-site nonessential activation plus linear intersecting noncompetitive inhibition. Data for cirazoline were consistent with involvement of a fourth site.

6 These results reveal an ability of imidazoline ligands to modulate BPAO kinetics allosterically. The derived mechanism may have functional significance with respect to modulation of MAO by I_2BS ligands.

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Abbreviations: α, factor by which K_M is altered by imidazoline drug; AM, amiloride; β, factor by which V_{max} is altered by imidazoline drug; 2-BFI, 2-(2-benzofuranyl)-2-imidazoline; BPAO, bovine plasma amine oxidase; BZ, benzylamine; CIR, cirazoline; D, imidazoline drug binding to any site on BPAO; E, enzyme; E_{max}, maximum degree to which measured parameter can be changed by drug of interest; γ, factor by which affinity of enzyme for substrate is reduced by a mixed inhibitor; H, imidazoline drug binding to high-affinity site on BPAO; I, imidazoline drug binding to (very low-affinity) inhibitory site on BPAO; IBS, imidazoline binding site; IDZ, idazoxan; I_1R, imidazoline type-1 receptor; k_p, rate constant for enzymatic formation of product; K_H, dissociation constant for ligand H from high-affinity site on BPAO; K_L, dissociation constant for ligand L from low-affinity site on BPAO; K_S, dissociation constant for substrate S from active site of BPAO; L, imidazoline drug binding to low-affinity site on BPAO; MA, methylamine; MOX, moxonidine; P, product (of an enzyme reaction); PEA, *β*-phenylethylamine; PKDAO, porcine kidney diamine oxidase; S, substrate; SPD, spermidine; SSAO, semicarbazide-sensitive amine oxidase; TYR, *p*-tyramine; v, enzyme reaction velocity; V_{max}, enzyme reaction velocity at saturating substrate concentration

Introduction

Following the discovery that clonidine exerts its hypotensive effect at least in part through an action at novel nonadrenergic receptors in the ventrolateral medulla (Bousquet *et al*., 1984), interest in these imidazoline receptors, as well as in other binding sites for clonidine and related drugs, has blossomed (see Eglen *et al*., 1998; Head, 2003 for reviews). At least three subclasses of binding sites have been claimed to exist, with only the imidazoline type-1 receptor (I_1R), which is thought to play a role in central control of blood pressure (see Bousquet &

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Feldman, 1999), having been shown thus far to be associated with a signal transduction system (Takada *et al*., 1997; Edwards *et al*., 2001). Imidazoline-3 binding sites (I_3BSs) encompass a nebulous group of loci associated predominantly with various ion channel subunits, and while ligands may modulate processes such as insulin secretion and catecholamine release (see Molderings, 1997; Morgan & Chan, 2001 for reviews), some of these effects may be due to drugs acting at two or more different binding sites in target tissues (Morgan & Chan, 2001).

While a variety of physiological effects can be ascribed to ligands acting at I_1Rs or I_3BSs, the same may not be said for those binding to imidazoline-2 binding sites (I_2BSs).

The observation that I_2BSs were located on the outer membranes of mitochondria (Tesson *et al*., 1991) quickly led to the realisation that many such sites were actually located on the monoamine oxidase (MAO) protein (Olmos *et al*., 1993; Alemany *et al*., 1995; Carpéné *et al*., 1995; Tesson *et al*., 1995). Imidazoline ligands bound with high affinity to these sites and were able to inhibit MAO and other amine oxidases *in vitro* (Carpéné *et al*., 1995), while chronic treatment of rats with amine oxidase inhibitors caused either a downregulation (Alemany *et al*., 1995) or an upregulation (Wiest & Steinberg, 1997) of I_2BSs in the brain or liver. Some functional interplay between the I_2BS on MAO and enzyme activity was thus presumed to occur, and the I_2BS became increasingly referred to as a modulatory or regulatory site on MAO (Tesson *et al*., 1995).

In support of such a designation, numerous studies have shown clearly that the I_2BS is distinct from the MAO active site (Raddatz *et al*., 1997; 1999) and that, in rats at least, MAO-B appears to be the predominant enzyme isoform upon which these sites might be found (Raddatz *et al*., 2000; Remaury *et al*., 2000). However, while imidazolines certainly bind with high affinity to the I_2BS on MAO, inhibition of enzyme activity requires rather higher ligand concentrations than are necessary to saturate the I_2BS (Ozaita *et al*., 1997).

A few other enzymes also show affinity for I_2BS ligands, including several amine oxidases of the EC 1.4.3.6 subclass (Carpéné *et al*., 1995; Holt & Baker, 1995; Holt *et al*., 2003). This family of copper-containing amine oxidases includes diamine oxidase, plasma amine oxidases and tissue-bound semicarbazide-sensitive amine oxidase (SSAO) (Callingham *et al*., 1991). It is likely that the active sites of these enzymes are responsible, at least in part, for binding imidazoline ligands, since several endogenous ligands for I_1Rs, I_2BSs and I_3BSs, including agmatine, tryptamine, histamine and polyamines, are substrates for one or more of these enzymes (Holt & Baker, 1995; Lyles, 1996). However, the possibility remains that an I_2BS separate from the active site may exist on semicarbazide-sensitive amine oxidase proteins.

If the high-affinity I_2BS on MAO, and perhaps on semicarbazide-sensitive amine oxidases, corresponds to a modulatory site and not simply to an artefactual binding site, then modulation of substrate turnover would necessarily take place on ligand binding to the enzyme. Some precedent does exist for rapid activation of amine oxidase activities. Human platelet MAO and rat brain MAO can be activated *in vitro* in a substrate-selective manner by a component of human plasma, as a result of a reduction in substrate K_M values (Yu & Boulton, 1979; Wahlund *et al*., 1984). Human plasma also activates human lung SSAO, once again in a substrate-selective fashion, by increasing substrate affinity (Dalfó *et al*., 2003), and captopril, although not possessing an imidazoline structure, activates rat MAO *in vitro* through increasing V_{max} (Raasch *et al*., 2002).

An extensive study by Carpéné *et al*. (1995) showed concentration-dependent inhibition of MAO by high concentrations of several imidazoline ligands; at low concentrations, MAO activity was similar to control values. However, in the same study, tranylcypromine and cirazoline, an imidazoline I_2BS/I_1R ligand, appeared to induce a small, statistically insignificant potentiation of bovine plasma amine oxidase (BPAO), an EC 1.4.3.6 semicarbazide-sensitive amine oxidase enzyme, at low concentrations of ligand. This effect was not commented upon by the authors. A related amine oxidase from porcine plasma was stimulated *in vitro*, in a pH-dependent manner, by including ammonia or imidazole in the enzyme reaction (Kelly *et al*., 1981; Yadav & Knowles, 1981).

Given the clear precedent for rapid modulation of several amine oxidase enzymes and the ability of imidazole, and perhaps cirazoline and tranylcypromine, to stimulate plasma amine oxidases *in vitro*, it was decided to examine the abilities of several imidazoline binding site (IBS) ligands to influence the activities both of BPAO and of diamine oxidase from pig kidney (PKDAO). Both of these enzymes are semicarbazide-sensitive amine oxidases of the EC 1.4.3.6 subclass, and they share a common quinone cofactor and similar reaction mechanisms (Klinman, 2003).

Methods

Enzyme preparation

Commercially available BPAO and PKDAO were dissolved in potassium phosphate buffer (0.2 M, pH 7.4), usually at 1 or 3 mg ml^{-1} (BPAO) or 7.5 mg ml^{-1} (PKDAO), without further purification. Final assay concentrations were 0.167 or 0.5 mg ml^{-1}, and 1.25 mg ml^{-1}, respectively. Enzyme solutions were prepared freshly on the day of assay and were retained on ice until required.

Amine oxidase activity measurements

Enzyme activities were measured by a modification of the colorimetric protocol of Holt *et al*. (1997). Assays were performed in 96-well polystyrene plates in a total volume of 300 μl and generally contained 50 μl enzyme, 50 μl chromogenic solution, 50 μl amine substrate or water (blanks) and 150 μl water (controls) or test compound. The chromogenic solution contained vanillic acid, 4-aminoantipyrine and horseradish peroxidase in potassium phosphate buffer (Holt *et al*., 1997), with the peroxide generated during amine turnover initiating the peroxidase-dependent formation of a red quinoneimine dye. Amine substrates were benzylamine (final concentration 740 μM) for BPAO, and putrescine (final concentration 288 μM) for PKDAO, concentrations similar to their respective K_M values. In some kinetic experiments (see below), a range of benzylamine concentrations was used, between 10 μM and 3 mM. In examinations of ligand effects upon turnover of different substrates, *p*-tyramine, β-phenylethylamine, methylamine and spermidine were also used as BPAO substrates, at 135, 275, 420 and 50 μM, respectively.

These final concentrations are equal to one-half of their K_M values.

Assays were performed in duplicate or triplicate wells, at 37°C, with reactions usually initiated by adding amine substrates that had been prewarmed to 37°C. The increase in absorbance at 498 nm was monitored continuously in a Molecular Devices SpectraMax Plus 384 plate reader for an appropriate period of time, and initial rates of enzyme activities were determined by linear regression of data (SOFTmax PRO, version 3.1.2).

BPAO and drug purity assessments

The BPAO used was purchased from Sigma and was included in assays without further purification. However, since more than one plasma amine oxidase activity *versus* benzylamine has been detected in other ruminants, such as the sheep (Elliott *et al*., 1992), it was necessary to clarify how many activities in the bovine plasma extract might contribute to amine metabolism.

Proteins in the solid obtained from Sigma (86% protein, $w\,w^{-1}$) were separated by SDS–polyacrylamide gel electrophoresis. Samples were separated under denaturing conditions and lanes were either stained with Coomassie blue or were electroblotted to a nitrocellulose membrane. The membrane was then incubated in 2 M potassium glycinate buffer (pH 10) containing nitroblue tetrazolium (0.24 mM) for 45 min in the dark. This procedure results in deposition of purple formazan on the nitrocellulose paper over protein bands, such as that for BPAO, containing a quinone or quinoid cofactor (Paz *et al*., 1991; Holt *et al*., 1998). Gel electrophoresis (not shown) revealed that the commercial enzyme preparation contained several major and numerous minor protein components. Quinone staining of an electroblot identified one major band, at 82 kDa, consistent with the mass of a single BPAO subunit (Mu *et al*., 1994). Very faint formazan staining was also observed at 101 kDa, similar to a minor band at 102 kDa seen with partially purified porcine aorta SSAO (Holt *et al*., 1998). Thus, although the commercial BPAO preparation contains only one major quinone amine oxidase activity, it is possible that the 101 kDa component represents a subunit from vascular smooth muscle SSAO. Nevertheless, kinetic evidence in this study did not support participation of a second enzyme activity in benzylamine turnover.

Since benzylamine is also a substrate for MAO-B, the possibility was examined that some platelet MAO-B activity might be present in the bovine plasma extract and thus contribute to reaction velocity. Colorimetric assays were performed as described above, but with benzylamine substrate present at a final concentration of 2 mM, and either in the presence or absence of the MAO inhibitor pargyline (10 μM), added 10 min before substrate. While electrophoresis indicated that one or more proteins were present with masses close to 60 kDa, not inconsistent with the potential presence of MAO-B, the failure of pargyline to have any effect on benzylamine turnover (not shown) indicated that no active MAO-B enzyme was present in the preparation.

In order to assess the purity of drug compounds, aqueous solutions of clonidine and cirazoline were diluted in HPLC-grade methanol and were injected directly onto a Waters Micromass ZQ quadrupole mass spectrometer, with positive electrospray ionisation. Mass spectra (not shown) revealed the presence of parent ions with m/z ratios of 229.93, 231.94 and 233.95 (clonidine, containing ^{35}Cl and ^{37}Cl) and 217.03 (cirazoline), consistent with calculated $M+H^+$ values for the drugs. No other charged species could be detected above baseline noise in either sample, suggesting that drugs were devoid of contaminants that might demonstrate efficacy in subsequent assays.

Kinetic analyses of BPAO activity

In kinetic analyses, BPAO was incubated in the presence of a range of concentrations of benzylamine substrate (10 μM–3 mM), in the absence (controls) or presence of imidazoline test drugs. In order to obtain kinetic constants, data were fitted to a Michaelis–Menten hyperbola with the nonlinear regression facility of GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, U.S.A.). Sigmoidal curves were fitted to data, where appropriate, with the nonlinear regression facility, and Dixon plots and kinetic replots with the linear regression facility of GraphPad Prism.

Based on results obtained from kinetic studies, a reaction mechanism was proposed and a novel equation formulated to describe the observed behaviour. Details of the derivation of this mechanism will be published elsewhere.

Assessment of IBS characteristics

I_2BSs are differentiated from I_1Rs partly on the basis of the former group's high affinity for idazoxan, while subclassification of I_2 sites is dependent on a high or low affinity for the diuretic amiloride (Michel & Ernsberger, 1992). Although data from Figure 1a (see Results) suggested that the affinities of moxonidine and idazoxan for BPAO were rather low, it was possible that the modest inhibition observed resulted from interactions of these drugs with the active site, and the possibility remained that moxonidine or idazoxan may bind to the 'high-affinity' cirazoline/clonidine site, but lack the intrinsic efficacy to alter substrate turnover. Therefore, in order to determine whether the high-affinity sites through which cirazoline and clonidine act resembled typical I_1, I_{2A} or I_{2B} sites, the abilities of cirazoline (10 μM) or clonidine (100 μM) to potentiate benzylamine turnover were assessed in the absence or presence of a range of concentrations of moxonidine, idazoxan or amiloride. Results would reveal whether or not the effects of cirazoline or clonidine could be antagonised in a concentration-dependent manner at lower concentrations of the selective ligands than those required to inhibit enzyme activity directly.

Materials

BPAO (marketed incorrectly as EC 1.4.3.4; bovine plasma MAO) and PKDAO were obtained from Sigma (Oakville, Ontario, Canada). BPAO is no longer available from Sigma.

Benzylamine hydrochloride, putrescine dihydrochloride, methylamine hydrochloride, spermidine trihydrochloride, vanillic acid, 4-aminoantipyrine, peroxidase (type II, from horseradish), amiloride hydrochloride, clonidine hydrochloride, efaroxan hydrochloride, guanabenz, idazoxan hydrochloride, moxonidine hydrochloride and oxymetazoline hydrochloride were from Sigma. 2-(2-Benzofuranyl)-2-imidazoline hydrochloride (2-BFI) was purchased from Tocris

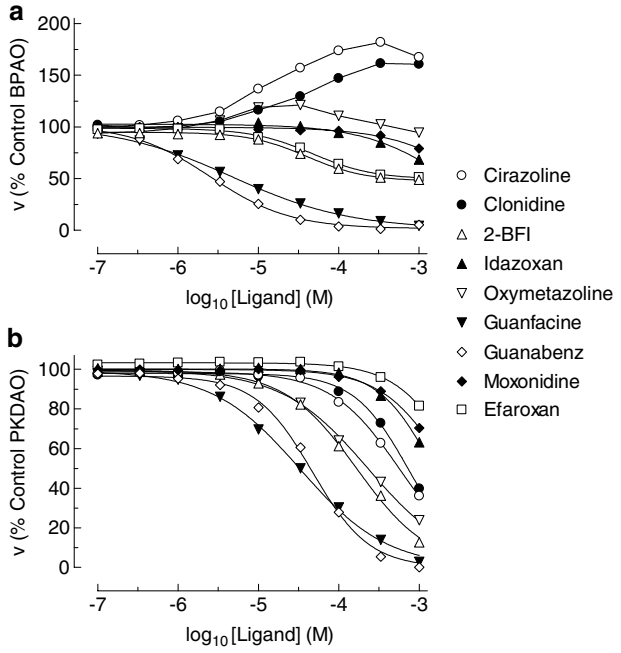

Figure 1 Preliminary screening results for effects of imidazoline ligands on benzylamine oxidation by BPAO (a) or putrescine oxidation by PKDAO (b). Benzylamine (740 μM) was incubated with BPAO (1 mg ml^{-1}), or putrescine (288 μM) with PKDAO (2.5 mg ml^{-1}) in the presence of test compounds (0.1 μM–1 mM) and the absorbance at 498 nm was monitored continuously. Initial rates, obtained from a single assay at each concentration of test compound, are expressed as a percentage of those in relevant control experiments. Estimates of IC_{50} values, obtained by nonlinear regression, for those drugs showing concentration-dependent inhibition are, *versus* BPAO, 2.5 μM (guanabenz), 4.8 μM (guanfacine), 41 μM (2-BFI) and 47 μM (efaroxan), and *versus* PKDAO, 33 μM (guanfacine), 47 μM (guanabenz), 168 μM (2-BFI), 233 μM (oxymetazoline), 576 μM (cirazoline) and 748 μM (clonidine).

(Ballwin, MO, U.S.A.). Cirazoline was a generous gift from Sanofi-Synthélabo (Bagneux, France), and guanfacine a gift from Sandoz (Basel, Switzerland; now Novartis Pharma). Sodium phosphate and potassium phosphate buffer salts were of molecular biology grade, and were obtained from Sigma. HPLC-grade methanol was purchased from Fisher Scientific (Nepean, Ontario, Canada). All other chemicals were of reagent grade.

Results

Preliminary screen for modulation of enzyme activities

Figure 1a shows the effects of several imidazoline ligands on turnover of benzylamine by BPAO, with the substrate present at 740 μM, close to its K_M concentration. Guanfacine and guanabenz inhibited activity completely, while 2-BFI and efaroxan appeared to be able to inhibit only around 50% of turnover. However, cirazoline and clonidine both caused a substantial increase in turnover rate, while oxymetazoline had a modest effect in this regard. The most efficacious activator was cirazoline, which increased turnover to 182% of control at 300 μM. Modest activation by oxymetazoline appeared to be lost as its concentration was increased, although no inhibition *per se* was evident. Moxonidine and idazoxan began to inhibit activity only at the highest ligand concentrations used. Similar results were obtained when the same compounds were included in an assay of benzylamine turnover by sheep plasma amine oxidase (not shown).

In contrast, PKDAO was inhibited by all compounds tested (Figure 1b), with no indication of activation at lower ligand concentrations. For those compounds that inhibited both enzymes, the rank orders of inhibitory potency were similar (guanfacine = guanabenz > 2-BFI), and in the absence of activation, weak inhibition of PKDAO by cirazoline, clonidine and oxymetazoline was evident. While 2-BFI appeared to be able to inhibit BPAO only by around 50%, inhibition of PKDAO by 2-BFI appeared uniphasic and largely complete at 1 mM. Efaroxan, a structural analogue of 2-BFI, which inhibited BPAO to a degree and with a potency similar to that of 2-BFI was virtually without effect on PKDAO.

Activation of BPAO-mediated benzylamine oxidation by cirazoline and clonidine was only observed above pH 6.00 and below pH 8.33 (data not shown). Although activity was highest between pH 7.33 and 7.67, both in control wells and in the presence of cirazoline or clonidine, the greatest degree of potentiation was seen at pH 6.33 for 30 μM cirazoline (152% of control activity at pH 6.33) and at pH 6.67 for 300 μM clonidine (164% of control activity at pH 6.67) in 167 μM sodium phosphate buffer.

The activation of BPAO in the absence of similar effects upon PKDAO suggests that the effects observed with BPAO are not due simply to nonspecific interactions of some imidazoline ligands with all copper-containing quinone cofactor amine oxidases. Furthermore, neither cirazoline nor clonidine interacted directly with components of the chromogenic solution, including peroxidase, or acted as BPAO substrates from which peroxide would be generated (results not shown).

Assessment of IBS characteristics

Figure 2 illustrates that idazoxan, amiloride and moxonidine did not compete with cirazoline and prevent the stimulation of enzyme activity that occurred in the presence of cirazoline at

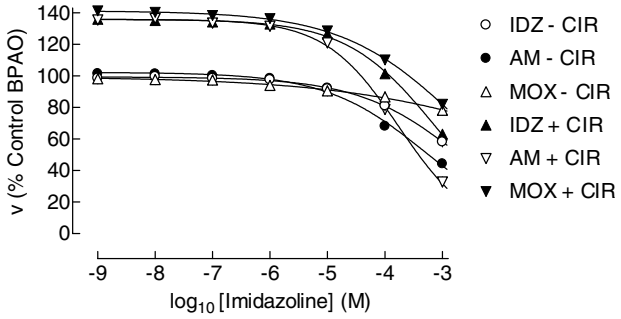

Figure 2 Effects of the I_1R ligand moxonidine (MOX), the I_2BS ligand idazoxan (IDZ) and the $I_{2A}BS$ ligand amiloride (AM) on the ability of cirazoline (CIR; 60 μM) to potentiate oxidation of benzylamine (300 μM) by BPAO. Moxonidine, idazoxan or amiloride was added to assay wells containing BPAO (0.167 mg ml^{-1}) and either water (−CIR) or cirazoline (+CIR). Plates were warmed to 37°C and reactions were started by the addition of benzylamine. Initial rates of change in absorbance at 498 nm are expressed as a percentage of that in a control experiment containing only BPAO and benzylamine. Data are the mean ± s.e. mean values from triplicate determinations; error bars do not exceed symbol size.

the low (nanomolar) concentrations at which these ligands have been shown to bind to I_1 or I_2 sites. Similar results were obtained when BPAO was instead stimulated with clonidine (not shown). These observations suggest that the sites through which cirazoline and clonidine act to stimulate enzyme activity do not possess characteristics typical of I_1, I_{2A} or I_{2B} sites (but see Discussion).

Although inhibition of BPAO by moxonidine, idazoxan and amiloride could only be observed at high concentrations of these drugs, with the consequence that IC_{50} values could not be determined with any certainty, it was nevertheless evident that inhibitory potencies of these compounds were increased in the presence of cirazoline (Figure 2). The effects of clonidine were less substantial in this regard, with the potency of amiloride in particular remaining entirely unaffected by the presence of clonidine (not shown). These data suggest that binding of cirazoline, and to a lesser extent clonidine, to BPAO increases the affinity of the enzyme for at least some imidazoline ligands.

Effects of imidazoline ligands on turnover of other substrates

In addition to benzylamine, BPAO can oxidise other aromatic amines such as *p*-tyramine and *β*-phenylethylamine, as well as aliphatic amines such as methylamine, and polyamines such as spermidine. Following preliminary experiments to determine kinetic constants for these substrates (not shown), BPAO was incubated with benzylamine, *p*-tyramine, *β*-phenylethylamine, methylamine or spermidine, at concentrations equal to one-half of their K_M values, in the presence of a range of concentrations of cirazoline (100 nM–3 mM). Figure 3 shows that cirazoline once again caused a concentration-dependent increase in benzylamine turnover, which was followed, at higher cirazoline concentrations, by enzyme inhibition. However, turnover of other aromatic and aliphatic amines was not potentiated. Rather, enzyme activity *versus* these substrates was inhibited; inhibition of oxidation of *p*-tyramine, *β*-phenylethylamine and methylamine was clearly biphasic, while inhibition of spermidine turnover appeared to be inhibited by cirazoline in a uniphasic manner. These observations suggest that cirazoline may have at least two binding sites on the BPAO enzyme, with binding of the drug to either site resulting in a distinct, substrate-dependent change in amine oxidation kinetics.

Modulation of kinetic constants by imidazoline ligands

To determine whether or not kinetic constants (K_M and V_{max}) were altered in a concentration-dependent manner by imidazoline ligands, a range of concentrations of benzylamine (10 μM–3 mM) was incubated with BPAO in the presence of a range of concentrations of cirazoline (1 μM–10 mM) or clonidine (1 μM–50 mM). Data were fitted initially to simple Michaelis–Menten hyperbolae and kinetic constants determined by nonlinear regression. Excellent fits for all data sets were generally obtained, with r^2 typically higher than 0.995.

Figure 4a is a representative plot illustrating that cirazoline caused a concentration-dependent reduction in the K_M of BPAO for benzylamine, with an EC_{50} value in this example of 5.9 μM and an E_{max} reducing K_M to 44.8% of control. At higher concentrations, the K_M value was seen to increase, due to inhibition of activity by cirazoline. V_{max} was increased by

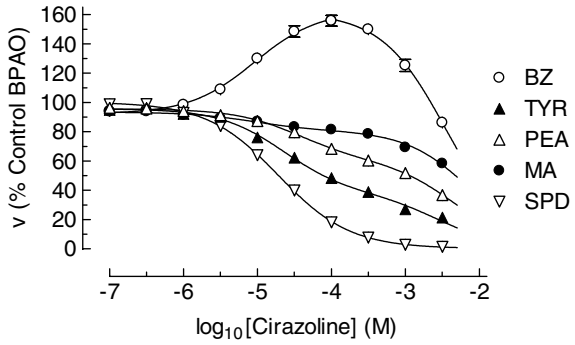

Figure 3 Effects of cirazoline (0.1 μM–3 mM) on oxidation by BPAO of benzylamine (BZ; 300 μM), *p*-tyramine (TYR; 135 μM), *β*-phenylethylamine (PEA; 275 μM), methylamine (MA; 420 μM) or spermidine (SPD; 50 μM), concentrations approximating 50% of their respective K_M values. Amines were incubated with BPAO (0.33 mg ml^{-1}) and water (controls) or cirazoline, and absorbance was monitored continuously at 498 nm. Data are mean ± s.e. mean initial rates from duplicate determinations and are expressed as percentages of relevant control values, determined in the absence of cirazoline. EC_{50} and IC_{50} values were obtained by nonlinear regression analyses of either partial data sets (0.1–100 μM, inclusive, and 100 μM–3 mM, inclusive) for benzylamine, or of the entire data sets for other substrates. Uniphasic sigmoidal curves were fitted to data for benzylamine and spermidine with a standard four-parameter logistic equation, while biphasic sigmoidal curves were fitted to data sets for other substrates with an equation describing two-site competition (GraphPad Prism 4). EC_{50} and IC_{50} values thus determined were as follows: BZ, 9.1 μM (activation) and 3.5 mM (inhibition); SPD, 19.1 μM; MA, 8.7 μM and 7.7 mM; TYR, 22.4 μM and 2.9 mM; PEA, 35.4 μM and 4.8 mM. For those substrates for which biphasic inhibition was apparent, the fraction of total amine turnover inhibited by the 'high-affinity' component of cirazoline's effect was 14% (MA), 60% (TYR) and 36% (PEA).

cirazoline to 110% of control (Figure 4b), with an EC_{50} of 44.0 μM. However, it was observed that at very low micromolar concentrations, cirazoline appeared already to have reduced V_{max} by around 6%; two separate experiments suggested that this minor effect was reproducible, and occurred with an EC_{50} of approximately 2 μM. Thus, the effect of cirazoline in increasing V_{max} was, in this representative experiment, to activate the enzyme to 117% of baseline activity.

Figure 5a illustrates results from a representative experiment, showing that clonidine reduced K_M in a concentration-dependent manner to 78.2% of the control value. The EC_{50} in this example was 7.0 μM. This stimulatory effect was rapidly obscured by the apparent increase in K_M as a result of enzyme inhibition by clonidine. The major effect of clonidine was to increase V_{max} (Figure 5b). The EC_{50} in this example was 133.6 μM, and the E_{max} of clonidine was apparent as an increase in substrate turnover to 156% of the control V_{max} value, consistent with the effect on *v* shown in Figures 1a and 3.

Table 1 lists mean effects of cirazoline and clonidine on kinetic constants for benzylamine turnover by BPAO.

The inhibition by cirazoline of spermidine oxidation (see Figure 3) was examined in greater detail (results not shown). Kinetic plots revealed a mixed pattern of inhibition, with an increase in K_M accompanied by a decrease in V_{max}. These observations are inconsistent with simple competitive inhibition at the BPAO active site, and instead indicate that cirazoline inhibits activity through binding to at least one site on the enzyme that is distinct from the active site.

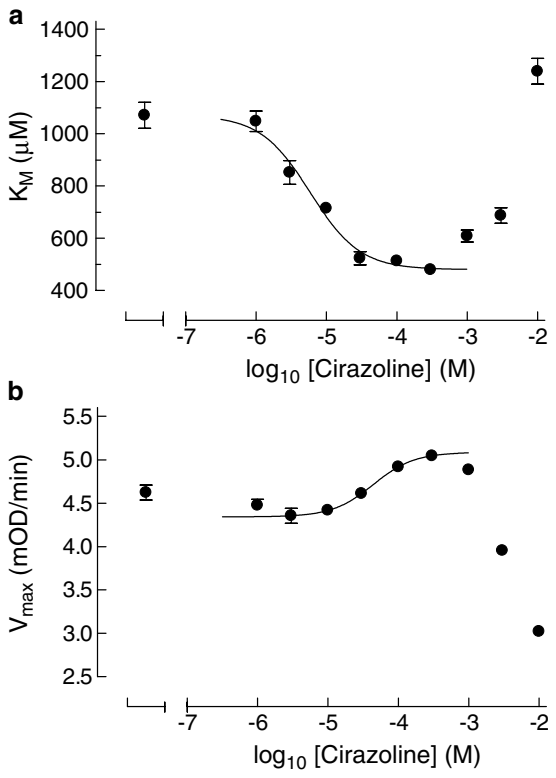

Figure 4 Effects of cirazoline on the kinetic constants K_M (a) and V_{max} (b) for benzylamine turnover by BPAO. Benzylamine (10 μM–3 mM) was incubated at 37°C with BPAO (0.167 mg ml^{-1}) in the presence of water (controls) or cirazoline (1 μM–10 mM) and absorbance at 498 nm was monitored continuously. Initial rates were plotted *versus* substrate concentrations and kinetic constants were obtained following fitting of rectangular hyperbolae by nonlinear regression to mean ± s.e. mean velocity data from triplicate measurements. Data, which are from one experiment representative of three similar experiments, thus show the best-fit ± s.e. kinetic constants, where standard errors are asymptotic standard errors of best-fit values. EC_{50} values in this representative experiment, obtained following nonlinear regression of the kinetic constant replots, are 5.9 μM (effect on K_M; 95% confidence intervals, 3.0–11.6 μM) and 44.0 μM (effect on V_{max}; 95% confidence intervals, 39.6–48.8 μM). Maximal effects are exhibited as a 55.2% reduction in K_M and a 10.0% increase in V_{max}.

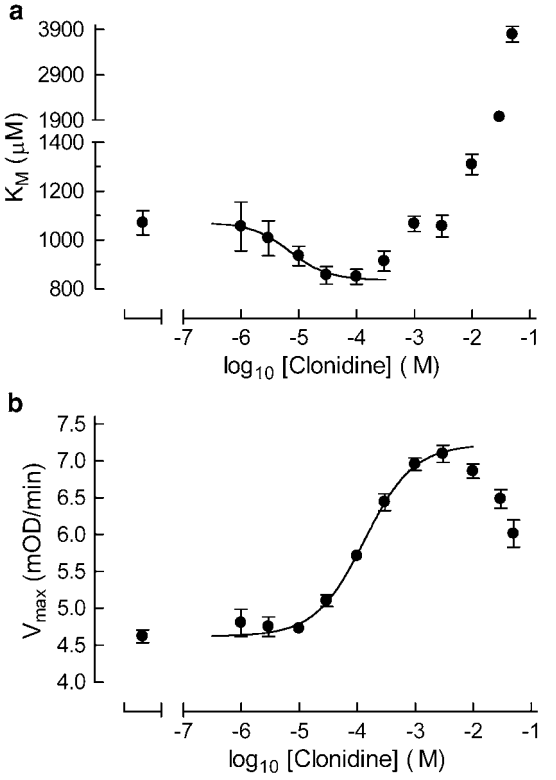

Figure 5 Effects of clonidine on the kinetic constants K_M (a) and V_{max} (b) for benzylamine turnover by BPAO. Benzylamine (10 μM–3 mM) was incubated at 37°C with BPAO (0.167 mg ml^{-1}) in the presence of water (controls) or clonidine (1 μM–50 mM) and absorbance at 498 nm was monitored continuously. Analyses were completed as described in the legend to Figure 4. EC_{50} values in this representative experiment, obtained following nonlinear regression of the kinetic constant replots, are 7.0 μM (effect on K_M; 95% confidence intervals, 3.3–14.6 μM) and 134 μM (effect on V_{max}; 95% confidence intervals, 90.1–198 μM). Maximal effects are exhibited as a 21.8% reduction in K_M and a 56.2% increase in V_{max}.

Mechanism of inhibition of benzylamine oxidation at high concentrations of imidazolines

At concentrations of imidazoline compounds above approximately 1 mM, the rate of benzylamine oxidation was seen to decrease, as a combined result of a reduced V_{max} value and an apparent increase in K_M. Lineweaver–Burk plots of velocity data obtained in the presence of ligand concentrations (cirazoline, 300 μM–10 mM; clonidine, 3–50 mM) at which activating effects were already maximal, and at which inhibition was clearly present, showed common intercepts to the left of the $1/v$ axis, and above the 1/[S] axis (not shown). These observations are indicative of linear mixed-type inhibition (Segel, 1993). Abscissal intercepts from replots of K_M/V_{max} *versus* [cirazoline] (Figure 6a) or [clonidine] (Figure 6c) indicated that inhibitor dissociation constant (K_i) values for these compounds in the absence of bound substrate were approximately 2.64 and 8.8 mM, respectively. However, as revealed by abscissal intercepts from replots of $1/V_{max}$ *versus* [cirazoline] (Figure 6b) or [clonidine] (Figure 6d), constants

Table 1 Effects of imidazoline ligands on kinetic constants for benzylamine turnover by BPAO

Ligand	*Effects on* V_{max}					*Effects on* K_M				
	Effect	EC_{50} (μM)	n	E_{max} (%)	n	*Effect*	EC_{50} (μM)	n	E_{max} (%)	n
Cirazoline	↓	2.15 ± 1.56	2	91.4 ± 2.8	3	↓	5.63 ± 1.43	3	42.6 ± 1.1	3
	↑	49.0 ± 3.8	3	114.4 ± 3.8	3					
Clonidine	↑	164.6 ± 17.6	3	154.1 ± 6.6	3	↓	8.82 ± 1.86	3	75.1 ± 4.2	3

Values represent the mean ± s.e.m. of *n* triplicate observations. E_{max} values indicate the maximal change induced by the drug, expressed as a percentage of the relevant baseline value. Baseline values are equivalent to the control value for that kinetic constant, except in the case where V_{max} was increased by cirazoline, where the baseline V_{max} value had already been reduced to 91.4% of the original V_{max} value.

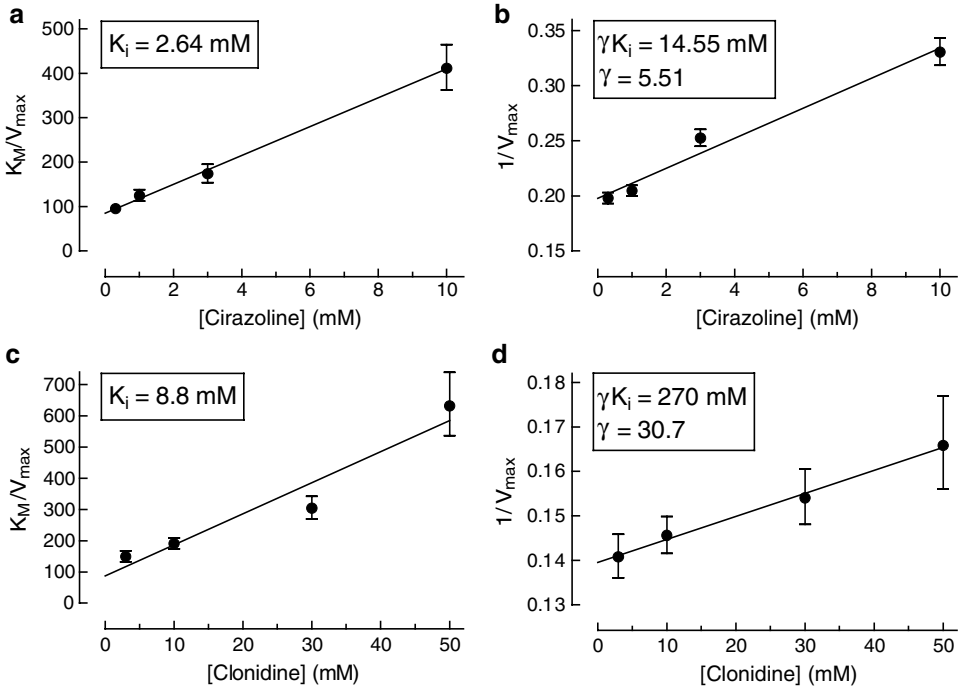

Figure 6 Replots of kinetic constants for oxidation of benzylamine by BPAO in the presence of high (inhibitory) concentrations of cirazoline or clonidine. K_M and V_{max} values for benzylamine oxidation were obtained by nonlinear regression analysis of velocity data, as described in the legend to Figure 4. Constants determined for assays carried out in the presence of cirazoline (300 μM–10 mM) or clonidine (3–50 mM) were replotted as K_M/V_{max} *versus* [I] for cirazoline (a) and clonidine (c), and yielded dissociation constants (abscissal intercepts) for loss of I from EI (in the absence of bound S) of 2.64 mM (cirazoline) and 8.8 mM (clonidine). Thereafter, reciprocals of V_{max} values were plotted *versus* [I] for cirazoline (b) and clonidine (d), and yielded dissociation constants (abscissal intercepts) for loss of I from ESI of 14.55 mM (cirazoline) and 270 mM (clonidine). The values for γ, the degree to which the presence of substrate reduces the affinity of enzyme for inhibitor, and thus to which the presence of inhibitor reduces the affinity of enzyme for substrate, were calculated as approximately 5.5 (cirazoline) and 30.7 (clonidine). The analysis was completed on one set of representative data from three similar experiments, and shows the best-fit ± s.e. kinetic constants, where standard errors are asymptotic standard errors of best-fit values.

increased by a factor γ if substrate was also bound, such that K_i values increased to approximately 14.55 mM (cirazoline) and 270 mM (clonidine). Thus, values for γ, which also reflect the increase in K_M that occurs on binding of the inhibitor to the enzyme–substrate (ES) complex, could be estimated as 5.51 (for E-benzylamine-cirazoline) and 30.7 (for E-benzylamine-clonidine) (Segel, 1993).

Dixon plots ($1/v$ *versus* [I]) at a range of substrate concentrations for a representative data set were linear for high (inhibitory) concentrations of both cirazoline and clonidine (not shown), suggesting that the complexes formed between enzyme, benzylamine and either cirazoline or clonidine were unable to yield product (Segel, 1993). However, while common intercepts of Dixon plots confirmed the estimated K_i value for cirazoline of 2.64 mM, they suggested that the K_i for clonidine may be two- to three-fold higher than the value of 8.8 mM, determined from Figure 6c, perhaps a consequence of the outlying point for 50 mM clonidine on that plot. Dixon slope replots (Figure 7) were thus used to confirm estimates for inhibitor constants, and yielded respective values for K_i, γK_i and γ of 2.85 mM, 15.95 mM and 5.6 (cirazoline) and 19.1 mM, 379 mM and 19.8 (clonidine).

The present data are consistent with intersecting linear noncompetitive inhibition (Segel, 1993), a simple mixed inhibition system in which the EI complex has a lower affinity for substrate than does free enzyme, and complexes containing both substrate and inhibitor are nonproductive.

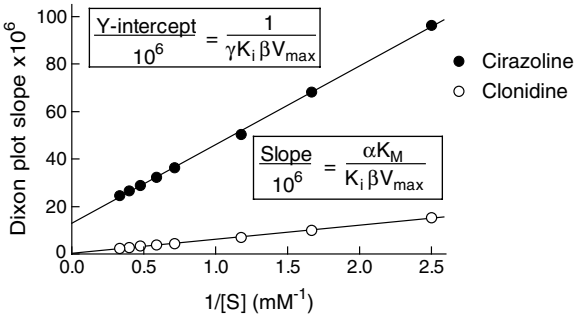

Figure 7 Replots of slopes obtained from Dixon plots ($1/v$ *versus* [I]) at several substrate concentrations and at high (inhibitory) concentrations of cirazoline and clonidine. Mean velocity data from triplicate determinations, representative of three similar experiments, were plotted on Dixon plots (not shown), and slopes from these plots were replotted *versus* 1/[S]. Values for K_i and γ were determined by applying the indicated equations. Respective estimates for αK_M and βV_{max} (the maximally activated K_M and V_{max} values), calculated by applying percentage values listed in Table 1 to K_M and V_{max} constants from parallel control data, were 456 μM and 4.834 mOD min^{-1} (cirazoline), and 804 μM and 7.124 mOD min^{-1} (clonidine). Calculated K_i and γK_i dissociation constants were 2.85 and 15.95 mM (cirazoline), and 19.1 and 379 mM (clonidine), respectively.

Proposed reaction scheme

The U-shaped (hormetic) changes observed in kinetic constants in the presence of clonidine and cirazoline could occur

as a result of binding, at higher ligand concentrations, of more than one drug molecule within a single binding site. Alternatively, the results obtained might also suggest that clonidine interacts with the enzyme at three distinct sites, while cirazoline might also interact with a fourth site. BPAO is a dimer composed of two identical subunits, each of which contains an active site. While it is highly improbable that each monomer also contains two or more distinct allosteric sites, it is far more likely that a single allosteric site exists on each monomer, and that a degree of communication takes place between the active site and the allosteric site within each monomer, and also between these sites on opposing monomers. Although we found no evidence for cooperativity with respect to substrate turnover in the present study, an extreme form of negative cooperativity, half-site reactivity, has been found to occur when phenylhydrazine binds to BPAO (Morpurgo *et al*., 1992) and when *p*-nitrophenylhydrazine binds to a copper amine oxidase from *Aspergillus niger* (Frébort *et al*., 1995), and we would have been unable to detect such an effect under the present experimental conditions. Such a mechanism would allow for oxidation kinetics at the active site on one monomer to be influenced allosterically by binding of an imidazoline ligand to the opposite active site, to an allosteric site on the same monomer and to an allosteric site on the opposite monomer. A reaction scheme modelling this mechanism has been devised, which reflects observations made with clonidine. Details of this mechanistic scheme and the equation derived therefrom will be published elsewhere. In this model, enzyme can bind with substrate, and/or clonidine at one, two, or all three of three drug binding sites. Binding of clonidine to site 'H' (in this case, a high-affinity site) with dissociation constant K_H alters the affinity for benzylamine substrate by a factor, α. Binding to site 'L' (in this case, a low-affinity site) with dissociation constant K_L alters the rate of release of product (V_{max}) by a factor, β. Binding to site 'I' with dissociation constant K_i reduces the affinity of the enzyme for benzylamine substrate by a factor, γ, and any complex of ES in which clonidine is also bound at site 'I' is unable to release product. The model makes no assumptions about the location of the drug binding sites on or within the enzyme protein, relative to each other or to the active site, other than that the dissociation constant for loss of clonidine from any one site remains unaffected by binding of clonidine to any combination of the other two clonidine binding sites.

A velocity equation (not shown) was devised based on this scheme by an approach that assumed the existence of rapid equilibrium conditions (Segel, 1993). The equation was entered into the user-defined nonlinear regression equations facility of GraphPad Prism, and was then applied to the representative *v versus* [S] data sets from which Figures 4 and 5 were generated. The variables α, β, K_H and K_L were constrained initially to the estimated values listed in parentheses in Table 2, which were deduced from the results listed in Table 1, while estimates for γ and K_i were obtained from Figure 7. Thereafter, β, K_L, α and K_H were allowed to vary in turn, in order to assess goodness of fit. However, the values for γ and K_i were held constant at their initial values, since the very low affinities of the imidazoline drugs for the inhibitory site meant that at most drug concentrations, large variations in K_i or γ would have a negligible effect on calculated values for v at any given substrate concentration.

Figure 8a shows that velocity curves at all concentrations of clonidine could be described successfully by the equation. Table 2 lists the best-fit variables obtained by nonlinear regression. With most data sets, it was subsequently found that when three of four variables were constrained to their best-fit values, the fourth could be re-obtained by regression even when the initial estimate was quite different from the calculated best-fit value, indicating that the calculated best-fit values were probably not localised minima or maxima.

The reaction scheme devised to describe reactions between BPAO, benzylamine and clonidine provides for one allosteric site, not including the low-affinity inhibitory site, through which modulation of V_{max} might be effected. However, cirazoline was observed to reduce V_{max} slightly at concentrations lower than those that subsequently decreased K_M and increased V_{max}, and a reaction scheme that would account for this further interaction would be rather more complex than that devised for clonidine. As such, the scheme for clonidine may be a little too simplistic with respect to providing an indication of all pathways that might alter reaction kinetics. Nevertheless, since the effect of cirazoline in reducing V_{max} is rather small, and since it occurs at cirazoline concentrations lower than those required to decrease K_M and increase V_{max}, it might be expected that the equation would provide an adequate fit for data obtained at higher drug concentrations. Representative data were subsequently analysed as described above for clonidine, and it was found that good fits could be obtained for velocity data in the presence of cirazoline at 100 μM or higher (Figure 8b). At 10 and 30 μM, fits were poor and could be improved only by altering α, K_H, β or K_L to values that were unrealistic. Fits could not be obtained for data obtained in the presence of 1 or 3 μM cirazoline. Table 2 lists the best-fit variables obtained from data sets for 100 μM cirazoline and higher.

The fits obtained support the contention that allosteric modulation of BPAO by cirazoline and clonidine occurs through interactions of these drugs with at least three distinct

Table 2 Variables determined for clonidine and cirazoline acting to induce two-site nonessential activation and intersecting linear noncompetitive (mixed) inhibition of BPAO, with benzylamine as substrate, by applying a novel equation (see text) to a representative data set (see Figure 8)

Ligand	K_H (μM)	α	K_L (μM)	β	n	K_i (mM)	γ
Clonidine	7.42±0.82 (8.82)	0.781±0.005 (0.751)	145±10 (164.6)	1.51±0.04 (1.541)	11	19.13	19.8
Cirazoline	6.20±0.39 (5.63)	0.445±0.002 (0.426)	54.1±8.0 (49.0)	1.12±0.01 (1.14)	5	2.85	5.59

Values of K_H, α, K_L and β represent the mean±s.e.m. of variables obtained by analysing n curves, as described in the text. Values for K_i and γ were determined from replots of Dixon plot data (Figure 7), and were held constant during nonlinear regression analyses. Initial estimates for K_H, α, K_L and β obtained from Figures 4 and 5 are shown in parentheses alongside computed values. Values for K_M and V_{max} in this experiment were determined as 1071 μM and 4.623 mOD min^{-1}, respectively.

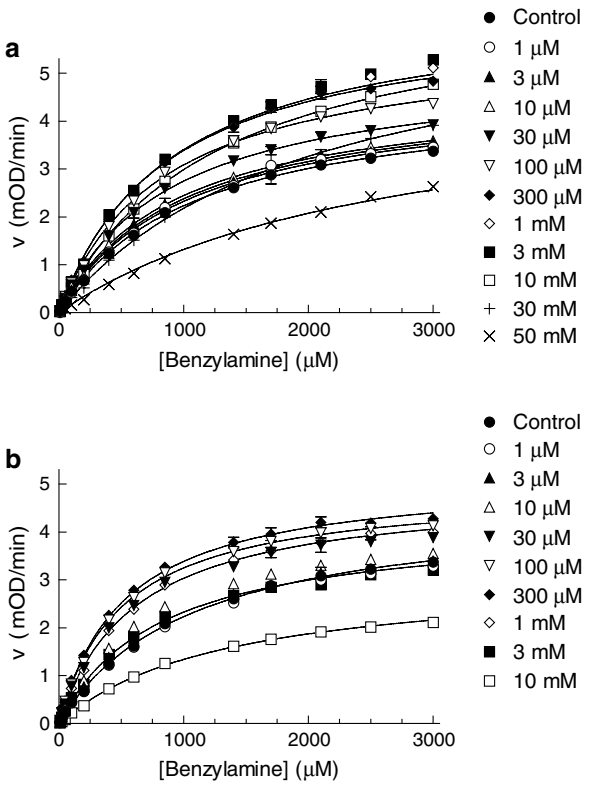

Figure 8 Representative velocity *versus* [S] data for clonidine (a) and cirazoline (b) obtained as described in the legend to Figure 4, and fitted to an equation describing enzyme activity in the presence of a drug that is a nonessential activator acting at two sites as well as an intersecting linear noncompetitive inhibitor acting at a third site. Data show mean ± s.e. mean initial rates from triplicate determinations, where error bars exceed symbol size. While nonlinear regression was successful at all concentrations of clonidine, analyses were unsatisfactory at concentrations of cirazoline below 100 μM, and failed at concentrations below 10 μM (see text). Mean values for variables calculated from these data are listed in Table 2.

sites, and thus that alternative explanations, such as binding of two or more ligands within a single site leading to a loss of efficacy, are unlikely to offer a satisfactory explanation for the present observations. Accordingly, these data also provide indirect support for communication between copper amine oxidase subunits.

Discussion

The present data provide the first conclusive evidence for allosteric modulation of amine oxidase enzymes by imidazoline receptor/binding site ligands. The effects observed with cirazoline and clonidine in this study occurred at micromolar concentrations or higher, and it is doubtful if, for these particular drugs, such effects would be of much consequence *in vivo*. Rather, these drugs have provided the first indication that imidazoline ligands are able to modulate SSAO enzymes through acting at novel binding sites on these enzymes. Consequently, it is anticipated that other ligands that are rather more potent in this regard will be identified or synthesised.

The present observations are of particular interest in view of the abilities of a number of imidazoline ligands to bind with high affinity to a site on MAO that corresponds, for a subset of human MAO-B enzymes, to a region between Lys 149 and Met 222 (Raddatz *et al*., 1997; 2000), and it has been proposed that MAO activity may be regulated in a tissue-specific manner by imidazoline ligands interacting with this site (Raddatz *et al*., 2000). However, with the exception of MAO inhibition through an action claimed to be at the active site (Carpéné *et al*., 1995; Raasch *et al*., 1999), effects of these ligands on MAO activity have not been demonstrated at low concentrations around the ligand K_D values for these sites. The enzyme examined in the present study is not a membrane-bound EC 1.4.3.4 flavin enzyme from the MAO family, but rather a soluble copper-containing EC 1.4.3.6 quinoprotein from the SSAO family (Janes *et al*., 1990). Nevertheless, despite numerous physicochemical differences, these enzyme classes share many substrates and inhibitors, and perhaps some functions (Lyles, 1984; 1996; Callingham *et al*., 1991). For example, chronic inhibition of both activities *in vivo* results in a downregulation of rat brain I_1R, presumably as a result of a prolonged increase in the levels of an endogenous I_1R agonist that is also a substrate for both MAO and SSAO (Holt *et al*., 2003).

Enzyme activation is usually essential in nature, and is most often evident as a requirement for a metal ion (activator) to form a complex with substrate before turnover can occur (Segel, 1993). Nonessential activation, where activity is simply enhanced following binding of the activator to the enzyme protein, is a common phenomenon in cooperative enzyme systems, and activation by low concentrations of a competitive inhibitor at low substrate concentrations is indicative of cooperative binding (MacRae *et al*., 2000). SSAO enzymes are homodimeric, with one active site per subunit. Crystal structures for EC 1.4.3.6 enzymes from *Escherichia coli* (Parsons *et al*., 1995), pea seedling (Kumar *et al*., 1996) and *Arthrobacter globiformis* (Wilce *et al*., 1997) reveal β-ribbon arms that extend from each subunit into the other, with residues from these arms partially lining a channel within the opposite subunit that extends from bulk solvent to the active site and which allows substrates to access the active site (Wilce *et al*., 1997). Thus, it is conceivable that binding of substrate (or inhibitor) to one active site might influence access of substrate (or inhibitor) to the second through an effect on the second substrate channel mediated through the β-ribbon arm. Since access of substrate to the active site is not thought to be rate limiting in catalysis by these enzymes (Mure *et al*., 2002), only by limiting access of substrate might a cooperative effect be induced; however, no evidence has been uncovered for negative (or, indeed, positive) cooperativity with respect to substrate turnover in SSAO dimers. This is also true for MAO-B, which is thought to exist as a dimer on the mitochodrial membrane (Binda *et al*., 2001). Nevertheless, half-site reactivity, an extreme form of negative cooperativity, has been shown to occur with hydrazine and hydrazide inhibitors of SSAO enzymes (Morpurgo *et al*., 1992; Frébort *et al*., 1995). Proof of the occurrence of half-site reactivity constitutes proof of crosstalk between subunits. Consequently, the possibility that cooperative substrate binding could occur in BPAO, and thus that low concentrations of an imidazoline ligand binding at one active site might alter substrate binding kinetics at the other active site, is very real.

While the existence of such crosstalk would provide an adequate explanation for the effects of a modulator that acts at a single site, the multisite effects observed with clonidine and cirazoline clearly implicate sites other than the dimer active sites, and the mechanism(s) responsible may thus include, but are not limited to, classical cooperativity. The present data may therefore describe a highly unusual and perhaps unique mechanism.

The model that has been proposed to explain the effects of imidazolines on the BPAO dimer contains a substrate binding site, two sites through which activity is modulated allosterically (either positively or negatively) and a site through which activity is inhibited. The model makes few assumptions, although, for simplicity, it was presumed that binding of an imidazoline drug to any of its sites was without effect on subsequent binding of the drug to the other vacant sites. Experimental data for clonidine fitted well to the derived equation. However, the possibility that cirazoline may bind to one more site than does clonidine indicates that the model may be too simplistic. While clonidine may have bound to an extra site that was not revealed experimentally, the drug failed to influence enzyme activity through binding to that site, and, for clonidine, the model therefore represents an acceptable simplification of the true system.

Cirazoline appears to bind to four sites, causing both a reduction and an increase in V_{max}, a reduction in K_M, and mixed inhibition at high concentrations. It is possible, but difficult to envisage, that a monomer could contain three or more different allosteric sites with affinities for one drug. Rather, the results can be explained adequately through a homodimeric model with one active site and one modulatory site on each monomer. In order that an explanation based on such a model might fit the present observations with benzylamine and BPAO, the existence of cooperativity between subunits would be necessary. With substrate bound at one active site, cirazoline might then bind to the active site on the second subunit, influencing turnover at the first active site, or at a regulatory site on the first subunit, influencing turnover at the first active site, or at a regulatory site on the second subunit, influencing either turnover of benzylamine at the first active site or binding or efficacy of the cirazoline at the second active site. The latter point is supported by the observation that the inhibitory potencies of cirazoline and clonidine appeared to be increased in the presence of other imidazoline ligands (Figure 2). This suggests that, while it is possible that inhibition is mediated through binding within the active site pocket at which substrate is oxidised, albeit not directly at the substrate binding site itself, it is equally likely that the mixed inhibition instead takes the form of negative cooperativity, with drugs binding to the active site at which substrate is not bound. In deriving an equation to describe this model, no allowance was made for the possibility that a ligand might influence its own affinity or efficacy at one site through binding to an adjacent allosteric site; the hypercubic model including such an interaction would be comprised of 32 enzyme species, 88 equilibrium constants and eight rate constants, and would be both highly complex and of little predictive usefulness.

The IBS on MAO fits the profile for an I_2BS, based partly on an affinity for idazoxan in the nanomolar range (Eglen *et al*., 1998). While the low micromolar affinity of at least one of the sites on BPAO for clonidine is consistent with binding to an I_2BS (Eglen *et al*., 1998), the low potencies of idazoxan and amiloride as antagonists of the potentiating effects of cirazoline and clonidine in the present study would seem to suggest that the sites to which cirazoline and clonidine bind are not I_2BSs. Although several of the ligands demonstrated here to have modulatory actions *versus* BPAO activity are I_1R ligands, binding profiles are also not entirely consistent with an involvement of I_1Rs, which in any case, like I_3BSs, are not associated with amine oxidase enzyme proteins. However, dissociation constants for the interaction of these ligands with BPAO, measured as K_i values *versus* amine turnover, were determined in the presence of amine substrates, and would not thus be expected to be similar to K_D values determined from radioligand binding assays. Consequently, the possibility that these allosteric sites on the BPAO protein possess characteristics typical of the I_2 sites already identified on MAO might not be precluded at the present time.

The very different effects of cirazoline on five different substrates indicate that this drug is unlikely to influence only the oxidative (second) half-reaction, since this copper- and oxygen-dependent step involves oxidation of an aminoquinol cofactor to regenerate topa quinone cofactor at the enzyme active site (Klinman, 2003); accordingly, this step should be similar with all substrates used. Rather, the reductive (first) half-reaction, which results in the release of aldehyde following binding and oxidation of amine substrate, is more likely to be the process affected by binding of low concentrations of imidazoline drugs, although a concomitant effect on the oxidative half-reaction may not be ruled out at this time. In support of this hypothesis, the rate-limiting step in benzylamine oxidation by BPAO is thought to be C–H bond cleavage during aldehyde production (Mure *et al*., 2002), an important step in the reductive half-reaction, and thus the rate of C–H bond cleavage must necessarily be increased in any activation process. This would not occur if the mechanism of action of imidazoline ligands was simply to control access of substrates to the hydrophobic channel, which, as has been shown in a bacterial SSAO enzyme, leads from bulk solvent to the active site (Wilce *et al*., 1997). The unusual temperature dependence of BPAO activity and the large kinetic isotope effect for benzylamine with this enzyme indicate that hydrogen tunnelling, a process by which activation energy requirements are anomalously reduced, occurs during this C–H bond cleavage step (Grant & Klinman, 1989) in a copper-dependent manner. The distance between the active site aspartate base (proton acceptor) and the substrate (proton donor) has a major influence on the degree to which hydrogen tunnelling may occur (Kohen & Klinman, 1999), and very small changes in the distances between donor, acceptor and active site copper following binding of an imidazoline drug to a binding site in the vicinity of the active site could be reflected in very large changes in rates of catalysis. This might explain the differential effects of cirazoline on oxidation of benzylamine, β-phenylethylamine, *p*-tyramine, methylamine and spermidine, since a minor spatial rearrangement of residues bordering the active site might facilitate hydrogen tunnelling when benzylamine is bound as substrate, but reduce the extent of tunnelling with the other amines.

Activation of SSAO enzymes *in vitro* is not entirely without precedent. Elegant kinetic work with porcine plasma amine oxidase by Kelly *et al*. (1981) revealed that this enzyme could be activated and inhibited by ammonia, a product of oxidation

of primary amines by SSAO, and by imidazole, a common constituent in biological buffers. Furthermore, the authors concluded that the simplest model that might explain their observations would possess two sites, in addition to the active site, at which modifiers might bind (Kelly *et al*., 1981). More recently, a component of human plasma was found to activate SSAO from human lung through increasing affinity for benzylamine substrate (Dalfó *et al*., 2003). The E_{max} in that instance was to reduce K_M to 36.5% of control, comparable with the E_{max} of 42.6% determined for cirazoline in this study. While lysophosphatidylcholine was shown also to activate lung SSAO, the efficacy of this plasma phospholipid was rather lower than that of plasma itself, and it was suggested that other components in the plasma may be of greater importance in this regard (Dalfó *et al*., 2003). Nevertheless, the demonstration that a tissue-bound SSAO enzyme can be activated *in vitro* through effects of an endogenous species on substrate affinity in a manner analogous to the present observations with soluble SSAO enzymes, and to those of others (Kelly *et al*., 1981), suggests that allosteric sites similar to those identified in the present study may be present on several different amine oxidase enzymes, and may be physiologically relevant. Furthermore, the findings here and by others of SSAO activation in enzymes from several species and tissue sources, and in both impure and purified enzyme preparations, argue against the likelihood that the imidazoline drugs bind to a second contaminating protein and that this protein–drug complex then interacts with the SSAO dimer to activate or inhibit substrate turnover.

Active site-directed inhibitors of SSAO are being developed as novel anti-inflammatory agents (Koskinen *et al*., 2004). Such drugs may also reduce atherosclerotic plaque deposition (Yu *et al*., 2002) and reduce formation of advanced glycation end products and thus vascular damage in diabetics (Yu *et al*., 2003). In addition, inhibition of adipocyte SSAO results in a reduction in glucose transport into fat cells, and substantial weight loss in obese mice (Yu *et al*., 2004). However, while selective SSAO inhibition would seem, in some patients, to be beneficial, there is a dearth of inhibitors that show good selectivity for SSAO *versus* MAO enzymes *in vivo*, while retaining high potency. The data presented here provide novel information regarding kinetic mechanisms of amine turnover by SSAO, and indicate that novel inhibitors might target sites on the protein separate from the active site, perhaps offering a means by which selectivity could be improved. That such drugs could act in a substrate-dependent manner is an intriguing thought. Furthermore, the observation that benzylamine oxidation by BPAO could be inhibited only by 50% by 2-BFI and efaroxan suggests that these compounds will prove to be useful tools in future examinations of possible cooperative behaviour in SSAO dimers.

In summary, the present study has revealed an ability of some imidazoline ligands to alter reaction kinetics of the EC 1.4.3.6 amine oxidase from bovine plasma in a manner dependent on the substrate used. Similar observations were made in a preliminary experiment with a related enzyme from sheep plasma. Effects, both activatory and inhibitory, appeared to be mediated through drugs binding to three or four sites, and the present results are consistent with some degree of crosstalk occurring between monomers in the SSAO homodimer. A review of the literature indicates that a number of drugs and chemicals can alter reaction kinetics *in vitro* and potentiate activity of SSAO, and also MAO enzymes, which are known to express a high-affinity I_2BS. An ability of imidazoline ligands to alter MAO reaction kinetics and influence substrate specificity in a tissue-specific manner through an action at the I_2BS on MAO would reveal a novel target for future antidepressant and neuroprotective compounds. Work continues in our laboratory to clarify the nature of the IBSs on SSAO enzymes, and to identify compounds that alter MAO reaction kinetics through binding to the imidazoline site on that enzyme.

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