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In vitro and in vivo effect of BU99006 (5-isothiocyanato-2-benzofuranyl-2-imidazoline) on I_2 binding in relation to MAO: Evidence for two distinct I_2 binding sites

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Abstract

BU99006 is an irreversible I_2 ligand which selectively inactivates I_2 binding sites, making it an ideal tool with which to study I_2 site mechanism. We sought to determine the effects of BU99006 on I_2 binding in relation to monoamine oxidase (MAO), and the time course of these effects. In vitro, rat brain membranes that were pre-treated with 10 μM BU99006 showed no change in MAO activity, despite suffering a significant reduction in [^{3}H]2BFI binding (52.5 ± 19.6 to 8.5 ± 3.8 fmol mg^{-1}, 84%). Furthermore, reversible I_2 ligands 2BFI and BU224 were able to inhibit MAO, whether treated with BU99006 or not. In vivo, a 5 mg kg^{-1} i.v. dose of BU99006 in rats rapidly reduced [^{3}H]2BFI binding with similar magnitude (85%, maximal reduction after 20 min), without effect on either MAO activity or the specific binding of selective MAO-A and MAO-B radioligands. Moreover, following this irreversible treatment, recovery of central [^{3}H]2BFI binding occurred with a rapid half-life of 4.3 h in rat brain (2.0 h in mouse), which is not consistent with a site on MAO. These data indicate that the high affinity site which is occupied by [^{3}H]2BFI and irreversibly binds BU99006, is not the same as that which causes inhibition of MAO, and may point to the existence of another I_2 binding site.

Keywords: Imidazoline binding site; Monoamine oxidase; MAO; BU99006; 2BFI; Autoradiography; I_2

1. Introduction

Imidazoline$_2$ (I_2) selective ligands have been shown to exert several interesting effects in vivo, such as the elevation of extracellular brain monoamine concentrations shown by microdialysis (Hudson et al., 1999; Nutt et al., 1995), efficacy in the Porsolt swim test (Finn et al., 2003; Nutt et al., 1995), and increased feeding behaviour in rats (Brown et al., 1995; Polidori et al., 2000). However, despite much study the mechanism by which these effects occur and the identity of the protein(s) responsible remains unknown. Although a number of proteins thought to represent I_2 binding sites have been described, including monoamine oxidase (MAO) (Raddatz et al., 1995), creatine kinase (Kimura et al., 2003) and semicarbazide-sensitive amine oxidase (SSAO) (Holt et al., 2004b), the functional significance of these interactions is not clear.

I_2 ligands are known to inhibit MAO (Carpéné et al., 1995; Lalies et al., 1999) and since this provides a plausible explanation for many of the functional effects of I_2 ligands, this has been the most studied interaction. A series of publications demonstrated that a high affinity I_2 binding site existed on MAO-B, between amino acids K149 to M222, and a similar lower affinity site on MAO-A (Raddatz et al., 1995, 2000;

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Remaury et al., 1999, 2000). Although the authors suggested that the I_2 binding domain probably represented a site distinct from the active centre of MAO, the subsequent publication of the crystal structure of MAO-A and MAO-B places the majority of these amino acid residues within the active site or entrance cavity of the enzyme (Binda et al., 2003, 2002; Ma et al., 2004). This is borne out by functional studies which report the likelihood of a competitive interaction (Carpéné et al., 1995; Ozaita et al., 1997), which is most likely explained by active site inhibition (for review see Holt et al., 2004a). If MAO represents a functional I_2 binding site, the efficacy of I_2 ligands to inhibit MAO and their affinity for I_2 sites should correspond. However, these two sets of values are inconsistent (Lione et al., 1998; Carpéné et al., 1995; Lalies et al., 1999). Furthermore, low concentrations of I_2 ligands that are sufficient to saturate the I_2 site have not yet been shown to affect enzyme activity. Therefore, clarification of the precise nature of the interaction between I_2 ligands and MAO was needed in order to determine its likely role in I_2 function.

2BFI is currently the ligand of choice for the study of I_2 binding sites (Lione et al., 1998), having both high affinity and selectivity. However, an irreversible ligand would lend itself much better to functional studies, since by definition, after any initial effects consequent to binding, it would then act as a functional antagonist at the I_2 site. Recently our group have shown that the isothiocyanate derivative of 2BFI, BU99006, a high affinity and high specificity I_2 ligand, can selectively and irreversibly bind I_2 sites both in vitro (Coates et al., 2000) and in vivo (Tyacke et al., 2002). Therefore, the in vivo kinetic profile of BU99006 could be used to estimate the 'turnover' of the I_2 binding protein. A radiochemical method for determining protein turnover has previously been devised, which utilises a compound's irreversible property by allowing it to bind in vivo, thus permanently removing the protein's ability to bind the radioactively labelled ligand of choice, until new protein is synthesised. This technique has been validated and used to determine the half-lives of proteins such as MAO (Goridis and Neff, 1971), acetylcholine receptors (Gardner and Fambrough, 1979), and β and α-adrenoceptors (Mckernan and Campbell, 1983; Neve and Molinoff, 1986).

Results presented here utilise the irreversible binding property of BU99006 to distinguish between MAO-associated I_2 binding sites and those that are MAO-independent, by using parallel MAO activity assays and radioligand binding experiments. The I_2 binding site turnover rate is also estimated by measuring the rate of recovery of specific [^{3}H]2BFI binding following irreversible inactivation by BU99006 in vivo. These data will not only provide essential kinetic information, but also provide a better understanding of the likely nature of the I_2 binding proteins.

2. Methods

2.1. Chemicals

BU99006 (5-isothiocyanato-2-benzofuranyl-2-imidazoline) and BU224 (2-(4,5-dihydroimidazol-2-yl)quinoline) were kindly synthesised by Dr S. Husbands, Department of Pharmacy and Pharmacology, University of Bath, UK. [^{3}H]Ro 41-1049 and [^{3}H]lazabemide were generously supplied by Dr E. Borroni, F. Hoffman-La Roche Ltd, Basel, Switzerland. [^{3}H]2BFI (2-(2-benzofuranyl)-2-imidazoline), [^{14}C]5-HT and [^{14}C]PEA (2-phenylethylamine) were obtained from Amersham Biosciences. The remaining chemicals were obtained from Sigma-Aldrich, Gillingham, UK or Tocris Cookson, Bristol, UK.

2.2. Membrane preparation

Whole rat brain membranes were prepared according to a previously described protocol (Lione et al., 1998). Male or female Wistar rats (240–300 g) were killed by stunning followed by decapitation. Whole brains were rapidly removed over ice and homogenised in 10 volumes (w/v) of assay buffer (50 mM Tris–HCl, 1 mM $MgCl_2$, pH 7.4 at 4 °C) containing 320 mM sucrose, using a motor driven Teflon-glass homogeniser. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C and the resulting supernatants pooled. These were re-centrifuged at 32,000 × *g* for 20 min at 4 °C, to obtain a crude P_2 membrane preparation. The supernatants were discarded and resultant pellets washed a further two times in 30 ml assay buffer, by re-suspension and centrifugation, at 32,000 × *g*, 4 °C for 20 min, before storage at −70 °C. Whole mouse brain pellets were prepared using male CBA/Ca mice (25–30 g) in a manner identical to that described above for rat brain, except the slower centrifugation step is omitted, leaving a more crude P_1 preparation. Before use in experiments, pellets were defrosted and washed a further two times in an appropriate assay buffer; 50 mM sodium orthophosphate, 1 mM $MgCl_2$, pH 7.4 for binding experiments or 50 mM potassium orthophosphate, pH 7.2 for MAO assays. Membrane preparations were re-homogenised immediately prior to incubations. The protein content of membrane preparations was determined using Coomassie Blue reagent with bovine serum albumin (BSA) as the standard (Bradford, 1976).

2.3. In vitro pre-treatment

Prior to binding and/or MAO assays, membrane samples were pre-treated with test compounds of interest (10 μM BU99006, 100 nM clorgyline or 100 nM deprenyl) or an appropriate vehicle, according to methods previously published by this laboratory (Coates et al., 2000; Tyacke et al., 2002). Membrane aliquots (2.7 ml) were homogenised in assay buffer and then incubated with 300 μl test compound (37 °C, 20 min) in a total volume of 3 ml. The reaction was quenched by dilution with 30 ml ice-cold assay buffer. Samples were vortexed and centrifuged at 32,000 × *g* at 4 °C for 20 min and the supernatants discarded. The pellets were then washed twice more in an assay buffer appropriate for the subsequent assay, which is sufficient to remove any unreacted ligand.

2.4. In vivo treatment

All animals were used according to the Animals Procedures Act 1986, housed on a 12 h light/dark cycle and given free access to food and water. Thirty-three male CBA/Ca mice (25–30 g) and 40 male Wistar rats (175–200 g) were housed in groups of 3 and 4, respectively. Mice were injected (i.v., via tail vein) with BU99006 (5 mg/kg, 5 ml/kg dose volume) dissolved in 0.9% saline at time zero ($t = 0$), and sacrificed by cervical dislocation at $t = 5$ min, and various time points up to 10 days after injection ($t = 1$, 2, 4, 8, 16, 32, 64, 128 and 256 h). Rats were injected (i.v., via tail vein) with BU99006 (5 mg/kg, 2 ml/kg dose volume), under halothane anaesthetic, and sacrificed via decapitation under terminal anaesthesia at $t = 5$, 20, 40, 60, 90, 120, 240, 480 and 960 min after injection. Control animals were dosed with 0.9% saline vehicle alone. All brains were carefully removed and frozen rapidly in isopentane on dry ice. These were stored at −70 °C until use.

2.5. Radioligand binding studies

Radioligand binding studies were carried out using the method of Lione et al. (1998). Membrane aliquots were incubated in ice-cold buffer (50 mM