Abstract 2-(2-Benzofuranyl)-2-imidazoline (BFI) is a highly selective ligand for imidazoline-type 2 (I₂) binding sites that are known to be associated with monoamine oxidase (MAO). Recently we demonstrated a potentiation of ³H-BFI binding in human but not in rat brain by the non-selective MAO inhibitor tranylcypromine. In the present studies, we evaluated the effect of tranylcypromine on the binding of ³H-BFI to human platelet inner membranes. Membranes were incubated with ³H-BFI at 22 °C in 50 mM Tris, 1.5 mM EDTA, pH 7.5. Saturation experiments with ³H-BFI (0.5–80 nM) were analyzed using non-linear curve fitting. Addition of tranylcypromine (0.1 mM) increased the number of ³H-BFI binding sites (Bₘₐₓ = 0.35±0.06 vs. 1.87±0.15 pmol/mg protein for vehicle and tranylcypromine, respectively) and increased ³H-BFI affinity slightly (Kᵢ = 16.0±4.1 vs. 6.5±0.3 nM for vehicle and tranylcypromine, respectively). In competitive binding experiments using the less selective I₂ ligand, ³H-idazoxan, tranylcypromine only weakly inhibited binding. Preincubation of platelet membranes with tranylcypromine (1 nM–10 μM) enhanced the Bₘₐₓ of ³H-BFI binding in a concentration-dependent manner peaking at 1 μM (13 × control) and returning to near baseline at 100 μM. ³H-BFI binding was displaced monophasically (in order of decreasing potency) by BFI ≥ 2-(4,5-dihydroimidazol-2-yl)quinoline (BU224) ≥ cirazoline > idazoxan >> (1,4-benzodioxan-2-methoxy-2-yl)-2-imidazoline (RX821002) = moxonidine. Amiloride, clorgyline, guanabenz and clonidine displayed biphasic curves with nanomolar high affinity components. Tranylcypromine altered the competition curves for all ligands (except BFI) by increasing the affinities for clonidine and RX821002 and decreasing affinities for BU224, cirazoline, guanabenz, idazoxan, clorgyline, moxonidine, and amiloride. Thus, in human platelets tranylcypromine exposes a high capacity ³H-BFI binding site distinct from previously described I₂ sites that retains high affinity for BFI but not other I₂ ligands. Our results suggest that ³H-BFI and ³H-idazoxan may not be considered as interchangeable probes for the I₂ binding site.

Key words ³H[2-(2-Benzofuranyl)-2-imidazoline] · ³H-BFI · Tranylcypromine · I₂ receptors · MAO · Human platelets

Introduction

Compounds containing an imidazol(ine) or guanidinium moiety bind with high affinity to sites that are distinct from classical adrenergic receptors and are termed imidazoline/guanidinium receptive sites (Michel and Insel 1989; Parini et al. 1996; Regunathan and Reis 1996). Imidazoline subtypes, I₁ or I₂, were defined initially by high affinity binding of ³H-clonidine and ³H-idazoxan, respectively (Ernsberger et al. 1987; Tesson and Parini 1991; Miralles et al. 1993; Ernsberger et al. 1995). Subsequently, new ligands were developed having increased selectivity for I₂ vs. a₂ sites as well as higher affinity at I₂ sites when compared to ³H-idazoxan. These ligands include the photoaffinity adduct ¹²⁵I-iodoazidophenoxymethylimidazoline (¹²⁵I-AZIPI) that binds covalently to the I₂ subtype (Lanier et al. 1993) as well as high affinity, reversible I₂ ligands such as 2-(2-benzofuranyl)-2-imidazoline (BFI), 2-(4,5 dihydroimidazol-2-yl)-quinoline (BU224) and 4-chloro-2-(imidazolin-2-yl)isoindoline (RS-45041-190) (MacKinnon et al. 1995; Hudson et al. 1997). ³H-BFI has been shown to selectively label I₂ binding sites in rat (King et al. 1998), rabbit (Nutt et al. 1995; Lione et al. 1996) and human brain (Wiest and Steinberg 1997) and has similar binding characteristics as ³H-idazoxan for the I₂ site in brain (Alemany et al. 1997). A subpopulation of imidazoline I₂ binding sites is directly associated with the mitochondrial enzyme monoamine oxidase (MAO) (Tesson et al. 1995; Raddatz and Lanier 1997). However, it remains unclear whether all I₂ binding can be attributed specifically to MAO since there
are both tissue specific (Raddatz et al. 1995) and quantitative (Sastre and García-Sevilla 1993; Raddatz et al. 1995) discrepancies between the density of I2 and MAO binding sites. Recently, we demonstrated that the nonselective MAO inhibitor tranylcypromine markedly potentiates binding of $^3$H-BFI but not $^3$H-idazoxan in human cerebral cortex and medulla (Wiest and Steinberg 1997). These observations suggested that $^3$H-BFI and $^3$H-idazoxan may differ in their ability to access or bind to a population of I2 sites. Interestingly, no such potentiation was seen in the rat cortex. This species effect might reflect a differential effect of tranylcypromine on MAO isoforms since the ratio of MAO-A to MAO-B is generally greater in rat brain compared to human (Garrick et al. 1979; Saura et al. 1992; Gerlach and Riederer 1993; Chen and Shih 1998). Therefore, the aim of the present study was to characterize the effects of tranylcypromine on $^3$H-BFI binding in human platelets, a tissue that contains I2 binding sites (Piletz and Sletten 1993) and only the MAO-B isoform (Donnelly and Murphy 1977; Saura et al. 1992).

Materials and methods

Human platelet membrane preparation. Packed human platelets out-dated for clinical use were obtained from the Central Indiana Regional Blood Center six days from donation. Each unit was a concentrate of platelets separated from a single unit of whole blood. Approximately 350 ml of platelets (six units) were mixed with 3 mg of phenylmethylsulfonylfluoride and centrifuged at 120,000 g for 90 min at 105,000 g in a SW28 rotor (Beckman XL-80 Ultracentrifuge). Pellets containing platelet inner membranes (pellet 4 °C for 10 min). Platelet membranes (5 ml) were layered on a 14.5% (15 ml)/34% (15 ml) sucrose gradient and centrifuged at maximum 10 strokes. Platelet membranes (5 ml) were layered on a 14.5% (15 ml)/34% (15 ml) sucrose gradient and centrifuged at 4 °C for 12 min to remove red blood cells. The supernatant was then centrifuged at 31,000 g for 12 min to pellet platelets. Pellets were flash frozen on ethanol/dry ice for 5 min followed by thawing at RT for 10 min, resuspension in 22 mM Hepes Na, pH 7.4 and then centrifugation at 31,000 g for 12 min at 4 °C. The freeze/thaw cycle was repeated a total of 3 times with the last centrifugation at 48,000 g for 15 min. The final pellet was resuspended in 5 mM Hepes Na, pH 7.4, 0.5 mM EGTA, 0.5 mM MgCl2 (HME) with gentle homogenization (glass homogenizer, maximum 10 strokes). Platelet membranes (5 ml) were layered on a 14.5% (15 ml)/34% (15 ml) sucrose gradient and centrifuged at 4 °C for 90 min at 105,000 g in a SW28 rotor (Beckman XL-80 Ultracentrifuge). Pellets containing platelet inner membranes (pellet from 105,000 g) were resuspended in cold distilled water and centrifuged at 120,000 g for 40 min. The final pellet was resuspended in HME and stored at –70 °C until used in binding or enzyme activity assays.

$^3$H-BFI and $^3$H-idazoxan binding assays. Radioligand binding assays with $^3$H-BFI or $^3$H-idazoxan were performed at 22 °C in 50 mM Tris HCl, pH 7.5, 5 mM EDTA, in 96-well polystyrene plates. After equilibration, incubates were filtered through GF/B filters using a 48-channel Brandel harvester (Gaithersburg, MD). Filters along with trapped radioactivity were washed 3 times for 3 seconds each with ice cold 50 mM Tris, pH 7.5. Filters were presoaked for 30–45 min in 0.1% polyethyleneimine to reduce nonspecific binding. Specific binding was determined in the presence of 0.1 mM guanabenz and represented >80% of total binding. Protein was determined by the biuret reaction using bichinchoninic acid for colorimetric detection of cuprous cation (Pierce; Rockford, IL). $^3$H-BFI was analyzed by thin layer chromatography (TLC) using 0.25 mm silica gel 60 plates (E. Merck, Darmstadt) with a solvent phase of chloroform/ ethylacetate/ methanol/ diethylamine (10/40/50/1). Plates were scraped in 0.5 cm slices and radioactivity counted.

MAO activity assays. MAO activity was measured with $^{14}$C-tyramine as substrate using a modification of a previously published method (Yu 1986). Membranes were incubated with $^{14}$C-tyramine (0.5 mM) at 22 °C, pH 7.5 for 30 min. Enzyme activity was terminated by addition of 2 M HCl. The radiolabeled metabolites of $^{14}$C-tyramine, 3-hydroxyphenylacetic acid and 3-hydroxymandelic acid, were extracted into a toluene/ethyl acetate mixture (1:1) by vortexing for 15 min and aliquots of the organic phase counted. One mg/ml carrier concentration of both end-products and a saturating amount of NaCl were present in the organic phase. Blank values were measured in incubates exposed to 2 M HCl and represented less than 1% label added (or less than 4% product generated).

Analysis of data. Binding data were calculated as fmol bound/mg protein. Kinetic constants: $k_{on}$=rate of onset (M$^{-1}$min$^{-1}$) and $k_{off}$=rate of offset (min$^{-1}$) and saturation constants: $B_{max}$=maximum binding (fmol/mg) and $K_d$=affinity (M) were calculated by nonlinear curve fitting, and an $F$ test was used to determine fit to a one or two site model using GraphPad software (San Diego, CA). Dissociation constants from kinetic data were determined by the ratio of $K_{on}/K_{off}$ in separate experiments or alternatively by a linear plot of $k_{obs}$ vs. $^3$H-BFI concentration on pooled data where $k_{obs}$ is the observed rate constant (min$^{-1}$). $k_{on}$=slope and $k_{off}$=Y-intercept. Significance between group means was determined by Student’s unpaired or paired t test as appropriate and defined as $P<0.05$.

Results

Saturation binding of $^3$H-BFI to platelet membranes

Binding of $^3$H-BFI to human platelet inner membranes was potent and saturable in the absence and presence of tranylcypromine (Fig. 1). The $B_{max}$ of $^3$H-BFI binding increased 5 fold in the presence of 0.1 μM tranylcypromine ($B_{max}=350±57$ and $1868±152$ fmol/mg in control and tranylcypromine, respectively) whereas affinity changed only slightly ($K_d=16.0±4.1$ and 6.5±0.3 nM in control and tranylcypromine, respectively; $P=0.0514$). Using TLC, the integrity of the labeled ligand after binding was determined. $^3$H-BFI (10 nM) was incubated with two different platelet preparations in the absence and presence of tranylcypromine (1 μM) followed by dissociation in the presence of 10 μM unlabelled BFI. The $R_2$ of the dissociated $^3$H-BFI in both the absence and presence of tranylcypromine was similar to the $R_2$ of native $^3$H-BFI (0.40) with >92% of total radioactivity found in one peak (data not shown).