Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 518 (2005) 10 – 17

www.elsevier.com/locate/ejphar

[^{3}H]linopirdine binding to rat brain membranes is not relevant for M-channel interaction

Christian Wolff *, Michel Gillard, Bruno Fuks, Pierre Chatelain

Department of in vitro Pharmacology, UCB SA, CNS Preclinical Research, chemin du Foriest R4, 1420 Braine l'Alleud, Belgium

Received 10 March 2005; received in revised form 3 June 2005; accepted 7 June 2005

Abstract

Linopirdine was developed as a cognitive enhancing molecule and demonstrated to specifically block the potassium current generated by the brain specific KCNQ2–KCNQ3 proteins (M-channel). In this study we investigated the relevance of [^{3}H]linopirdine binding in rat brain extracts to the interaction with the M-channel proteins. Our results confirm the presence of a high affinity site for [^{3}H]linopirdine in rat brain tissues (K_D=10 nM) but we also identified a high affinity binding site for [^{3}H]linopirdine in rat liver tissues (K_D=9 nM). Competition experiments showed that [^{3}H]linopirdine is displaced by unlabelled linopirdine with comparable affinities from its binding sites on rat brain and rat liver membranes. [^{3}H]linopirdine was completely displaced by a set of cytochrome P450 (CYP450) ligands suggesting that [^{3}H]linopirdine binding to rat brain and liver membranes is linked to CYP450 interaction. The testing of CYP450 ligands on the M-channel activity, using a Rb^+ efflux assay on cells expressing the KCNQ2–KCNQ3 proteins, demonstrated that [^{3}H]linopirdine binding results cannot be correlated to M-channel inhibition. The results obtained in this study demonstrate that [^{3}H]linopirdine binding to rat brain and rat liver membranes is representative for CYP450 interaction and not relevant for the binding to the M-channel proteins.

Keywords: Linopirdine; Cytochrome; M-channel; Liver; Brain

1. Introduction

Linopirdine is a cognitive enhancing molecule which was shown to induce neurotransmitter (e.g., acetylcholine) release in vitro and in vivo (Aiken et al., 1996). The molecule entered phase III clinical trials in Altzheimer's patients but was discontinued due to its low efficacy. Several structural analogues of linopirdine, like DMP 543 and XE-991, showed increased potency in stimulating acetylcholine release but were discontinued since 2001. Linopirdine and its analogue XE-991 were shown to selectively inhibit the M-current (Lamas et al., 1997; Wang et al., 1998), a slowly activating and non-inactivating potassium current predominantly found in sympathetic neurons (Brown, 1988) and in the central nervous system (CNS) (Schroeder et al., 1998). The binding site for linopirdine was identified and shown to be the M-current ion channel protein encoded by two genes: KCNQ2 and KCNQ3 (Wang et al., 1998). Mutations affecting the KCNQ2 or KCNQ3 genes were identified to cause a rare form of neonatal epilepsy (BFNC) and hence the development of potent M-channel activators may lead to new anticonvulsant drugs (Gribkoff, 2003). Retigabine, currently in clinical phase II trials for epilepsy, is a selective M-channel activator and has been shown to induce a leftward shift of its current activation curve (Dailey et al., 1995; Wickenden et al., 2000).

A high affinity binding site for [^{3}H]linopirdine has been previously identified in rat brain membranes (Tam et al., 1991). This binding was shown to be saturable, reversible, time and temperature dependent. These authors also showed that a wide range of drugs were unable to displace [^{3}H]linopirdine from its binding site and thus concluded to the existence of a novel binding site for linopirdine in the

* Corresponding author. Tel.: +32 2 386 2332.
E-mail address: christian.wolff@ucb-group.com (C. Wolff).

0014-2999/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2005.06.005

brain. Based on autoradiography experiment on rat brain slices it was shown that the highest specific binding for linopirdine was found in the cortex, hippocampus and amygdala (De Souza et al., 1992) which correlates with the expression pattern of the KCNQ2 and KCNQ3 proteins (Tinel et al., 1998). In this study we investigated the relevance of [^{3}H]linopirdine binding in rat brain membranes to the interaction with the M-channel proteins.

2. Materials and methods

2.1. Drugs and radioligand

Mivazerol (3-[1(H-imidazol-4-yl)methyl]-2-hydroxy-benzamide hydrochloride) and retigabine (N-(2-amino-4-[fluorobenzylamino]-phenyl)) were synthesized at UCB S.A. (Braine-l'Alleud, Belgium). [^{3}H]linopirdine (62 Ci/mmol) (3,3-bis(4-pyrindinylmethyl)-1-phenyl-2H-indolin-2-one) was custom labelled by Amersham Biosciences (Roosendaal, The Netherlands). XE-991 (10,10-bis(4-pyridinylmethyl)-9-anthracenone) was purchased from Tocris (Avonmouth Bristol, UK) and all other reference compounds were purchased from Sigma-Aldrich (Bornem, Belgium).

2.2. Brain and liver tissues

Spargue–Dawley male rats (200–300 g) from Iffa-Credo (Belgium) were sacrificed by decapitation. Brain and liver were quickly removed and the tissues were dissected on ice. All subsequent operations were performed at 4 °C. The tissues were homogenized (10% w/v) in 20 mM Tris–HCl buffer (pH 7.4) containing 250 mM sucrose (buffer A). The homogenates were centrifuged at 30 000 $\times g$ at 4 °C for 15 min and the pellets resuspended in the same buffer. After incubation at 37 °C for 15 min, the membranes were washed three times using the same centrifugation protocol. The final pellets were resuspended in buffer A at a protein concentration of 10 to 15 mg/ml and stored in liquid nitrogen.

2.3. Binding studies

Binding experiments of [^{3}H] linopirdine were performed as previously described with some minor modifications (Hofner and Schmidt, 1996). Membrane proteins (brain: 0.25 mg/assay, liver: 0.01 mg/assay) were incubated 60 min at 25 °C in 0.5 ml of a 50 mM Tris–HCl buffer (pH 7.4) containing 2 mM $MgCl_2$, [^{3}H]linopirdine (3 nM) and increasing concentrations of unlabelled competition drugs (DMSO final concentration of 1% v/v). Non specific binding was defined as the residual binding observed in the presence of 100 μM of unlabelled linopirdine. At the end of the incubation period, the membrane-bound radioligand was recovered by rapid filtration through GF/C glass fibre filters pre-soaked in 0.1% polyethyleneimine. The membranes were washed with 4 × 2 ml of ice-cold 50 mM Tris–HCl buffer pH 7.4. The total filtration procedure did not exceed 10 s per sample. The filters were dried and the radioactivity determined by liquid scintillation.

For saturation binding studies, membranes were incubated 60 min at 25 °C with concentrations of [^{3}H]linopirdine ranging from 1 to 500 nM (concentrations above 35 nM were obtained by isotopic dilution). Membrane protein concentration for brain and liver tissues was 0.5 and 0.05 mg/assay, respectively. Data analysis was performed by computerised nonlinear curve fitting (Graphpad Prism® software, San Diego, CA), according to equations describing several binding models (Molinoff et al., 1981).

For association kinetics, specific [^{3}H]linopirdine binding was measured at the indicated times after addition of the membranes. For dissociation studies, membranes were first incubated 60 min (25 °C) with [^{3}H]linopirdine and dissociation was initiated by adding 100 μM of unlabelled linopirdine. The samples were then filtered after increasing time intervals.

2.4. CYP450 inhibition study

Cytochrome P450 (CYP450) inhibition experiments were performed by CEREP (France) on the two human recombinant isoforms CYP2D6 and CYP3A4 according to the procedures previously described (Ono et al., 1996; Stresser et al., 2002). 7-MFC (7-methoxy-4-trifluoromethylcoumarin) and 7-benzoyloxyresorufin were used as substrates for CYP2D6 and CYP3A4 activity, respectively. Inhibition of CYP450 activity was measured by fluorimetry in the presence of test compound (from 0.1 nM to 10 μM) and pIC_{50} values were determined by non-linear regression analysis of the concentration–response curve using Hill equation curve fitting.

2.5. Rubidium efflux assay

Chinese hamster ovary cells (CHO) were stably transfected with a human KCNQ2/KCNQ3 tandem construct as previously described (Wickenden et al., 2000) and grown in Ham-F12 medium supplemented with 10% heat-inactivated fetal bovine serum and 400 μg/ml G418 sulfate at 37 °C (5% CO_2 atmosphere). The non-radioactive Rb^+ efflux assay was performed as described by Scott et al. (2003) with some minor modifications. Briefly, cells were seeded in 96 well plates at a density of 50,000 cells/well and cultured for 24 h. The culture media was then removed by aspiration and the cells were loaded with RbCl for 3 h at 37 °C using 100 μl of Rb^+ load buffer (25 mM Hepes, 150 mM NaCl, 5.4 mM RbCl, 1 mM $MgCl_2$, 0.8 mM NaH_2PO_4, 2 mM $CaCl_2$, 5 mM glucose, pH 7.4). Compounds were added in 50 μl of Rb^+ load buffer (1% dimethyl sulfoxide (DMSO) final concentration) during the last 30 min. Cells were subsequently washed three times with 200 μl of Phosphate buffered saline (PBS) pH 7.4 and incubated at room temperature in the presence of compound diluted in 130 μl of Depolarization buffer (25 mM Hepes, 130 mM NaCl, 20 mM KCl, 2 mM $CaCl_2$, pH 7.4). After 15 min the supernatant was removed and transferred to another 96 well plate and the cells were lysed during 20 min at 37 °C in the presence of 130 μl of 0.15% SDS solution. The Rb^+ concentration in the cell lysate (Rb_L) and cell supernatant (Rb_S) was determined by using an ICR 8000 flame atomic absorption spectrometer (Aurora Biomed Inc., Vancouver, Canada) and under conditions defined by the manufacturer. The net Rb^+ efflux was calculated as follows: $\%\ \text{efflux} = [Rb_S] \times 100 / ([Rb_S] + [Rb_L])$. Drugs were assayed from 10 nM to 100 μM and data analysis was performed as described for binding studies.