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

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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

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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.

3. Results

3.1. [^{3}H]linopirdine binding in whole rat brain membranes

The binding of [^{3}H]linopirdine to rat brain membranes was investigated according to the method previously described (Hofner and Schmidt, 1996) (see Materials and methods). Specific binding represented about 60–40% of total binding in the range of 1–200 nM (Fig. 1A). The Scatchard plot from the transformed data (Fig. 1A, inset) shows that [^{3}H]linopirdine binds to a heterogeneous population of binding sites (see Table 2): a high affinity binding site (K_{D1}=10±1 nM, B_{max1}= 78±27 fmol/mg protein, n=3) and a low affinity binding site (K_{D2}=2313±857 nM, B_{max2}=4360±1050 fmol/mg protein, n=3). To further characterize the binding sites of [^{3}H]linopirdine, we performed association and dissociation kinetics at 25 °C in the presence of 3 nM [^{3}H]linopirdine (Fig. 1B). We obtained rate constants of $27\pm12\times10^6$ min^{-1} M^{-1} for k_{on} (n=3) and 0.06±0.01 min^{-1} for k_{off} (n=2) and the calculated K_D value using k_{off}/k_{on} was 2 nM, which is very similar to the value obtained from Scatchard analysis for the high affinity binding site (see Table 2). Homologous competition experiments with unlabelled linopirdine in the range of 100 pM to 10 μM generated a pIC_{50} value of 7.6 (Fig. 2). The binding of [^{3}H]linopirdine to rat brain membranes was further investigated by competition experiments in the presence of two M-channel modulators: retigabine and XE-991. The M-channel opener retigabine did not compete with the [^{3}H]linopirdine binding site in the range of 0.1 nM to 1 μM but showed a significant inhibition of 33% at 10 μM (Table 1). The linopirdine analogue and M-channel blocker XE-991 was able to displace [^{3}H]linopirdine with a pIC_{50} of 7.8 (Table 1).

In order to validate the [^{3}H]linopirdine binding assay we subjected the assay to competition experiments in the presence of compounds from a diverse chemical library. The outcome of these experiments indicated that about 50% of the active compounds presented imidazole ring structures. Such compounds are known to interact with CYP450 proteins and to verify whether [^{3}H]linopirdine binding to rat brain membranes is linked to CYP450 interaction, we performed competition experiments in the presence of known CYP450 ligands. The selected references have been shown to act on different human CYP450 isoforms: ketoconazole (CYP3A4) (Moody et al., 2004), miconazole (CYP2E1) (Tassaneeyakul et al., 1998), ellipticine (CYP1A1) (Tassaneeyakul et al., 1993), mepyramine (CYP2D6) (Fukui et al., 1995), quinine (CYP2D6) (Ellis et al., 2000) and mivazerol (isoform not identified) (Flamez et al., 1997). As shown in Table 1, the compound mivazerol at a concentration of 10μM was able to displace 90% of the [^{3}H]linopirdine specific binding on rat brain membranes and presented a 3 to 4 fold higher affinity than unlabelled linopirdine or XE-911. Ellipticine was as potent as XE-

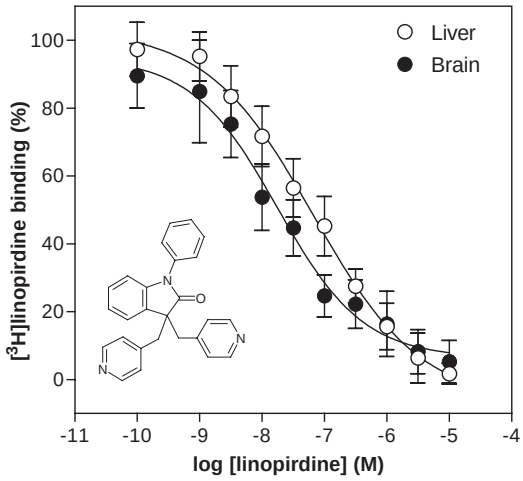

Fig. 2. Competition experiments of [^{3}H]linopirdine in rat tissue membranes. Unlabelled linopirdine was incubated at increasing concentrations in the presence of [^{3}H]linopirdine for 60 min at 25 °C (see Materials and methods). Data were analyzed by non-linear regression according to a model with variable slope and normalized between 0–100% as calculated from results obtained with 0 (100%) or 100 μM linopirdine (0%). Results are presented as the average of three independent experiments (δstandard deviation). Inset: chemical structure of linopirdine.

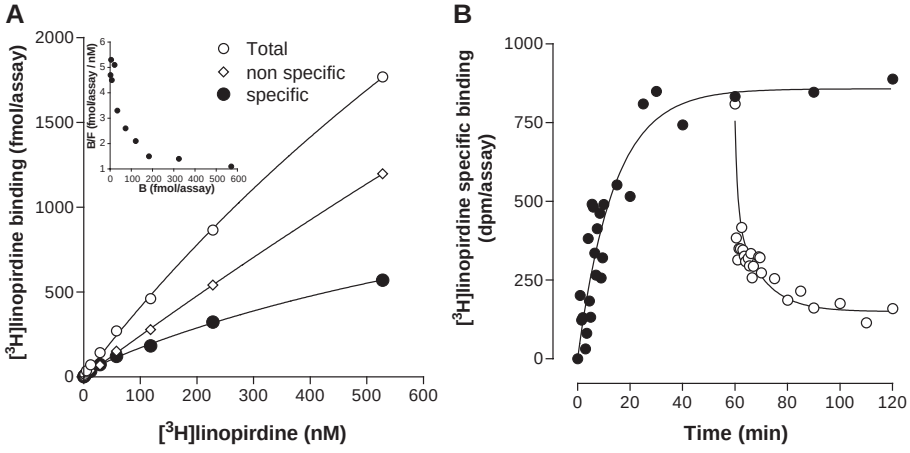

Fig. 1. Binding characteristics of [^{3}H]linopirdine to rat whole brain membranes. A. Saturation isotherm of [^{3}H]linopirdine to rat brain membranes (see Materials and methods). Membranes were incubated with increasing concentrations of [^{3}H]linopirdine for 60 min at 25 °C. Non specific binding was determined as the residual binding observed in the presence of 100 μM unlabelled linopirdine. Results are representative of three experiments. Inset: Scatchard plot from the transformed data. B. Binding kinetics of [^{3}H]linopirdine to rat brain membranes at 25 °C (see Materials and methods). Association kinetics (closed circles) were obtained in the presence of 3 nM [^{3}H]linopirdine and dissociation (open circles) was initiated after 60 min by adding 100 μM of unlabelled linopirdine. Results are representative of three separate experiments.

Table 1
Affinity of selected drugs for [^{3}H]linopirdine binding sites in rat brain and rat liver membranes

Drug	Brain			Liver		
	Inhibition (%)[a]	pIC_{50}	n_H	Inhibition (%)[a]	pIC_{50}	n_H
Linopirdine	95±6	7.6±0.2	0.49±0.12	97±2	7.4±0.2	0.63±0.13
XE-991	88±3	7.8±0.4	0.95±0.41	90±1	7.3±0.4	0.74±0.17
Mivazerol	90±5	8.3±0.4	0.63±0.24	92±4	7.4±0.2	0.89±0.18
Ketoconazole	75±15	5.8±0.3	0.58±0.25	97±1	7.3±0.4	0.47±0.08
Miconazole	90±8	6.7±0.2	0.46±0.03	99±1	7.8±0.2	1.01±0.11
Retigabine	33±17	*n.c.*	*n.c.*	7±27	*n.c.*	*n.c.*
Ellipticine	68±6	7.9±0.4	0.96±0.19	97±1	7.1±0.3	0.84±0.03
Mepyramine	12±6	*n.c.*	*n.c.*	12±5	*n.c.*	*n.c.*
Quinine	0	*n.c.*	*n.c.*	13±10	*n.c.*	*n.c.*

Drugs were assayed from 0.1 nM to 10 μM at 25 °C in the presence of 3 nM [^{3}H]linopirdine. Results are shown as the average of at least three independent experiments (±standard deviation).
[a] Maximal inhibition of [^{3}H]linopirdine specific binding at 10 μM expressed as the percentage of non-specific binding in the presence of 100 μM linopirdine, *n.c.* not calculated.

991 but only displaced 68% of the [^{3}H]linopirdine binding at 10μM (Table 1). Miconazole and ketoconazole also competed with [^{3}H]linopirdine but were 10 to 100 fold less potent than linopirdine or XE-991. Mepyramine and quinine did not displace [^{3}H]linopirdine from rat brain membranes.

3.2. [^{3}H]linopirdine binding in rat liver membranes

The high affinity of mivazerol and ellipticine for the [^{3}H]linopirdine binding site on rat brain membranes suggest a possible interaction of linopirdine with CYP450 proteins. To assess whether [^{3}H]linopirdine binds to CYP450 proteins we further investigated the binding of [^{3}H]linopirdine in rat liver membranes. Liver membranes contain high amounts of CYP450 enzymes but not the M-channel proteins (Schroeder et al., 1998) and binding of [^{3}H]linopirdine to such membranes in the presence of CYP450 ligands should thus allow to verify whether the binding of [^{3}H]linopirdine is linked to CYP450 proteins.

Fig. 3A shows that [^{3}H]linopirdine presents a specific (80% of total binding) and saturable binding to rat liver membranes in the concentration range of 1 to 500 nM. A Scatchard plot of the transformed data (Fig. 3A, inset) indicates the presence of at least two binding sites (see Table 2): a high affinity site with a K_{D1} of 9±2 nM and a B_{max1} of 8400±2400 fmol/mg protein (n=3) and a low affinity binding site characterized by a K_{D2} of 447±46 nM and a B_{max2} of 125 000±18 000 fmol/mg protein (n=3). Association and dissociation kinetics at 25 °C in the presence of 3 nM [^{3}H]linopirdine (Fig. 2B) gave rate constants of 336 ± 150 × 10^6 min^{-1} M^{-1} for k_{on} (n=3) and 1.0 ± 0.4 min^{-1} for k_{off} (n=3) and the calculated K_D value of 3 nM is in agreement with the value obtained from Scatchard analysis for the high affinity binding site (see Table 2). Competition experiments in the presence of unlabelled linopirdine (Fig. 2) resulted in a dose dependent inhibition of [^{3}H]linopirdine binding and characterised by a pIC_{50} value of 7.4 (see Table 1). The linopirdine analogue XE-991 was also able to displace [^{3}H]linopirdine from rat liver membranes and generated a pIC_{50} value of 7.3 whereas the M-

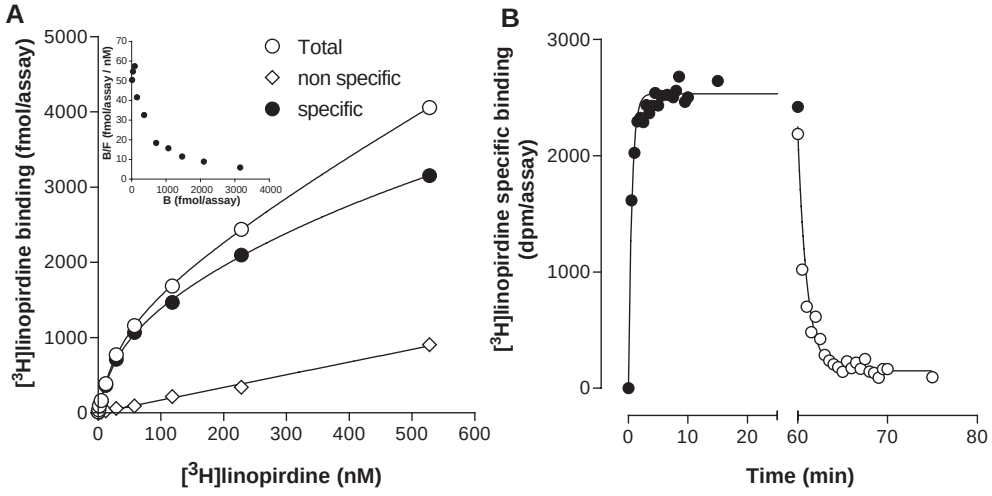

Fig. 3. Binding characteristics of [^{3}H]linopirdine to rat liver membranes. A. Saturation isotherm of [^{3}H]linopirdine to rat liver membranes (see Materials and methods). Membranes were incubated with increasing concentrations of [^{3}H]linopirdine for 60 min at 25 °C. Non specific binding was determined as the residual binding observed in the presence of 100 μM unlabelled linopirdine. Results are representative of three experiments. Inset: Scatchard plot from the transformed data. B. Binding kinetics of [^{3}H]linopirdine to rat brain membranes at 25 °C (see Materials and methods). Association kinetics (closed circles) were obtained in the presence of 3 nM [^{3}H]linopirdine and dissociation (open circles) was initiated after 60 min by adding 100 μM of unlabelled linopirdine. Results are representative of three separate experiments.

channel activator retigabine did not show any activity in this assay. Mivazerol, ketoconazole, miconazole and ellipticine also competed with the binding site for [^{3}H]linopirdine in rat liver membranes and present pIC_{50} values comparable to those obtained with unlabelled linopirdine (see Table 1). The drugs mepyramine and quinine did not displace [^{3}H]linopirdine from its binding site in rat liver membranes.

3.3. Measurement of CYP450 activity

To verify that the reference compounds linopirdine and XE-991 interact with cytochrome P450 (CYP450) proteins we measured their effect on the activity of the two human isoforms CYP2D6 and CYP3A4. These two CYP450 isoforms are highly abundant in liver tissues and are the most representative in the metabolic pathway of chemical drugs. The drug induced inhibition of these enzymes allows predicting CYP450 interaction. Results from CYP450 inhibition experiments are shown in Table 3. Linopirdine and its analogue XE-991 strongly inhibited the isoform CYP3A4. The pIC_{50} values measured for the two compounds are comparable to those obtained with the potent CYP3A4 inhibitor ketoconazole and therefore suggest a strong interaction of these drugs with the CYP3A4 isoform. The two compounds however didn't show any activity on the CYP2D6 isoform.

3.4. Effect of CYP450 ligands on M-channel activity

Linopirdine and its analogue XE-991 are potent and selective inhibitors of the M-current generated by the KCNQ2–KCNQ3 channel proteins (M-channel). Based on the finding that some of our selected CYP450 ligands are able to displace [^{3}H]linopirdine from its binding sites in rat brain membranes, we further investigated their effect on the activity of the KCNQ2–KCNQ3 channel proteins. The inhibition of the M-channel activity was measured by using a Rb^+ efflux assay on CHO cells expressing the KCNQ2–KCNQ3 proteins (see Materials and methods). Fig. 4 shows the dose–efflux relationship of the M-channel reference compounds and the CYP450 ligands used in this study. We observed that XE-991 (pIC_{50}=6.4) was ten fold more potent than linopirdine (pIC_{50}=5.4) in blocking the M-channel mediated Rb^+ efflux. Mepyramine and ketoconazole induced a significant inhibition of the Rb^+ efflux at concentrations above 30 μM whereas the CYP450 ligands mivazerol, ellipticine, miconazole and quinine did not inhibit the M-channel activity in our rubidium efflux assay. The presence of 10 μM mivazerol did not alter the dose-efflux relationship of the M-channel blockers linopirdine and XE-991 (Fig. 4, inset).

Table 2
Characteristics of the [^{3}3H]linopirdine binding sites

	Brain	Liver	Brain (literature)
K_{D1} (nM)	10±1	9±2	7.8[a], 19[b]
B_{max1} (fmol/mg protein)	78±27	8400±2400	100[a], 102[b]
K_{D2} (nM)	2313±857	447±46	
B_{max2} (fmol/mg protein)	4360±1050	125,000±18000	
pIC_{50}^*	7.6±0.2	7.4±0.2	7.3[a]
n_H^*	0.49±0.12	0.63±0.13	0.59±0.07[a]

Results are obtained from experiments carried out on rat tissue extracts (see Materials and methods) (±standard deviation, n=3). *calculated from homologous competition experiments in the presence of unlabelled linopirdine (see Table 1), References: [a](Hofner and Schmidt, 1996), [b](Tam et al., 1991).

Table 3
Inhibition of CYP450 activity

	CYP 3A4	CYP 2D6
	pIC_{50}	pIC_{50}
Linopirdine	6.1	<5
XE-991	5.5	<5
Ketoconazole	6.0	*n.t.*
Quinidine	*n.t.*	7.0

The inhibition of the activity of the two human CYP450 isoforms was measured as described under Materials and methods in the presence of drug concentrations ranging from 0.1 nM to 10 μM. Ketoconazole and quinidine were used as internal references. (*n.t.* not tested).

4. Discussion

Our results obtained from saturation isotherms of [^{3}H]linopirdine binding on whole rat brain membranes are in agreement with those obtained previously by two other groups, showing two binding sites of low and high affinities and with dissociation constants in the nanomolar and micromolar range, respectively (see Table 2). Characterization of the [^{3}H]linopirdine binding sites on rat brain membranes using homologous competition experiments allowed us to confirm that linopirdine presents a high affinity binding site with a K_i value of 19 nM (calculated according to Cheng and Prusoff, 1973), which is very similar to the K_i value of 11 nM observed in a previous study (Hofner and Schmidt, 1996). Although the low Hill coefficient value of 0.49 (see Table 1) indicates the presence of at least two binding sites, the high variability of the experimental binding levels did not allow us to perform an accurate F test in order to identify the low affinity binding sites. Moreover, the use of [^{3}H]linopirdine at a concentration of 3 nM allowed us to label 80% of the high affinity binding sites and consequently a precise characterisation of the low affinity sites remains difficult.

We used [^{3}H]linopirdine binding to rat brain membranes in order to identify novel modulators of the M-channel proteins and the outcome from an internal screening campaign indicated that [^{3}H]linopirdine binding is strongly inhibited by a diverse class of compounds containing imidazole ring structures. Such compounds are often related to CYP450 interaction (Testa and Jenner, 1981) and to evaluate the extent of CYP450 interaction we measured the binding of [^{3}H]linopirdine to rat liver membranes.

In the present study we demonstrate the presence of a high affinity binding site for [^{3}H]linopirdine in rat liver membranes characterised by a dissociation constant similar to the one found in the rat brain tissue. We observed a significant difference in the association/dissociation kinetic parameters suggesting that the molecular identity of the [^{3}H]linopirdine binding sites is different on the two tissue

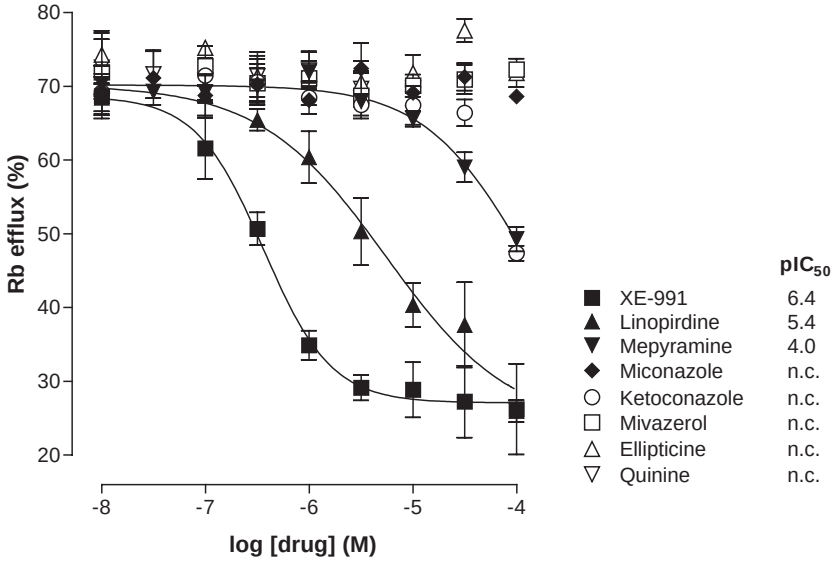

Fig. 4. Effect of CYP450 ligands on the activity of KCNQ2–KCNQ3 channels stably expressed in CHO cells. Cells were loaded with RbCl for 3 h and efflux was stimulated with 20 mM KCl during 15 min. The amount of Rb^+ in the cell lysate and cell supernatant was quantified as described in Materials and methods and results are given as the percentage of total Rb^+ detected in the supernatant. Data were analyzed by non-linear regression according to a model with variable slope and the calculated pIC_{50} values are 6.4 (XE-991), 5.4 (Linopirdine), 4.0 (Mepyramine) (*n.c.*: not calculated). Data are presented as the average of three independent experiments and the error bars represent the standard deviation. Inset: Dose-efflux relationship of M-channel blockers in the presence (open symbols) or absence (closed symbols) of 10 μM mivazerol (average of triplicate values).

membranes. These results indicate that [^{3}H]linopirdine may bind with high affinity to other molecular targets than the M-channel proteins which are brain specific and not expressed in the liver (Schroeder et al., 1998). The distribution of these proteins is restricted to the central nervous system and the dorsal root ganglion neurons and we therefore exclude that the binding site on rat liver membranes is mediated by the M-channel proteins.

To investigate the possibility that linopirdine may bind to CYP450 proteins in brain tissue we used a set of selected CYP450 ligands and measured their affinity for the [^{3}H]linopirdine binding sites. Some reference drugs were able to displace [^{3}H]linopirdine from its binding sites on rat brain and rat liver membranes. The drugs ellipticine and mivazerol were respectively 2 to 5 times more potent than linopirdine in displacing [^{3}H]linopirdine on rat brain membranes whereas they showed comparable potencies on rat liver membranes. Mepyramine and quinine, which are both inhibitors of the human CYP2D6 isoform did not compete with [^{3}H]linopirdine in the rat liver or rat brain extracts. Moreover, linopirdine and XE-991 did not inhibit the activity of the human CYP2D6 isoform and we therefore conclude that linopirdine binding is not linked to the CYP2D1 protein, the rat homologue of CYP2D6. However, ketoconazole is a potent CYP3A4 inhibitor and we showed that this drug binds to [^{3}H]linopirdine binding sites in rat liver extracts with 40 times higher affinity than in rat brain extract. Furthermore, inhibition experiments carried out with the human CYP3A4 enzyme indicated that linopirdine and its analogue XE-991 are potent inhibitors of this cytochrome isoform. Moreover, the number of high affinity binding sites found in the liver extract is about 100 times higher than in the brain tissue. Such a difference in number of binding sites between liver and brain tissues is also found for most of the CYP450 isoforms which were shown to present at least a 100 fold higher abundance in liver membranes (Nishimura et al., 2003).

The results obtained from [^{3}H]linopirdine binding on rat liver membranes suggest that linopirdine and its analogue XE-991 bind with high affinity to CYP450 proteins. We also observed that a selected set of CYP450 ligands are able to displace with high affinity [^{3}H]linopirdine from its binding sites in the brain and it remains therefore conceivable that these sites could be restricted to CYP450 proteins.

Similarly, the results from the KCNQ2–KCNQ3 rubidium efflux assay suggest that inhibition of M-channel activity cannot be directly correlated to [^{3}H]linopirdine binding in the rat brain. Indeed, linopirdine and its analogue XE-991 inhibit the M-channel activity with a ten fold difference in potency in agreement with the results obtained previously by Rb^+ efflux (Scott et al., 2003; Wang et al., 2004) and electrophysiology (Wang et al., 1998). Furthermore, mivazerol and ellipticine which were shown to displace [^{3}H]linopirdine from its binding sites in the brain have no effect on M-channel activity whereas ketoconazole and mepyramine, which respectively show a complete or no inhibition of [^{3}H]linopirdine binding in rat brain and liver membranes (see Table 1), inhibited the M-channel mediated Rb^+ efflux at concentrations above 10 μM. Previous reports indicated that ketoconazole and mepyramine are able to inhibit the activity of various potassium channels in the concentration range of 10–100 μM (Dumaine et al., 1998; Salata et al., 1995).

In order to exclude that the binding site of mivazerol might be allosterically linked to the [^{3}H]linopirdine site, we performed Rb^+ efflux inhibition experiments in the presence of 10 μM mivazerol, a concentration which completely displaces [^{3}H]linopirdine from rat brain and liver membranes. Our results show that mivazerol does not affect the inhibition curves of linopirdine and XE-991 and we therefore conclude that mivazerol does not bind to M-channel proteins. Moreover, since mivazerol is able to displace the specific binding of [^{3}H]linopirdine in the rat brain, we suggest that the binding observed in our experiments is not relevant for KCNQ2–KCNQ3 proteins. A recent report demonstrated that the effects of linopirdine and XE-991 on M-channel inhibition are voltage dependent (Romero et al., 2004) but one cannot infer from these results that the binding mechanism of linopirdine to the M-channel proteins is also voltage dependent. These aspects of the [^{3}H]linopirdine binding conditions should be further investigated.

Although the results obtained in this study indicate that [^{3}H]linopirdine binding to rat brain is linked to CYP450 proteins, they do not allow us to determine which of the CYP450 isoforms are involved. Nevertheless, the difference in pIC_{50} values observed for the various drugs between rat liver and rat brain membranes leads to the conclusion that [^{3}H]linopirdine binds to multiple CYP450 isoforms that are differentially distributed in the two tissue extracts. Similar observations have already been reported by other groups such as for [^{3}H]mepyramine (Leurs et al., 1989; Fukui et al., 1995) and [^{3}H]GBR12935 (Niznik et al., 1990) which bind to the H_1 receptor and the dopamine transporter, respectively and were both identified to bind with high affinity to CYP450 isoforms. We propose that [^{3}H]linopirdine binding to rat brain membranes under the experimental conditions used in the present and previous studies does not represent the M-channel binding sites and therefore that the identification of M-channel modulators is hampered by the high affinity of [^{3}H]linopirdine for CYP450 proteins.

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