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Species differences in [^{3}H]linopirdine (DuP 996) binding to brain membranes

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Abstract

Binding of [^{3}H]linopirdine was evaluated in membranes prepared from rat, mouse, calf, pig, and human brain tissue. Saturation and homologous competition experiments with freshly prepared and subsequently frozen brain membranes of young adult rats yielded biphasic binding curves. Analysing binding data with two-site models confirmed the existence of specific, high-affinity binding sites for [^{3}H]linopirdine with a K_d value of 7.8 ± 3.5 nM and revealed that another site with micromolar affinity for the radioligand may exist. Almost identical data were obtained with mouse brain membranes. However, high-affinity binding of [^{3}H]linopirdine could not be detected in cerebral cortical membranes from calf, pig or an aged human subject, respectively. In these tissues [^{3}H]linopirdine bound only with moderate affinity (K_d about 200 nM). In subsequent experiments using brain membranes either freshly prepared from aged (25-month-old) rats or prepared from young adult (3-month-old) rats after a post-mortem delay of up to 15 h, it could be excluded that the factors age or post-mortem delay were responsible for the lack of high-affinity [^{3}H]linopirdine binding sites in calf, pig or human brain. It is concluded that [^{3}H]linopirdine binding data obtained from rodent studies, and consequently physiological drug effects mediated by this drug target, cannot be readily extrapolated to other species including man.

Keywords: Linopirdine; DuP 996; Brain; Membrane binding; Species difference

1. Introduction

Neuroscientific research has led to various therapeutic strategies for the treatment of neurodegenerative diseases, in particular Alzheimer's disease. Linopirdine (DuP 996, 3,3-bis(4-pyridinylmethyl)-1-phenylindolin-2-one) has been tested in phase III clinical trials as a putative palliative treatment against Alzheimer's disease. A large number of investigators found that this compound possesses cognitive enhancing capabilities (Cook et al., 1990; DeNoble et al., 1990; Brioni et al., 1993; Baxter et al., 1994; Buxton et al., 1994; Fontana et al., 1994; Murai et al., 1994), although conflicting results also emerged recently (Flagmeyer and Van der Staay, 1995; Nordholm et al., 1995). Linopirdine is thought to mediate its behavioural effects by an interesting novel mechanism of action, i.e. stimulation-dependent potentiation of presynaptic acetylcholine release, and to a lesser extent of other neurotransmitters also (Nickolson et al., 1990; Smith et al., 1993). According to the cholinergic hypothesis of geriatric memory dysfunction (Bartus et al., 1982) improved synaptic transmission caused by a depolarisation-evoked release of acetylcholine is considered to increase cognitive performance (Vickroy, 1993; Fontana et al., 1994).

In a search for the molecular site of action of linopirdine, binding studies were performed using [^{3}H]linopirdine as a probe. These experiments revealed a specific and saturable binding site for the radioligand in rat brain membranes, binding [^{3}H]linopirdine with a K_d value of 19 nM and a B_{max} value of 102 fmol/mg protein (Tam et al., 1991). This binding site displays only very little affinity towards a large number of common neurotransmitters and channel blocking compounds, and shows a characteristic distribution pattern in rat brain with high densities in brain areas associated with cognition such as cortex and hippocampus (Tam et al., 1991; De Souza et al., 1992).

Hence this novel drug target protein could open new possibilities for the treatment of cognitive deficits, if it is not unique for rats. Up to now, all successful pharmaco-

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logical approaches to demonstrate beneficial effects of linopirdine in the therapy of cognitive dysfunctions used rodents (Zaczek and Saydoff, 1993), and there is no direct evidence for the existence of this binding site in the brain of other species. We therefore compared the characteristics of [^{3}H]linopirdine binding to membranes from rats, mice, calf, pig, as well as in a sample of human frontal cortex.

2. Materials and methods

2.1. Membrane preparation

Whole brains were obtained after decapitation of young adult 3-month-old or aged, 25-month-old, male Wistar rats weighing about 250 g and male NMRI mice weighing about 20 g. At least five brains from each animal species and each different condition relating to age or within a post-mortem delay were processed immediately if not stated otherwise. Fresh calf and pig brains were obtained from the local slaughterhouse. Cortex and hippocampus were prepared after a post-mortem delay of 3–4 h. Human frontal cortical tissue (age 74 years, female, no signs of central nervous system pathology, obtained at autopsy after a post-mortem delay of 18 h) was kindly donated by Dr. K. Zilles (Anatomy Department, University of Cologne). All brain membranes were prepared according to the procedure of Tam et al. (1991) and stored frozen in liquid nitrogen until the time of the experiments.

2.2. [^{3}H]Linopirdine binding

Binding studies were conducted in the way described by Tam et al. (1991) with minor modifications. Frozen membranes were thawed on the day of the assay and aliquots of about 250 μg protein, estimated by the method of Bradford (1976), were incubated in the presence of [^{3}H]linopirdine in a final volume of 500 μl at 25°C for 60 min. Samples were rapidly filtered through Whatman GF/C filters pre-soaked in 50 mM Tris-HCl buffer pH 7.4 containing 1% polyethyleneimine, 0.1% dimethyl sulfoxide, and 10 μM unlabeled linopirdine in order to minimise filter binding. Saturation experiments were performed with 10–12 concentrations ranging from about 1 nM to 200 nM [^{3}H]linopirdine. Homologous competition experiments with unlabeled linopirdine were done with 25–30 concentrations from 100 pM to 100 μM in the presence of 30 nM [^{3}H]linopirdine. Non-specific binding was determined in the presence of 100 μM unlabeled linopirdine.

2.3. Data analysis

In saturation experiments, values of the dissociation constant (K_d) and of the density of binding sites (B_{max}) were calculated using the method of Scatchard (1949) and Rosenthal (1967) or using AccuFit Saturation-Two Site (Beckman Instruments). In homologous competition experiments, values of IC_{50} (concentration of competing drug that inhibits 50% of specific binding binding) and Hill coefficients (n_H) were calculated using InPlot 4.0 (GraphPad Software). Statistical analysis was performed using an F-test to determine whether data could be described better by a one- or a two-site model. If not stated otherwise, the data are expressed as means ± S.E.M. of three separate experiments, each carried out in triplicate.

2.4. Materials

Linopirdine and [^{3}H]linopirdine were synthesised in the chemical facilities of the Bayer Pharma Research Center (Wuppertal, Germany). All other materials and reagents were purchased from Sigma (Deisenhofen, Germany).

3. Results

[^{3}H]Linopirdine bound to whole brain membranes from young rats in a concentration-dependent manner. Specific binding accounted for 70–45% of total binding in the concentration range of 1–70 nM [^{3}H]linopirdine. Saturation of binding sites was not attained up to 200 nM [^{3}H]linopirdine. Analysis of binding data yielded curvilinear Scatchard plots (Fig. 1) which, in each of six independent experiments, fitted better to a two-site model than to a one-site model, as indicated by the sum of squares of residual errors. The high-affinity binding site thus calculated had a K_d value of 7.8 ± 3.5 nM and a B_{max} value of 100 ± 31 fmol/mg protein. The K_d value attributed to the low-affinity site seemed to be in the μM range but could not be estimated exactly within [^{3}H]linopirdine concentrations up to 200 nM. The complex binding of [^{3}H]linopirdine to fresh whole brain membranes from young rats was confirmed in experiments investigating the homologous competition of total [^{3}H]linopirdine binding by unlabeled linopirdine (Fig. 2). A two-site model compared to a one-site model fitted the competition data significantly better as indicated by the F-test ($P < 0.05$). Calculations of homologous competition data yielded a n_H value of 0.59 ± 0.07, IC_{50} values of 53 ± 13 nM for the high-affinity binding sites and 11 ± 5 μM for the low-affinity interaction, respectively.

[^{3}H]Linopirdine binding to mouse whole brain membranes exhibited properties similar to those in rat tissue, although the recovery of specific binding was slightly lower compared to that in rat brain (Table 1). Scatchard analysis of saturation experiments yielded biphasic curves and Hill coefficients calculated from homologous competition studies were smaller than unity, again necessitating the use of a two-site model, which fitted homologous competition data significantly better as indicated by the F-test ($P < 0.05$). The K_d and IC_{50} values of the high-affinity binding sites were thus determined to be 29.5 and

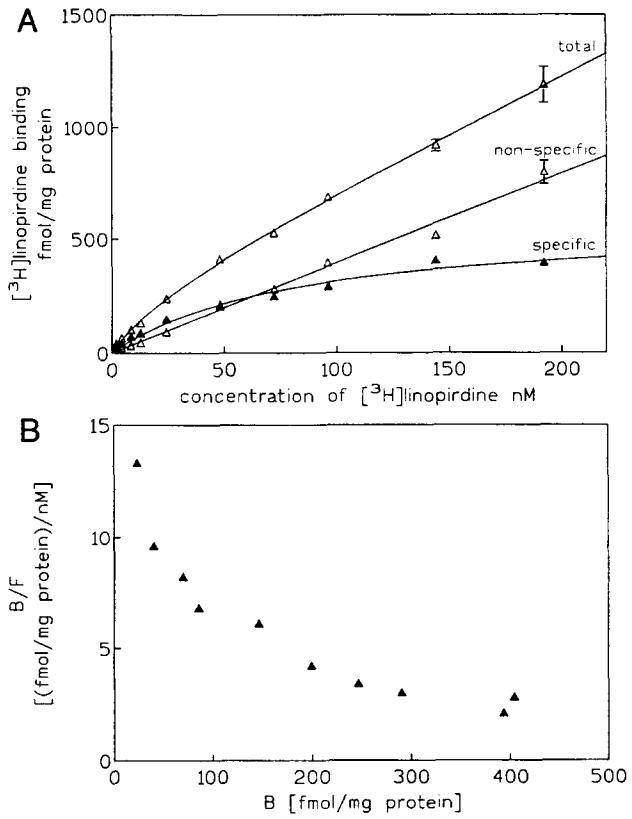

Fig. 1. A: Representative saturation isotherm of [^{3}H]linopirdine binding to rat whole brain membranes in six experiments (total and non-specific binding expressed as mean ± S.E.M. calculated from triplicates, specific binding calculated as difference from the means of total and non-specific binding). B: Scatchard plot.

108 nM, respectively (means of two experiments each). As in rats, the corresponding values for the low-affinity site were in the μM range (data not shown).

In contrast to the biphasic binding in rodents, equilibrium binding of [^{3}H]linopirdine to membranes from calf brain was apparently monophasic (Fig. 3). Only moderate affinity was observed, however (Table 1). Dissociation constants of 117 ± 21 nM for cortex and 243 ± 38 nM for

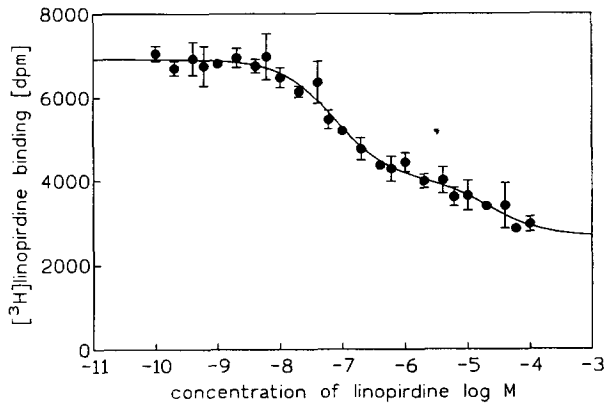

Fig. 2. Representative competition of [^{3}H]linopirdine and unlabeled linopirdine in rat whole brain membranes in three experiments (mean ± S.E.M. calculated from triplicates).

Table 1
Characteristics of [^{3}H]linopirdine binding to brain membranes from various species

Tissue	n	K_d (nM)	B_{max} (fmol/mg)	Specific binding (% of total binding at K_d)
Rat whole brain [a]	6	7.8 ± 3.5	100 ± 31	60
Mouse whole brain [a]	2	29.5 ± 4.5	90 ± 0	50
Calf cortex [b]	3	117 ± 21	450 ± 38	35
Calf hippocampus [b]	3	243 ± 38	718 ± 79	40
Pig cortex [b]	2	252 ± 61	550 ± 110	25
Human frontal cortex [b]	3	252 ± 64	2295 ± 417	45

[a] K_d calculated for the high-affinity site; [b] K_d calculated with one-site model.

hippocampus – both brain regions with reportedly high densities of linopirdine binding sites in rats (Tam et al., 1991; De Souza et al., 1992) – were determined. Similar results were obtained in saturation experiments with pig cortical membranes (Table 1). Note that the extent of specific binding in all these tissues reached only 25–40% of total binding. Moreover, the binding capacity was higher

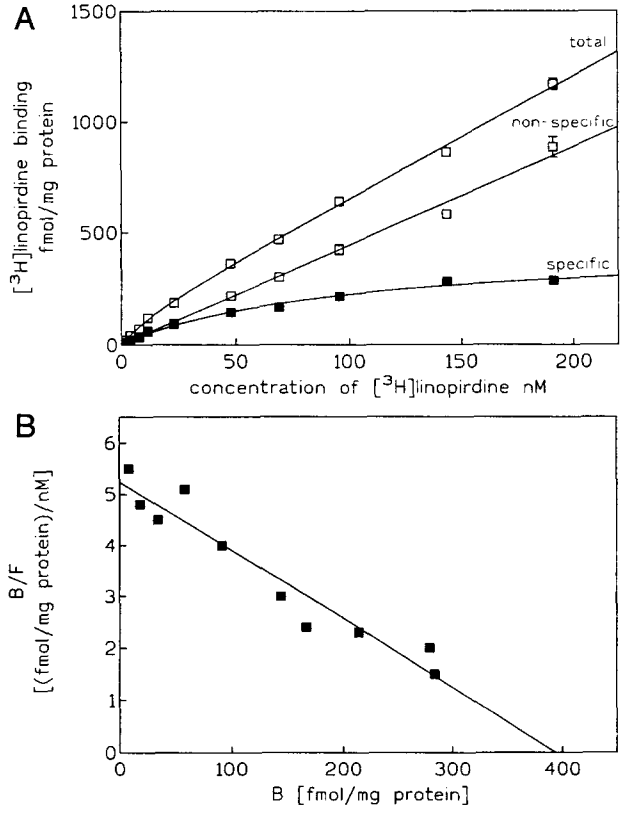

Fig. 3. A: Representative saturation isotherm of [^{3}H]linopirdine binding to calf cortical membranes in three experiments (total and non-specific binding expressed as mean ± S.E.M. calculated from triplicates, specific binding calculated as difference from the means of total and non-specific binding). B: Scatchard plot.

Table 2
Dependence of [^{3}H]linopirdine binding to rat brain membranes on age and post-mortem delay in preparation

Age	n	Post-mortem delay in h (decapitation/preparation)	K_d (nM) [a] (fmol/mg)	B_{max}
Young	6	0	7.8 ± 3.5	100 ± 31
Young	2	4	4.9 ± 1.1	101 ± 28
Young	2	15	2.2 ± 0.4	69 ± 4
Old	2	0	8.1 ± 4.8	63 ± 16

[a] Calculated for the high-affinity site.

than that calculated for high-affinity binding of [^{3}H]linopirdine in rodent brain. This could, however, be related to the use of defined brain regions with possibly higher densities of drug binding sites instead of whole brain preparations.

In view of the apparent species differences regarding the binding affinity of [^{3}H]linopirdine binding to brain membranes, we also measured [^{3}H]linopirdine binding to membranes prepared from human prefrontal cortex. Specific binding of the radioligand was indeed found (Table 1). The Scatchard curves obtained appeared linear, similarly to those from pig and calf membranes. Also, the affinity constant of [^{3}H]linopirdine was comparable to the results in these latter species. Only the density of binding sites appeared much higher than in any other tissue tested.

One important difference in test material from the different species was the post-mortem delay in membrane preparation. While rodent brains could be processed immediately after decapitation, membranes from calf and pig brains obtained from the slaughterhouse could only be prepared within 3–4 h after killing. The autopsy material from the human case had an even longer post-mortem delay, 18 h. In addition, the human case was aged (74 years old), while the animal brains originated from young adult subjects. In order to exclude the possibility that the high-affinity binding site for [^{3}H]linopirdine could suffer from long post-mortem delays or during ageing, saturation isotherms were run with whole brain membranes from young adult (3-month-old) rats prepared 4 or 15 h after killing, as well as with whole brain membranes freshly prepared from aged (25-month-old) rats. As with freshly prepared membranes from young adult rats, curvilinear Scatchard plots were obtained, which again fitted better to a two-site model than to a one-site model, as indicated by the sum of squares of residual errors. K_d and B_{max} values calculated for the high-affinity binding sites were of the same order of magnitude as those obtained from fresh whole brain membranes of young rats (Table 2).

4. Discussion

The present study revealed distinct differences among the five mammalian species investigated in their properties to specifically bind [^{3}H]linopirdine. In membranes isolated from rat brain, we could confirm the existence of high-affinity [^{3}H]linopirdine binding sites. The K_d value of 7.8 nM for [^{3}H]linopirdine as well as the IC_{50} value of 53 nM for unlabeled linopirdine to high-affinity sites are well in accordance with the data from Tam et al. (1991) and De Souza et al. (1992), respectively. Although the rough estimation of a K_i value of about 10 nM from the IC_{50} value of 53 nM for unlabeled linopirdine at high-affinity binding sites determined in homologous competion experiments according to Cheng and Prusoff (1973) is not strictly correct for the protocol used here, the affinity constants for linopirdine and [^{3}H]linopirdine seem to be of the same order of magnitude.

However, in contrast to the apparently homogenous population of [^{3}H]linopirdine binding sites in rat brain indicated by the linear Scatchard plots reported by Tam et al. (1991), we obtained curvilinear plots, showing the existence of an additional class of low-affinity binding sites. Saturation and homologous competition experiments with mouse brain membranes also yielded biphasic graphs, suggesting heterogeneity of linopirdine binding sites, with a K_d value of 29.5 nM for [^{3}H]linopirdine and an IC_{50} value of 108 nM for unlabeled linopirdine corresponding to a K_i value of 50 nM (approximative estimation according to Cheng and Prusoff, 1973) for the high-affinity binding site. The observed affinity for the high-affinity linopirdine binding sites was somewhat lower than calculated from experiments with rat brain, but still of the same order of magnitude.

A possible explanation for the more complex binding data we obtained could be the use of 10-fold higher concentrations of unlabeled linopirdine used to determine non-specific binding (100 μM instead of 10 μM) in the study of Tam et al. (1991). Additionally we kept our membranes frozen and thawed them on the day of the assay whereas Tam et al. (1991) processed the membranes immediately before the binding assay. These changes in membrane preparation were necessary in order to keep the test conditions as similar as possible for all species investigated. For the same reason the influence of the post-mortem delay in membrane preparation as well as of brain ageing was also studied in rats. Apparently neither a post-mortem delay of up to 15 h between decapitation and preparation nor the age of the animals seems to substantially influence affinity constants in saturation experiments. The high-affinity component of [^{3}H]linopirdine binding in particular was not sensitive to these modifications. This finding enables us to compare the binding data obtained with rat brain membranes to data from brain membranes of other species, which could not be prepared immediately.

Despite the general possibility of detecting high-affinity [^{3}H]linopirdine binding sites with our protocol, no such sites could be determined in saturation isotherms as well as in homologous competition studies (data not shown) using membranes from non-rodent brain tissue. Even though it

has to be considered that hypothetical experimental artefacts or scattered data may lead to misinterpretations of both biphasic and linear Scatchard plots, the results of 14 saturation experiments with rodent brain membranes yielding biphasic Scatchard plots and of 11 identically designed experiments with non-rodent brain membranes consistently showing a monophasic shape suggest a species difference in the affinity of brain [^{3}H]linopirdine binding sites. This is the most remarkable finding of the present study. Although we used brain regions with potentially high densities of [^{3}H]linopirdine binding sites (De Souza et al., 1992), the affinity constants in calf cortex and hippocampus and pig cortex were less by about one order of magnitude than those for rodents. Scatchard plots were linear, indicating only one class of non-interacting, medium-affinity binding sites.

Essentially the same situation was found in a sample from human frontal cortex. Since all brain membranes, irrespective of the species used, were prepared identically, and preparation artefacts originating from differential post-mortem delays or age could be excluded (see above), these results suggest strongly that the high-affinity binding site for [^{3}H]linopirdine which is found in rat and mouse brain is not expressed in bovine, porcine and human cortex, although the restriction in the last case, that there was only a single sample, has to be mentioned. Clearly, further studies are warranted to exclude the possibility that high-affinity binding sites may exist in other regions of bovine, porcine, or human brain. However, it should be kept in mind that in case of rat brain, cortex is particularly enriched in these binding sites (De Souza et al., 1992; Zaczek and Saydoff, 1993), and the cortex is among the brain regions which are most affected by cholinergic denervation in Alzheimer's disease (Coyle et al., 1983), the target indication for linopirdine. Additional support for species-specific differences in the pharmacology of linopirdine comes from a recent study by Nordholm et al. (1995), in which linopirdine failed to enhance cognitive function in squirrel monkeys and pigeons performing an operant behavioural task. In this context, it would be interesting to determine whether the high-affinity site for [^{3}H]linopirdine is present in monkey and bird brain, respectively.

Of course it is conceivable that the low-affinity [^{3}H]linopirdine binding sites in rodents or the medium-affinity sites found in calf, pig, and human cortex may also contribute to the physiological and behavioural effects of linopirdine. This assumption is however very difficult to verify. The behavioural studies demonstrating cognition enhancing properties of linopirdine (Cook et al., 1990; DeNoble et al., 1990; Brioni et al., 1993; Baxter et al., 1994; Buxton et al., 1994; Fontana et al., 1994; Murai et al., 1994) were all carried out with rats or mice, which express both the high- and the low-affinity classes of binding sites. Similarly, the neurotransmitter release-enhancing effects of linopirdine were usually studied in rodent tissue (Nickolson et al., 1990; Tam et al., 1991; Zaczek et al., 1993; Vickroy, 1993; Fontana et al., 1994), with the exception of the study by Provan and Miyamoto (1994), who determined the action of linopirdine on quantal acetylcholine release at frog neuromuscular junctions. In these functional in vitro studies, pharmacological effects of linopirdine were consistently noticed at the low micromolar range, with an EC_{50} value of 5 μM (Zaczek and Saydoff, 1993). Although there appears to be a reasonable correlation between effective drug levels in vitro and in vivo (Zaczek and Saydoff, 1993), evidence for the involvement of the high-affinity binding site in the release-stimulating effect is not compelling.

On the other hand, it is well possible that functional studies require higher doses than binding experiments, and it should be considered that Tam and his co-workers (Tam et al., 1991) showed an excellent correlation between the respective potencies of 30 structural analogues of linopirdine to displace [^{3}H]linopirdine binding from its high-affinity binding site on rat brain membranes and to enhance K^+-stimulated acetylcholine release from rat cerebral cortical slices. Moreover, linopirdine was reported to improve cognitive function in rodent models of learning and memory within the rather low dose-range of 0.01–2.5 mg/kg (for review see Zaczek and Saydoff, 1993). At such low doses, it is rather unlikely that a drug binding site with micromolar affinity is involved. Hence, the present state of knowledge supports the view that the behavioural and physiological effects of linopirdine in rodents are in fact mediated by an interaction with the high-affinity binding site for the compound. Further investigations are required in order to determine if there is a physiological role for the low-affinity binding site for [^{3}H]linopirdine in rat and mouse brain and, in particular, one for the medium-affinity binding site found in this study to be present in the brain of non-rodent mammals.

In summary, we could confirm the existence of high-affinity binding sites for [^{3}H]linopirdine in rat brain membranes. An additional low-affinity site was detected. While very similar binding data were obtained for mouse brain, membrane preparations from calf, pig, or human frontal cerebral cortex only showed a single, medium-affinity binding component under identical conditions. These species-dependent differences in the characteristics of [^{3}H]linopirdine binding justify the conclusion that [^{3}H]linopirdine binding data from rodent studies – and hence physiological effects of linopirdine believed to be mediated by this drug target – are not readily applicable to other species, possibly including ageing humans.

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