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Lack of effects of prolonged treatment with phenobarbital or phenytoin on the expression of P-glycoprotein in various rat brain regions

Ulrike Seegers, Heidrun Potschka, Wolfgang Löscher *

Department of Pharmacology, Toxicology and Pharmacy, School of Veterinary Medicine, Bünteweg 17 Building 218, D-30559 Hannover, Germany

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Abstract

P-glycoprotein is an ATP-dependent drug transport protein that is predominantly found in the apical membranes of various epithelial cell types in the body, including the blood luminar membrane of the brain capillary endothelial cells that make up the blood–brain barrier. Increased P-glycoprotein expression in the blood–brain barrier has been described in epileptogenic brain tissue of patients with pharmacoresistant epilepsy, suggesting that overexpression of P-glycoprotein may be involved in multidrug resistance of epilepsy. The mechanisms underlying the overexpression of P-glycoprotein in brain tissue of epileptic patients are not clear. Two antiepileptic drugs, phenobarbital and phenytoin, have been reported to up-regulate P-glycoprotein in cell cultures, so that chronic treatment with antiepileptic drugs may enhance P-glycoprotein expression in the blood–brain barrier. To directly address this possibility, we treated rats with phenobarbital or phenytoin over 11 days and subsequently determined expression of P-glycoprotein by immunohistochemistry in endothelium and parenchyma of several brain regions, including regions of the temporal lobe, which is often involved in pharmacoresistant types of epilepsy. Except for a moderate increase in the intensity of P-glycoprotein expression in the piriform/parietal cortex and cerebellum of phenobarbital-treated rats, no significant P-glycoprotein increases were seen after prolonged treatment with phenobarbital or phenytoin in any brain region examined. In view of recent findings that seizures lead to a transient induction of P-glycoprotein in the brain of rats, it seems reasonable to suggest that the overexpression of P-glycoprotein in brain regions of patients with intractable epilepsy is a consequence of uncontrolled seizures rather than of chronic treatment with antiepileptic drugs.

Keywords: Epilepsy; Pharmacoresistance; Multidrug resistance gene; Antiepileptic drug

1. Introduction

The blood–brain barrier is a physical and metabolic barrier between the brain and the systemic circulation, which serves to regulate and protect the microenvironment of the brain (Pardridge, 1999; Huber et al., 2001). Tight junctions between brain capillary endothelial cells and the lack of fenestrations enable the blood–brain barrier to regulate brain parenchyma composition. Furthermore, the brain microvasculature is ensheated by astrocytic end feet, which play an essential role in the functioning of the blood–brain barrier (Huber et al., 2001). In addition to limitation of blood–brain barrier permeability because of morphological characteristics, a number of ATP-dependent efflux transport proteins located in the apical membranes of brain capillary endothelial cells and pericapillary astrocytes act as a defense mechanism by limiting entry of many compounds, including a variety of drugs, into the brain (Fromm, 2000; Lee et al., 2001). Limitation of blood–brain barrier permeability by efflux pumps such as P-glycoprotein is an increasingly recognized determinant of drug disposition (Fromm, 2000). The drug-transporting P-glycoprotein is a product of the multidrug resistance (*MDR1*) gene and was originally identified by its capacity to confer multidrug resistance to tumor cells against anticancer drugs (Ling, 1997). However, apart from anticancer drugs, P-glycoprotein accepts a variety of lipophilic drugs of diverse structures as substrates and has recently been suggested to confer multidrug resistance in normal tissues including the brain (Golden and Pardridge, 2000). In line with

* Corresponding author. Tel.: +49-511-856-8721; fax: +49-511-953-8581.

E-mail address: wolfgang.loescher@tiho-hannover.de (W. Löscher).

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this suggestion, overexpression of P-glycoprotein was found in capillary endothelial cells and astrocytes in brain specimens obtained during resective surgery of patients with pharmacoresistant epilepsy, drawing on parallels between drug resistance in cancer and epilepsy (Tishler et al., 1995; Dombrowski et al., 2001; Sisodiya et al., 2002).

Several major antiepileptic drugs, including phenytoin and phenobarbital, have recently been shown to be transported by P-glycoprotein in the blood–brain barrier (Löscher and Potschka, 2002). Thus, overexpression of P-glycoprotein in the blood–brain barrier of epileptogenic brain tissue is likely to reduce penetration of these drugs into the parenchyma and thereby reduce their antiepileptic effect. It would therefore be important to know why overexpression of P-glycoprotein develops in a subpopulation of patients with epilepsy. Theoretically, P-glycoprotein overexpression could be a consequence of epilepsy, of uncontrolled seizures, of chronic treatment with antiepileptic drugs, or it could be constitutive, i.e., present before onset of epilepsy, for instance related to hereditary polymorphisms in *MDR1* (Löscher and Potschka, 2002). In cell lines, P-glycoprotein is strongly up-regulated by many drugs, including antiepileptic drugs such as phenobarbital and, to a lesser extent, phenytoin (Schuetz et al., 1996). This prompted us to examine whether prolonged treatment of rats with phenobarbital or phenytoin increases the expression of P-glycoprotein in the brain. P-glycoprotein was determined by immunohistochemistry in endothelium and parenchyma of several brain regions, particularly regions of the temporal lobe, which is often involved in pharmacoresistant epilepsy (Regesta and Tanganelli, 1999).

2. Materials and methods

2.1. Animals

Female Wistar outbred rats (Harlan-Winkelmann, Borchen, Germany), weighing 200–220 g, were used. The animals were purchased from the breeder at an age of about 10 weeks. Following arrival in the animal colony, the rats were kept under controlled environmental conditions (ambient temperature 24–25 °C, humidity 50–60%, 12:12 h light/dark cycle, light on at 6:00 a.m.) for at least 1 week before being used in the experiments. Standard laboratory chow (Altromin 1324 standard diet) and tap water were allowed ad lib. All experiments were done between 10:00 and 12:00 h to minimize the bias of circadian variations. All animal care and handling was conducted in compliance with the German Animal Welfare Act and was approved by the responsible governmental agency in Hannover.

2.2. Experiments with phenobarbital and phenytoin

After adaption to our animal colony, groups of rats were once daily in the morning treated with either phenobarbital ($n = 9$) or phenytoin ($n = 8$) for 11 days. Phenobarbital was i.p. administered at a daily dose of 30 mg/kg. Based on a half-life of 12 h in female Wistar rats, this treatment protocol leads to anticonvulsant effects and plasma drug levels above or within the "therapeutic range" known from patients with epilepsy (Löscher and Hönack, 1989). Treatment with phenytoin was started with an i.p. injection of 75 mg/kg on the first day, followed by once daily 50 mg/kg on the subsequent days. This treatment schedule was based on previous experiments in female Wistar rats, taking into account the nonlinear saturation elimination kinetics of phenytoin (Rundfeldt and Löscher, 1993). Although phenytoin is more rapidly metabolized in rats than in humans after administration of single doses (half-life is about 2–4 h in rats compared to about 20 h in humans), the long-lasting saturation of phenytoin-metabolizing enzymes after repeated administration of high doses of phenytoin in rats results in a dramatic retardation of its elimination, so that plasma phenytoin levels in the therapeutic range known from patients with epilepsy are maintained in rats by the treatment protocol used in the present experiments (Rundfeldt and Löscher, 1993). At the drug levels reached and maintained by these dosing protocols in rats, both phenobarbital and phenytoin have been shown to induce hepatic drug metabolizing enzymes in rodents and humans (Frey, 1986), so that these dosing protocols were considered to represent an adequate drug stimulus to test whether P-glycoprotein expression is altered by prolonged treatment with these drugs.

On day 11 of the treatment period, rats were killed 2 h (phenobarbital) or 1 h (phenytoin) after the drug administra-

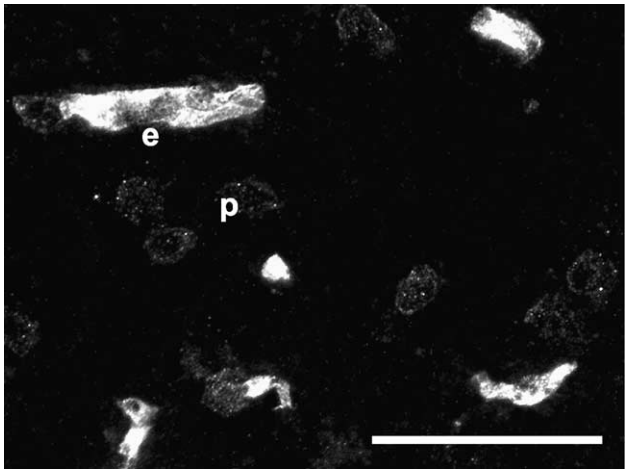

Fig. 1. Immunohistochemical staining for P-glycoprotein on a section from the posterior piriform cortex (at −3.8 mm from bregma) of a drug-naive rat. The monoclonal P-glycoprotein antibody C219 was used for P-glycoprotein staining. Prominent immunoreactivity is evident in endothelial cells of capillaries ("e") as well as in several parenchymal cells ("p"). The scale bar indicates 50 μm. Scoring of staining intensity in this section by the grading scale described in Section 2 yielded an intensity score of 4 for P-glycoprotein-immunostaining in endothelial cells and a score of 1–2 for staining in parenchymal cells.

tion in the morning. Blood was sampled for drug analysis in plasma. Two control groups of eight rats each were treated with vehicle (saline) in parallel to the drug-treated groups, so that each drug-treated group had its own control. Controls were killed together with drug-treated rats and were concurrently used for P-glycoprotein expression analysis.

2.3. Immunohistochemistry

After decapitation, the brains were rapidly removed and frozen by means of liquid nitrogen. Frozen transverse sections of forebrain and midbrain were cut at 10 μm by means of a cryomicrotom (HM 500 OM, Microm Zeiss; Walldorf, Germany). Sections were then fixed in acetone. Three to six sections of the following regions were chosen for immunohistochemistry: +3.7, +2.2, −0.8, −2.2, −3.8, −5.8, and −11.0 mm relative to bregma (Paxinos and Watson, 1998).

P-glycoprotein expression was detected with the primary monoclonal mouse antibody C219 (Calbiochem, Bad Soden, Germany) in a 1:100 dilution. The antibody C219 has been used to detect P-glycoprotein in capillaries isolated from rat brain (Jette et al., 1993) and does not discriminate between the *mdr1a* and *mdr1b* isoforms of P-glycoprotein. We have recently confirmed the selectivity of the reaction of the antibody C219 against P-glycoprotein in rat brain sections by comparison with P-glycoprotein staining by the polyclonal rabbit antibody *mdr*-Ab-1 (1:50, Oncogene Research Products, San Diego, CA, USA), which, as C219, labels the *mdr1a* and *mdr1b* isoforms of P-glycoprotein (Seegers et al., 2002a).

Labelling of the C219 antibody was visualised by utilising biotinylated goat anti-mouse immunoglobulin G (1:500, DAKO, Hamburg, Germany) coupling the antibody to a fluorochrome (carbocyanin 3, 1:1000, Jackson Immunoresearch Laboratories) using a conventional streptavidin–biotin technique. Epitope-specificity of the antibody C219 against P-glycoprotein was tested by preincubation of the primary antibody with the mdr-peptide (a product of the human multidrug resistance *mdr-1* gene) (1.5 μg in 100 μl; Oncogene Research Products).

P-glycoprotein expression was investigated in fields of the frontal and parietal cortex, basolateral amygdala, hippocampus, dentate gyrus, piriform cortex, substantia nigra pars reticulata, and cerebellum by a person who was not aware whether the coded sections were from drug-treated rats or controls. For each region, P-glycoprotein expression was separately determined for left and right hemisphere. In each region, fields of 38,320 μm^2 were randomly chosen for analysis of P-glycoprotein

Table 1
Quantitative immunostaining for P-glycoprotein (PGP) in control and phenobarbital-treated rats

Brain region	Bregma	Area (%) labelled for PGP		Intensity scores of PGP-immunostaining in			
		Control	Phenobarbital	Endothelium		Parenchyma	
				Control	Phenobarbital	Control	Phenobarbital
Piriform cortex	3.7	1.67 ± 0.14	1.93 ± 0.11	4.11 ± 0.13	4.21 ± 0.08	1.16 ± 0.09	1.44 ± 0.17
	2.2	1.88 ± 0.07	2.10 ± 0.16	3.91 ± 0.09	4.19 ± 0.11*	0.94 ± 0.08	1.24 ± 0.08*
	−0.8	1.60 ± 0.06	1.62 ± 0.09	4.17 ± 0.11	3.97 ± 0.15	1.15 ± 0.11	0.92 ± 0.17
	−2.2	1.20 ± 0.11	1.20 ± 0.11	3.93 ± 0.13	3.99 ± 0.23	1.17 ± 0.17	1.03 ± 0.11
	−3.8	1.49 ± 0.08	1.27 ± 0.08	3.98 ± 0.11	3.88 ± 0.14	1.22 ± 0.09	1.24 ± 0.08
Frontal cortex	3.7	2.59 ± 0.15	2.42 ± 0.17	4.05 ± 0.12	4.18 ± 0.14	1.42 ± 0.09	1.68 ± 0.20
	2.2	2.05 ± 0.19	2.23 ± 0.17	3.75 ± 0.11	3.92 ± 0.11	0.98 ± 0.13	1.07 ± 0.10
Parietal cortex	2.2	2.62 ± 0.18	2.69 ± 0.11	3.86 ± 0.09	3.94 ± 0.11	1.02 ± 0.12	1.39 ± 0.13*
	−0.8	2.50 ± 0.17	2.14 ± 0.15	4.08 ± 0.08	3.88 ± 0.15	1.05 ± 0.11	1.01 ± 0.12
	−2.2	2.25 ± 0.13	2.26 ± 0.28	3.88 ± 0.13	4.07 ± 0.16	1.12 ± 0.18	1.00 ± 0.16
	−3.8	2.67 ± 0.21	2.55 ± 0.21	3.89 ± 0.08	3.70 ± 0.15	1.12 ± 0.07	1.29 ± 0.13
Dentate gyrus	−2.2	1.23 ± 0.05	1.25 ± 0.13	3.79 ± 0.13	3.79 ± 0.26	0.54 ± 0.10	0.39 ± 0.13
	−3.8	1.66 ± 0.09	1.53 ± 0.16	3.80 ± 0.07	3.65 ± 0.16	0.50 ± 0.08	0.34 ± 0.08
Hilus	−2.2	0.91 ± 0.08	1.06 ± 0.11	3.65 ± 0.09	3.55 ± 0.15	0.78 ± 0.15	0.89 ± 0.09
	−3.8	1.02 ± 0.09	1.15 ± 0.11	3.81 ± 0.16	3.75 ± 0.09	1.00 ± 0.13	0.75 ± 0.13
CA3	−3.8	1.29 ± 0.10	1.23 ± 0.12	4.06 ± 0.11	3.81 ± 0.13	0.70 ± 0.10	0.53 ± 0.05
	−5.8	1.40 ± 0.10	1.28 ± 0.08	3.48 ± 0.09	3.57 ± 0.09	0.21 ± 0.15	0.14 ± 0.07
CA1	−5.8	2.00 ± 0.17	2.08 ± 0.20	4.36 ± 0.14	4.20 ± 0.13	0.67 ± 0.22	0.71 ± 0.14
Amygdala	−2.2	1.04 ± 0.07	0.91 ± 0.05	3.97 ± 0.12	3.96 ± 0.23	1.05 ± 0.23	1.08 ± 0.10
Substantia nigra	−5.8	1.69 ± 0.10	1.55 ± 0.12	4.34 ± 0.07	3.96 ± 0.29	0.32 ± 0.09	0.25 ± 0.10
Cerebellum	−11.0	2.25 ± 0.18	2.34 ± 0.17	4.08 ± 0.1	4.31 ± 0.08*	0.89 ± 0.10	0.90 ± 0.08

In sections of the brain regions shown in the table, the area of positive P-glycoprotein-immunostaining relative (in %) to the total area of the fields randomly chosen for P-glycoprotein analyses and the intensity of positive P-glycoprotein-immunostaining in capillary endothelial cells and parenchyma were determined per region, hemisphere, and rat, using three to six sections per region and at least 10 fields per region. The average values of each rat from left and right hemisphere were used for calculation of group values. Data are means ± S.E.M. of eight controls and nine phenobarbital-treated rats. For each region, the anterior–posterior coordinate (in mm from bregma) of the section(s) is indicated. Significant increases in P-glycoprotein expression compared to controls are indicated by asterisk ($P < 0.05$).

expression, using at least 10 fields per region. Analysis was performed by using the Kontron KS 300 image analysis system. In each field, the area of P-glycoprotein-staining relative to the total area [%] of the field was measured per region, side, and animal. For analysis of intensity of P-glycoprotein staining, a semi-quantitative scoring system was used, similar to that used by Tishler et al. (1995) for grading the intensity of endothelial and astrocyte staining of P-glycoprotein in epileptic patients treated with antiepileptic drugs. The scoring system used in the present study has been described in detail elsewhere (Seegers et al., 2002a). In short, the intensity of the reaction was graded on a scale of 0–5; 0 = no reaction is visible, 1 = very weak, 2 = weak, 3 = moderate, 4 = strong, 5 = very strong reaction. The mean values per region determined for each rat were used for calculating group means. An example for values obtained by the grading scale is shown in Fig. 1 (see legend to figure).

For positive control (i.e., sections with significant increase in P-glycoprotein immunostaining), brain sections from kainate-treated rats had been used in recent experiments, showing that both the scoring system and percent area labeled measurements used in the present experiments reliably demonstrated significant increases in P-glycoprotein (Seegers et al., 2002a,b). Furthermore, the reliability of the scoring system and percent area labeled measurements have been checked by comparing results from these measurements with results from a computer-assisted automatic analysis of staining intensity and P-glycoprotein-labeled area by an image analysis system, using diaminobenzidine-stained sections (Seegers et al., 2002b). For the present experiments, we preferred to use fluorescence staining which did not allow automatic staining analysis but yielded a much better staining of parenchymal P-glycoprotein than the diaminobenzidine staining method (Seegers et al., 2002b).

2.4. Drug analysis in plasma

Phenobarbital and phenytoin were determined in plasma by high-performance liquid chromatography with ultraviolet detection as described recently (Potschka and Löscher, 2001).

2.5. Drugs

Phenobarbital (sodium salt) was purchased from Serva (Heidelberg, Germany). Phenytoin was obtained from

Table 2
Quantitative immunostaining for P-glycoprotein (PGP) in control and phenytoin-treated rats

Brain region	Bregma	Area (%) labelled for PGP		Intensity scores of PGP-immunostaining in			
		Control	Phenytoin	Endothelium		Parenchyma	
				Control	Phenytoin	Control	Phenytoin
Piriform cortex	3.7	2.62 ± 0.71	2.32 ± 0.58	4.45 ± 0.17	4.48 ± 0.22	1.35 ± 0.17	1.53 ± 0.13
	2.2	3.09 ± 0.25	3.35 ± 0.27	4.67 ± 0.09	4.17 ± 0.21	1.15 ± 0.07	1.10 ± 0.05
	−0.8	3.29 ± 0.24	3.00 ± 0.40	4.56 ± 0.13	4.61 ± 0.21	1.20 ± 0.08	1.03 ± 0.02
	−2.2	2.98 ± 0.16	2.45 ± 0.19	3.69 ± 0.13	3.80 ± 0.13	1.77 ± 0.13	1.77 ± 0.12
	−3.8	1.44 ± 0.22	1.55 ± 0.17	3.72 ± 0.09	3.68 ± 0.07	1.47 ± 0.09	1.52 ± 0.09
Frontal cortex	3.7	2.36 ± 0.31	2.86 ± 0.27	3.98 ± 0.10	4.15 ± 0.23	1.50 ± 0.10	1.50 ± 0.07
	2.2	2.37 ± 0.22	2.30 ± 0.33	4.15 ± 0.20	3.75 ± 0.13	1.23 ± 0.10	1.12 ± 0.06
Parietal cortex	2.2	2.44 ± 0.10	2.86 ± 0.39	4.13 ± 0.17	3.85 ± 0.18	1.34 ± 0.14	1.13 ± 0.08
	−0.8	3.51 ± 0.34	3.46 ± 0.37	4.06 ± 0.14	4.28 ± 0.16	1.02 ± 0.13	1.13 ± 0.14
	−2.2	3.95 ± 0.19	3.48 ± 0.36	3.66 ± 0.13	3.80 ± 0.11	1.61 ± 0.18	1.72 ± 0.13
	−3.8	2.58 ± 0.15	2.36 ± 0.20	3.45 ± 0.05	3.54 ± 0.10	1.19 ± 0.15	1.41 ± 0.12
Dentate gyrus	−2.2	2.49 ± 0.12	2.13 ± 0.18	3.64 ± 0.13	3.38 ± 0.15	1.16 ± 0.07	1.00 ± 0.11
	−3.8	1.59 ± 0.11	1.45 ± 0.16	3.50 ± 0.11	3.39 ± 0.07	0.91 ± 0.12	0.93 ± 0.12
Hilus	−2.2	1.28 ± 0.09	1.31 ± 0.09	4.06 ± 0.09	3.89 ± 0.10	0.69 ± 0.10	0.47 ± 0.07
	−3.8	1.10 ± 0.04	1.05 ± 0.03	4.28 ± 0.09	4.23 ± 0.09	0.40 ± 0.13	0.63 ± 0.10
CA3	−3.8	1.38 ± 0.13	1.09 ± 0.15	3.53 ± 0.10	3.39 ± 0.07	0.92 ± 0.11	0.89 ± 0.09
	−5.8	1.52 ± 0.11	1.49 ± 0.07	3.89 ± 0.14	4.04 ± 0.14	0.95 ± 0.11	0.75 ± 0.11
CA1	−5.8	1.69 ± 0.11	1.66 ± 0.06	3.90 ± 0.14	4.06 ± 0.13	0.98 ± 0.10	0.91 ± 0.09
Amygdala	−2.2	1.56 ± 0.12	1.42 ± 0.12	3.69 ± 0.13	3.72 ± 0.08	1.63 ± 0.13	1.59 ± 0.17
Substantia nigra	−5.8	2.38 ± 0.23	2.40 ± 0.12	4.00 ± 0.11	3.89 ± 0.11	0.77 ± 0.12	0.49 ± 0.10
Cerebellum	−11.0	2.85 ± 0.11	2.86 ± 0.14	4.25 ± 0.06	4.28 ± 0.07	0.59 ± 0.08	0.53 ± 0.13

In sections of the brain regions shown in the table, the area of positive P-glycoprotein-immunostaining relative (in %) to the total area of the fields randomly chosen for P-glycoprotein analyses and the intensity of positive P-glycoprotein-immunostaining in capillary endothelial cells and parenchyma were determined per region, hemisphere, and rat, using three to six sections per region and at least 10 fields per region. The average values of each rat from left and right hemisphere were used for calculation of group values. Data are means ± S.E.M. of eight controls and eight phenytoin-treated rats. For each region, the anterior–posterior coordinate (in mm from bregma) of the section(s) is indicated. Compared to controls, no significant increases in P-glycoprotein expression were found.

Aldrich (Steinheim, Germany). They were dissolved in saline, phenytoin by means of dilute NaOH. Injection volume was 3 ml/kg. Controls received the same volume of saline. The pH of saline in controls was adjusted to the pH of the respective drug solution (pH 10–11 in case of phenytoin).

2.6. Statistics

All values were expressed as means ± S.E.M. In view of the score system used, all data were statistically analysed by nonparametric tests. Within-group differences were analyzed by the Wilcoxon rank test for paired replicates. Differences between different groups were analyzed by the Mann–Whitney *U*-test or, in case of more than two sets of data, by Kruskal–Wallis analysis of variance (ANOVA) followed by the *U*-test. Because our hypothesis was that treatment with phenobarbital or phenytoin would increase P-glycoprotein expression, all tests were used one-sided and a $P < 0.05$ was considered significant.

3. Results

A comparison of P-glycoprotein immunostaining in left and right brain regions did not indicate any significant difference between hemispheres, so that the regional values for left and right hemisphere were averaged for each rat and used for calculation of group values. As shown in Fig. 1, immunolocalization of the P-glycoprotein antibody C219 was observed mainly in microvessel endothelial cells. Furthermore, cells in the parenchyma were labelled (Fig. 1). Usually, the intensity of parenchymal P-glycoprotein expression was much lower (score 1–2) than P-glycoprotein expression in capillary endothelial cells (score 3–4), but parenchymal P-glycoprotein expression could be clearly differentiated from background. With respect to the type of parenchymal cells showing P-glycoprotein expression, no clear differentiation between glia and neurons (or other cell types) could be made on morphological grounds under the conditions of P-glycoprotein immunohistochemistry at the light microscopic level (see Fig. 1). Because P-glycoprotein was predominantly located in capillary endothelial cells, the major part of the P-glycoprotein-positive area determined by image analysis in each region was related to endothelial rather than parenchymal P-glycoprotein.

As reported recently (Seegers et al., 2002a), the distribution of P-glycoprotein across brain regions in controls was not homogeneous. In terms of P-glycoprotein labelled area, the highest values were seen in cortical areas and the lowest values in the hippocampal formation (Tables 1 and 2). The intensity of endothelial P-glycoprotein expression varied less markedly across the regions examined in the present study, but marked differences were seen in parenchymal P-glycoprotein expression with lowest values in substantia nigra, cerebellum, and part of the hippocampal formation (Tables 1 and 2).

Prolonged treatment with phenobarbital or phenytoin did not induce any robust increases in P-glycoprotein expression in the brain (Tables 1 and 2). In terms of P-glycoprotein labelled area, no significant increases were seen after drug treatment in any brain region (Tables 1 and 2). Furthermore, phenytoin did not significantly increase the intensity of endothelial or parenchymal P-glycoprotein expression in any brain area (Table 2). In phenobarbital-treated rats, a small (6–7%) but statistically significant percent increase of endothelial P-glycoprotein expression was determined in the piriform cortex and cerebellum (Table 1). Furthermore, the intensity of parenchymal P-glycoprotein expression was significantly increased by about 30% in the piriform cortex and parietal cortex (Table 1). However, because no such increases were seen in any other brain area, including the hippocampal formation, after prolonged treatment with phenobarbital, it is not very likely that these differences to control have any functional meaning. Furthermore, as shown in Fig. 2 for the piriform cortex, although the average increases in P-glycoprotein expression in piriform cortex and parietal cortex of phenobarbital-treated rats were statistically significant, most individual data for P-glycoprotein expression were within the range of control

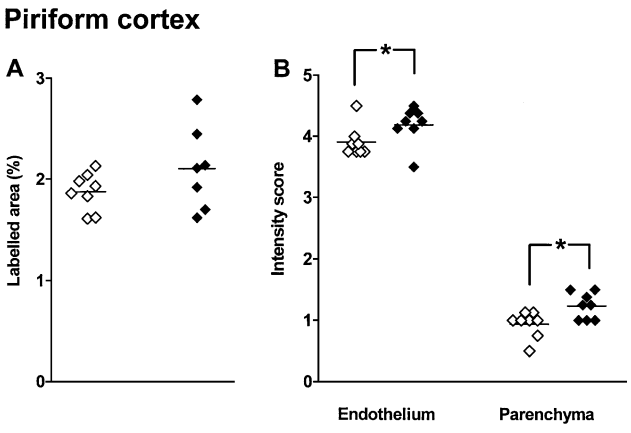

Fig. 2. Quantitative data from P-glycoprotein immunostaining in the piriform cortex of individual control rats (open diamonds) and phenobarbital-treated rats (closed diamonds). All data are from sections that are +2.2 mm from bregma. Each symbol reflects data from one rat. "A" shows individual data for the area of positive P-glycoprotein-immunostaining relative to the total area of the fields randomly chosen for P-glycoprotein analyses. For each rat, the labeled areas were averaged from analyses of three to six sections using at least 10 fields per region. "B" shows the intensity of positive P-glycoprotein-immunostaining in capillary endothelial cells and parenchymal cells, using a rating system with maximal intensity scores of 5. For each rat, the intensity scores were averaged from analyses of three to six sections with 10 fields per region. Significant differences between phenobarbital-treated rats and controls are indicated by asterisk ($P = 0.033$ for endothelial and 0.019 for parenchymal P-glycoprotein staining, respectively).

values, demonstrating the lack of any robust effect of drug treatment on P-glycoprotein expression.

The prolonged treatment with phenobarbital or phenytoin resulted in plasma concentrations within or above the therapeutic range known from patients with epilepsy. Plasma levels of phenytoin were 25.0 ± 1.3 μg/ml (mean ± S.E.M.) while those of phenobarbital were 56.2 ± 3.2 μg/ml.

4. Discussion

The present data indicate that prolonged treatment of rats with phenobarbital and phenytoin does not induce any functionally relevant increases in endothelial or parenchymal P-glycoprotein in the brain. The only significant increases were seen at one section level in the piriform and parietal cortices and cerebellum after treatment with phenobarbital, but only in terms of staining intensity. Furthermore, these increases, albeit statistically significant, were only moderate (6–36%). In view of recent findings that seizures lead to a transient induction of P-glycoprotein in the brain of rats (Zhang et al., 1999; Seegers et al., 2002b), it seems reasonable to suggest that the overexpression of P-glycoprotein in brain regions of patients with intractable epilepsy is a consequence of uncontrolled seizures rather than of chronic treatment with antiepileptic drugs.

Drugs frequently induce proteins involved in their detoxification (Michalets, 1998; Worboys and Carlile, 2001). P-glycoprotein is highly expressed in tissues that participate in drug detoxification, such as in the liver and intestine (Silverman, 1999). Several drugs that are metabolized by cytochrome *P*450 (CYP) 3A, the most prominent CYP in humans, are also transported by P-glycoprotein, and such drugs have been shown to coordinately up-regulate both CYP3A and P-glycoprotein in human colon carcinoma cells (Schuetz et al., 1996). In these cells, incubation with 10 μM phenobarbital for 48–72 h resulted in a 14-fold increase in P-glycoprotein and a sixfold increase in CYP3A4/5, whereas phenytoin (10 μM) caused only a modest (30%) increase in the expression of P-glycoprotein and no increase in CYP3A4/5 (Schuetz et al., 1996). In contrast to the data from colon carcinoma cells, phenobarbital induced CYP3A4 but not P-glycoprotein in cultured human hepatocytes (Runge et al., 2000). Both phenobarbital and phenytoin are known to induce different hepatic CYP isoforms and thereby to increase their own metabolism, but phenobarbital is a much more potent enzyme-inducer than phenytoin (Tanaka, 1999). Phenytoin has been shown to induce CYP isoforms not only in the liver but also in the brain (Volk et al., 1995). In contrast to the in vitro data showing up-regulation of P-glycoprotein by phenobarbital and phenytoin in human colon carcinoma cells (Schuetz et al., 1996), the present in vivo study with these drugs did not indicate any marked up-regulation of P-glycoprotein in brain endothelial and parenchymal cells.

Frequently, drugs that up-regulate P-glycoprotein are also substrates for this protein (Silverman and Thorgeirsson, 1995; Fardel et al., 2001). The data of Schuetz et al. (1996) with phenytoin and phenobarbital from human colon carcinoma cells were therefore an indication that these drugs are substrates for P-glycoprotein. This is substantiated by data showing that incubation of P-glycoprotein-expressing neuroectodermal cells with phenytoin resulted in intracellular phenytoin levels that were only one fourth of that in P-glycoprotein-negative cells (Tishler et al., 1995). Furthermore, in a kidney epithelial cell line transfected with *mdr1* cDNA, phenytoin was transported by P-glycoprotein, which could be blocked by a P-glycoprotein inhibitor (Schinkel et al., 1996). Transport of phenytoin and phenobarbital by P-glycoprotein in the blood–brain barrier was demonstrated by showing that P-glycoprotein inhibitors increase penetration of these drugs into the brain (Löscher and Potschka, 2002). In line with this finding, Rizzi et al. (2001) reported that systemic administration of phenytoin in *mdr1* knockout mice, which lack P-glycoprotein in the blood–brain barrier, resulted in a 50% increase in hippocampal drug levels compared to wild-type mice. The latter authors also reported that treatment of mice with phenytoin for 4 days did not change *mdr1* expression in the hippocampus, which substantiates the present findings with P-glycoprotein immunostaining.

Most clinical studies on P-glycoprotein expression in brain tissue of pharmacoresistant patients with epilepsy demonstrated overexpression of P-glycoprotein in the hippocampus, i.e., the region which most often includes the epileptic focus in temporal lobe epilepsy (Löscher and Potschka, 2002). Following administration of kainate in rats, a popular model of temporal lobe epilepsy, transient up-regulation of P-glycoprotein and *mdr1* was determined in the hippocampus (Zhang et al., 1999; Rizzi et al., 2001; Seegers et al., 2002b). In contrast, neither phenobarbital nor phenytoin increased expression of P-glycoprotein in this brain region in the present experiments.

In conclusion, although both phenytoin and phenobarbital are substrates for P-glycoprotein (Löscher and Potschka, 2002), these drugs do not increase the expression of this multidrug transporter in the brain. This is important because otherwise, by up-regulation of P-glycoprotein, phenytoin and phenobarbital would restrict their own penetration into the brain. Furthermore, the present data strongly indicate that the P-glycoprotein overexpression in epileptogenic brain tissue of patients with drug-resistant epilepsy is not a consequence of chronic treatment with antiepileptic drugs such as phenytoin or phenobarbital. However, because normal (nonepileptic) rats or mice were used in the present and previous (Rizzi et al., 2001) experiments on the effects of drug treatment on P-glycoprotein/*mdr1* expression, it cannot be ruled out that epileptic animals or humans exhibit different responses to antiepileptic drug treatment.

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