Send Orders of Reprints at reprints@benthamscience.net

Current Pharmaceutical Design, **2013,** *19,* **000-000** **1**

P-Glycoprotein Contributes to Cell Membrane Depolarization of Hippocampus and Neocortex in a Model of Repetitive Seizures Induced by Pentylenetetrazole in Rats

Jerónimo A. Auzmendi[a], Sandra Orozco-Suárez[b,£], Ivette Bañuelos-Cabrera[c], María Eva González-Trujano[d], Eduardo Calixto González[d], Luisa Rocha[c,✱,£] and Alberto Lazarowski[a,e,✱,£]

[a]*Instituto de Biología Celular y Neurociencias "Prof. Dr. E. de Robertis" Fac. de Medicina, UBA. Argentina.* [b]*Unidad de Investigación Médica de Enfermedades Neurológicas. Centro Médico Nacional Siglo XXI, México, D.F., México.* [c] *Depto. Farmacobiología. Centro de Investigación y de Estudios Avanzados, Sede Sur. México D.F., México.* [d] *Instituto Nacional de Psiquiatría "Ramón de la Fuente", México, D.F.;* [e]*Instituto de Investigaciones en Fisiopatología y Bioquímica Clínica. Fac. de Farmacia y Bioquímica. Universidad de Buenos Aires. Argentina*

Abstract: P-glycoprotein (P-gp) has been associated with pharmacoresistance and mechanisms regulating the membrane potential. However, at present it is unknown if P-gp overexpression in brain is associated with changes in membrane depolarization in refractory epilepsy. Experiments were designed to evaluate the membrane depolarization and P-gp overexpression induced by repetitive pentilenetetrazole (PTZ)-induced-seizures. Wistar rats were daily treated with PTZ during 4 to 7 days (PTZ4 and PTZ7 groups), and the brain was used to evaluate membrane potential by *in vitro* electrophysiological procedures and using bis-oxonol dye, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)), a fluorescence dye voltage-sensitive to membrane potentials. Rats with repetitive PTZ-induced seizures demonstrated lower phenytoin-induced anticonvulsant effects, increased number of DiBAC4(3) fluorescence cells and P-gp overexpression in hippocampus and neocortex, as well as augmentation of the induced fEPSP in CA1 field. These changes were more evident in PTZ7 group. Phenytoin or phenytoin plus nimodipine (a P-gp antagonist) avoided the enhanced fEPSP and decreased DiBAC4(3) fluorescence in animals from PTZ4 group. However, in PTZ7 group these effects were evident only when phenytoin was combined with nimodipine. An additional flow cytometry study demonstrated increased intracellular accumulation of DiBAC4(3) in K562 leukemic cells that overexpress MDR-1 and COX-2 genes, and are refractory to specific cytotoxic agents. These results represent the first evidence supporting the notion that brain P-gp overexpression contributes to a progressive seizure-related membranes depolarization in hippocampus and neocortex. Further experiments should be carried out to confirm the role of P-gp on membrane depolarization and epileptogenesis process.

Keywords: P-glycoprotein; membrane depolarization; PTZ; refractory epilepsy; epileptogenesis.

1. INTRODUCTION

Epilepsy is one of the most prevalent neurological disorders affecting between 1% and 2% of the world population. It is characterized by recurrent, spontaneous, and unpredictable seizures. At cellular level, epilepsy is associated with altered intrinsic membrane excitability and excessive synchronous neuronal activity [1], alterations explained by augmented excitatory amino acid neurotransmission [2].

Approximately one third of patients with epilepsy do not have satisfactory control of seizures with antiepileptic drugs (AEDs) and it is suggested that drug resistant phenotype could be present at the early stage of the disease [3]. Several experimental and clinical reports indicate that pharmacoresistance in epilepsy is associated with overexpression of brain P-glycoprotein (P-gp), the product of MDR-1 gene [4,5]. P-gp is a membrane protein with transmembrane efflux activity responsible for transporting a wide variety of unrelated drugs from cells [6]. Through glutamate/NMDA receptor/cyclooxygenase-2 (COX-2) signaling, glutamate induces overexpression of P-gp in brain [7]. Indeed, P-gp as well as COX-2/prostaglandin E(2) pathway have been considered targets for increasing clinical response to current epilepsy treatments [8].

It is reported that MDR-1 gene upregulation could be acquired as a consequence of repetitive non-controlled seizures [9,10]. On the other hand, P-gp upregulation has been associated with pathological conditions such as inflammation tumors or hypoxia [11-13], supporting the idea that silent mechanisms could overexpress P-gp in brain before epilepsy and refractoriness becomes clinically evident. Indeed, Chengyun *et al*. [14] found P-gp overexpression in brain of patients with long-lasting refractory epilepsy associated with different causes, such as trauma, vascular injuries, encephalitis, cortical dysplasia, cavernous angioma and Sturge-Weber disease. Interestingly, seizures are an important neurologic sequel of brain hypoxia-ischemia [15], a condition associated with overexpression of P-gp [16,17].

On the other hand, a group of evidence indicates that P-gp can also decrease plasma membrane potential of several cell types [18,19] and modulates the swelling-activated Cl^- currents [20], both physiologic disturbances observed during brain hypoxia [21] and convulsive stress [22]. However, at present there are no studies focused to determine if P-gp over-expression correlates with changes in membrane potential in brain areas of subjects and/or experimental models of pharmacoresistant epilepsy.

For the present study, we proposed that the progressive brain P-gp overexpression associated with pharmacoresistance correlates with lower membrane potential. Using rats, we investigated the effects on membrane potential and pharmacosensitivity after repetitive seizures induced by Pentylenetetrazol (PTZ).

In brain slides, the membrane depolarization associated with P-gp expression was also investigated during the effects of phenytoin (PHT), an anticonvulsivant substrate of P-gp [23], nimodipine (NIMO), a P-gp blocker [24] and their combination (NIMO+PHT).

We also investigated if leukemic cells refractory to specific cytotoxic agents (Imatinib-resistant-K562 or IR-K562 cells) and overexpressing MDR-1 and COX-2 genes [25] show low membrane potential and its modification after NIMO administration.

*Address correspondence to this author at the Av. Caseros 1944 #9B, CABA (1152) - Argentina; Tel: 54-11-4304-2611; Emails: nadiatom@ffyb.uba.ar or alazarowski@gmail.com

✱L. Rocha and A. Lazarowski contributed equally to this study.

£.Members of GENIAR (Cyted 610RT0405)

1381-6128/13 $58.00+.00

2. METHODS

2.1. Animals

The experimental subjects were male Wistar rats, weighing 250-300 g at the beginning of the experiments. Animals were housed individually under controlled environmental conditions (22°C; 12; 12-h light-dark cycle; food and water *ad libitum*). Rats received a daily administration of saline solution (1 ml/kg, i.p.) for 1 week for habituation to manipulation. Experimental procedures were conducted according to the NIH guidelines for the Care and Use of Laboratory Animals and the principles presented in the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience and the CICUAL (Comite Institucional de Cuidado y Uso de Animales de Experimentación) from University of Buenos Aires (Argentina) and Center for Research and Advanced Studies (Mexico). All efforts were made to reduce the suffering by, and the number of animals used.

2.2. Pentylenetetrazol Induced Seizures and Effects of PHT *In vivo*

Animals received a daily administration of saline (1 ml/kg, i.p.) for 1 week in order to habituate to manipulation. Then, rats were submitted to one of the following protocols:

a) PTZ7+PHT group (n=8). Animals were daily treated with PTZ (45 mg/kg i.p.) during 7 days. Sixty minutes previous at the moment of the seventh PTZ application, rats received PHT (50 mg/kg i.p., freshly dissolved in saline, pH 11.2, in a volume of 3 ml/kg)

b) PTZ7 group (n=16). Rats were manipulated as previously described for PTZ7+PHT group, except that they received vehicle instead of PHT administration.

c) PTZ4+PHT group (n=8). Rats were manipulated as described above for the PTZ7+PHT group, except that PTZ was daily applied during 4 days. Sixty minutes previous at the moment of the fourth PTZ application, rats received PHT at the doses previously indicated.

d) PTZ4 group (n=16). Rats were manipulated as previously described for PTZ4+PHT group, except that they received vehicle instead of PHT administration.

e) PTZ1+PHT group (n=8). Rats received daily saline solution administration during seven days. Then, they received PHT at the doses previously indicated and sixty minute later, PTZ was applied.

f) PTZ1 group (n=8). Rats were manipulated as previously described for PTZ1+PHT group, except that they received vehicle instead of PHT administration.

g) Control group (n=10). Control animals were manipulated as described above for PTZ7 group but animals received daily vehicle administration instead of PTZ.

Immediately after each PTZ or vehicle administration, animals were located in individual cages during 30 min for behavioral evaluation. Latency to seizure activity was determined. After experiments, animals from PTZ4 and PTZ7 groups were used for measuring membrane potential and evaluation of P-gp protein expression in hippocampus and neocortex (see below). These brain areas were evaluated because they are involved in seizure activity expression and propagation. In addition, neocortex shows significant benzodiazepine receptor changes as consequence of repetitive PTZ administration [26].

2.3. Evaluation of Membrane Potential *In vitro*

2.3.a. Hippocampal Slices Preparation

Immediately after the last PTZ administration and according to procedures previously described [27], animals from PTZ4 (n=6) and PTZ7 (n=6) groups were anesthetized and then perfused transcardially with cold, Artificial Cerebral Spinal Fluid (CSF, pH 7.4) containing (in mM): 229 sucrose, 2.0 KCl, 10 dextrose, 26 $NaHCO_3$, 1.2 Na_2HP0_4-$7H_2O$, and 1.5 $MgCl_2$, bubbled with a mixture of 95%O_2/ 5%CO_2, (carbogen). Control tissue was obtained from a control group of animals (n=5) manipulated as described above but without seizure activity.

Hippocampal coronal slices, 450 μm thick, were incubated for 60 min at room temperature in CSF containing (in mM): 125 NaCl, 2 KCl, 10 dextrose, 26 $NaHCO_3$, 1.2 Na_2HP0_4-$7H_2O$, 3 $MgCl_2$, and 3 $CaCl_2$, bubbled with carbogen at pH 7.4. Slices were transferred to a recording chamber maintained at 22±2°C and constantly perfused (2.5–3.0 ml/min) with CSF.

2.3.b. Measuring Membrane Potential by In vitro Electrophysiological Techniques

To evoke CA1 field excitatory postsynaptic potentials (fEPSPs), stimulation pulses were applied with bipolar electrodes placed in Collateral Shaffer fibers. fEPSPs were recorded in the *stratum pyramidale* of CA1 using glass micropipettes (4–6 MΩ) filled with NaCl solution (0.5 M). Responses were displayed on an oscilloscope as well as digitalized in a PC computer for storage and off-line analysis (Lab View system, National Instruments, Austin TX). Test stimuli (100 msec duration, 0.1 Hz) were administered at a stimulation intensity (20 –70 μA) that elicited a fEPSP with an amplitude that was 50% of maximum fEPSP amplitude. The identification of CA1 fEPSPs was based on the following criteria: (1) negative (sink) waveform restricted to the *s. lucidum*; (2) medium latency (10 msec); and (3) slow time course (average 7.5 msec). CA1 fEPSPs were monitored for 60 min according with pilot experiments in which we found that exposure to the vehicle solutions and electrical stimulation for 120 min had no effect on baseline fEPSPs (data no shown).

In vitro electrophysiology experiments were designed to investigate changes in fEPSP amplitude alone or in presence of PHT (5 μM), NIMO (5 μM) or PHT plus NIMO, of brain samples obtained from animals of PTZ4 and PTZ7 groups (see above). Effects were calculated as percentage of the average fEPSP amplitude recorded during the baseline period (5-15 min). Drugs were dissolved as stock solutions and working solutions were prepared fresh daily.

2.3.c. Imaging Membrane Potential Using DIBAC4(3)

The present experiment was designed to evaluate changes in membrane potential using bis-oxonol dye, bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC4(3)), a fluorescence dye voltage-sensitive to membrane potentials. The dye partition between the cell membrane and the cytosol is a function of membrane potential. Depolarization of the membrane leads to a sequestration of the dye into cytosol and is associated with an increase in the fluorescence intensity. In contrast, the dye concentrates in the cell membrane during membrane hyperpolarization, leading to a decrease of cytoplasmic fluorescence intensity [28]. Due to these characteristics, DiBAC4(3) represents a selective marker for plasma membrane [29] and can reveal the depolarized condition of cells irrespective the underlying mechanisms [30].

Immediately after the last PTZ administration, rats from PTZ4 (n=6) and PTZ7 (n=6) groups were sacrificed under light ether anesthesia and brains were quickly removed. Coronal sections (80 μm thick, containing both, hippocampus and neocortex) were obtained.

Control tissue was acquired from a control group of animals (n=6) manipulated as described above but without seizure activity. In the dark for at least 30 min at 37°C, sections were soaked in the voltage-sensitive dye DiBAC4(3) (2 μM, Molecular Probes ®) in slides alone or in presence of PHT (5 μM), NIMO (5 μM) or PHT plus NIMO. Then, they were imaged with an Olympus BX51W1 fluorescent microscope (excitation at 488 nm, emission at 530 nm). For fluorescent imaging and collection of the data, Image Pro Plus 5.1 software was used for cell counting (40X).

2.4. Evaluation of P-gp Expression

Brain tissue obtained from control group (n=6), PTZ4 (n=6) and PTZ7 (n=6) groups was fixed with glutaraldehide (0.2%) and paraformaldehide (4%) at 4°C during 2 days, and then cryoprotected in sacarose (30%). Frozen sections of 10 µm were obtained with a cryostat, and mounted on gelatin-coated slides, then washed with PBS buffer containing 0.025% Triton X-100, for 15 minutes. The blockage was made with normal horse serum (1: 200) in buffer PBS during 1 h. Antibodies were dissolved in PBS containing 1% v/v normal horse serum and 0.3% v/v Triton X-100, pH 7.4. The slides were incubated for 48 h at 4°C with anti-P-gp primary antibody (C-494, Signet Laboratories, Dedham, MA) (1: 500) and then incubated with the secondary antibody anti-mouse conjugated with fluorescein isothiocyanate during 3 hours (Zymed Lab Inc., USA) (1: 200). The contrast was made with propidium iodide (Sigma Chemical, St. Louis, MO). Finally the sections were mounted with Vectashield mounting medium (Vector lab. USA). Controls were obtained by omitting the primary antibody. A microscope Olympus BX-50 equipped with a digital cooled camera (Coolpix) was used to analyze the pictures.

2.5. Flow Cytometry Measurements of Membrane Potential in Cell Culture

As it was previously described, DiBAC4(3) is a fluorescence dye voltage-sensitive to membrane potentials that may allow the evaluation of changes in membrane potential. Increased fluorescence intensity results from depolarization of the membrane leading to a sequestration of the dye into cytosol [28]. On the other hand, it is known that multidrug resistance gene (MDR-1) that encodes the drug efflux transporters such as P-gp, has been reported to be highly expressed in brain of patients with pharmacoresistant epilepsy [4,31].

A complementary flow cytometry study was focused to evaluate DiBAC4(3) intracellular accumulation under basal conditions in IR-K562 leukemic cells that overexpress MDR-1 and COX-2 genes. Flow cytometry assays were set up with 2.5 to 3.0 × 10^6 cells/ml in buffer with DiBAC4(3) at 5 µM (Molecular Probes, B438). The intracellular and extracellular dye concentrations were allowed to equilibrate at 37°C/5% CO2 for 30 min. In order to determine the role of P-gp expression in DiBAC4(3) fluorescence, NIMO was added in the culture (35 µM), 30 min before DiBAC4(3) in an additional flow cytometry assay. In controls, the assays were performed in the absence of DiBAC4(3). The excitation and emission wavelengths were optimized prior to analysis and set at 488 and 525 nm, respectively, with a 515 nm cutoff wavelength. The fluorescent readings were stored in a computer using SoftMax Pro Version 3.1.1 (Molecular Devices). The concentration to produce 50% of maximal change (EC50) was calculated by using Prism version 2.0 (GraphPad). The experiment was repeated three times with a similar trend and results of representative experiments are depicted in the results.

2.6. Statistical Analysis

Values were expressed as mean ± S.E.M. The latencies to PTZ-induced seizures were evaluated by an unpaired t-test. Analysis of Variance (ANOVA) followed by a post hoc Dunnet test were applied to examine changes on fEPSP amplitude and number of cell labeled with DiBAC4(3) dye. In all statistical comparisons, a *p* value of less than 0.05 was considered statistically significant.

3. RESULTS

3.1. Pentylenetetrazol Induced Seizures and Effects on Membrane Potential

3.1.1. Control Conditions

In all control animals without previous experience with seizure activity (PTZ1), PTZ induced clonic seizures, with a latency of 58±2.8 s. Animals pretreated with PHT (PTZ1+PHT group) demonstrated a significant increase in the seizure latency (174%, $^{@}p<0.001$, Fig. **1**). Concerning fEPSPs recordings in CA1 In brain slices of control animals, orthodromic stimulation applied in Shaffer collaterals induced a response of 0.26 ± 0.012 mV. This response was not modified throughout the 60 min of experimental procedure (101 ± 0.02% at the beginning versus 98 ± 12% at the end) (Fig. **2**). Under control conditions, hippocampus and neocortex showed immunoreactivity to P-gp exclusively located in capillary endothelial cells (Fig. **1b**). These brain areas also demonstrated low DiBAC4(3) fluorescence, suggesting that the plasma membrane potential was intact and the cells can exclude the dye (Fig. **3a** and Fig. **4**).

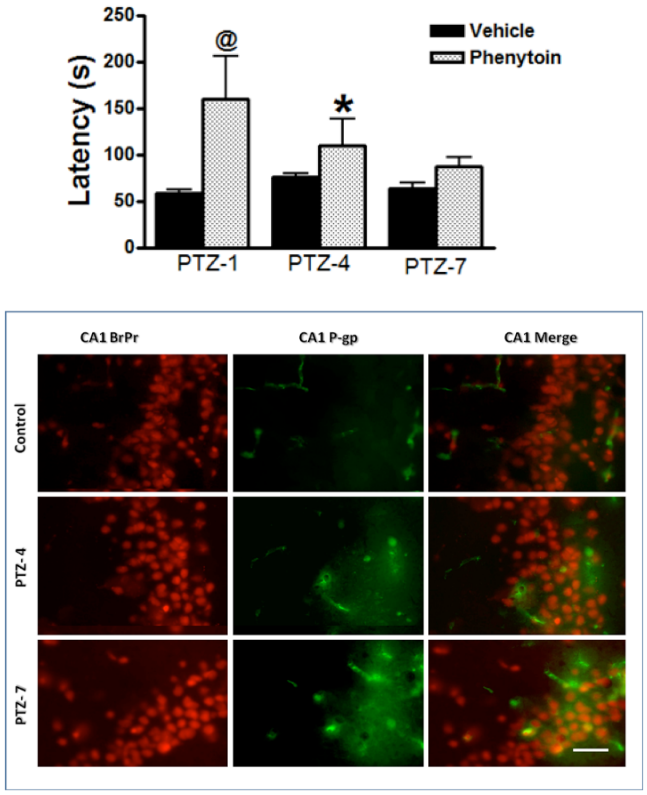

Fig. (1). a. Latencies to PTZ-induced seizure alone and after PHT administration. Values indicate the latency to seizure activity evaluated after a single PTZ injection (PTZ-1), or following 4 (PTZ-4) or 7 administrations (PTZ-7), alone or in animals treated with PHT before the last PTZ injection. Notice that PHT augmented the latency PTZ-1 group ($p<0.001$), an effect that was lower in PTZ-4 and no evident in PTZ-7 group.

b. Double immunostaining of CA1 hippocampal sections of animals from Control (upper panels), PTZ4 (middle panels) and PTZ7 (lower panels) groups. Cell nuclei were stained in red with propidium iodide (left panels), P-glycoprotein expression is shown in green (middle panels) and the merged images are presented on the right panels. Scale bar, 30 µm.

3.1.2. PTZ4 Group

Daily PTZ administration induced clonic seizures. As consequence of the fourth PTZ administration, latency for clonic seizures was similar to that showed by PTZ1 group, whereas PHT pretreatment (PTZ4+PHT group) significantly delayed their appearance ($*p<0.05$, Fig. **1**). The initial fEPSP amplitude values obtained from PTZ4 group (0.35 ± 0.12 mV) during the *in vitro* electrophysiology experiments were analogous to those recorded under control situation. Thereafter, a significant increase in the amplitude of the fEPSP (42%, $p<0.001$) was detected at 30 min and remained constant throughout the experimental procedure. This effect

was avoided when slices were incubated with PHT or PHT plus NIMO, whereas NIMO alone did not induce significant effects on the progressive augmentation of the fEPSP (Fig. **2B**). When compared with control group, sections obtained from PTZ4 group demonstrated intense P-gp staining located in microvessels (Fig. **1b**), as well as higher number of DiBAC4(3) fluorescence cells ($p<0.001$) in hippocampus and neocortex (Fig. **3b** and Fig. **4**). This last effect was avoided when slices were incubated with NIMO, PHT or NIMO plus PHT (Fig. **4**).

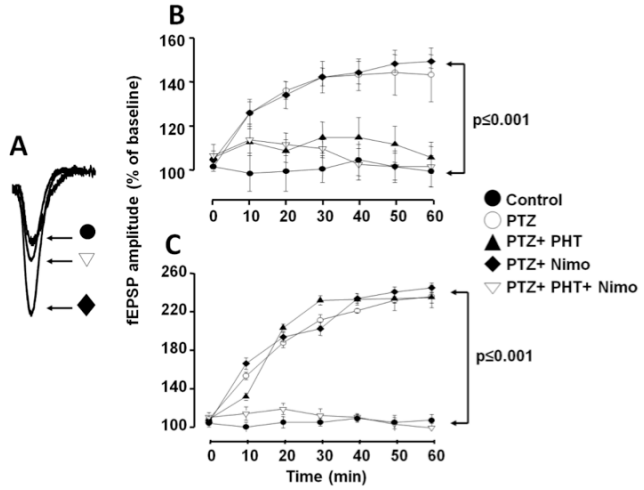

Fig. (2). Evaluation of the synaptic response (**A**) based on the percentage of change of the fEPSP amplitude in hippocampus of rats that received daily Pentylenetetrazol (PTZ) administration during 4 (PTZ4, **B**) and 7 (PTZ7, **C**) days. Notice the hyperexcitability in CA1 field of both, PTZ4 and PTZ7 groups (PTZ). This effect was avoided in PTZ4 group when slices were incubated in presence of phenytoin (PTZ+PHT) or phenytoin plus nimodipine (PTZ+PHE+Nimo), but not with nimodipine alone (PTZ+Nimo). In contrast, hyperexcitability of PTZ7 was blocked only when slices were incubated in presence of phenytoin plus nimodipine (PTZ+PHT+Nimo). The values represent the average ± SEM.

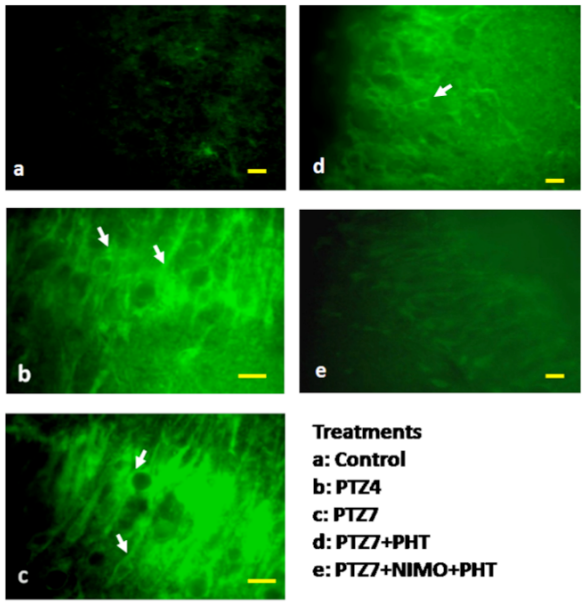

Fig. (3). Demonstration of membrane depolarization of CA1 cells labeled with DIBAC4(3). Hippocampal sections obtained from Control, PTZ4, PTZ7, and loaded with fluorescent DiBAC4(3) alone (**a**, **b** and **c**, respectively). Samples from PTZ7 were also loaded with PHT (**d**) or Nimodipine plus PHT (**e**). Arrows indicate cells loaded with DiBAC4(3). Scale bars: 10 μm (**a**, **d** and **e**) and 20 μm (**b**, and **c**).

3.1.3. PTZ7 Group

Daily PTZ administration during seven days induced clonic seizures in all animals, and their expression was similar to that observed following one (PTZ1) or four injections (PTZ4 group). The clonic seizures produced after the seventh injection demonstrated latencies similar to those recorded for the PTZ1 and PTZ4 groups. PHT pretreatment (PTZ7+PHT group) did not modify the seizure expression and latency (Fig. **1**). The initial fEPSP amplitude values obtained from PTZ7 group (0.35 ± 0.12 mV) during the *in vitro* electrophysiology experiments were analogous to those recorded under control situation. Thereafter, there was a 136% increase of the induced fEPSP in CA1 field at 60 min of the experimental procedure, an effect significantly higher when compared with both, control and PTZ4 groups ($p<0.001$). Indeed, the augmentation of fEPSP was only avoided when slices were incubated in PHT plus NIMO, but not in PHT or NIMO alone (Fig. **2C**). Brain sections obtained from PTZ7 group demonstrated higher P-gp immunoreactivity in capillary endothelium and surrounding cells, as well as higher DiBAC4(3) fluorescence (Fig. **1b**; Fig. **3c**) as compared with other groups cells. This increased DiBAC4(3) intensity of fluorescence was mildly modified by PHT (Fig. **3d**) but clearly diminished after the treatment with NIMO+PHT (Fig. **3e**). An increased number of DiBAC4(3) positive cells ($p<0.001$) in hippocampus and neocortex was observed (Fig. **4**). Although a low number of DiBAC4(3) fluorescence cells was detected when tissue was incubated with NIMO plus PHT, but not in presence of PHT or NIMO alone, none drug treatment was able to completely restore the basal conditions for DiBAC4(3) fluorescence, suggesting partial restitution of the membrane potential in slices from animals receiving repetitive PTZ administration during 7 days (Fig. **4**).

3.2. Membrane Depolarization in Cell Culture

Flow cytometry, which permits the simultaneous examination of multiple cellular characteristics at the single cell level, was used to determine the relationship between cellular depolarization and cell size by examining DiBAC4(3) fluorescence and the forward light scattering property of MDR-1 positive IR-K562 cells, respectively. A population of IR-K562 cells with plasma membrane depolarization was detected, a situation not observed under control situation or in presence of NIMO ($p<0.001$). Data from DiBAC4(3) fluorescence contour plot suggest that cellular depolarization occurs in a homogeneous population of cells (Fig. **5**).

4. DISCUSSION

The results obtained from the present study support the notion that daily PTZ-induced seizures produces the progressive acquisition of pharmacoresistant phenotype, a situation associated with an increased brain P-gp expression [32] and high membrane depolarization in brain areas such as hippocampus and cortex. A similar situation was previously detected after daily 3-mercaptopropionic acid-induced seizures during 7 days, resulting in increased P-gp expression in blood brain barrier, astrocytes and neurons as well as loss of anticonvulsant effects of PHT and phenobarbital [31, 33,34].

In agreement with the results obtained, a greater membrane depolarization is found in animals exposed to repetitive PTZ administration during seven, but not four days, an effect associated with P-gp overexpression and acquisition of pharmacoresistant phenotype. This condition was associated with membrane depolariztion according with the larger fEPSPs found during electrophysiological experiments and high DiBAC4(3) fluorescence. The hypothesis that the P-gp overexpression is involved in membrane depolarization in pharmacoresistance was supported by the following results: a) the high membrane depolarization found in animals with pharmacoresistant phenotype and overexpressing P-gp (PTZ7) was restored when PHT was combined with NIMO, a P-gp antagonist; b) the elevated plasma membrane potential revealed by high DIBAC4(3) fluorescence in cells refractory to specific cytotoxic

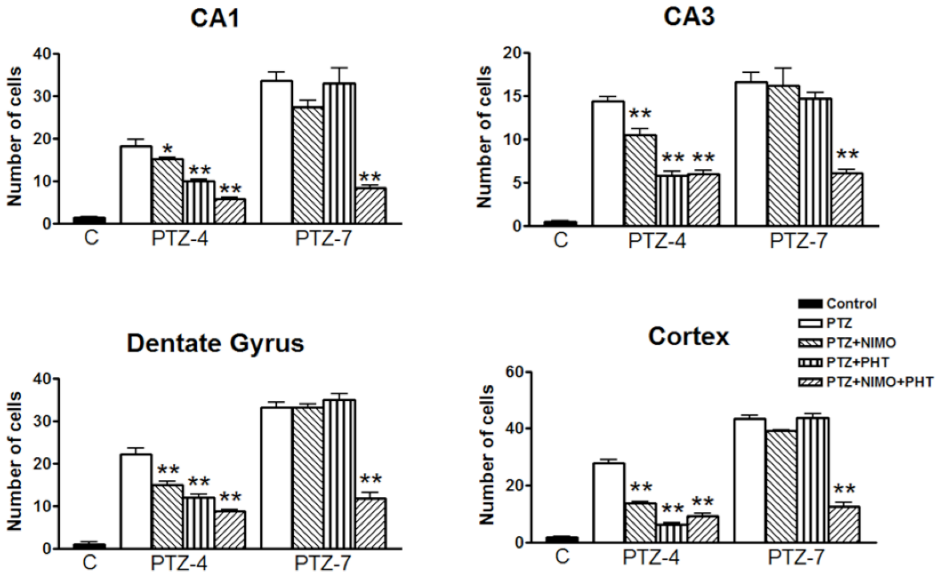

Fig. (4). Number of cells labeled with DIBAC4(3) demonstrating membrane depolarization in hippocampal sections obtained from Control and PTZ groups (PTZ-4 and PTZ-7) alone or in presence of nimodipine (PTZ+NIMO), phenytoin (PTZ+PHT) or both (PTZ+NIMO+PHT). *p<0.05 versus PTZ group; **p<0.01 versus PTZ group.

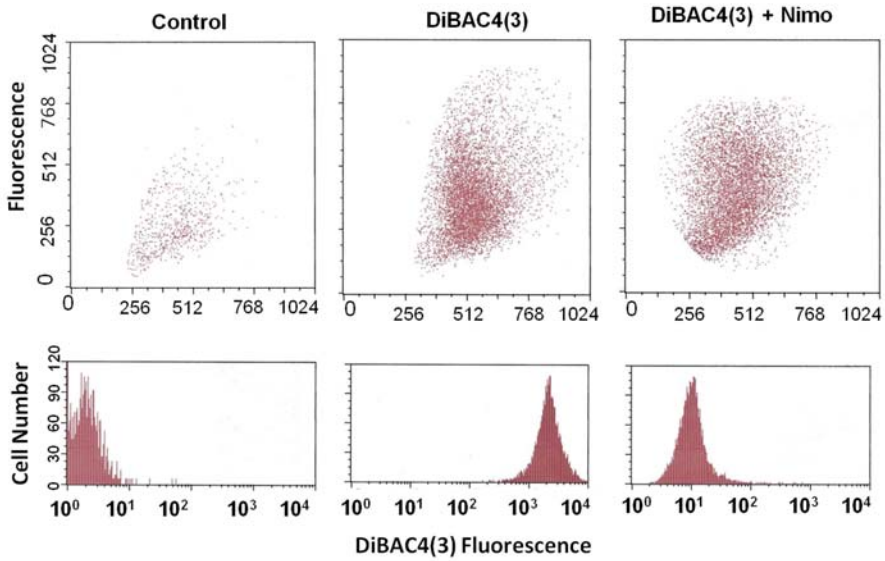

Fig. (5). Flow cytometry study of IR-K562 cells that overexpress MDR-1 and COX-2 genes and stained with DiBAC4(3) as described in Materials and Methods. Representative histograms for DiBAC4(3) fluorescence (lower panels) are shown for each condition. An increase in DiBAC4(3) fluorescence (2544 ± 338) indicating that cells have depolarized membranes (middle panels), as compared with the low accumulation of DiBAC4(3) detected after P-gp inhibition with nimodipine (right panels) (p<0.01). Negative control (left panels) showed little number of cells detected.

agents and overexpressing MDR-1 and COX-2 genes (IR-K562 leukemic cells) was avoided in presence of NIMO.

P-gp belongs to the ABC superfamily of ABC transporters encoded by different genes. In addition to the very popular drug pump model, studies indicate that P-gp may mediate drug resistance through channels function regulation [35]. Supporting this idea, it has been described that MDR1 protein behaves as some type of Cl^- transporter, stimulates Cl^- channel activity and may alter cell volume in several cell types [36]. Its overexpression leads to decreased plasma membrane electrical potential by increasing Cl^- permeability and thus decreasing the dominance of membrane potential by K^+ conductance, and leads to altered pHi regulation by disrupting normal Cl^-/ H^+ CO_3 exchange processes [18,19]. According to this information, the P-gp overexpression in hippocampus and neocortex induced by repetitive seizures could contribute to crucial factors detected in several types of epilepsy such as the intracellular acidosis associated with altered Na^+/H^+ exchange [37], enhanced extracellular concentration of potassium [38], and particularly in the increased membrane depolarization [39]. The P-gp-induced depolarization associated with high glutamate and low GABA interictal release in pharmacoresistant epilepsy [40] could be sufficient for worsening the clinical seizures. We suggest that a variety of phenomena reported on the MDR literature should be re-examined using experimental models of pharmacoresistant epilepsy to determine whether they are unequivocally due to MDR protein functional overexpression.

Contradictory results have been described concerning pro- and anticonvulsant effects of NIMO, a Ca^{2+} channel antagonist of the dihydropyridine type, as observed in both patients with epilepsy and in experimental models [41-44]. Here, we demonstrated that NIMO alone was able to reduce the membrane depolarization in leukemic IR-K562 cells overexpressing P-gp. However, the recovery of potential membrane of depolarized slides in hippocampus and neocotex (*in vitro* experiments) was induced only when NIMO was combined with PHT. Similar effects with complete control of seizures and normalized flow of PHT in hippocampus were previously reported using 3-mercaptopropionic acid-induced seizures [33]. These observations indicate that membrane depolarization as well as pharmacoresistant phenotype are the result of several simultaneous factors where P-gp may play an important role.

Antiepileptic drug refractoriness has been explained by the "drug transporter overexpression" and the "reduced drug-target sensitivity" hypotheses [9]. On the other hand, it is so mentioned that "seizures induce seizures" and "seizures without control induce refractoriness". In these contexts, brain overexpression of P-gp inducing lower membrane potential could first help to develop the seizures of different epileptic syndromes secondary to wide spectrum of etiologies, some of them initially without or with sporadic seizures, and also contribute with a progressive increases of seizure frequency and severity that later develops a epileptic refractory phenotype, worsening the clinical features and prognostic of the disease. This group of evidence supports that brain P-gp overexpression correlates with a progressive acquisition of refractoriness and sustain a third theory to explain pharmacoresistant epilepsy based on the inherent severity of epilepsy [45].

The challenge ahead is to define and understand the effects associated with brain P-gp brain overexpression, both previously and during epilepsy development. For example, it is known that PTZ-induced chemical kindling is associated with a rise in glutamate release which is apparent early in the epileptogenic process [46]. It is possible that the high glutamate release induced by epileptic seizures since the early stages of the epileptogenic process induces a progressive over-expression of P-gp in brain through glutamate/NMDA receptor/cyclooxygenase-2 (COX-2) signaling [7]. On the other hand, COX-2 inhibition could represent a good strategy to reduce or avoid the epileptic refractory phenotype through downregulation MDR-1 expression [7,8,25].

Finally, it is also important to distinguish between the truly drug-transporter properties of this protein leading to the pharmacoresistant phenotype, from those related with plasmatic membrane depolarization inducing pro-epileptic effects. Perhaps both features of P-gp are simultaneously acting as left and right hands of the same boxer fighting against cell death. Future therapeutic strategies should include combined treatments of antiepileptic drugs with P-gp inhibitors to restore the normal membrane potential and reduce seizure activity [8,47].

HIGHLIGHTS

► We studied the membrane depolarization in rat brain slides after PTZ-induced seizures

►Highlight a progressive P-gp overexpression associated with a membrane depolarization

► Highlight the recovery of membrane potential after combined PHT+NIMO administration

► We postulate a potential role for P-gp brain overexpression in epileptogenesis

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

We thank Valeria Sismondi (Center of Molecular Diagnostic-Bs.As), for her assistance to perform flow cytometry study, and Mr. Hector Vazquez Espinosa for their excellent technical assistance. This study was supported by the National Council for Sciences and Technology of Mexico (grant 98386), Red GENIAR from Programa Iberoamericano de Ciencia y Tecnología para el Desarrollo (CYTED), CECYT-CONACyT ME-PA03-SV-027 and Instituto de Ciencia y Tecnología del Distrito Federal (PFTUt-08027).

ABBREVIATIONS

P-gp	=	P-glycoprtein
MDR	=	Multidrug resistance
PTZ	=	Pentylenetetrazole
PHT	=	Phenytoin
NIMO	=	Nimodipine
DiBAC4(3)	=	Bis-oxonol dye, bis-(1,3-dibutylbarbituric acid) trimethine-oxonol
fEPSPs	=	Field excitatory postsynaptic potentials

REFERENCES

[1] Cohen I, Navarro V, Clemenceau S, Baulac M, Miles R. On the origin of interictal activity in human temporal lobe epilepsy *in vitro*. Science 2002; 298 (5597): 1418-21.

[2] Avoli M, Bernasconi A, Mattia D, Olivier A, Hwa GG. Epileptiform discharges in the human dysplastic neocortex: *in vitro* physiology and pharmacology. Ann Neurol 1999; 46(6): 816-26.

[3] Eger Ch. Pharmacoresistance: modern concept and basic data derived from human brain tissue. Epilepsia 2004; 44: Suppl. 5: 9-15.

[4] Lazarowski A, Sevlever G, Taratuto A, Massaro M, Rabinowicz A. Tuberous sclerosis associated with MDR1 gene expression and drug-resistant epilepsy. Pediatr Neurol 1999; 21: (4): 731-4.

[5] Rizzi M, Caccia S, Guiso G, *et al.* Limbic seizures induce P-glycoprotein in rodent brain: functional implications for pharmacoresistance. J Neurosci2002; 22: 5833-9.

[6] Endicott JA, Ling V. The biochemistry of Pglycoprotein mediated multidrug resistance. Ann Rev Biochem 1989; 58: 137-71.

[7] Bauer B, Hartz AM, Pekcec A, Toellner K, Miller DS, Potschka H. Seizure-induced up-regulation of P-glycoprotein at the blood-brain barrier through glutamate and cyclooxygenase-2 signaling. Mol Pharmacol 2008; 73: 1444-53.

[8] Robey RW, Lazarowski A, Bates SE. P-glycoprotein-a clinical target in drug-refractory epilepsy? Mol Pharmacol 2008; 73: 1343-6.

[9] Sisodiya, SM. Mechanisms of antiepileptic drug resistance. Curr Opin Neurol 2003; 16: 197-201.

[10] Kwan P, Sills GJ, Butler E, Gant TW, Meldrum BS, Brodie MJ. Regional expression of multidrug resistance genes in genetically epilepsy-prone rat brain after a single audiogenic seizure. Epilepsia 2002; 43(11): 1318-23.

[11] Roberts DJ, Goralski KB. A critical overview of the influence of inflammation and infection on P-glycoprotein expression and activity in the brain. Expert Opin Drug Metab Toxicol 2008; 4: 1245-64.

[12] Kunishio K, Okada M, Matsumoto Y, Nagao S, Nishiyama Y. Technetium-99m-sestamibi single photon emission computed tomography findings correlated with P-glycoprotein expression in pituitary adenoma. J Med Invest 2006; 53: 285-91.

[13] Comerford KM, Wallace TJ, Karhausen J, Louis NA, Montalto MC, Colgan SP. Hypoxia-inducible factor-1-dependent regulation of the multidrug resistance (MDR1) gene. Cancer Res 2002; 62: 3387-94.

[14] Chengyun D, Guoming L, Elia M, Catania MV, Qunyuan X. Expression of multidrug resistance type 1 gene (MDR1) P-glycoprotein in intractable epilepsy with different aetiologies: a double-labelling and electron microscopy study. Neurol Sci 2006; 27: 245-51.

[15] Björkman ST, Miller SM, Rose SE, Burke C, Colditz PB. Seizures are associated with brain injury severity in a neonatal model of hypoxia-ischemia. Neuroscience 2010; 166: 157-67.

[16] Lazarowski AJ, García-Rivello HJ, Vera Janavel GL, *et al.* Cardiomyocytes of chronically ischemic pig hearts express the MDR-1

gene-encoded P-glycoprotein. J Histochem Cytochem 2005; 53: 845-50.

[17] Lazarowski A, Caltana L, Merelli A, Rubio M, Ramos A, Brusco A. Neuronal mdr-1 gene expression after experimental focal hypoxia: a new obstacle for neuroprotection?. J Neurol Sci 2007; 258: 84-92.

[18] Wadkins RM, Roepe PD. Biophysical aspect of P-glycoprotein mediated multidrug resistance. Int Rev Cytol 1997; 171: 121-65.

[19] Roepe PD. What is the precise role of human MDR 1 protein in chemotherapeutic drug resistance? Curr Pharm Des 2000; 6: 241-60.

[20] Vanoye C, Castro A, Pourcher T, Reuss L, Altenberg G. Phosphorylation of P-glycoprotein by PKA and PKC modulates swelling-activated Cl- currents. Am J Physiol 1999; 276: C370-C378.

[21] Müller M. Effects of chloride transport inhibition and chloride substitution on neuron function and on hypoxic spreading-depression-like depolarization in rat hippocampal slices. Neuroscience 2000; 97: 33-45.

[22] Le Duigou C, Bouilleret V, Miles R. Epileptiform activities in slices of hippocampus from mice after intra-hippocampal injection of kainic acid. J Physiol 2008; 586: (Pt 20): 4891-904.

[23] Neerati P, Ganji D, Bedada SK. Study on in situ and *in vivo* absorption kinetics of phenytoin by modulating P-glycoprotein with verapamil in rats. Eur J Pharm Sci 2011; 44: 27-31.

[24] Onod, JM, Nelson KK, Taylor JD, Honn KV. *In vivo* characterization of combination antitumor chemotherapy with calcium channel blockers and cis diamminedichloroplatinum(II). Cancer Res 1989; 49: 2844-50.

[25] Arunasree KM, Roy KR, Anilkumar K, Aparna A, Reddy GV, Reddanna P. Imatinib-resistant K562 cells are more sensitive to celecoxib, a selective COX-2 inhibitor: role of COX-2 and MDR-1. Leuk Res 2008; 32: 855-64.

[26] Rocha L, Ackermann RF, Engel JJr. Chronic and single administration of pentylenetetrazol modifies benzodiazepine receptor-binding: an autoradiographic study. Epilepsy Res 1996; 24: 65-72.

[27] Calixto E, Thiels E, Klann E, Barrionuevo G. Early maintenance of hippocampal mossy fiber-long-term potentiation depends on protein and RNA synthesis and presynaptic granule cell integrity. J Neurosci 2003; 23: 4842-9.

[28] Wilson H, Chused T.. Lymphocyte membrane potential and $Ca2^{+}$-sensitive potassium channels described by oxonol dye fluorescence measurements. J Cell Physiol 1985; 125: 72-81.

[29] Bortner CD, Gomez-Angelats M, Cidlowski JA. Plasma membrane depolarization without repolarization is an early molecular event in anti-Fas-induced apoptosis. J Biol Chem 2001; 276: 4304-14.

[30] Willmott NJ, Wong K, Strong AJ. Intercellular Ca^{2+} waves in rat hippocampal slice and dissociated glial-neuron cultures mediated by nitric oxide. FEBS Letters 2000; 487: 239-47.

[31] Lazarowski A, Ramos AJ, García-Rivello H, Brusco A, Girardi E. Neuronal and glial expression of the multidrug resistance gene product in an experimental epilepsy model. Cell Mol Neurobiol 2004; 24: 77-85.

Received: January 29, 2013 **Accepted: March 18, 2013**

[32] Liu X, Yang Z, Yang J, Yang H.Increased P-glycoprotein expression and decreased phenobarbital distribution in the brain of pentylenetetrazole-kindled rats. Neuropharmacology 2007; 53: 657-63.

[33] Höcht C, Lazarowski A, Gonzalez NN, *et al.* Nimodipine restores the altered hippocampal phenytoin pharmaco-kinetics in a refractory epileptic model. Neurosci Lett 2007; 413 168-72.

[34] Höcht C, Lazarowski A, Gonzalez NN, *et al*. Differential hippocampal pharmacokinetics of phenobarbital and carbamazepine in repetitive seizures induced by 3-mercaptopropionic acid. Neurosci Lett 2009; 453(1): 54-7.

[35] Mizutani T, Masuda M, Nakai E, *et al.* Genuine functions of P-glycoprotein (ABCB1). Curr Drug Metab 2008; 9: 167-74.

[36] Valverde MA, Diaz M, Sepulveda FV, Gill DR, Hyde SC, Higgins CF. Volume-regulated chloride associated with the human multidrug-resistance P-glycoprotein. Nature 1992; 355: 830-3.

[37] Siesjö BK, von Hanwehr R, Nergelius G, Nevander G, Ingvar M. Extra- and intracellular pH in the brain during seizures and in the recovery period following the arrest of seizure activity. J Cereb Blood Flow Metab 1985; 5: 47-57.

[38] Durand DM, Park EH, Jensen AL. Potassium diffusive coupling in neural networks. Philos Trans R Soc Lond B Biol Sci 2010; 365: 2347-62.

[39] Prince DA, Connors BW. Mechanisms of interictal epileptogenesis.. Adv Neurol 1986; 44: 275-99.

[40] Luna-Munguia H, Orozco-Suarez S, Rocha L. Effects of high frequency electrical stimulation and R-verapamil on seizure susceptibility and glutamate and GABA release in a model of phenytoin-resistant seizures. Neuropharmacology 2011; 61: 807-14.

[41] Czuczwar SJ, Gasior M, Janusz W, Kleinrok Z. Influence of flunarizine, nicardipine and nimodipine on the anticonvulsant activity of different antiepileptic drugs in mice. Neuropharmacology 1992; 31: 1179-83.

[42] De Falco FA, Bartiromo U, Majello L, Di Geronimo G, Mundo P. Calcium antagonist nimodipine in intractable epilepsy. Epilepsia 1992; 33, 343-5.

[43] Kulak W, Sobaniec W. Anticonvulsant effects of calcium antagonists in the experimental seizures in the rat. Neurosci Jpn1993; 19: 107-12.

[44] Gasior M, Kamiñski R, Brudniak T, Kleinrok Z, Czuczwar SJ. Influence of nicardipine, nimodipine and flunarizine on the anticonvulsant efficacy of antiepileptics against pentylenetetrazole in mice. J Neural Transm 1996; 103: 819-31.

[45] Rogawski MA, Johnson MR. Intrinsic severity as a determinant of antiepileptic drug refractoriness. Epilepsy Curr 2008; 8: 127-30.

[46] Rocha L, Briones M, Ackermann RF, *et al.* Pentylenetetrazol-induced kindling: early involvement of excitatory and inhibitory systems. Epilepsy Res 1996; 26: 105-13.

[47] Lazarowski A, Czornyj L, Lubienieki F, Girardi E, Vazquez S, D'Giano C. ABC transporters during epilepsy and mechanisms underlying multidrug resistance in refractory epilepsy. Epilepsia 2007; 48 Suppl 5: 140-9.