CHAPTER V

DISCUSSION

The epilepsy model induced by systemic administration of pilocarpine in rats has been proposed as an animal model resembling some aspect of human temporal lobe epilepsy (Cavalheiro et al., 1991; Mello et al., 1993). It produces morphological, electroencephalographic and behavioral features similar to the most form of human complex partial seizures (Mello et al., 1993). Pilocarpine is a muscarinic agonist which induced partial seizures that secondarily generalize, leading to status epilepticus (SE). Several studies suggested that muscarinic cholinergic transmission is involved in the onset of seizures (Maslansky, Powelt, and Diemengiant, 1994; Hamilton, Loose, and Qi, 1997) and causes an imbalance between excitatory and inhibitory amino acid transmittion that can result in the generation of SE observed in animals acutely treated with pilocarpine (Priel and Albuquerque, 2002). They also found that the suppression of M-current, a voltage-dependent K^+ current, by pilocarpine results in an increase of firing rate (Hamilton, Loose, and Qi, 1997; Priel and Albuquerque, 2002). SE starts within 30-50 min after the administration of pilocarpine and lasts for 8-15 h. This acute period is followed by recurrent seizures over a period of 24-48 h (Cavalheiro et al., 1991; Turski et al., 1989). SE is accompanied by widespread brain damage is followed by a silent (seizure-free) period and, finally, by a chronic period characterized by spontaneous recurrent seizures SRSs, whose characteristic resemble those of human temporal lobe epilepsy. The SRSs occur at a frequency of about two to five per week and last for the remaining life of the animal (Turski et al., 1983; Cavalheiro et al., 1991; Leite et al., 2002; Loscher, 2002). The key to the chronic phase seems to be the occurrence of SE, because those rats without SE or with short-lasting SE do not have seizures or have just a few seizures (Lemos and Cavalheiro, 1995). The distinct features, in a single animal preparation, of an acute damage induced by status epilepticus, a silent interval between injury and the onset of spontaneous seizures, and a chronic epilepticus state have allowed the possibility to study antiepileptic drugs (AEDs) for different purposes (Leite, Garcia-Cairasco, and Cavalheiro, 2002). In this study we

investigate the efficacy and mechanisms of VPU against seizure activity and neuroprotection against damage induced by sustained seizure in the acute phase of pilocarpine model.

Anticonvulsant activity and possible underlying mechanisms of VPU and VPA in pilocarpine-induced seizure in rats

Behavioral alterations after a high dose of pilocarpine observed in the present study agreed well with data that were previously described by several studies (Turski et al., 1983; Leite, Garcia-Cairasco, and Cavalheiro 2002; Loscher, 2002). Intraperitoneal administration of pilocarpine produced motor limbic seizures and SE which could be prevented by VPU and VPA with the ED_{50} of 49 and 322 mg/kg B.W., respectively. Thus VPU was approximately 6 times more effective than VPA. Similar finding was previously observed, VPU was found to be more effective than VPA in protecting experimental animals against maximal electroshock seizure (MES) and the pentylenetetrazole (PTZ) tests (Tantisira et al., 1997). Therefore the anticipation that VPU could become a broad spectrum AEDs clinically was further strengthened by the present observation.

The involvement of various neurotransmitter systems (particularly excitatory and inhibitory amino acids) in the generation of sustained local epileptic hyperactivity (epileptic foci), as well as the initiation and spread of seizures is well documented (Meldrum, 1989; Bardford, 1995). Brain microdialysis is an interesting technique to study changes in extracellular amino-acid levels during seizures in different animal models (Lehmann et al., 1985; Zhang et al., 1991; Bruhn et al., 1992; Millan, Chapman, and Meldrum, 1993) and also in human epileptic foci (Carlson et al., 1992; During and Spencer, 1993). The pilocarpine-induced behavioral alteration was also found to elicit alterations in the extracellular levels of the neurotransmitters (Smolders et al., 1997; Khan et al., 1999). During the pilocarpine-induced status epilepticus, the sustained augmentation of extracellular glutamate concentrations, observed in the present study, is in agreement with those previously reported (Smolders et al., 1997; Khan et al., 1999). Elevations of extracellular concentration of glutamate were also demonstrated in seizures induced by the cholinesterase inhibitor, soman (Lallement et al., 1991) and in epileptic patients (Carlson et al., 1992; During and

Spencer, 1993). Furthermore pilocarpine induced status epilepticus was found to increase glutamate release in rat hippocampal synaptosomes (Costa et al., 2004). This augmentation of extracellular concentrations of glutamate concentrations corresponded well with the appearance of pilocarpine-evoked seizures, as manifested by changes in behavior and movement states. The sustained increase in glutamate levels may therefore play a key role in the maintenance and spread of the seizures which continue until the end of the experiment.

Furthermore we investigated the effects of VPU on the level of hippocampal amino acid neurotransmitters in pilocarpine-induced seizure model in freely moving rats with microdialysis technique. VPU elicited rather similar profile to that of VPA on hippocampal amino acid levels. VPU markedly decreased the enhancement of glutamate level induced by pilocarpine. Like VPA, VPU also significantly reduced the level of aspartate, glycine and GABA. This is consistent with the finding that VPU significantly decreased the levels of cortical excitatory (aspartate and glutamate) and inhibitory (GABA and glycine) amino acid neurotransmitters in dose dependent manner in anesthetized rats. However, the depression was greatest on glutamate and least on glycine (Sooksawate, 1995). Normally, direct or indirect enhancement of inhibitory GABA neurotransmission usually leads to an anticonvulsant effect (Soderpalm, 2002). Therefore, it is unlikely that the reduction of GABA and glycine level is responsible for the anticonvulsant activity of VPU. However if this effect was encountered by a stronger reduction of excitatory amino acid, an anticonvulsant activity would then be accomplished. This may be the case of VPU which significantly decreased both excitatory and inhibitory aminó acid neurotransmitters but reduction was most prominent on glutamate. It seems likely, therefore, that the anticonvulsant activity of VPU in pilocarpine-induced seizure rats is due, at least inpart, to reduction of an abnormally high extracellular level of excitatory amino acid neurotransmitters. Additionally the non-selective depressant effect of VPU suggested that VPU seems to exert its anticonvulsant activity by any mechanism other than those involving the GABAergic system. VPA which has been found to block sodium and, probably, T-type calcium channels (Kwan et al., 2001) demonstrated non selective depressant effect on brain amino acid neurotransmitters as VPU, it is suggestive that VPU might exert its effect on those ion channels as well. Some

anticonvulsant drugs inhibited voltage-activated Ca^{++} channels which one consequence of inhibition could be blockade of Ca^{++} entry into presynaptic nerve terminals and inhibitory effect on the release of neurotransmitters such as glutamate, resulting in net reduction of excitatory synaptic transmission (McNamara, 2001).

Neuroprotective effect and possible underlying mechanisms of VPU and VPA on pilocarpine-induced seizure in rats

In addition to the induction status epipticus, pilocarpine was found to elicit neuronal damages which are consistent with previous reports showing that systemic administration of a large dose of pilocarpine leads to excessive cholinergic stimulation in the brain which leads unequivocally to epilepsy-related brain damage (Turski et al., 1983; Lemos and Cavalheiro, 1995; Fujikawa, 1996).

Pretreatment with VPU and VPA exhibited protection of neurons in CA1 and CA3 region of hippocampus against damages induced by pilocarpine. This neuroprotection is correlated with the behavioral alterations which VPU and VPA prolonged the onset of motor limbic seizure and decreased severity of seizure induced by pilocarpine. Similar to previous studies, the anticonvulsant drugs, which prevented behavioral seizures produced by pilocarpine, such as clonazepam, phenobarbital, trimethadione and valproic acid, protected rat also against epilepsy-related brain damage (Turski et al., 1987). Several drugs have proved to efficiently abort or attenuate status epilepticus when injected either before or right after pilocarpine or kainate administration. In the pilocarpine model, interruption of status epilepticus by anticonvulsant drugs such as pentobarbital and diazepam in different periods of time demonstrated that early status epilepticus suppression can prevent spontaneous recurrent seizures. In addition, severe cell loss and synaptic reorganization are prevented if treatment is established with 30 min of status epilepticus (Lemos and Cavalheiro, 1995). It suggests that the ability of VPU to reduce neuronal injury induced by pilocarpine may prevent the mortality associated with prolonged seizures and spontaneous recurrent seizures.

Oxidative stress and mitochondrial dysfunction has been reported as a consequence of prolonged epileptic seizures and may contribute to seizure-induced brain

damage (Dal-Pizzol, et al., 2000; Patel, 2004). Excessive activation of excitatory amino acid (glutamate) receptors is believed to result in pathological increases in $[Ca^{++}]_i$ concentration and oxidative stress (Ortiz et al., 2000; Freitas et al., 2004). Lipid peroxidation in tissue is an index of irreversible biological damage of the cell membrane phospholipid, which in turn leads to inhibition of most of the sulphydryl and some nonsulphydryl enzymes (Gilbert and Sawas, 1983). Lipid peroxidation can be induced in many tissue injuries by many chemicals, and has been suggested as a possible mechanism for the neurotoxic effects of convulsive process (Sawas and Gilbert, 1985; Dal-Pizzol et al., 2000). Level thiobarbituric acid reactive substances (MDA levels) was measured as an index of lipid peroxidation after the status epileticus induced by pilocarpine. It was found that there was a significant enhancement of the MDA levels in hippocampus after pilocarpine-induced status epileticus. This result is in accordance with several previous reports (Dal-Pizzol et al., 2000; Freitas et al., 2004). These results suggest a putative role of reactive oxygen species in pilocarpine induced epilepsy. Reactive oxygen species are a part of normal human metabolism. When produced in excess, reactive oxygen species can cause tissue injury such as lipid peroxidation, DNA damage, and enzyme inactivation (Dal-Pizzol et al., 2000). Our results demonstrated that, pilocarpine induced neuronal damage were accompanied by an increment in the level of lipid peroxidation of the hippocampus. These suggest that reactive oxygen species could involve in the neuronal damage induced by pilocarpine.

In the present study, different doses of VPU and VPA produced a significant decrease in hippocampal lipid peroxidation level which was elevated as a result of the administration of pilocarpine. This finding is in agreement with a recent study that treatment with VPA at therapeutically relevant concentrations decreased lipid peroxidation and protein oxidation induced by the oxidant ferric chloride in primary cultured rat cerebral cortical cells (Wang, Azzam, and Young, 2003). Furthermore pretreatment of rats with all the anticonvulsant drugs tested (phenobarbital, lamotrigine, and phenazepam) as well as antioxidant substances such as α-tocopherol significantly decreased seizure manifestations and partially prevented both enhancement of NO generation and increase in TBARS formation in PTZ-induced seizures (Bashkatova et al., 2003). VPU and VPA demonstrated an attenuation of lipid peroxidation which corresponded well with the

prevention of neuronal loss in hippocampus. These findings indicated that suppression of pilocarpine-induced lipid peroxidation enhancement may underlie protective effect of VPU against neuronal damage-induced by pilocarpine.

Defects in oxidative phosphorylation in the central nervous system are the characteristic sign of mitochondrial encephalopathies, which are observed in variety of diseases with epileptic phenotype (Finsterer, 2004). Mitochondrial respiration is conventionally classified into several states, which can be measured via oxygen consumption (Chance and Williums, 1956). State 3, termed active respiration, is defined as respiration in the presence of an oxidizable substrate and ADP and thus is a measure of the respiration that is coupled to ATP synthesis. State 4, or resting, respiration is the rate of respiration in the presence of substrate, but without ADP, and thus is a measure of the rate of respiration that is not coupled to ATP synthesis. The present study have shown that pilocarpine treatment inhibited state 3 respiration while unaffect state 4 respiration when both glutamate plus malate and succinate were used as substrate resulting in the reduction of ATP synthesis. It is well known that glutamate plus malate are NAD^+-linked substrates which donate electrons to mitochondrial respiratory chain via complex I and succinate is substrate which donates electrons to the respiratory chain via complex II. These results indicate that pilocarpine may depress oxidative phosphorylation through effect on the respiratory chain since inhibition pattern via complex I and II. Deficiency of mitochondrial respiratory chain complex I has been reported in patients with temporal lobe epilepsy and epileptic rat (Kunz, Kudin, and Vielhaber, 2000; Kudin et al., 2002). Intense seizure activity causes massive influx of Ca^{++} through voltage-gated and *N*-methyl-D-aspartate (NMDA)-dependent ion channels (Van den Pol, Obrieten, and Belousov, 1996) which results in elevated intracellular and intramitochondrial Ca^{++} thus leading to mitochondrial membrane depolarization, thereby resulting in energy failure and superoxide production (Gupta and Dettbarn, 2003, Liang, Ho, and Patel, 2000; Schuchmann et al., 1999). This could trigger the acute neuronal cell death that occurs after status epilepticus (Fujikawa, Shinmei, and Cai, 2000). The present reports demonstrate inhibition of oxidative phosphorylation and ATP synthesis in the brains of animals following limbic SE induced by pilocarpine, and subsequently evolution of mitochondrial dysfunction. Inhibition of mitochondrial respiration

correspond accordingly with the well-established pattern of acute neuronal damage following SE. These results suggest that inhibition of oxidative phosphorylation of mitochondria in pilocarpine-induced SE lead to energy failure which may contribute to seizure-induced neuronal damage.

VPU behaves as an uncoupler by stimulating state 4 respiration whereas it was succesfully restored pilocarpine-induced inhibition of state 3 respiration when glutamate plus malate were used as the substrates, leading to slightly increase ATP synthesis. When succinate was used as substarte, VPU treament had no effect on the state 3, state 4 and ATP synthesis. These results indicated that VPU appeared to improve inhibitory effect of pilocarpine at the early part of mitoctochondrial respiratory chian such as the segment from NADH to CoQ or site I. Furthere experiments with isolated complex I are needed to prove. During seizures, mitochondria depolarize, so there is the risk of increased production of free radicals. Because of the increase in blood flow during seizures, oxygen supply may be augmented, and consequently the risk of ROS generation could be further enhanced (Heinemann et al., 2002). The improvement by VPU on mitochondria inhibition induced by pilocarpine may, at least in part, involve the attenuation of lipid peroxidation and neuronal damage. In contrary, VPA did not show any effects to improve the efficacy of mitochondrial respiration on state 3, state 4 and ATP synthesis. Previous study demonstrated that the rate of respiration of state 3 and state 4 of cerebral and hepatic mitochondria were reduced by VPA, suggesting that VPA impaired the properties of the internal mitochondrial membrane (Rumbach et al., 1983; Hayasaka et al., 1986) and then play an important pathogenic role in hepatotoxic effect. However a reductive oxidative phosphorylation cannot alone explain the hepatotoxicity of VPA, for oxidative phosphorylation is consistently inhibited, but hepatotoxic complications are rare (Hayasaka et al., 1986). Our finding did not show a correlation between mitochondria function and neuronal pathology produced by VPA. This suggests that other factors may account for the increased survival of neurons. Previous study demonstrated that some potential antiseizure actions of VPA is blocking of T-type calcium channels which lead to a reduction of Ca^{++} entry into cell (McNamara, 2001). Furthermore blockade of Ca^{++} entry into presynaptic nerve terminals leads to inhibition of release of neurotransmitters such as glutamate, resulting in net reduction of excitatory synaptic

transmission (McNamara, 2001) and thus directly lead to a suppression of seizures. These events may account for ability of VPA to protect neuronal damage induced by pilocarpine.