Indian J Physiol Pharmacol 1998; 42 (3) : 359–368

DEVELOPMENT OF SELF-SUSTAINING LIMBIC STATUS EPILEPTICUS BY CONTINUOUS VENTRAL HIPPOCAMPAL STIMULATION FOLLOWED BY LOW DOSE PILOCARPINE IN RATS

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(Received on December 11, 1997)

Abstract : Sequential treatment of rats with low doses of lithium and pilocarpine, a high dose of pilocarpine, or continuous hippocampal stimulation [CHS] (9 epochs, 10 min each) is reported to result in status epilepticus (SE). We report a novel method to establish SE based on continuous ventral hippocampal stimulation (5 epochs) followed by low dose pilocarpine (40 mg/kg) challenge. Motor limbic seizures occured in all the control rats. The latency to spike activity was 15 ± 1 min after pilocarpine administration. Ventral hippocampal [VHc] and cortical EEG recordings were used to monitor the protective effect of diazepam (5 mg/kg). Except phenobarbital, all the three drugs completely prevented all the phases of seizure activity. Initiation of spikes was significantly prolonged by phenobarbital pretreatment. Further study on the characteristics of these convulsions offers a unique possibility for the recognition of brain regions, pathways, and neurotransmitters engaged in the spread of seizures in this model.

Key words : status epilepticus, benzodiazepines, phenobarbital, MK-801

INTRODUCTION

Excessive activation of the cholinergic system in the CNS can result in seizures. In rodents, a high dose of pilocarpine (400 mg/kg) given systemically is believed to activate muscarinic receptors in the hippocampus (1) thereby initiating spontaneous recurrent motor limbic seizures and SE that have electographic, behavioural and neuropathological features in common with complex partial seizures in man (2). Sequential injections of lithium chloride and pilocarpine (separated by 20 – 24 h) also reliably induces SE in rats (3, 4). It is widely believed that the action common to these agents is an excitatory effect on limbic system structures (5) and that both the seizures and the neuropathology are a response to excessive excitation. Glutamatergic neurotransmission is particularly important in the hippocampus

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where epileptic-like brain injury has been induced both by electrical stimulation of the hippocampal afferents (6). Antagonists for the NMDA preferring excitatory amino acid receptor subtype (MK-801) show neuroprotective properties in a number of seizure models including lithium-pilocarpine-induced SE (7, 8). Animal studies suggest that efficacy with NMDA antagonists is more probable in primary generalized seizures and in SE rather than in complex partial seizures.

Attenuation of seizures is frequently achieved with drugs that increase neuronal inhibition. Consequently, inhibitory neurotransmitters such as GABA have been suggested to play an important role in regulating neuronal excitability and seizure production (9). Clonazepam and phenobarbital are anticonvulsants used clinically that enhance inhibition by modifying GABA responses through their actions at the benzodiazepine and barbiturate receptor sites, respectively. Several electrical paradigms have been suggested for producing SE in rats. Taber et al (10) produced a syndrome of recurrent seizures by repeated hippocampal stimulation in mice. Vicedomini and Naddler (11) produced SE using pulse trains to give maximal synaptic response in CA_3. Neural stimulation of dentate granule cells was reported to produce a highly specific pattern of cell damage which appeared identical to that caused by the administration of kainic acid. McIntyre et al (12) reported self sustaining SE in previously kindled rats by continuous hippocampal stimulation. Lothman et al (13) reported self-sustaining limbic SE induced by continuous hippocampal stimulation (CHS) in previously non-kindled rats. They produced limbic SE after 9 epochs of CHS using 10 s train of 1 ms, biphasic square wave pulses at 50 Hz, at 400 μA for 9 min followed by 1 min gap (1 epoch). After 9 such successive trains of stimulation's (9 epochs = 90 min) they could achieve SE in over 90% of the rats tested. We studied the effect of decreasing, period of CHS (from 9 to 5 epochs, i.e. 50 min instead of 90 min) and challenging those rats with low dose of pilocarpine (40 mg/kg,ip), and explore the action of known anticonvulsants on the emergence of seizures. The model produced a syndrome of convulsive SE in all the control rats tested with reproducible behavioural and electrographic changes characteristic of SE.

METHODS

Animals and Surgery : Male Wistar rats (Experimental Animal house facility, AIIMS, New Delhi) weighing 200–250 g were used. Under ketamine anaesthesia (80 mg/kg, i.m.) rats were stereotaxically (David Kopf, model – 1404, USA) implanted with both cortical, unilateral watch screw electode (2 mm posterior to bregma, 2 mm lateral, and 1.5 mm ventral to surface of skull), and depth unipolar electode (for recording and stimulating) unilaterally in the left ventral hippocampus (VHc) (2.8 mm posterior, 2 mm lateral and 3.5 mm ventral) according to the Paxinos and Watson atlas (14). An indifferent lead over the frontal sinus. served as the ground. All electrodes were insulated with epoxyliate except for 1 mm tip. Electrodes were soldered to a connecting plug using radio wires. After 5 days of recovery each animal was allowed atleast 30 min for habituation to the transparent

recording cage (modified skinner box, 25 × 25 × 29 cm) permitting free movement with the experimental set up.

Experimental procedure : On the day of experiment, basal EEG activity was obtained before stimulation was begun for each rat. The stimulus intensity was then set to 400 µA to override the postictal refractoriness (15). A standard stimulus consisting of a 10 s train of 1 ms, biphasic square wave pulses at 50 Hz, was given into the left VHc using Nihon Kohden electonic stimulator (SEN-3301) with isolator (SS 201J), Japan. The stimulus-on segment was determined by the 10 s train length. The stimulus-off segment was set at 1 s except as indicated below. Thus the stimulation interval was typically 11 s. The stimulus pattern was continuously delivered for 9 min, a time designated as a stimulus period. At the end of a stimulus period, stimulation was suspended for 1 min gap to allow a stimulus-free observation period. Together, the stimulus period and the observation period constituted an epoch (10 min). Soon after 5 epochs, hippocampal and cortical EEG was alternately recorded on the polygraph, then immediately these animals were challenged with pilocarpine (40 mg/kg, i.p.), which invariably resulted in convulsive SE in 100% of the control rats tested. For verifying the electrode placement, rats were anaesthetised with ketamine and later transcardially perfused first with normal saline followed by 10% formalin. Randomly selected brains from control group were serially sectioned (10 µ thickness) with rotary microtome [E.Leitz, Wetzlar, Germany] and stained with hematoxylin and eosin. The electrode tracks were deciphered by observing the stained sections under a light microscope.

Electrical and Behavioural recordings : Rats were monitored for occurrence of behavioural and electroencephalographic seizures till 90 min post pilocarpine. SE was defined as continuous convulsions (associated with forelimb clonus with rearing) for a period longer than 30 min associated with continuous spikes of high amplitude. EEG was also recorded 24 h post pilocarpine in the drug treated groups. No video monitoring was done. EEG signals were recorded on the Grass Model 7D polygraph. Prior to recording EEG, calibration of EEG signals was done by determining the height of signal having voltage of 50 µv.

Drugs : Pilocarpine nitrate (Boehringer Ingelheim, Germany), ketamine (Themis, India), diazepam (Ranbaxy Labs, New Delhi, India), clonazepam (Sauter Labs, U.K.), MK–801 (Merck Sharp and Dohme), phenobarbitone (BDH, Poole, Dorset, U.K.). All drugs were administered in a volume of 1 ml/kg body weight and were given i.p. 30 min prior to the CHS protocol. Doses selected for each drug were based on previous studies done in our laboratory (9, 16).

Statistical analysis : Descriptive statics for amplitude and frequency in the cortex and hippocampus at various time points was calculated by mean and standard deviation for all the groups separately. Freidmans test (non-parametric test) was applied to find out at what time from the basal value changes become significant. This was done for both the parameters in the cortex and hippocampus group wise. Average maximum value attained for amplitude and frequency in all the groups was compared using

Kruskal Wallis test. Area under the curve was compared among the groups by Kruskal Wallis test. In case of overall significance, multiple range test was applied. *P < 0.5 was considered statistically significant. Mortality was recorded during the 48 h period following pilocarpine challenge.

RESULTS

Five epochs of CHS produced mild electographic seizures which subsided within 20–30 min post stimulation (in non-pilocarpine treated rats) in both the regions tested and none of these rats went into SE during the 48 h period. However, when another group of rats exposed to the same duration of CHS were given pilocarpine 30 mg/kg, i.p. they showed SE, but as the results were not reproducible and consistent so we increased the dose of pilocarpine to 40 mg/kg. This resulted in SE in all the tested rats with 100% mortality within 48 h. To evaluate the possible effect of side of stimulation on the development of SE, another group of rats were stimulated on the right VHc and recordings were taken from the left VHc (bilaterally implanted) and

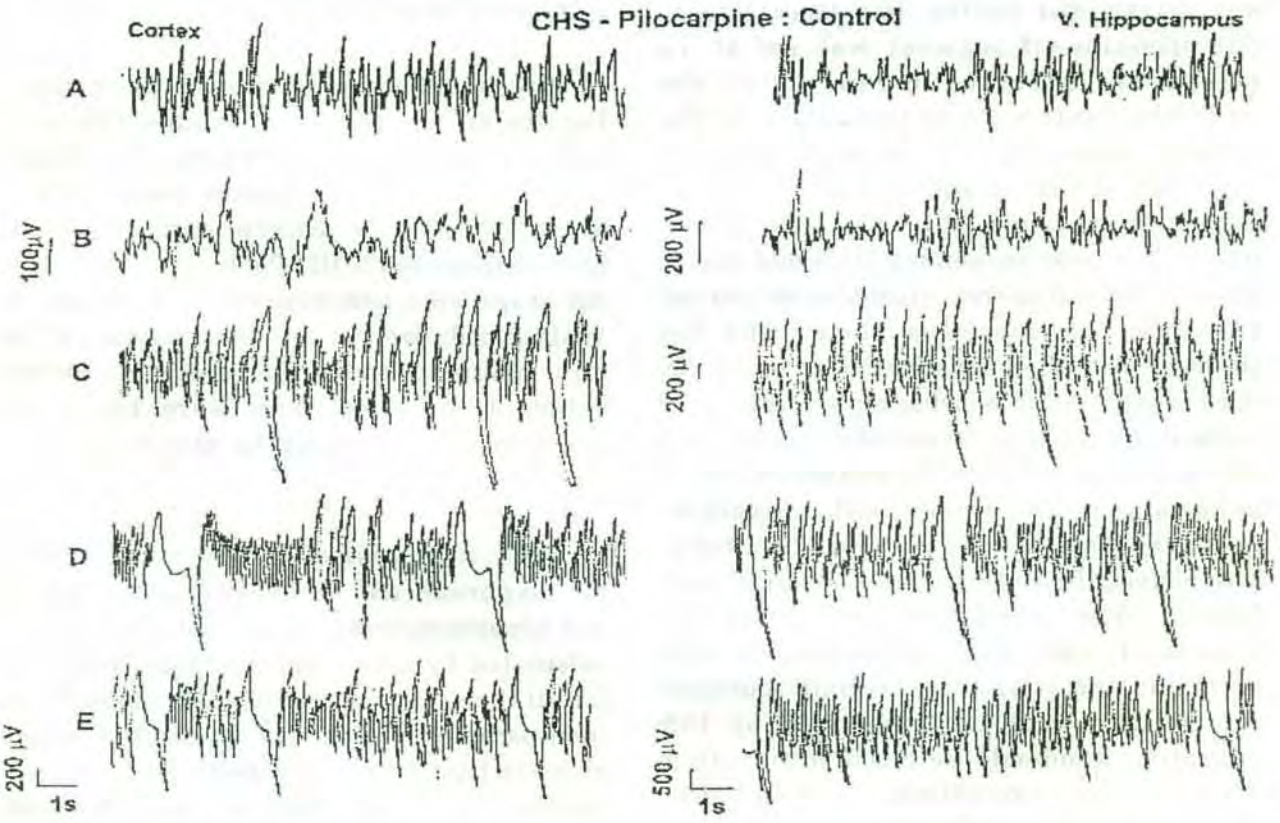

Fig.1 : Representative cortical and ventral hippocampal (VHc) EEG recordings in the control rats showing the development of SE at different time intervals. A : Basal recording; B: After CHS (5 epochs of stimulation); C, D, E: 0.5, 1 and 1.5 h post pilocarpine (40 mg) challenge, respectively. The cortical amplitude corresponding to A, B (100 µV); C, D, E (200 µV) and VHc amplitude corresponding to A, B (200 µV); C (200 µV); D, E (500 µV), respectively are indicated.

cortex. The proportion of animals developing SE was found to be the same in both the groups and there was no difference in the EEG recordings taken from both sides.

In the control group, within 5–15 min post pilocarpine (first phase) signs of cholinergic stimulation and stereotypic behaviour was noted with animals exhibiting staring and intense chewing, teeth chattering, sniffing, scratching with rear paws, head nodding. Within 15 min of pilocarpine, high voltage fast activity superimposed over the hippocampal theta rhythm and isolated high voltage spikes were registered both in the VHc and the cortex. The stereotyped behaviour soon gave way to motor seizures which was characterized by very frequent and vigorous wet dog shakes leading to occasional forelimb clonus with rearing (by approximately 30th min) resembling stage IV of kindling, as per Racine scale (17). After 60–70 min post pilocarpine, stage IV frequency was very much reduced. Once started seizure activity was found to occur in both the regions unabated. Significant increase in the amplitude was registered both in the cortex and VHc from the basal value during the 30th, 60th and 90th min post pilocarpine (Fig. 1, 2). Cortical and hippocampal frequency gradually increased (statistically insignificant change from the basal value) and reached to a maximum during the 90th min record (not shown).

Protective effect of drugs: Our results demonstrate a remarkable anticonvulsant efficacy (against SE induced by partial CHS followed by low dose pilocarpine) of drugs acting to potentiate GABA-mediated inhibition in the CNS. Pretreatment with

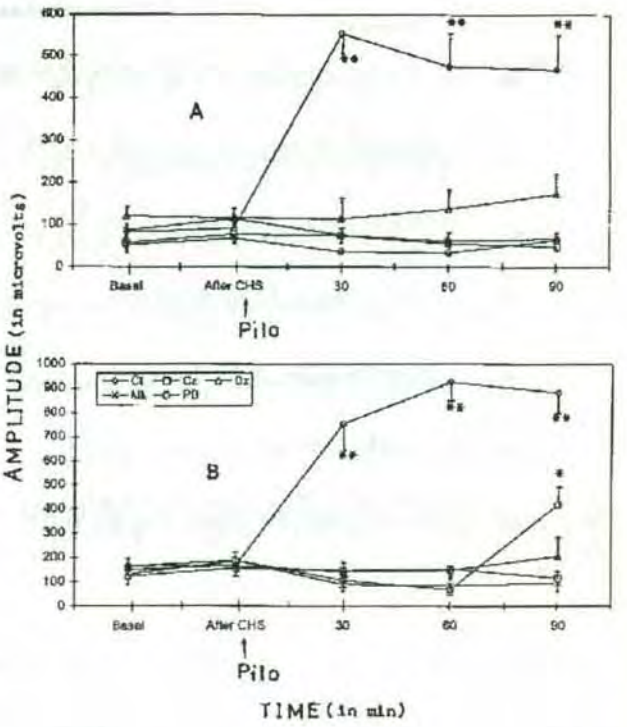

Fig. 2: Time course changes in the cortical (3A) **A, B** : and VHc (3B) EEG amplitude in CHS – Pilocarpine model of SE following different drug pretreatment in rats. n=8 in control and 4 in each of the drug pretreated group. Amplitude: in μV, Ct: Control, Cz: Clonazepam (1 mg), Dz: Diazepam (5 mg), MK: MK–801 (0.5 mg), PB: Phenobarbitone (50 mg). *p<0.01 (significance with respect to the basal values by applying Freidmans test). Also there was significant difference in the area under the curve (AUC) between Ct-Cz, Ct-Dz at *p<0.05 in Fig. 3A. In Fig. 3B between Ct-Cz, CT-MK at **p<0.01, and Ct-Dz at *p<0.05 using Kruskal Wallis test.

clonazepam (1 mg/kg) (Fig. 3A) and diazepam (5 mg/kg) (Fig. 3B) completely prevented the buildup of convulsive activity as evident from the amplitude and frequency which did not increase significantly from their respective basal values. Behavioural signs like wet dog shakes, forelimb clonus with rearing were absent. Generalized spikes were seen in the 90th min cortical recording taken from the VHc in two out of the four rats pretreated with the classical

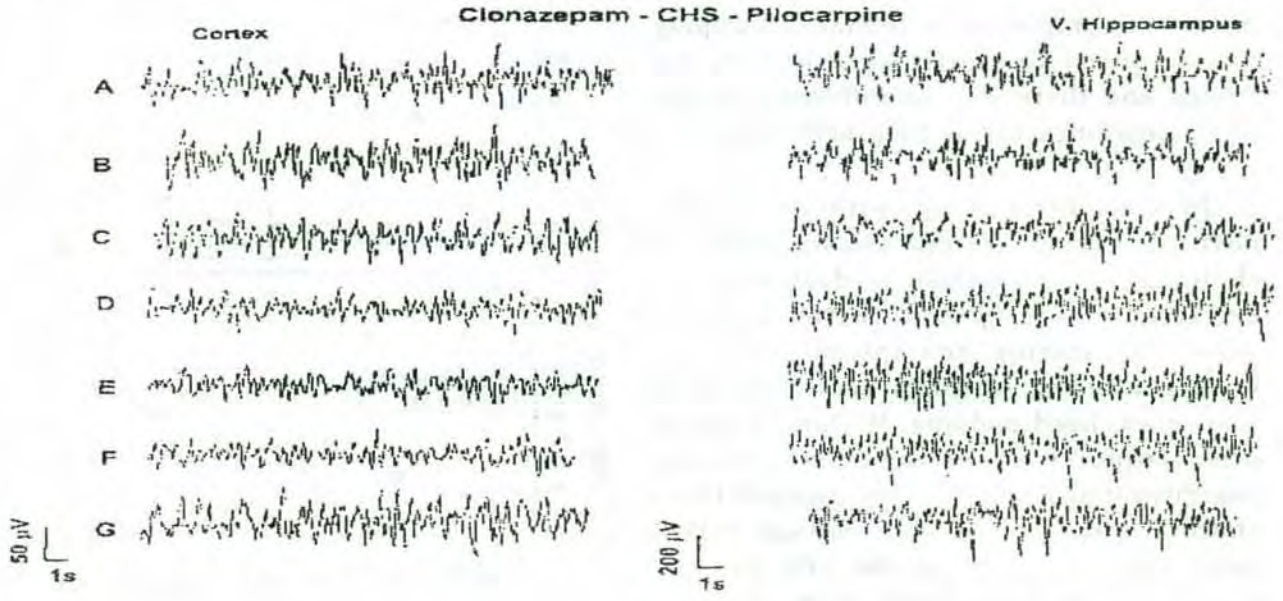

Fig. 3 A

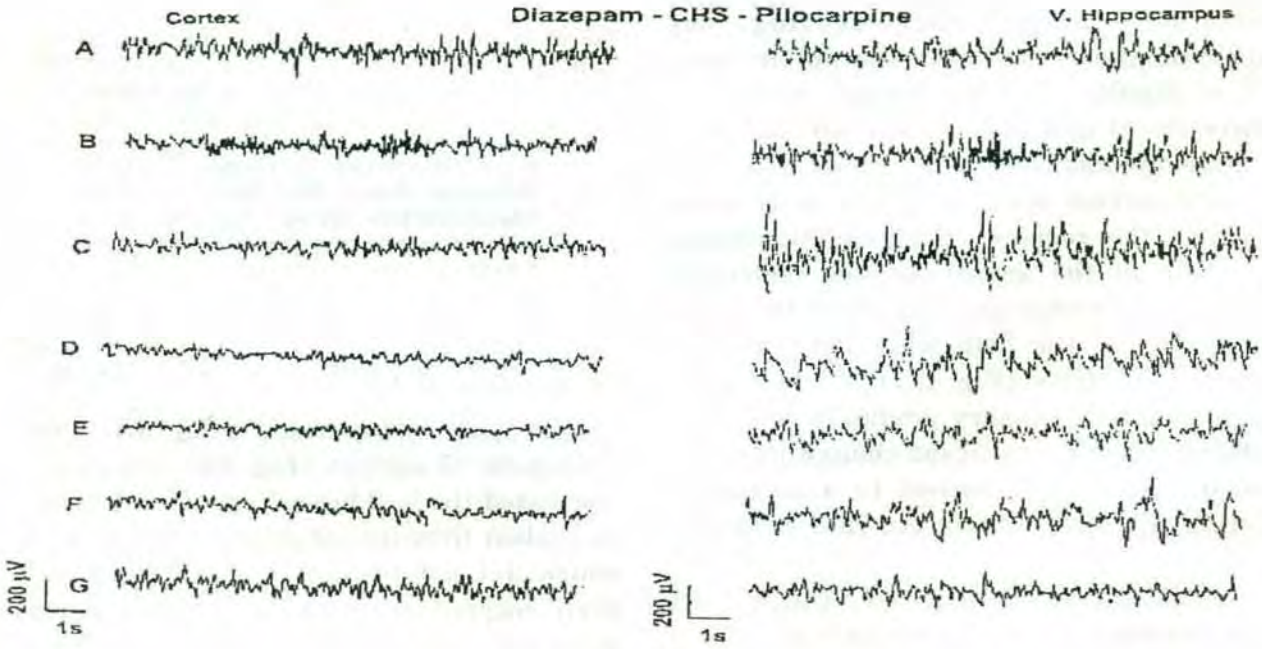

Fig. 3 B

Fig. 3 : Representative cortical and VHc EEG in clonazepam (1 mg) & diazepam (5 mg) pretreated rats showing .A, B : the recordings taken at different time intervals. A: Basal recording; B: 30 min after clonazepam (A) / diazepam (B); C: soon after CHS (5 epochs of stimulation); D, E, F, G: 0.5, 1, 1.5 and 24 h post pilocarpine (40 mg) challenge, resepectively.

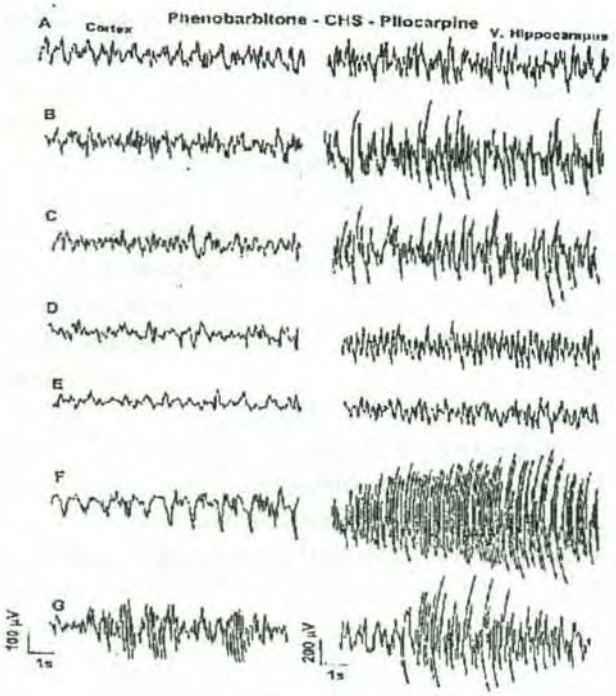

Fig. 4 A

barbiturate anticonvulsant, phenobarbital (50 mg/kg). Phenobarbital effectively delayed the onset of seizure activity in the VHc with none of the rats displayed rearing with forelimb clonus and all of the rats survived for over 48 h (Fig 4A). Average maximum value of amplitude attained in the cortex and VHc of clonazepam, diazepam and phenobarbitone pretreated groups were significantly lower than the control group (not shown).

Administration of MK-801 (0.5 mg) caused generalized spiking activity within 10 min, which virtually ceased 4–5 min after pilocarpine administration. None of the MK – 801 pretreated rats developed SE, and there was no associated mortality, in contrast to 100% lethality observed in the control rats. The cortical and VHc EEG

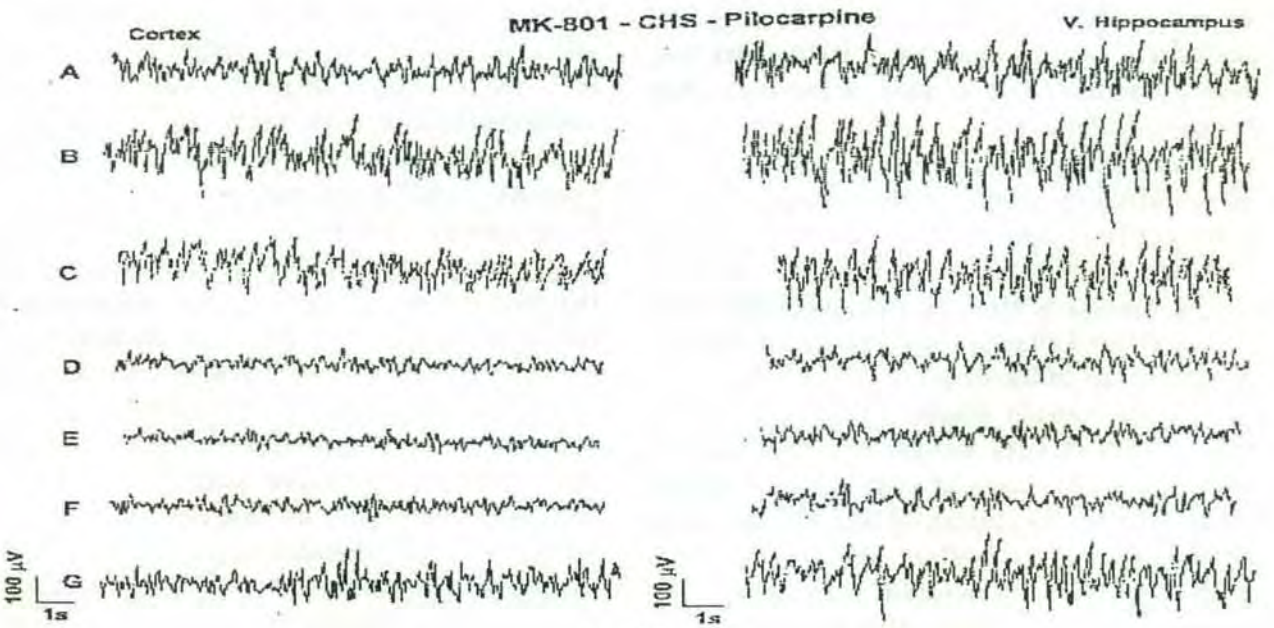

Fig. 4 B

Fig. 4 : Representative cortical and VHc EEG recordings in phenobarbitone (50 mg) & MK-801 (0.5 mg) **A, B** : pretreated rats showing the recordings taken at different time intervals. A: Basal recording; B: 30 min after phenobarbitone **(A)** / MK-801 **(B)**; C: soon after CHS (5 epochs of stimulation); D, E, F, G: 0.5, 1, 1.5 and 24 h post pilocarpine (40 mg) challenge, respectively.

recordings taken 24 h post pilocarpine was without any generalized spikes suggesting there was neuroprotection at the given dose (Fig. 4B). The average maximum value for amplitude reached in the cortex and VHc with the MK–801 pretreatment is also significantly lower than the control group. The histology verified the eletrode placement to be in the VHc as per the atlas of Paxinos and Watson (14).

DISCUSSION

Lithium pretreatment results in a nearly 15 fold potentiation of pilocarpine-induced seizures (3, 4). Likewise, aminophylline (18) or morphine (19) pretreated rats when subjected to low dose of pilocarpine elicit SE. Similarly, here partial CHS results in the reduction of dose of pilocarpine by nearly 10 fold. Lothman et al (13) were able to induce SE reliably in over 90% of the tested rats after 9 epochs of CHS (with low mortality rate) and in none of the rats after 5 epochs alone. We could establish SE by partial continuous ventral hippocampal stimulation (5 epochs) followed by low dose pilocarpine (40 mg/kg) challenge with absolute mortality. Our study finds support in the results of Buterbaugh et al (20), who demonstrated that pilocarpine facilitates the development of SE in amygdala kindled rats. A suprathreshold electrical stimulation of fully kindled rats subjected to definitely subconvulsant doses of pilocarpine triggers an immediate evolution of SE. The seizures produced by this method are reproducible, prolonged and associated with very high mortality rate. The benzodiazepines, clonazepam and diazepam which efficiently terminate either SE or complex partial seizures in humans and block PTZ, maximal electroshock (MES), or amygdala kindled seizures in rodents, were found to block the initiation of SE itself. Phenobarbital, an anticonvulsant barbiturate, effective against generalized tonic-clonic and complex partial seizures in man (21) delayed the latency of seizures. None of the phenobarbital treated rats died during the observed period of 48 h post pilocarpine. The non-competitive NMDA antagonist, MK-801, which acts by binding to the NMDA receptor gated calcium channel (22) is reported to be neuroprotective in lithium-pilocarpine model (as it does not block the electrographic seizures, but prevents the subsequent mortality (8). MK-801 (0.5 mg/kg) was also found to be protective in this model, supporting the theory that NMDA receptors play an important role in SE and brain damage.

It is reported that during hippocampal stimulation, seizures may begin unilaterally in the hippocampus but become synchronized in both hippocampi, creating a positive feedback wherein the seizures become self-sustaining within the hippocampi. Thereafter, probably both hippocampi interact to maintain seizures in the limbic circuits as the projections out of the hippocampus would then disseminate seizure activity in parallel to extra-hippocampal regions (13). The cortical recordings of rats support this concept. Previous studies have pointed out that the brain areas of amygdala, basal forebrain, and substantia nigra disseminate hippocampal-initiated seizures. Animal models that closely mimic pathological features of human seizure disorders are useful for studying the mechanisms as well as therapeutic approach to human epilepsy.

The data presented here furnishes supportive evidence that pilocarpine (40 mg/kg) administration in CHS rats provides an animal model of epilepsy with which therapeutic approaches to drug-resistant forms of complex partial seizures can be investigated. The model is a rapid and reliable method of inducing SE in rats and is responsive to standard anticonvulsants tested. Electrographic and behavioural data can be collected within a convenient time frame. Our procedure lends itself to examination of the biochemical and physiological basis of seizures, SE and neuronal cell death, because the timing of these events during the stimulation protocol in control rats is fairly predictable. Further studies will have to identify the morphological networks active in the motor expression of these generalized seizures and also look into the metabolic, neurochemical and neuroanatomical changes in the brain due to the prolonged seizure activity and the protective effect with the different antiepileptic drugs on the initiation as well as on ongoing SE.

ACKNOWLEDGEMENTS

The Senior Research Fellowship (B.G.) of the Council of Scientific and Industrial Research (CSIR), New Delhi, is gratefully acknowledged. The technical assistance provided by the faculty of department of Biostatistics, AIIMS, New Delhi during statistical analysis is duly acknowledged.

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