

Motor and electrographic response of refractory experimental status epilepticus in rats and effect of calcium channel blockers

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Effects of various calcium channel inhibitors have been studied in lithium-pilocarpine model of status epilepticus. Status epilepticus was induced by administration of lithium chloride (3 meq/kg) followed 21 hr later by pilocarpine (30 mg/kg). Diltiazem (5 and 10 mg/kg) was not effective in delaying onset of convulsions. Verapamil (20 mg/kg) showed protection against lithium-pilocarpine-induced convulsions. The dihydropyridine nifedipine (2.5 and 5 mg/kg) did not show any protection in this model. Amlodipine (5 and 10 mg/kg) as partially protective. Flunarizine (10 and 20 mg/kg) delayed the onset of forelimb clonus and rearing and only 60% of the rats underwent status in the 20 mg/kg group. Pre-treatment of MK-801 led to a potentiation of the antiserizure activity of calcium channel inhibitors. The percent increase in amplitude at various time points with amlodipine pretreatment was significant only at the 30th min recording, and at the rest of the time frames was practically similar as the controls. It can be concluded that the anticonvulsant action of MK-801 can be enhanced by centrally acting calcium channel inhibitors.

Accumulating experimental evidence suggest that abnormalities in calcium-regulated ion channels or enzymatic processes may underlie alterations in neuronal excitability and result in seizure activity. Increased Ca^{2+} influx in epileptogenesis is well established, and it is assumed that both receptor-operated and voltage-sensitive Ca^{2+} channels take active part in these processes. Desmedt *et al.*¹ demonstrated that two piperazine derivatives, flunarazine and cinnarizine antagonized PTZ and electroshock-induced seizures. These observations were confirmed and extended for calcium channel inhibitors (CCIs) from two other classes, dihydropyridine and benzothiazepines. Consistent with these findings, BAY K 8644, an L-type Ca^{2+} channel agonist, can elicit seizures by itself and potentiate audiogenic, PTZ- or NMDA-induced convulsions. CCIs are the only effective drugs for BAY K 8644-induced seizures. Representatives of the class I CCIs (verapamil, gallopamil) do not appear to exhibit significant anticonvulsant activity in any of the above mentioned models, with the notable

exception of BAY K 8644-induced seizures². BAY K 8644 augments NMDA-induced seizures whereas CCIs such as diltiazem, nifedipine or verapamil antagonize NMDA- and kainate-induced convulsions in mice. Convulsions elicited with intrahippocampal injections of quinolinic acid, an endogenous agonist acting preferentially at NMDA-type receptors are suppressed by flunarizine or verapamil, but not by nifedipine³.

The development and evaluation of drugs which block Ca^{2+} channels has therefore been an important rational strategy for anticonvulsant drug design⁴. Most selective CCIs which have been evaluated for anticonvulsant effects primarily block the L-type channel. Evidence suggest that dihydropyridines (nifedipine) may act on T-type channels at higher doses, and some CCIs may act at other non-calcium binding sites as well⁵. Flunarizine is able to block T-type as well as L-type Ca^{2+} channels in several tissues including brain membranes. Nifedipine and flunarizine, partially protects the kindled rats from subsequent kindling stimuli³. CCI nimodipine, has been shown to block amygdalar and hippocampal kindling⁶.

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The metabolic demands and physiological changes associated with SE indicate a major role of Ca^{2+} in modulating continued excitability and cytotoxicity⁷ and this high $[\text{Ca}^{2+}]$ has been repeatedly shown to cause cell death⁸. Griffiths *et al.*⁹ were the first to suggest this to be the mechanism of status-induced cellular damage. They showed that during 2 hr of status induced by bicuculline or L-allylglycine in rats, Ca^{2+} accumulates in the swollen and disrupted mitochondria in basal dendrites and selected neuronal cell bodies within the hippocampus. *In vitro* studies indicate that hippocampal bursting activity is Ca^{2+} dependent and dihydropyridine channels are localized in regions such as the hippocampus which are related

to epileptogenesis, CCIs acting at CNS sites may be useful in treating epilepsy.

Materials and Methods

Animals—Wistar rats of either sex, 200–250 g from Central Animal house facility, Panjab University were used. Animals were housed in cages and received standard laboratory chow and water. Animals were acclimatized to the laboratory conditions and all experiments were carried out between 9.00 and 17.00 hrs.

Drugs—The following drugs (with their respective source) were used during the entire study: lithium

Li - Pilocarpine : Control

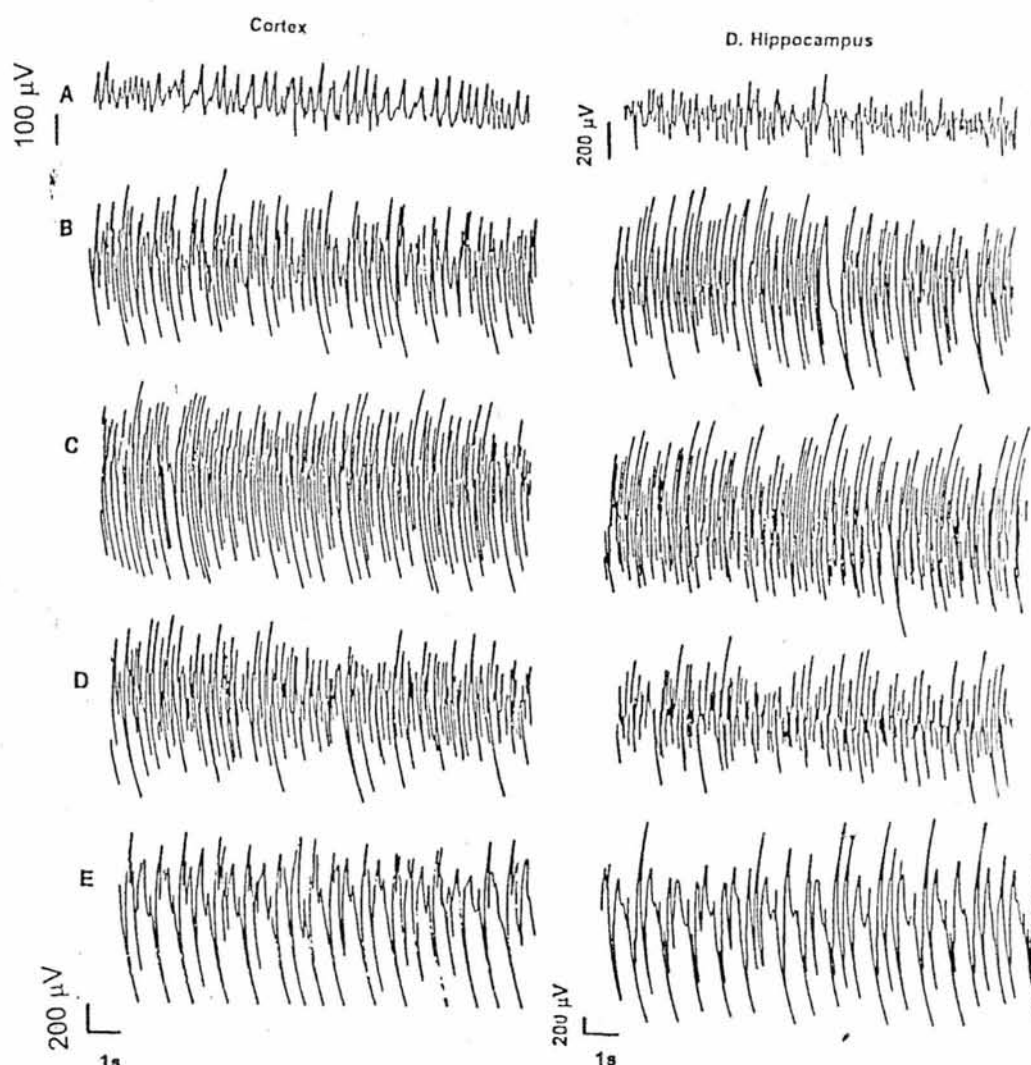


Fig. 1—Representative cortical and dorsal hippocampal (DHe) EEG in Li-pilocarpine control rats showing the development of SE at different time intervals. A: Basal recording; B, C, D, E: 0.5, 1, 1.5 and 4 hr post pilocarpine (30 mg) challenge ($n=6$). The cortical amplitude corresponding to A (100 μV); B→E (200 μV) and DHe amplitude corresponding to A (200 μV); B→E (200 μV), respectively are indicated.

chloride (Merck, Germany), atropine sulphate, pilocarpine nitrate, (Boehringer Ingelheim, Germany), ketamine (Themis, India), MK-801 (Merck Sharp and Dohme, UK), nifedipine (Torrent Labs, Ahmedabad), diltiazem (Torrent Labs, Ahmedabad), verapamil (Torrent Labs, Ahmedabad), amlodipine (Systopic Laboratories Ltd., Faridabad, India), flunarizine (Torrent Pharmaceutical Ltd, Baroda, India). The doses used in the present study are based upon the previous studies carried out in our laboratory.

Experimental procedure—SE was induced by the method of Honchar and colleagues¹⁰ by administering lithium chloride (3 meq/kg, ip) followed 21 hr later by pilocarpine (30 mg/kg, sc), following which rats were

observed continuously for occurrence of behavioural and electroencephalographic (EEG) seizures (Fig. 1). In the interaction studies, the drugs were administered 30 min prior to pilocarpine challenge. The latency to attain forelimb clonus with rearing was noted and seizures were monitored till 90 min post pilocarpine behaviourally.

For electrophysiologic study, under ketamine anaesthesia (80 mg/kg, im) rats were stereotaxically¹² (David Kopf, Model-1404, USA) implanted with both cortical bilateral watch screw electrodes and unilateral unipolar electrode in the right dorsal hippocampus (−2.8 mm posterior, 2 mm lateral and 3.5 mm ventral). Prior to ketamine atropine sulphate (7 mg/kg, ip) was given to rats to reduce respiratory

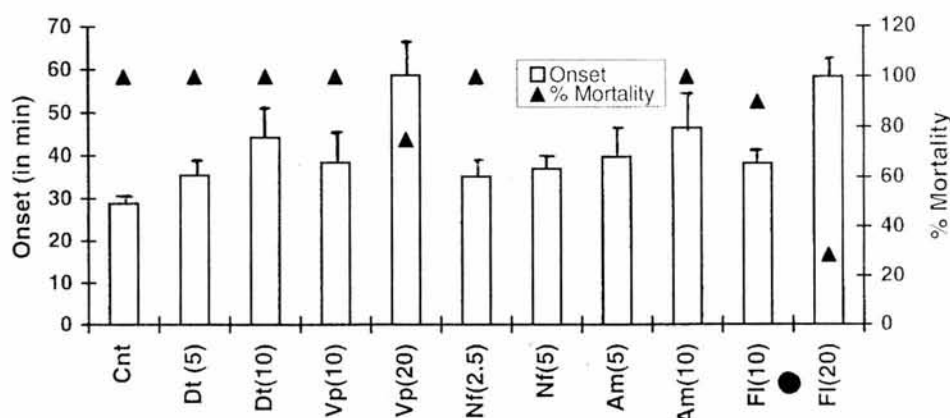


Fig. 2—Effect of different calcium channel blockers diltiazem (Dt: 5 and 10 mg/kg), verapamil (Vp: 10 and 20 mg/kg), nifedipine (Nf: 2.5 and 5 mg/kg), amlodipine (Amd: 5 and 10 mg/kg), and flunarizine (Fl: 10 and 20 mg/kg) administered 30 min before pilocarpine (30 mg/kg) challenge in Li-pretreated rats. Severity was assessed as onset of F.C+R and percent mortality. Each value represents mean $\pm$ S.E. (n=7-9). * P <0.05 [compared w.r.t. control (Cnt), Kruskal Wallis test followed by Student's t-test].

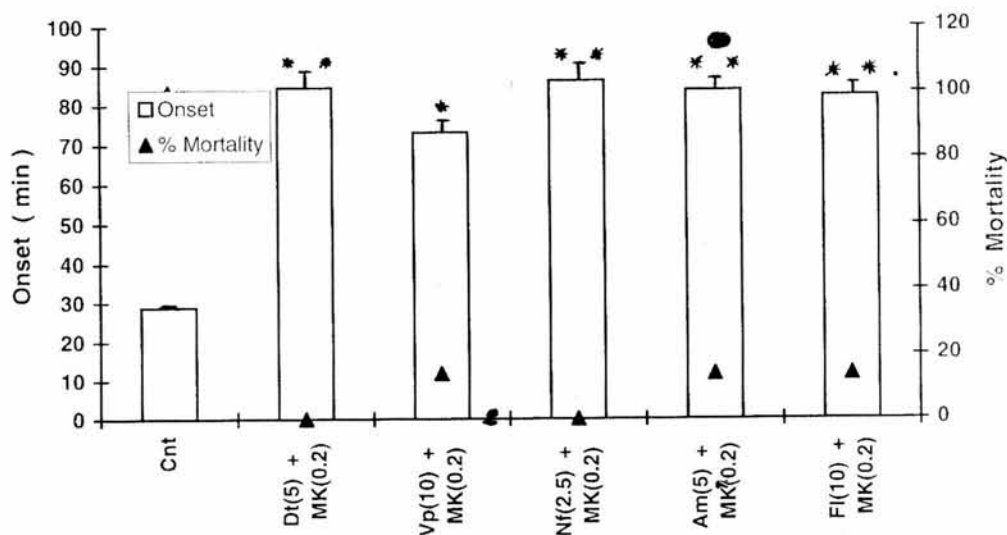


Fig. 3—Effect of combination of MK-801 (0.3 mg/kg) with different calcium channel blockers administered 45 and 30 min before pilocarpine (30 mg/kg), respectively in Li-pretreated rats. Diltiazem (Dt: 5 mg/kg), verapamil (Vp: 10 mg/kg), nifedipine (Nf: 2.5 mg/kg), amlodipine (Amd: 5 mg/kg), and flunarizine (Fl: 10 mg/kg). Severity was assessed as onset of F.C+R and percent mortality. Each value represents mean $\pm$ S.E. (n=7-9). * P <0.05, ** P <0.01 [compared w.r.t. control (Cnt), Kruskal Wallis test followed by Student's t-test].

secretions. After 5 days 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 following which SE was induced⁶. Onset of seizures was characterised electrographically by the occurrence of generalized spikes and polyspikes for longer than 20 s associated with sniffing, chewing, eye blinking and head nodding as reported by Hirsch *et al*¹¹. SE was defined as continuous convulsions for a period longer than 30 min associated with continuous spikes of high amplitude. EEG signals were recorded on the chart paper of Grass Model 7D polygraph (Grass Instruments Co., Quincy, Mass, USA). Prior to

recording, calibration of EEG signals was done by determining the height of signal having voltage of 50 μ V. Frequency was determined by calculating the average number of peaks recurring per second taken up from three 5 s intervals, similarly for amplitude, determined by measuring peak to peak voltage manually. The changes in EEG were recorded alternately for cortex and hippocampus 30 min before lithium (basal), 30 min after amlodipine (20.5 hr post lithium), and after administering pilocarpine (21 hr post lithium) at 30 min intervals up to 90th min, and finally after 4 hr of pilocarpine.

Statistical analysis—In behavioural studies data on onset of forelimb clonus with rearing was subjected to Kruskal-Wallis one-way analysis of variance output-

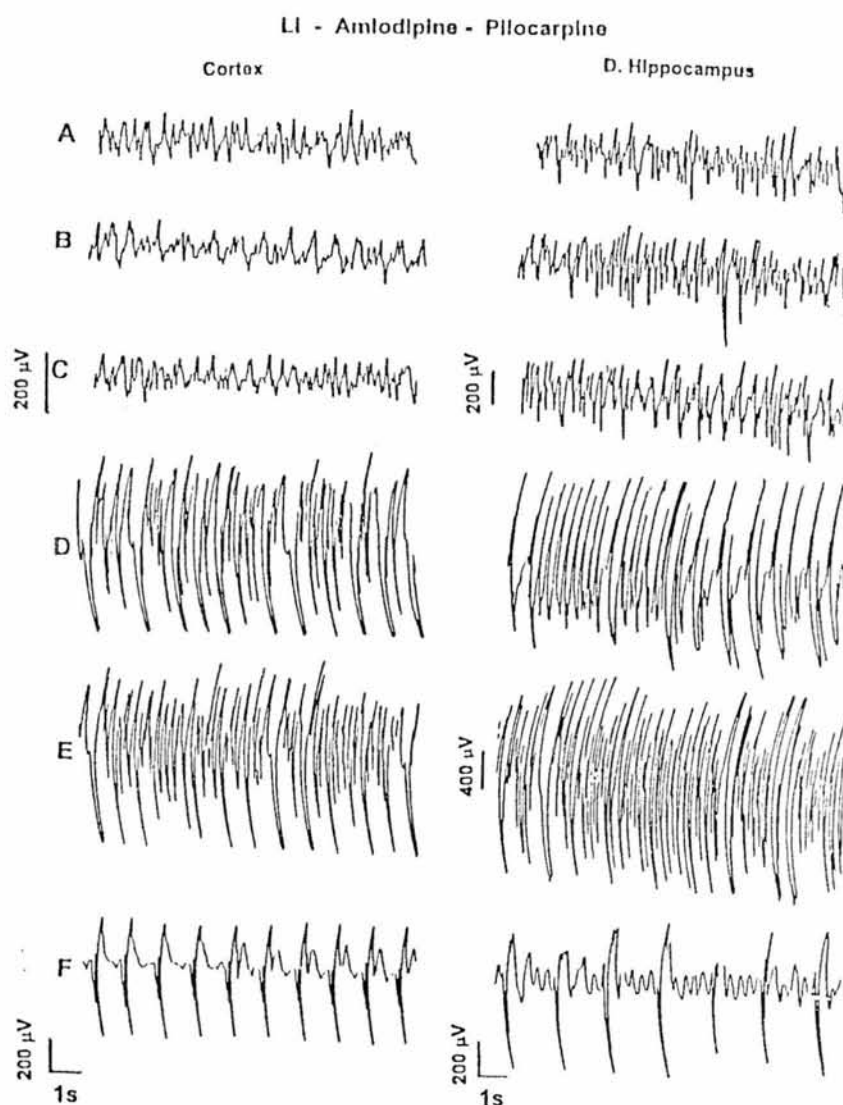


Fig. 4—Representative cortical and dorsal hippocampal (DHc) EEG, showing the development of SE in amlodipine (10 mg/kg) pretreated rats at different time intervals. A: Basal recording; B, C, D, E: 0.5, 1, 1.5 and 4 hr post pilocarpine (30 mg/kg) challenge. The cortical amplitude corresponding to A→C (200 μ V), D→E (400 μ V), F (200 μ V), respectively, are indicated.

test followed by Student's *t*-test. Descriptive statistics 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. Area under the curve (AUC) was compared among the groups by Kruskal Wallis test. In case of overall significance, multiple range test was applied. The data on the percent change in amplitude and frequency was analyzed by one way analysis of variance (ANOVA) amongst the groups. Further application of least significant difference procedure was performed for testing significance of each group. $P < 0.05$ was considered statistically significant.

Results

Behavioural studies—Among the CCI, diltiazem (5 and 10 mg/kg) was not effective in delaying onset

of status. Representatives of the class I CCI, verapamil (10 and 20 mg/kg) which is effective against kindling was effective only at the higher dose. The dihydropyridine nifedipine (2.5 and 5 mg/kg) did not show any protection in this model. Amlodipine (5 and 10 mg/kg) was partially protective. Flunarizine (10 and 20 mg/kg, po), a diphenylalkylamine and a potent CCI, able to block T-type as well as L-type Ca^{2+} channels in several tissues including brain membranes was successful in delaying the onset of F.C+R, and only 60% of the rats underwent status in the 20 mg/kg group (Fig. 2).

MK-801 when given 15 min before the sub-effective doses of CCI studied [diltiazem (5 mg/kg), verapamil (10 mg/kg), nifedipine (5 mg/kg), amlodipine (5 mg/kg), and flunarizine (10 mg/kg, po)] led to a potentiation of the antiseizure activity (Fig. 3).

EEG studies—However, amlodipine (10 mg/kg) neither reduced the severity of SE nor the subsequent mortality apart from the fact that it delayed the onset

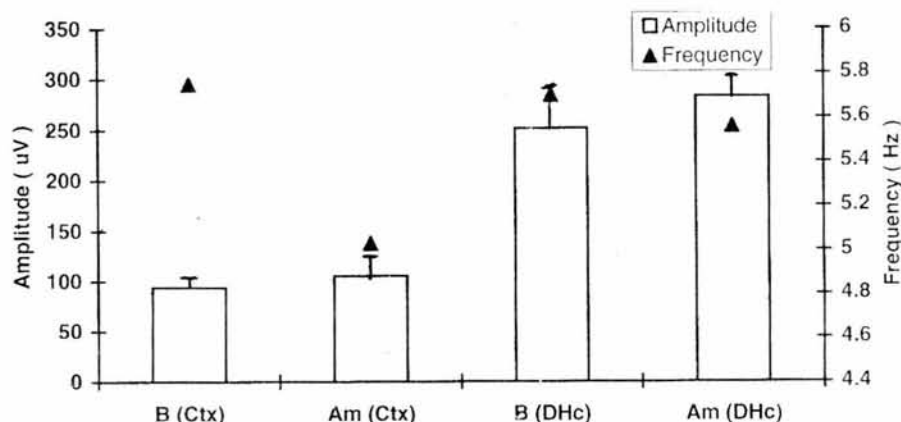


Fig. 5—Effect of amlodipine (Amd: 10 mg/kg) pretreatment in Li-pretreated rats on cortical (Ctx) and dorsal hippocampal (DHc) amplitude and frequency, compared with respect to basal (B) values. Each value represents mean $\pm$ S.D. ($n=4$). (Friedmans test).

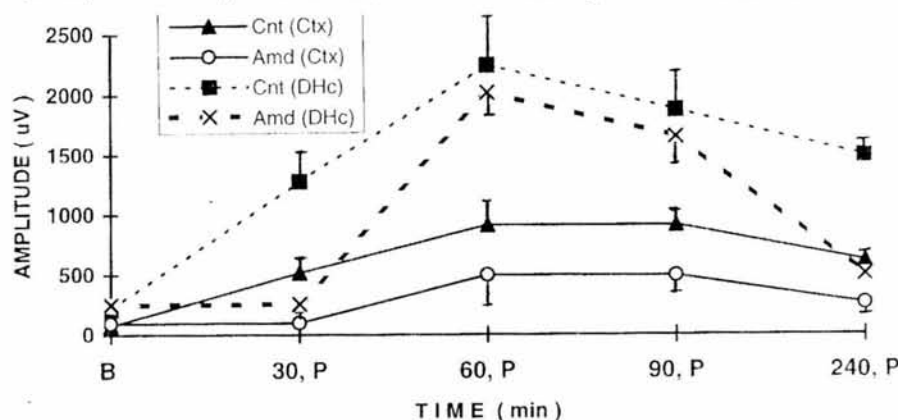


Fig. 6—Time course changes in the cortical (Ctx) and dorsal hippocampal (DHc) EEG amplitude in amlodipine (Amd: 10 mg/kg) pretreated rats as compared to the controls in Li-pilocarpine model. Each value represents mean $\pm$ S.D. ($n=4-6$). The AUC of Cnt (Ctx) v/s Amd (Ctx) and Cnt (DHc) v/s Amd (DHc) showed no significant difference (Kruskal Wallis test).

of status (Figs 4-6). The % increase in amplitude (with reference to the basal value) at various time points with amlodipine pretreatment was significant only at the 30th min recording, and at the rest of the time frames was practically similar as the controls (Fig. 7A). The hippocampal frequency in amlodipine group remained more or less at the basal value till 60th min. record, whereas in the control rats the DHc frequency increased right from the 30th min. record till the 90th min post pilocarpine. Recording taken at the 4th hr (240 min) showed a drop in frequency (Fig. 7B).

Discussion

Intracellular Ca^{2+} plays a crucial role in the initiation and regulation of a variety of physiological cellular processes, which include release of neurotransmitters and hormones, neuronal electrical activity, muscle contraction, gene expression, cell growth and differentiation and cell death¹³.

This potentiation of the antiseizure activity could be the result of simultaneous blockade of excitatory

events and voltage-dependent calcium channels. Because of the reported role of neuronal Ca^{2+} channels in seizure disorders, various CCI were examined for anticonvulsant activity in Li-pilocarpine model of SE.

The CCI, diltiazem (5 and 10 mg/kg), a potent coronary dilator, was not protective in the present model of SE. Verapamil and D-600 (methoxy-verapamil) are known to reduce the amplitude of Ca^{2+} spikes induced by iontophoretic pulses of NMDA in hippocampal slice preparation. Verapamil also partially protects the kindled rats from subsequent kindling stimuli³. In the present study verapamil (10 and 20 mg/kg) was protective only at the higher dose. The dihydropyridine CCI, nifedipine (2.5 and 5 mg/kg) was also ineffective and did not show any respite from the seizures. Amlodipine (5 and 10 mg/kg), another dihydropyridine CCI, with a long duration of action was found to be partially effective at 10 mg/kg dose in delaying the onset of seizures. In the EEG studies, the percent increase in amplitude at various time points with amlodipine (10 mg/kg)

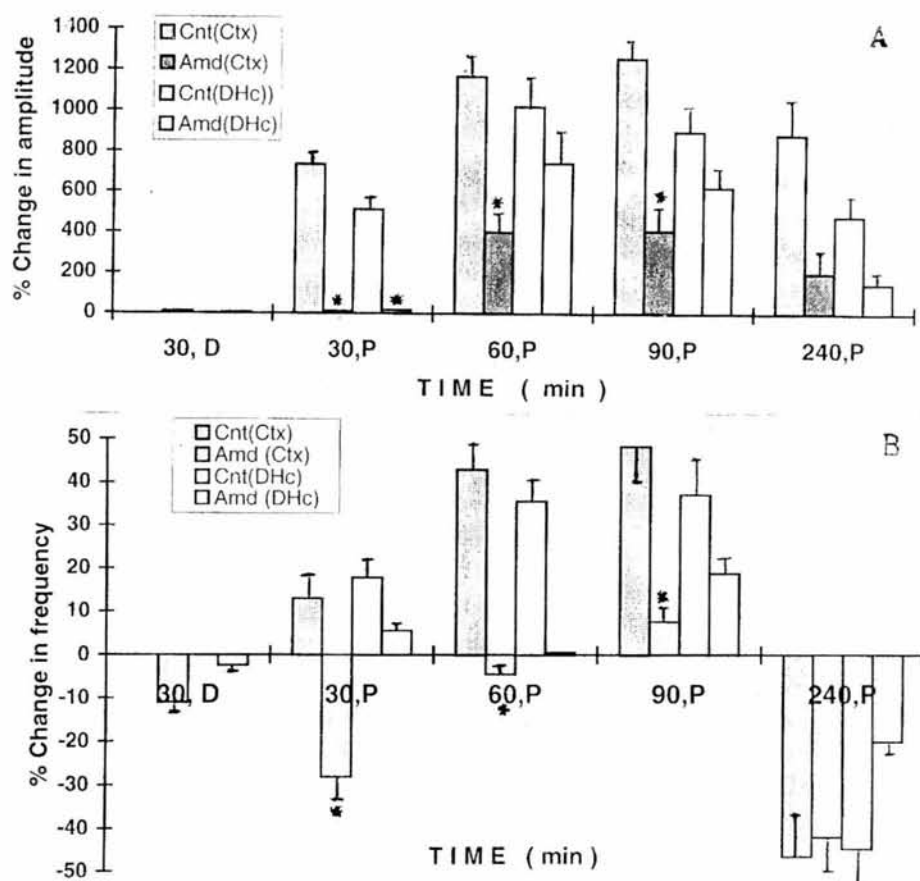


Fig. 7—A. The percent change in amplitude observed after amlodipine (Amd: 10 mg/kg) pretreatment on cortical (Ctx) and dorsal hippocampal (DHc) EEG activity. B. Similarly, for percent change in frequency in Li-pilocarpine model. Each value represents mean $\pm$ S.D. (n=4-6). $P < 0.05$ [compared with respect to control values, one way ANOVA followed by Student's t-test]

pretreatment was practically similar as that of controls.

Flunarizine (difluoro derivative of cinnarizine), a diphenylalkylamine, has a wide spectrum of pharmacological activity. Among these, actions which may account for its anticonvulsant properties are suppression of N- and T-type Ca^{2+} channel conductance, phenytoin-like effects on sodium channels, and inhibition of adenosine reuptake. Flunarizine (10 and 20 mg/kg) dose dependently delayed the onset of F.C+R. The 20 mg/kg dose also brought down the mortality rate significantly.

The mechanism of action of CCI in epilepsy is not well understood. The antiepileptic effects may be by either decreasing neuronal recruitment or abolishing the paroxysmal depolarization shift (PDS), an excitatory field potential recorded in groups of neurons during the process of seizure initiation. The PDS is believed to be the underlying generator of burst firing of neuronal groups that synchronize firing in discrete areas of the brain. CCI, such as verapamil have been reported to reduce the amplitude of the PDS or possibly to decrease generation of such spikes in hippocampal slice preparation¹⁴. Ca^{2+} entry into neurons is involved in initiation of epileptiform activity by activating Ca^{2+} and calmodulin-dependent protein kinases. The subsequent cell damage is mediated largely through second messenger mechanisms.

Ca^{2+} ions also play a pivotal role in EAA-mediated events¹⁵. Combination therapy, opens new possibilities for some of the EAA receptor antagonists in the treatment of epilepsy¹⁶. In the present study, MK-801 (0.2 mg/kg) administered before the subeffective doses of CCI in Li-pilocarpine model [diltiazem (5 mg/kg), verapamil (10 mg/kg), nifedipine (5 mg/kg), amlodipine (5 mg/kg), and flunarizine (10 mg/kg)] led to a potentiation of the antiseizure activity. The exact nature of the interaction between voltage-dependent calcium channels and EAA receptors remains to be determined. NMDA and non-NMDA receptors differ substantially in ion selectivity¹⁷. It is known that the NMDA receptor-mediated effects are calcium dependent due to the high permeability of the NMDA receptor to Ca^{2+} ions¹⁸. CCI, therefore, may synergistically increase the protective action of competitive NMDA receptor antagonists against convulsions. It can be concluded that the anticonvulsant action of MK-801 can be enhanced by centrally acting CCI. Such combinations may prove

useful in rationalizing polypharmacological approaches in the treatment of epilepsy.

Dynamic regulation of Ca^{2+} signal transduction system may provide insight into the molecular mechanisms of altered neuronal excitability which are important not only in the development of better therapeutic strategies in pathologic processes such as epilepsy, but also in understanding the physiological development of neuronal plasticity.

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