Effect of Ethanol on Nuclear Kinases and Protein Phosphorylation in Rat Brain

Thesis Submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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Enrolment No. PL-5985 November 1996

DEDICATED

TO MY

FAMILY MEMBERS

CONTENTS

DECLARATION

I hereby declare that the work presented in this thesis entitled ***"Effect of ethanol on nuclear kinases and protein phosphorylation in rat brain"*** has been carried out by me under the supervision of **Dr. Mohan C. Vemuri** and that this has not been submitted for any degree or diploma of any University.

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CERTIFICATE

This is to certify that **Mr. K. S. G. Haviryaji** has carried out the work in the present thesis under my guidance for a full period prescribed under the Ph.D. ordinance of the University. I recommend his thesis entitled *"Effect of ethanol **on** nuclear kinases **and protein phosphorylation in rat brain"*** for submission for the award of the degree of *Doctor of Philosophy* of this University.

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ACKNOWLEDGEMENTS

I thank, and appreciate my research supervisor Dr.Mohan C.Vemuri, for introducing me to the exciting field of Protein phosphorylation. His suggestions and constant encouragement helped me in having a better understanding of the subject.

My sincere thanks to Prof. A.R.Reddy, Dean, School of life Sciences and Prof. P.R.K. Reddy, Head, Department of Animal Sciences, for extending all the necessary facilities for my research work.

I thank Prof. Ch.R.K.Murthy for his suggestions and help rendered to me during his tenure as my In-charge supervisor and Prof.T.Suryanarayana for his help and encouragement. I also thank Prof. Michael E.Dahmus, Department of Biochemisry and Biophysics, University of California at Davis, USA for providing me with polyclonal antibodies for Casein kinase II., and my colleague, Dr. N. Nagaraju, for providing me monoclonal antibodies against phosphotyrosine and Dr. P.Reddenna for arachidonic acid.

My sincere thanks are also due to my colleagues, , Dr A. .Raghunathan, Dr. Prakash Babu, Mr.Rajgopal, and Ms Shailaja for the encouragement during my work in the lab. I also thank Kiran, Naresh, Ramarao, Goud, Kasyapa and Kranthi, for their constant moral support which made this work easier. My special thanks are due to my colleague Mr.A. Shanavas for helping me in photography and Dr. Md. Mustafa, Mr.Mahadev and Ms. Sheela Pai for scanning my thesis for typographical errors.

I would be failing in my duty if I do not mention the encouragement given by my father, sisters and brother in-law. I owe much to my Uncle K.S.K Satyam for encouraging me to pursue research as a career. Financial assistance provided by University Grants Commission is also gratefully acknowledged.

Haviryaji

ABBREVIATIONS

AA	: Arachidonic acid
ATP	: Adenosine 5'-Triphosphate
BCIP	: 5Bromo-4Chloro-3Indolyl Phosphate
$CaCl_2$	: Calcium Chloride
CaM	: Calmodulin
CPM	: Counts per minute
DAG	: Diacylglycerol
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra acetic acid
EGTA	: Ethylene glycol-bis(P-aminoethyl ether)- N,N,N'N' tetra acetic acid
GTP	: Guanosine 5'-Triphosphate
IEF	: Isoelectric focusing
kDa	: Kilo dalton
KH_2PO_4	: Potassium dihydrogen phosphate
µ	: Micron
M	: Molar
µci	: Micro curie
$MgCl_2$	: Magnesium Chloride
mg	: Milligram
µM	: Micro molar
mM	: Milli molar
mA	: Milli amps
$MnCl_2$	: Manganous chloride
$Na_4P_2O_7$	: Tetra sodium pyrophosphate
NaCl	: Sodium Chloride
PAGE	: Poly Acryiamidc Gel Electrophoresis
TBS	: Tris buffered saline

TCA	: Trichloroacetic acid
PGT	: Poly Glutamate Tyrosine
pl	: Isoelectric point
PMSF	: Phenyl Methyl Sulfonyl Fluoride
PS	: Phosphatidyl serine
PY20	: Phosphotyrosine antibodies
SDS	: Sodium Dodecyl Sulfate
Ser	: Serine
TBS	: Tris Buffered Saline
Thr	: Threonine
Tris	: Tris [hydroxymethyl] amino methane
V/V	: Volume/Volume

INTRODUCTION

Introduction

Ethyl alcohol is formed in nature by the fermentation of sugar, and serves as a disinfectant, drug, food and preservative. Ethanol being soluble in both water and lipid diffuses rapidly through cell membranes into cells. Most of the alcohol consumed is metabolised by liver into carbon dioxide and water liberating energy. It was shown that energy liberated by the oxidation of ethanol can be utilised by the body. Depending on the amount of alcohol intake and the capacity of the liver to detoxify it, the levels of ethanol or its chief metabolite acetaldehyde increase in the blood and reach different parts of the body and exert ill effects. The complex effects of ethanol are primarily due to changes in the function of CNS as a result of direct ethanol action on cerebral tissue. The immediate effects of ethanol ingestion are euphoria, sedation, intoxication and narcosis (Mello, 1968).

General effects of Ethanol

Ethanol is a neurotoxin which affects every organ and tissue of the body. Heavy alcohol consumption has been associated with brain damage (Parsons 1977). Clinical studies have established deleterious effects which include brain dysfunction, increased tolerance and physical dependence, encephalopathies and defects in learning, memory and conceptual abilities. Similarly irreversible impairments in brain function have been shown even after abstinence following long term ethanol administration in rodents. Brain damage in alcoholics is a multifactorial phenomenon. The neuropsychological disturbances are a consequence of addiction and toxicity from chronic ethanol exposure

together with associated systemic disturbances. Chronic ethanol abuse produces biochemical adaptations in the nervous system, which underlie the development of tolerance and dependence (Charness et al., 1989). Addiction following alcohol consumption results in neuroadaptational changes within CNS involving neurochemical and neurophysiological process. The problems involved in studying the effect of alcohol on brain damage are due to several factors like, 1. lack of linear relationship between the dose of alcohol and brain damage, and the presence of mechanisms in the brain for dealing with injury (Freund 1985), 2. the increased risk of brain damage due to wide array of reasons like poor nutrition, liver disease, and head trauma. 3. the presence of cognitive impairment prior to the onset of abusive drinking (Tarter and Edwards 1985), which make the study of ethanol induced brain damage more complicated and a challenging one.

Ethanol and its oxidative metabolite, acetaldehyde may directly damage the developing and mature nervous system (Arendt et al., 1988, Streissguth et al., 1980, Lieber 1988). Acetaldehyde has been shown to covalently bind to plasma or hepatic proteins (George and Hoberman 1986) to form adducts which are expected to potentiate its toxicity through the inhibition of microtubule assembly and enzyme dysfunction (Lieber 1988). On the other hand it can also form adducts with biogenic amines to form opioid like compounds tetrahydroisoquinones, which might be responsible for the addiction of ethanol (Cohen and Collins 1970). Pathological studies of the brains of alcoholics have shown evidence for ethanol-induced cerebral atrophy and reduction in neuronal density (Victor et al., 1989), which may result from increase in intracellular

calcium concentration. Interestingly, similar changes were associated with neuronal loss accompanying ageing, hypoxia and cerebral ischemia. Cholinergic neurons in nucleus basalis of basal fore brain, which innervate cerebral cortex are preferentially depleted in dementia due to Alzheimer's disease as well as in patients with Korsakoff syndrome (Arendt et al., 1983). Long term administration of ethanol to rats causes memory deficits, reduction in choline acetyl transferase and a slight loss of neurons in the nucleus basalis (Arendt et al., 1988). Transplantation of cholinergic neurons into hippocampus and neocortex corrects both cholinergic deficits and memory abnormality, implicating ethanol in the damage of cholinergic neurons (Arendt et al., 1983). Furthermore, ethanol is also involved in the disruption of serotonergic and adrenergic pathways in amnestic alcoholics (Charness et al., 1989). Neuronal density in the superior frontal cortex was reduced by 22% in alcoholics which was accompanied by selective glial proliferation. Chronic ethanol treatment inhibits superoxide dismutase activity, thereby increasing the vulnerability of the brain to free radical damage (Ledig et al., 1980 and Mandel et al., 1980). Recent findings have proposed that the alcohol induced neuronal death could be due to apoptosis (Freud 1994).

Ethanol and Neurotransmission

Ethanol is known to affect the functioning of neurotransmitters, receptors, ionchannels and transport process (Tabakoff et al., 1979). Binding of specific neurotransmitter molecule to its corresponding receptor initiates signal transduction process in the neurons. Ethanol has been shown to have both acute and chronic effects on the function of several receptor systems. At low concentration, ethanol prevents NMDA

activated currents in primary cultures (Lovinger et al., 1989, White et al., 1990), while 5mM ethanol inhibits Long Term Potentiation in rat hippocampal neurons, providing biological correlate of ethanol induced memory impairments (Blitzer et al., 1990). In dissociated brain cells, ethanol inhibits the NMDA stimulated increase in intracellular Ca^{2+} concentration (Dildy and Leslie 1989). Similarly it also inhibits the release of ^{3}H-Noradrenaline from rat brain cortex (Fink and Gother 1990) and endogenous dopamine from striatal slices (Woodward and Gonzales 1990) in response to NMDA stimulation. The inhibitory effects of ethanol are explained by possible modulation of NMDA-glycine interaction at NMDA receptor (Rabe and Tabakoff 1990). Earlier reports showed that chronic ethanol consumption lead to increased glutamate binding to synaptic membranes (Michaelis et al., 1978). The increased L-glutamate sites reported in human alcoholics and rats treated chronically with ethanol appear to represent an increase in the agonist binding site on NMDA ion channel. Further studies using cultured cerebellar neurons have found that chronic ethanol treatment can significantly increase NMDA stimulated Ca^{2+} influx (Iorio et al., 1992).

Increase in NMDA-receptor ion channels and NMDA mediated calcium flux are not the only changes that would sensitize neurons to excitotoxicity. Chronic ethanol treatment of cells in culture *in vitro* (Brennan et al., 1989, Messing et al., 1986) and *in vivo* in animals (Brenne et al., 1990) resulted in an increase in dihydropyridine sensitive voltage dependent calcium channels. In addition, ethanol can directly increase $(Ca^{2+})_i$ by releasing intracellular stores (Daniell and Harris 1989 a, b., Machu et al., 1989). Furthermore, recent studies suggest that chronic ethanol increases receptor stimulated

production of nitric oxide (Fulton and Chandler 1993). An increase in NMDA-stimulated nitric oxide production would be expected to increase excitotoxicity. However, ethanol doesn't change the sensitivity of neurons to nitric oxide mediated toxicity (Greenberg et al., 1992) Thus a variety of data suggest that chronic ethanol treatment may disrupt calcium homeostatic mechanisms and enhances exitotoxicity.

Earlier studies have shown the interference of ethanol with glutamate metabolism, because of decrease in glutamine synthetase activity of astrocytes in culture (Shanley and Wilce 1993), leading to an increased sensitivity to withdrawal seizures by NMDA. There was a significant decrease in glutamate decarboxylase activity in NMDA injected hippocampi suggesting increased sensitivity of GABAergic neurons to death (Shanley and Wilce 1993). Chronic ethanol treatment decreased the efficiency of GABAergic transmission in synaptoneurosomes (Morrow et al., 1990), and decreases GABA stimulated chloride flux (Harris and Allan 1989). It was shown that the down regulation of a subunit of $GABA_A$ receptor might be responsible for the decreased efficacy of the receptor (Molina et al., 1993). This down regulation could be an adaptive mechanism in response to functional disturbance caused by ethanol and may be leading to tolerance and symptoms of withdrawal. The decrease in GABA inhibition would reduce homeostatic mechanisms that normally prevent excess of excitation. In addition to receptor gated channels, ethanol exposure increases the maximum velocity of $Na\text{-}Ca^{2+}$ antiporter (Michaelis 1989b). This change in antiporter might protect, or it could increase Ca^{2+}, as the influx in sodium might actually reverse the antiporter leading to further increase in calcium. Alterations in dopaminergic and muscuranic cholinergic

receptors after chronic ethanol exposure may be the result of ethanol induced impairment of receptor effector coupling. Since the neurotransmission process is affected involving different neurotransmitters and their receptors, the ultimate action of ethanol leading to cognitive impairment may lie in the signal transduction process.

Ethanol and Lipids

Post natal ethanol exposure has shown that myelin accumulation is delayed in the optic nerve (Phillip 1989) and conduction velocity of impulses was decreased on ethanol treatment (Miller et al., 1990). Sun et al.,(1978) reported reduction of myelin in the brains of chronic ethanol fed animals. Myelin phospholipid/protein ratio and cholesterol/protein ratio was also lowered by ethanol (Sun et al., 1980). Phosphatidyl serine and Phosphatidyl choline contents were found to be decreased after prolonged ethanol ingestion, while an increase in the Phosphatidyl inositol was oberved in the experimental group (Vrbaski et al., 1984). Further studies demonstrated a delayed differentiation and maturation of Purkinje neurons in rats exposed prenatally to ethanol (Mohammad et al., 1987). No specific receptor for any actions of ethanol *per se* has yet been identified and it is possible that the many central effects of ethanol are produced by a generalized effect on cellular membranes (Goldstein. and Chin 1981). Following chronic administration of alcohol it was found that the membranes became resistant to the fluidizing effects of alcohol, associated with an increase in the cholesterol content (Chin et al., 1979) of the membranes and replacement of unsaturated by saturated fatty acids (Littleton 1977). The changes in membrane lipids witnessed after chronic ethanol administration would be expected to affect the function of proteins embedded in such

membranes. Results indicative of conformational changes in membrane proteins have been obtained with cells treated with ethanol in vitro (Noble et al., 1976) and with brain microsomes prepared from rats consuming ethanol in their drinking fluid (Dinovo et al., 1976). The disturbance in the membrane proteins could results in the abnormal functioning of receptor and effector coupling and there by affecting neurotransmission.

Ethanol, RNA and Protein Synthesis

The synthesis of proteins in brain, depends on the proper interaction of several factors. The availability of precursor amino acids as well as generation and accurate function of RNA can be altered by the presence of ethanol. Mice injected with ethanol solution for prolonged periods were found to have a marked decrease in the synthesis of nuclear RNA, a substantial reduction in mitochondrial RNA synthesis (Tewari and Noble 1975), and a decrease in the Poly (A+) RNA content in rats ingested with alcohol (Haviryaji, 1990). Boylan and Tewari (1983) showed the reduced ability of aminoacyl tRNA synthetase to charge tRNA in the brains of ethanol-dependent rats. Extensive studies indicate that chronic ingestion of ethanol produces a significant inhibition of brain protein synthesis (Kuriyama et al., 1971; Noble and Tewari 1973). When different cell types were separated, glial protein synthesis was found to be sensitive to inhibition by ethanol (Jarlstedt and Hamberger 1972). Lower ethanol levels did not reduce rat brain protein synthesis after 3 days of ethanol ingestion (Tewari and Noble, 1971). However rats made physically dependent of ethanol were found to have a significantly decreased capacity to synthesize proteins compared with control animals. (Noble and Tewari 1975), which could be reversed after ethanol withdrawal (Tewari et al., 1977). The decreased

capacity to synthesize proteins was attributed to the malfunctioning of ribosomes (Tewari et al., 1978), which was shown to be due to the defect in the reassociation of ribosomal subunits. Further it is shown that the integrity of ribosomes might have been affected during chronic ethanol treatment (Haviryaji et al., 1992). All these observations have strongly established the vulnerability of cerebral protein synthesis to ethanol exposure, a disturbance, which could lead to cerebral dysfunction.

Protein Phosphorylation in the Central Nervous System

Phosphorylation is now recognized as the most common post-translational modification involved in cellular regulation (Girault 1993). A wide variety of regulatory agents, both extracellular and intracellular messengers, produce different types of biological responses in the nervous system. Phosphorylation and dephosphorylation of proteins play a cardinal role in the regulation of these signals in the cell. Most extra cellular signals like hormones, growth factors, neurotransmitters and electrical potentials alter the state of phosphorylation of intracellular proteins. The fact that protein phosphorylation converts the extracellular signals into intracellular biochemical reactions and integrates the signals over a period of time, emphasizes the importance of protein phosphorylation in information processing cells like neurons in brain.

Protein phosphorylation system consists of three components namely; a protein kinase, a protein phosphatase and a substrate protein. The physiological activity of the substrate protein depends on either the transfer of y-phosphate from ATP or other phosphate donors, by a kinase or removal of phosphate from substrate protein by a

phosphatase. Hence the phosphorylation status of substrate protein can be modulated in one of the following ways.

1. By change in the activity of the protein kinase while the activity of the protein phosphatase remaining constant.
2. By change in the activity of the protein phosphatase while the activity of the protein kinase remaining same.
3. Activity of both the protein kinase and phosphatase changing.
4. The phosphorylation of a protein can also be affected by a change in the conformation of protein itself with no change in the kinases or phosphatases.

Protein kinases catalyse the transfer of phosphate group from ATP or sometimes GTP (Walsh, 1979) to the hydroxyl group of the side chain of either serine, threonine or tyrosine residues. The transfer of phosphate is achieved by the binding of Mg^{2+} and ATP or sometimes GTP complex to the catalytic site. The activation of protein kinase molecule requires either binding of small regulatory molecules or phosphorylation of the regulatory domain by another kinase or dephosphorylation by a phosphatase. Activation of many protein kinases is accompanied by an autophosphorylation reaction, which could be activatory, inhibitory, or neutral. It was shown that autophosphorylation of CaM kinase subunits at $Threonine_{286/287}$ turns the enzyme into calcium calmodulin independent form, whereas autophosphorylation at $threonine_{305/306}$ and $serine_{314/315}$ has an inhibitory effect on enzyme activity (Hanson and Schulman 1992).

A second mechanism of activation is by covalent modification of catalytic domain. When p34cdc kinase is phosphorylated on the catalytic site on tyrosine and

threonine residues, it becomes inactive while the activation requires the dephosphorylation of same residues(Gautier et al., 1991; Krek and Nigg, 1991). Another type of mechanism by which a kinase might get activated is linked to changes in the protein substrates. The binding of epinephrine to p-adrenoreceptor induces a conformational change that could unmask a potential phosphorylation site (Palczewski and Benovic. 1991). Alternatively, phosphorylation by a kinase might facilitate the phosphorylation by a second kinase. The last mechanism is related to the accessibility of relevant substrates to the kinase. Several kinases undergo intracellular translocations either to the membranes (Kraft and Anderson, 1983; Mochly-Rosen et al., 1991) or into the nucleus(Meinkoth et al., 1990; Adams et al., 1991).

Protein phosphorylation plays a paramount role in the regulation of gene expression. Several oncogenic viruses transform infected cells by synthesizing protein kinase that phosphorylates specifically on tyrosine residue of substrate protein (Erikson et al., 1980; Hunter and Sefton, 1982). Protein phosphorylation has been implicated in the neurotransmitter synthesis and their release, axoplasmic transport, ion channel conductance and protein synthesis. Some effects of protein phosphorylation underlie the biochemical events of short term memory and long term memory (Greengard and Kuo 1970). The basis of short term memory involves phosphorylation of presynaptic or post synaptic proteins, while biochemical events in long term memory involve the phosphorylation of proteins involved in the regulation of gene expression. It has been proposed that phosphorylation of histones (Langan 1969; Johnson 1982), non-histone nuclear proteins (Johnson 1982), and RNA polymerase (Krebs and Beavo 1979) might

regulate DNA transcription in nervous and in non-nervous tissues. Similarly, it has been proposed that phosphorylation of both aminoacyl-tRNA synthetase (Berg 1978) and a variety of ribosomal proteins might regulate messenger RNA translation in nervous (Roberts, 1982) and in non-nervous tissues (Hunt, 1980; Leader 1980). All these factors suggest the involvement of cytoplasmic and nuclear kinases in the phosphorylation of these substrates and phosphatases in their dephosphorylation.

Protein Phosphorylation in the CNS diseases

Diseased state of nervous system appears to be associated with changes in the phosphorylation state of phosphoproteins in specific cell types of CNS. A number of disorders of the nervous system like Huntington's chorea, Tardive dyskinesia, Parkinson's disease have been reported to involve alterations in neurotransmission. Since the neurotransmission is affected, the signal transduction mechanisms get perturbed resulting in an altered phosphorylation of concerned proteins. The microtubule assembly promoting activity of "tau" protein was found to be reduced due to hyperphosphorylation in alzheimer's brain tissue (Iqbal et al., 1986). Cytosolic Casein Kinase II activity was decreased in the brains of schizophrenics and Alzheimer's patients (Aksenova et al., 1991). Similarly CKII and PKC activity were altered during complete cerebral Ischemia (Hu and Weiloch 1993; Monika and Wieloch 1993). Hence the study of the phosphorylation state of proteins might serve as an index in understanding the pathophysiology of the disease condition.

Effect of Ethanol on Protein Phosphorylation in the CNS

It is well known that ethanol causes disturbance in the neuronal activity due to derangement in the neurotransmitter function, ion channel conductivity, RNA metabolism and protein synthesis. Most of these activities are regulated by protein phosphorylation and dephosphorylation reactions. Ethanol exposure alters several parameters which can influence PKC activity like in the intracellular calcium (Daniell et al., 1987; Rabe and Weight. 1988; Davidson et al., 1988a). PKC is also sensitive to changes in the redox state of cellular environment (Kass et al., 1989). Since ethanol alters the redox state and the intracellular calcium levels, it will lead to the modulation of PKC activity. It is also shown that acute ethanol decreases the calcium uptake while chronic ethanol increases intracellular calcium (Messing et al., 1986). The increase in intracellular calcium was associated with upregulation of dihydropyridine sensitive calcium channels in the membranes of ethanol dependent animals (Dolin et al., 1987), and in PC 12 cells exposed to ethanol for many days (Marks et al., 1989). The upregulation of calcium channels was sensitive to protein kinase C inhibitors (Messing et al., 1990). It was further shown that chronic ethanol treatment of neural cells increases the levels of PKC 8 and c and protein kinase mediated phosphorylation implicating PKC in the upregulation of Ca^{2+} channels (Messing et al., 1991). In contrast, the activity of PKC was decreased in the hippocampal membranes of ethanol treated rat synaptosomcs, while the binding of labelled phorbol esters remained same implicating a reduction in the response of the enzyme to its stimulators (Kruger et al., 1993).

The effect of phorbol ester induced increase in Ca^{24} uptake, had suggested that ethanol might alter some other pathways in addition to PKC to enhance Ca^{2+} uptake in presence of phorbol esters. Possible mechanisms include alterations in cAMP levels (Gordon et al., 1986), and cAMP dependent protein phosphorylation. Chronic ethanol exposure reduces adenosine stimulation of cAMP content in cultures (Rabin et al., 1993). This heterologous desensitization is due to decrease in Gsα mRNA and protein, leading to decreased cAMP production (Charness et al., 1988; Mochly-Rosen et al., 1988). In an acute ethanol treatment, phosphorylation of a 50kDa nuclear protein in G_0/G_1 phase of cell cycle in glial cultures and in G_0 cells of adult rat cerebrum has also been reported (Hinson et al., 1991).

All these changes suggest that the phosphorylation/dephosphorylation cascade might be playing considerable role in ethanol induced damage in the nervous system and hence can influence the cognitive functions.

Scope of the present study

Alcohol is a proven neurotoxin. Its abuse results in brain damage, and cognitive dysfunction. A variety of responses like learning, memory, long term potentiation and cell survival are associated with protein phosphorylations and these responses are impaired in alcoholics. Therefore it is likely that ethanol might elicit its responses by altering the phosphorylation status of specific cytosolic and nuclear proteins. Prolonged exposure to ethanol increased the activity of PKC 6 and *z* as well as PKC mediated phosphorylation in neural cells, while a decreased activity of PKC in the synaptosomcs of CA1 region of hippocampus has been reported in alcohol ingested rats. Studies have been carried out on membrane and cytosolic protein phosphorylation in neurons and astroglial cells of brain in chronic ethanol treatment and hyperphosphorylation of a 116kDa protein has been reported. However, information on nuclear protein phosphorylation during chronic ethanol toxicity is limited, although it is very important in terms of cellular regulation of several neuronal and neurochemical events in brain. Since most of the enzymes of RNA, DNA and protein metabolism are regulated by protein phosphorylation, an attempt is made in this study to ascertain the relation between nuclear protein phosphorylation and cognitive impairment in chronic alcoholism.. The present work is designed to identify the nuclear protein species phosphorylated under calcium dependent and calcium independent phosphorylation conditions. The different nuclear protein kinases involved in these events have also been characterized by specific protein kinase assays as well as by immunodetection.

METHODS

Ethanol Treatment

Wistar strain rats of 30 ± 2 days age weighing 80±10gms were used for the experiments. The animals were housed in groups of four each with access for food and water. The conditions in the animal house were adjusted for constant temperature and day night cycle of 12hrs with light from 7.A.M. to 7.P.M. The food given to the animals was standard laboratory rat feed. Control rats were given tap water in a bottle while the experimental animals consumed ethanol as 5% (V/V) prepared from a stock solution of absolute ethanol diluted with tap water as their only drinking fluid throughout the experimental period which was upto 3rd month of their age. Another set of control animals were also maintained on tap water containing glucose and NaCl for maintaining osmolarity and isocaloric status as described earlier(Babu et al., 1990; Saito.et al., 1987). The animals had the possibility to eat and drink throughout the day. The food consumed was determined by weighing the food when it was provided and reweighing it just before fresh feed was arranged. Similarly the amount of fluid consumed was measured at the end of the drinking period. For every three days body weights of the animals was recorded. All the protocols were performed regularly between 9.00 A.M. to 10.00 A.M. everyday.

Preparation of Nuclei

On the last day of the experiment, the animals were killed by decapitation and the brains were quickly dissected out, rinsed in cold 0.32M sucrose made in 10mM Tris-HCl (pH 7.5), and the blood capillaries were removed. The nuclei were isolated by the method of Giufrida et al., (1975). All the operations were carried out at 4°C. Cerebral cortex was separated from the brain tissue, minced and homogenized in 0.32M sucrose containing 1mM EDTA and lmM KH_2PO_4 buffer (pH 6.4) in Dounce

homogenizer using pestle 'B' by 5 up and down strokes. The homogenate was filtered through nylon mesh and centrifuged at 1000 X g for 10min. The crude nuclear pellet was suspended in 2 M sucrose containing 3mM $MgCl_2$ and 1mM potassium phosphate buffer (pH 6.4) and centrifuged at 75,000 X g for 60 min., using SW-28 rotor. The nuclear enriched pellet obtained was suspended in 10mM Tris-HCl (pH 7.4), 3mM $MgCl_2$ containing 0.32M sucrose and used for further analysis.

Protein Estimation

The concentration of protein in the nuclear samples was estimated by the method of Lowry et al., (1951) using bovine serum albumin as standard.

Scanning Electron Microscopy (SEM)

A small aliquot of nuclear suspension was taken and fixed with glutaraldehyde at a final concentration of 4% for 90 mins at room temperature. The nuclei were washed thrice with phosphate buffered saline (10mM Sodium phosphate buffer pH 7.4 and 150mM sodium chloride), to remove glutaraldehyde. Finally the nuclei were suspended in a 1% ammonium molybdate solution and used for electronmicroscopy. The nuclear suspension was spread on a metal stub and scanned in an electron microscope (Joel JSM-35) after gold vapour coating. Photographs were taken at 20KV with a 60μ objective aperture in a Joel JSM-35 scanning electron microscope equipped with a roll film camera.

Sodium Dodecyl Sulphate-Poly Acryiamidc Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed essentially as done by Laemmli (1970) with modifications as suggested by Thomas and Kornberg (1975). These include (1) concentration of Tris in the resolving gel was increased to 0.75M from 0.375M, (2)

the ratio of acrylamide to N, N' Bis-acrylamide was changed to 40:0.4 and (3) the electrode buffer contained 0.05M Tris, 0.19M Glycine and 0.1% SDS. The proteins were separated on a 1mm thick, 15cms long, 12% acrylamide gel at 150 volts for approximately 5hrs or till the tracking dye front reached the bottom of the gel.

Two-Dimensional Gel Electrophoresis (JEF- SDS-PAGE)

Two dimensional gel electrophoresis was done as described by O'Farrell (1975). Protein sample was solubilized in 50µl of 9.5M Urea, 2% ampholines (pH 5-7 = 1.6% and pH 3-10= 0.4%) and 5% 2-mercaptoethanol. Isoelectrofocusing (IEF) was done in glass tubes (12cms x 0.3cms) in a disc gel electrophoresis unit. Prefocusing was performed at 200volts for 15mins., 300volts for 30mins. and 400volts for 30mins. with 30mM sodium hydroxide as anode buffer and 10mM phosphoric acid as cathode buffer. At the end of prefocusing, the power is turned off, the upper reservoir is emptied, sample buffer and NaOH are removed from the surface of the gels, and the protein samples were loaded. The samples are overlaid with 10µl of 8M urea then 0.03M NaOH, and the chamber is refilled. One of the tube gels was loaded with sample buffer and was used for determining the pH of the gel matrix (blank gel). Electrophoresis was carried out at 600volts for 12hrs, 700volts for 1hrs and finally for 1hr at 800volts. The gels were extruded out of the tube and equilibrated in "equilibration buffer" (0.0625 Tris-HCl pH 6.8, 10% glycerol, 0.005M DTT and 2.3% SDS) for 1 hour.

These gels were subjected to second dimension electrophoresis. The IEF gels were placed on stacking gel (4.75% polyacrylamide/bisacrylamide in 0.125M Tris-HCl pH6.8, and 0.1% SDS) and sealed with 1% agarose made in equilibration buffer. The resolving gel was 12% polyacrylamide/bis-acrylamide in 0.55M Tris-HCl pH 8.8

and 0.1% SDS. Electrophoresis was performed at 15mA/gel till the marker dye entered the resolving gel followed by 20mA till the end of run. The running buffer contained 0.025M Tris, 0.192M Glycine and 0.1% SDS. Standard molecular weight markers were electrophoresed to determine molecular weight of the proteins.

Measurement of pH gradient

The isoelectric focusing gel was cut into 0.5cm sections which were placed in individual test tubes containing 1ml of double distilled water. These test tubes were vortexed, capped and kept overnight at room temperature; then the pH was measured on a pH meter.

Silver Staining of Proteins

Silver staining of proteins in the gels was done as described by Blum et al., (1987). Briefly, after the electrophoresis the gels were fixed in 50% methanol containing 12.5% acetic acid and 0.5ml of 37% formaldehyde/ liter, for 1 hour. Fixed proteins were washed with 50% ethanol thrice for 20 mins. each. Gels were then treated with sodium thiosulphate (0.2g/litre) precisely for 1 min and washed with double distilled water to remove excess thiosulphate. Freshly prepared silver nitrate (2gms/litre of double distilled H_2O containing 0.5ml of 37% formaldehyde) was impregnated in to the gels for 20 mins on a shaker. Excess silver nitrate was washed thrice with double distilled water and finally the gel was developed using 6% Na_2CO_3 containing 0.5ml 37% formaldehyde/litre. As soon as the protein spots appeared, gels were removed from developing solution, washed with water, followed by wash in 50% methanol and 12% acetic acid to stop the reaction. Finally the gels were kept in

50% methanol for 3hours and dried between cellophane sheets in a Bio-Rad gel drier at 80^0C **for 1 hour.**

A utoradiography

The dried gels were placed in a X ray cassette containing intensifying screen. An X ray film was exposed to the radiation coming out of the protein spots and kept over night at -80°C. The exposed film was developed to detect the phosphorylated proteins. The pI and molecular weight of proteins were determined by calculating from the standard values.

Immunodetection

Western blot was performed by the method of Towbin et al.,(1979). The proteins were resolved by SDS-PAGE and transferred onto a nitrocellulose sheet (0.45u) using TGSM buffer (25mM Tris, 192mM Glycine, 01% SDS and 20% Methanol) as electrode buffer at 0.8mA/ cm - transunit (total gel length x breadth) in a LKB-Multiphor II Nova blot for 4 hrs. Blots were stained with ponceau's solution to make molecular weights markings, washed with TBS (Tris buffered saline) and then were blocked with 5% milk powder made in TBS for 2 hrs. After blocking, primary antibody was diluted (1:100 in case of phosphotyrosine and 1:1000 for casein kinase II) in TBS and blots incubated with them over night at 4°C on a shaker. The anti body solution was removed and the blots were washed thrice with TBS, and then incubated with Alkaline phosphatase coupled secondary antibodies for 2 hrs. Finally, the nitrocellulose sheet was washed thrice with TBS and developed using 5Bromo-4Chloro-3Indolyl-Phosphate as substrate.

Endogenous Phosphorylation of Nuclear Proteins

Calcium independent phosphorylation

Phosphorylation assays of nuclear proteins were carried out by following standard methods (Ali et al.,1988; Babu et al., 1994). 100µgs of nuclear protein was used in the phosphorylation reaction, in a buffer containing 10mM Tris-HCl (pH 7.0), 10mM $MgCl_2$ and lmM DTT and lOOfiM EGTA to chelate calcium. The mixture in a final volume of 100µl was incubated at 30°C and the reaction was initiated by the addition of 10µCi of γ ^{32}P-ATP (3000Ci/ mmole). The reaction was allowed for 30 seconds. At the end of assay, the reaction was terminated by adding 4X SDS-PAGE sample buffer followed by boiling in water for 3min.

Calcium dependent phosphorylation

The calcium dependent phosphorylation was performed exactly as described above, excepting that the EGTA in the reaction mixture was replaced with Calcium (100µM).

PKC mediated phosphorylation

The PKC mediated phosphorylation was done as described above by including Calcium (100µM), Phosphatidyl serine (10µg/ml) and Diacyl glycerol (100µM) in the reaction mixture. Prior to inclusion in the assay, Phosphatidyl serine and Diacyl glycerol were dissolved in chloroform separately, evaporated to dryness under the stream of nitrogen. The residual lipids were sonicated in a small volume of 10mM Tris-HCl (pH 7.5), and then used to stimulate PKC in the nucleus. The nuclei were

pre incubated with sonicated lipids for 5 mins and the phosphorylation reaction was performed as described already.

PKC a and y mediated phosphorylation

The composition of reaction mixture was same as for PKC mediated phosphorylation, excepting that the phospholipids and diacly glycerol were replaced with arachidonic acid 100μM and 10μM respectively. Arachidonic acid was dissolved in ethanol (0.05%) as vehicle, and the nuclear samples were preincubated for 5 mins with arachidonic acid 100μM and 10}iM to stimulate PKC a and *y* respectively.

The phosphorylated proteins were subjected to two dimensional gel electrophoresis and autoradiography as described.

Assays for Nuclear Protein Kinases

Calcium independent kinases

Assay for total nuclear kinase activity

The total nuclear kinase activity was determined using dephosphorylated casein as substrate (Girault et al. 1990). The reaction mixture in a final volume of 100μl contained 10μg of nuclear protein, 10mM Tris-HCl (pH 7.0), 100μM ATP, 10mM magnesium chloride, and 1μCi γ^{32}P-ATP(3000Ci/mmol) and 100μg casein. The reaction was performed at 30°C for 15min and stopped by spotting on whatman No. 1 (2 x 2 cms) chromatographic papers and the protein was immediately precipitated with 10% TCA/ 1% sodium pyrophosphate, washed twice with the same solution for 10 mins each, finally rinsed in ethanol and air dried. The dried filter papers were placed in scintillation vials and 5 mL of scintillation fluid was

added, and the radioactivity was measured in a Beckman liquid scintillation spectrophotometer. The total kinase activity was expressed as cpm x 10^{-7} / mg protein.

Assay for Casein Kinase II Activity (CK II)

The CK II assay was performed exactly as done for total kinase activity, but the nuclei were preincubated for 5 min with heparin at a concentration of 0.1 jig/ml to inhibit casein kinase II. CK II activity was calculated by subtracting the heparin-insensitive activity from total kinase activity and expressed as cpm x 10^{-7} / mg protein.

Incorporation of GTP by casein kinase II

Casein Kinase II activity was assayed as described above. However, cold GTP was also included in the reaction mixture to monitor GTP utilization by CK1I. GTP different concentrations (5μM, 10μM, 20μM, 50μM, 100μM) were used to ascertain the optimal incorporation. The reaction was initiated by adding 1μCi ATP(3000Ci/mmol), and remaining steps were carried out as described already.

Immunodetection of nuclear casein kinase-II

Quantitative changes in the nuclear casein kinase II were detected by western analysis using polyclonal antibodies against CK II (gift from Dr. Michael E. Dahmus, University of California) as described already.

Assay for Tyrosine Kinases

The endogenous tyrosine kinase activity was determined using synthetic peptide, Polyglutamate tyrosine (PGT). Phosphorylation of the synthetic substrate was carried out following earlier procedures (Trembley et al., 1994). The reaction mixture consisted of 10mM Tris-HCl(pH 7.4), 10mM $MgCl_2$, 10mM $MnCl_2$, 50μgs

poly PGT, 10μM ATP, 1μCi ^{32}P ATP 3000Ci/mmol), 200μM Sodium orthovanadate and 10μg protein. Phosphorylation was performed for 15 min at 30°C and stopped by spotting the sample on Whatman No 1 (2 x 2cms) filter papers. The protein was precipitated with 10%TCA containing 1% $Na_4P_20_7$. The filter papers were washed twice with the same solution for 10 min. each, rinsed with ethanol and dried. The radioactivity associated with the filter papers was measured in a liquid scintillation spectrometer. Specific tyrosine kinase activity was calculated by subtracting the incorporation of labelled phosphate into the endogenous protein from the total activity measured in the presence of endogenous protein and PGT. The tyrosine kinase activity is expressed as CPM/mg protein.

Detection of Tyrosine Phosphorylated Endogenous proteins

Endogenous phosphorylation of nuclear proteins was carried out as described above except that the PGT was omitted and unlabelled ATP(100μM) was used instead of labelled ATP. The reaction was terminated by adding sample buffer (2X) (Laemmli, 1970) and western analysis was performed using anti-phosphotyrosine Monoclonal antibodies (PY20) to detect the phosphorylated tyrosine residues,

Assay for Calcium Dependent Kinase

Assay of Calcium Calmodulin Dependent Protein Kinase (CaM Kinase II)

CaM kinaseII activity was measured following standard protocol (Zhang et al., 1993). Calcium and Calmodulin sensitive protein phosphorylation in the nuclei was determined in an assay mixture containing 50mM Tris-HCl (pH 7.0), 10mM $MgCl_2$, 0.1 mM DTT, 0.12mM EGTA, 3μCi γ ^{32}P-ATP (3000 Ci/mmol), 5μg of protein and either various concentrations of $CaCl_2$, with fixed concentration of calmodulin(2μM) or various concentration of calmodulin with fixed concentration of $CaCl_2$ (250μM).

After preincubating the samples for 5min at 30°C, the reaction was initiated by adding (^{32}P ATP). The reaction was performed for one min at 30°C, and was stopped by spotting on Whatman No 1 filter paper (2 x 2 cms). The protein was precipitated with 10% TCA containing 1% Sodium Pyrophosphate, washed twice with the same solution for 10 mins each and finally rinsed with ethanol. The filters were air dried and counted in a liquid scintillation spectrometer.

Detection of CaM Kinase II substrate proteins in the nucleus

The CaM Kinase II substrates were identified by analysing the calcium calmodulin dependent incorporation of phosphate into endogenous nuclear proteins. The assay conditions were same as described for the CaM Kinase II assay. The phosphorylation reaction was stopped by adding 1/3 vol. of sample buffer [125mM Tris-HCl (pH-6.8), 3% SDS, 10% glycerol, and 5% 2 mercaptoethanol] and the mixture was heat denatured for 2 min in boiling water. The total protein was precipitated by five volumes of chilled acetone, and the samples were subjected to electrophoresis followed by autoradiography as described.

Statistical Analysis

All enzyme assays were carried out in four individual experiments. The ultimate data represented is a mean and standard deviation. The students 't' test was carried out to arrive at the significance of values and has been shown in concerned figures.

RESULTS

General Condition and Behavioural Symptoms of Animals

In the present study, animals were habituated to intake by giving them 5% ethanol (V/V) as the only drinking fluid throughout the experimental period. The general condition of the experimental animals was normal when compared to that of controls, except that the ethanol treated animals showed a slightly slower growth rate. A decrease (8%) in the body weight of experimental animals was noticed. This decrease can be correlated with the quantity of ethanol ingested, which was ~ 1.5gm ethanol/day/animal. The blood alcohol content (BAC) as measured by ADH activity was 0.15%. Initially the experimental animals consumed less quantity of ethanol which gradually increased with time, indicating that the volume of ethanol consumed is indirectly proportional to the weight of the animal, i.e. higher the ethanol slower the growth rate. Normal behavioural movements such as standing posture and physical functions like reaching for food etc. were normal. The growth rate, food consumption, body weight of control rats were normal and the data agrees with the earlier results (Klemn and Engen 1978). The loss in body weight of ethanolic rats also agrees with previous reports. (Matsubara *et al,* 1987).

The nuclei were checked for purity by scanning electron microscope.. There was no change in the structure and external morphology of the nuclei isolated from the control and the experimental animals the nuclei appeared to be pure and intact(Fig. 1). An earlier report has shown a decrease in the size of the neuronal nuclei in the entorhinal cortex in the post mortem brain of alcoholic patients (Ibanez et al., 1995). This difference between our results and earlier findings might be due to the heterogeneous nature of nuclear samples. The former study has used neuronal nuclei

for its investigation, while we have used both neuronal as well as glial nuclei for our experiments.

Protein Phosphorylation Pattern in the Brain Nuclei

The experiments carried out in the absence of calcium using EGTA in the reaction mixture showed phosphorylation of a group of proteins (Fig.2A). The two dimensional electrophoresis of total phosphorylated nuclear proteins could separate ~ 20-30 proteins in a pH gradient of 4.3 to 6.8. Several proteins got phosphorylated above the pI value of 6.8 which could not enter the gel and these proteins are visualized as a streak rather than clearly resolved proteins on the autoradiogram. Majority of the phosphorylated proteins were distributed between pI range of 5.3 to 6.3. The details of these proteins such as molecular weight and isoelectric point are shown in a tabular form (Table 1). The protein numbers indicated in the table correspond to the protein numbers marked in the autoradiograms. These proteins 1-8 as shown in the table (Table I and Fig. 2A-2E) show a consistent status of phosphorylation even when the phosphorylation conditions are altered i.e. when agents like Calcium(C), Phosphatidyl serine and Diacylglycerol (P), Arachidonic acid 10µM (AT) and Arachidonic acid 100µM (AH) were included in the phosphorylation assays (Fig 2A-2E). Proteins 1 and 5 are hyperphosphorylated while protein 3 alone exhibited a hypophosphorylation in samples incubated with AAlOuM (AT). The other proteins showed more or less no changes inspite of the presence of varying concentrations of EGTA, Calcium, Phosphatidyl serine/Diacylglycerol, AA10µM, and AA100µM.

Besides the general pattern described above, it is noticed clearly from this study, that three proteins which are shown as E35, E45 and El 16, light up in samples

containing EGTA i.e. in calcium independent phosphorylation (Fig. 2A). These proteins are not phosphorylated under any other conditions studied except in the presence of EGTA. These substrate proteins in turn prove to be good candidates of calcium independent phosphorylatable proteins. On the contrary, at least three proteins namely C52, C56, C116 appear in the presence of Calcium, Phosphatidyl serine/Diacylglycerol, of these three proteins C52, C56 also get phosphorylated in the presence of AA10|iM and AA100μM (Fig. 2D-2E). A protein namely C32 is exclusively phosphorylated only in the presence of calcium showing very high calcium dependency (Fig. 2B). The disappearance of C32 in the presence of PS/DAG and AA could be due to the activation of PKC stimulated phosphatases, which might be one of the responsible factors for this change.

In addition to the changes noticed in specific phosphorylated proteins, which are highly resolved on a 2D gel, there are specific groups of proteins which get phosphorylated. Each group consists more than four proteins in each of the experimental conditions such as in the presence of Phosphatidyl serine/Diacyl-glycerol. This particular group is named as Pgps (45-116), similarly another group named as ATgps(50-85), and AHgps 85-116 and gps>l 16 have been noticed in the samples containing Arachidonic acid at a concentrations of 10μM(AT) and 100μM(AH) respectively.

Effect of Ethanol Treatment on Calcium Independent Protein Phosphorylation

Chronic exposure to ethanol is known to increase the calcium channels so that the cell can recruit more calcium into the cell (Messing et al., 1986). The relation between PKC mediated phosphorylation and the upregulation of calcium channels by ethanol is not very clear. Under these conditions, it is very essential to understand as

to what happens to the nuclear protein phosphorylation status when calcium is restricted to the absolute minimum.. To pursue this aspect, nuclear protein phosphorylation assays were carried by including EGTA, which will chelate all the calcium ions in the nucleus. This study showed almost no changes in the proteins numbered from 1-7 (Fig. 3; Table II). However a group of proteins # 8 (gps116-50) and E45 have very conspicuously failed to get phosphorylated in chronic alcoholic condition. This indicates a possible inhibitory role inflicted by ethanol on calcium independent nuclear kinases.

Effect of Ethanol on Calcium Dependent Protein Phosphorylation

Unlike the changes in calcium independent phosphorylation, the experiments carried out using 100μM calcium showed almost no changes in the phosphorylation pattern of all the 1-8 proteins (Fig.4 ; Table III). However, the group of proteins (gps # 8) which have disappeared in ethanolic samples under conditions of calcium absence are restored when calcium is included in the assay conditions. This observation at the out set appears puzzling because these proteins are not phosphorylated in calcium deficient conditions but are restored when calcium is used. So the results can be interpreted that these proteins might be undergoing calcium dependent phosphorylation in alcohol treated samples.

Basing on these data (Fig.3 ; Table II), it can be concluded that calcium independent kinases are active in the control samples while they get affected on alcoholic treatment leading to the disappearance of whole group of proteins, which otherwise are seen to be phosphorylated in controls. It can be noticed that when calcium is included (Fig.4), calcium dependent kinases are active in control and experimental conditions. In the presence of calcium, the same group of proteins were

hyperphosphorylated, which were not phosphorylated in the presence of EGTA in the experimental samples. This result suggests the possibility that, during chronic ethanol ingestion the activity of calcium independent kinases might have decreased while that of calcium dependent kinases must have increased. This implies a greater involvement of calcium independent and calcium dependent protein kinases in ethanolic condition and hence a thorough search on the type of calcium dependent and calcium independent nuclear kinases is very essential and therefore studies were carried out in this particular direction.

*Role of **PKC** in the Ethanol Induced Nuclear Protein Phosphorylation*

Protein kinase C has been shown to be very important in normal neuronal function. Prolonged exposure to ethanol has been shown to increase PKC mediated phosphorylation in neurons (Babu et al., 1994). However, the specific substrates involved in PKC mediated phosphorylation in chronic ethanol treated cells remain obscure. In this attempt to identify the kinases involved, the experiments having PS/DAG, revealed clearly (Fig.5 ; Table IV) that a group of nuclear proteins P1, P2, P3, P4 and P5 with MW ranging from 40-66kDa and pI 4.4-4.8 get specifically phosphorylated under the present set of experimental conditions. Ethanol is known to increase several nuclear/cytosolic proteins. The group of nuclear proteins noticed in the experimental condition might involve PKC mediated regulation of nuclear events in chronic ethanol exposure.

***Stimulation of PKC a and** y isoforms by Arachidonic acid (AA)*

The PS/DAG happen to be a general activator of PKC. PKC is known for its multiple gene families consisting of (~ 8) different isoforms (Huang and Huang. 1993). Specific isoform of PKC can be stimulated by AA, in a concentration

dependent manner. By using 10μM AA the y form of PKC and at 100 μM concentration the a form of PKC have been shown to be stimulated (Huang. 1989). Hence in these experiments by using these two concentrations of AA, the phosphorylated protein profile by these two PKC isoforms has been worked out in control and ethanol treated samples. The γ form of PKC mediated phosphorylation (Fig.6 ; Table V) showed a protein profile more or less similar to what has been observed in sample where PS/DAG has been included. However, a set of nuclear proteins with molecular weight >116kDa (pI 5.6-6.2) get specifically phosphorylated in nuclei from ethanol treated samples. When 100μM arachidonic acid (AH) was used in both control and experimental conditions, no significant differences were noticed in the nuclear protein phosphorylation pattern, indicating sustained activity levels of PKC a both in control and experimental conditions (Fig. 7 ; Table VI).

Nuclear Protein Kinases

Calcium independent kinases

Casein Kinases

In the present work, since the focus is on identifying the involvement of different kinases, CK I and II activities were monitored in the nuclei isolated from control and experimental samples. The activity of casein kinase I and II can be measured either by using specific synthetic substrates or by making use of the fact that they are inhibited differentially by heparin at 100μg/mL and 0.1μg/mL respectively (Rose et al., 1981). Moreover, CKII can also use GTP instead of ATP as phosphate donor. These two properties of CK I and CK II were used to measure the activity of these enzymes in the nuclei of control and alcohol treated rats. In the present study enzyme assays were carried out using heparin.

The total incorporation of labelled phosphate into casein did not show any change between the control and experimental samples (Fig.8). Heparin inhibited the enzyme activity (Fig.9) in a concentration dependent mariner. The decrease in the incorporation of ^{32}P labelled phosphate into the substrate, suggests the presence of casein kinase I and II in the nucleus. The activities of casein kinase I and II were calculated by subtracting heparin insensitive activity from the total activity and expressed as CPM x 10^{-7}/mg protein. The nuclear CK II activity in the brains of control and ethanol treated rats is shown in Fig. 10. The CK II activity in particular decreased by 30% in experimental nuclei (Fig. 10). The CK I did not show any significant change between the two groups hence the data is not shown.(Result not shown).

To further confirm the presence of CK II in the nuclear preparations, GTP was included in the phosphorylation assays. A concentration dependent decrease in the incorporation of labelled phosphate into casein was noticed, suggesting that CK II was using GTP as phosphate donor. As the concentration of GTP increased from 5μM to 20μM, there was a steep decline, then the slope decreased from 20μM to 50μM reaching a plateau between 50 to 100μM (Fig. 11). The activity of CK II using GTP as phosphate donor also showed a decrease in experimental animals (Fig. 12). To understand the reason behind decreased activity of CK II, western analysis was performed to determine the quantitative changes of the enzyme. The immuno detection has shown a single band with a molecular weight of 45kDa, corresponding to the a subunit of CK II molecule (Fig. 13). The β subunit of the enzyme could not be detected which could be either due to its low immunogenicity or individual differences between species (Munstermann, et al., 1990). Although the enzyme

activity has decreased, the quantity of the enzyme appears to be not significantly affected, suggesting that there is neither a down regulation of enzyme synthesis nor the transport of the enzyme from cytosol to the nucleus is affected in ethanol fed animals.

Tyrosine Kinase activity

Tyrosine kinase activities though are quite general in cell transformation, proliferation and cell growth related events, they have been found over expressed in normal cells also in several adult mammalian tissues including central nervous system (Ullrich and Schlessinger 1990). Though tyrosine kinases have been purified from mammalian tissues not much is known about the phosphotyrosine containing protein substrates. Work on these lines is rather scarce because of the limitations in assaying and identifying protein tyrosine kinase substrates. The phosphoamino acid analysis of tyrosine is tedious and therefore the technique has to be relied mostly on monoclonal antibodies highly specific for phospho-tyrosine. In the present study the protein tyrosine kinase assays were carried out and the tyrosine residues of phosphorylated proteins were visualized by highly sensitive immunochemical analysis using phosphotyrosine monoclonal antibodies (PY20).

The nuclear protein tyrosine kinase activity was assayed in the presence and absence of a synthetic substrate (poly Glu^{80}-Tyr^{20}). The activity obtained in the absence of synthetic substrates represents the total sum of kinase activities. On the other hand the tyrosine kinase activity obtained in the presence of synthetic substrate represents the total kinase activity plus synthetic substrate dependent tyrosine phosphorylation. The difference between the two directly represents the protein tyrosine kinase activity. Tyrosine kinase activities were also monitored in the

presence and absence of sodium vanadate (200μM) which is a known inhibitor of protein tyrosine phosphatase, to prevent the dephosphorylation of exogenous substrate. The incorporation of labelled phosphate was more in the presence of vanadate in both control and experimental samples, indicating the presence of possibly very active protein tyrosine phosphatases in the nucleus. The endogenous protein tyrosine phosphorylation was less and addition of substrate (poly Glu-Tyr) could increase the tyrosine kinase activity very moderately. The tyrosine kinase activity in the presence of vanadate showed a subtle increase in the experimental samples over the controls (Fig. 14), while there was no difference between the two groups when the reaction was performed in the absence of vanadate (Fig. 15). The possible substrate proteins phosphorylated by tyrosine kinase in the nucleus were examined by western analysis with monoclonal phosphotyrosine antibodies(PY20). The tyrosine protein phosphorylation patterns are shown (Fig. 16 ; Table VII). Specific phosphotyrosine containing proteins were detected in the nuclei of control and experimental rats in the presence and absence of ATP as well as vanadate separately. The data obtained from these experiments showed an interesting result. In absence of ATP there were not many tyrosine phosphorylated proteins (Fig. 16: lane 1,2). In the presence of ATP several proteins were phosphorylated on tyrosine residues but there was no significant change between the control and ethanol treated samples (Fig. 16: lane 3, 4). However, when sodium vanadate was included, at least 4 proteins were highly phosphorylated in the nuclei of ethanol treated samples (Fig. 16; lane 5,6). A phospho protein of 40kDa was specifically reported in brain, spleen and thymus of tissues of rat following the addition of vanadate (Trembley and Beliveau1994). However in this study we could not find such a protein in the control

sample while we could find 4 major substrates i.e. T1,T2, T3, T4, (Fig. 16 ; Table VII) which are specifically hyperphosphorylated in ethanol treated samples.

Calcium dependent kinases

Calcium Calmodulin Dependent Protein Kinase 11 (CaM Kinase 11)

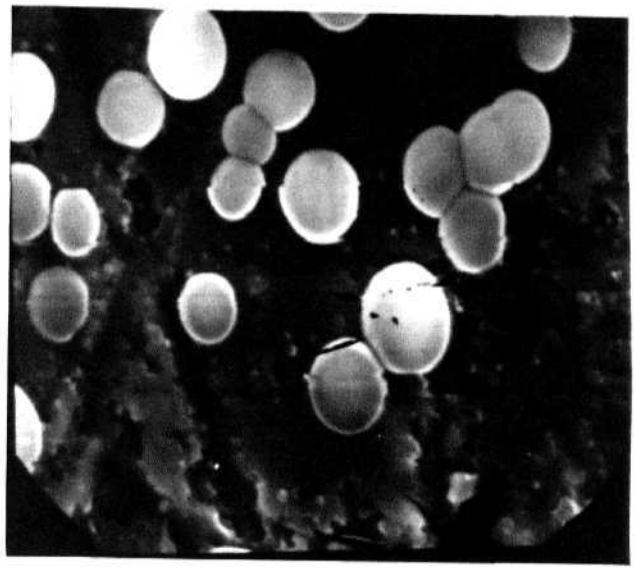
The cognitive impairment in alcoholics has been reported to have a molecular basis involving alteration in the long term potentiation. Among the neurobiological events that lead to LTP, the role of PKC, CK II and calcium calmodulin dependent kinase II has been well documented. Hence in the present study besides PKC and CK II, the activity levels of nuclear CaM kinase II have also been assayed to assess its contribution to ethanol induced neurotoxicity. In order to standardise the optimal conditions for CaM kinase II activity, the incorporation of labelled phosphate into nuclear proteins was monitored as a function of ATP(Fig.17), calmodulin (Fig. 18) and calcium (Fig. 19). The results showed that 3μCi ATP (3000Ci/mmol), 2μM calmodulin and 250μM calcium were required for an effective and maximal activity. Using the enzyme assay conditions, the CaM kinase II activity was determined in the nuclei of control and experimental samples. The CaM kinase II activity and the endogenous protein phosphorylation pattern are shown in Fig.20 and Fig.21 respectively. The electrophoretic gel pattern of CaM kinase II substrates in control and ethanolic samples did not show any significant variations. Nevertheless, the lighting up of several protein bands confirmed the presence of CaM kinase II and its substrates in the nucleus The addition of calcium and calmodulin to the reaction mixture induced hyperphosphorylation of two proteins with molecular weights 50-60 kDa. When KN-62, an specific inhibitor of CaM kinase II was added, the intensity of phosphorylation of 60 and 55kDa proteins decreased considerably. The calcium

calmodulin dependent phosphorylation of nuclear proteins did not show any significant alteration between the control and the experimental samples (Fig.21) suggesting that CaM kinase II mediated phosphorylation reactions in the nucleus might have not been altered in ethanol toxicity.

FIGURES

Fig. 1: Scanning electron micrographs of nuclei isolated from control (C) & experimental (E) samples. The nuclear suspension was spread on a metal stub and scanned in an electron microscope . Photographs were taken at 20KV with a 60μ objective aperture in a Joel JSM-35 scanning electron microscope . Magnification 10000 x.

C



E

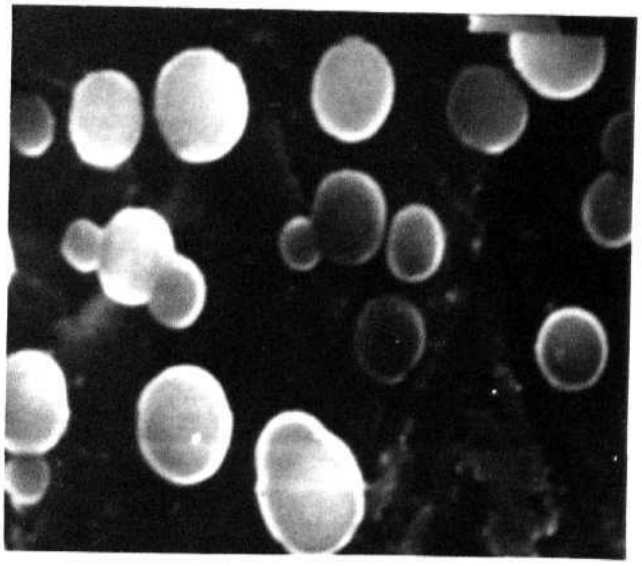
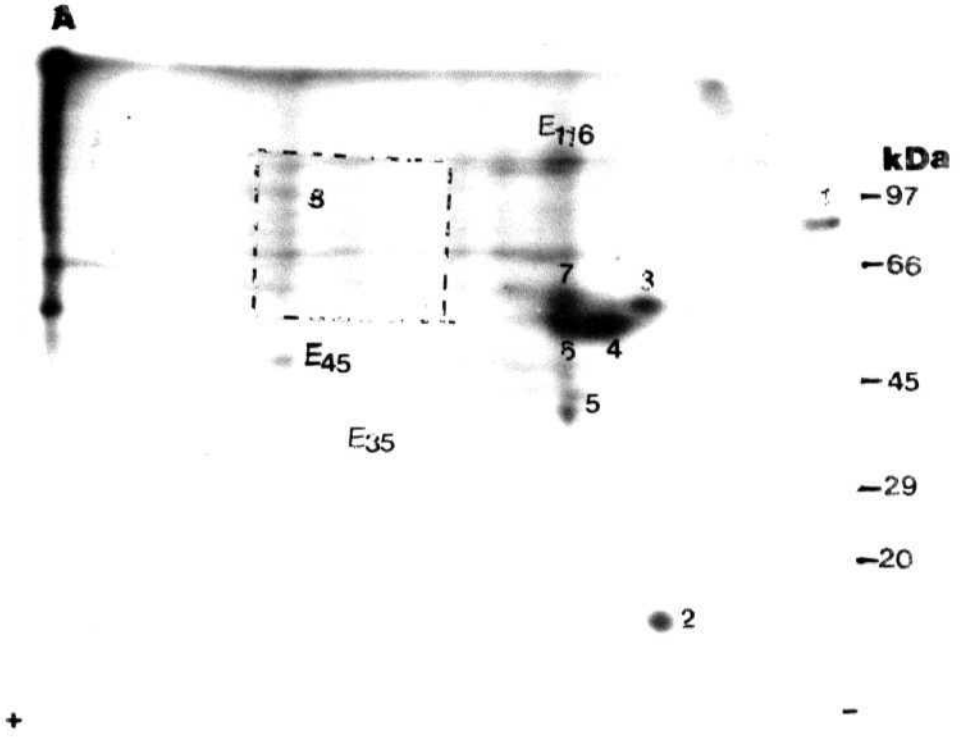


Fig. 2A: Autoradiograms of nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from rat brain control samples. After phosphorylation 100µgs of nuclear protein was loaded on to 2DE gel. EGTA (100µM) was included in phosphorylation assays to check calcium independent phosphorylation. Molecular weight is shown on the right side of the panel. Proteins numbered I to 8 are common, while proteins E35, E45, E116 represent proteins specifically phosphorylated under the experimental conditions. The pI and M.W of these proteins are shown in Table I

Fig. 2B: Autoradiograms of nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from rat brain control samples. After phosphorylation 100µgs of nuclear protein was loaded on to 2DE gel. Calcium (100µM) was included in phosphorylation assays to check calcium dependent phosphorylation. Molecular weight is shown on the right side of the panel. Proteins numbered 1 to 8 are common, while proteins C32, C52, C56, C116 represent proteins specifically phosphorylated under the experimental conditions. The pI and M.W of these proteins are shown in Table I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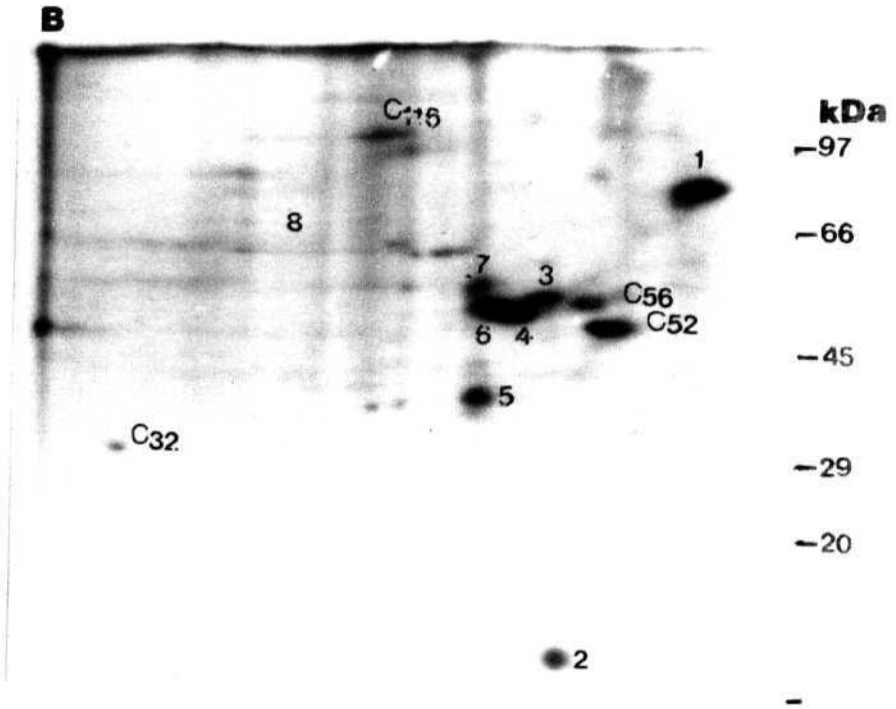
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Fig. 2C: Autoradiograms of nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from rat brain control samples. After phosphorylation 100µgs of nuclear protein was loaded on 2DE gel. Calcium(100µM), PS(10µg/ml), DAG(100µM) were included in phosphorylation assays to stimulate PKC mediated phosphorylation. Molecular weight is shown on the right side of the panel. Proteins numbered 1 to 8 are common, while proteins Pgps represent proteins specifically phosphorylated under the experimental conditions. The pI and M.W of these proteins are shown In Table I

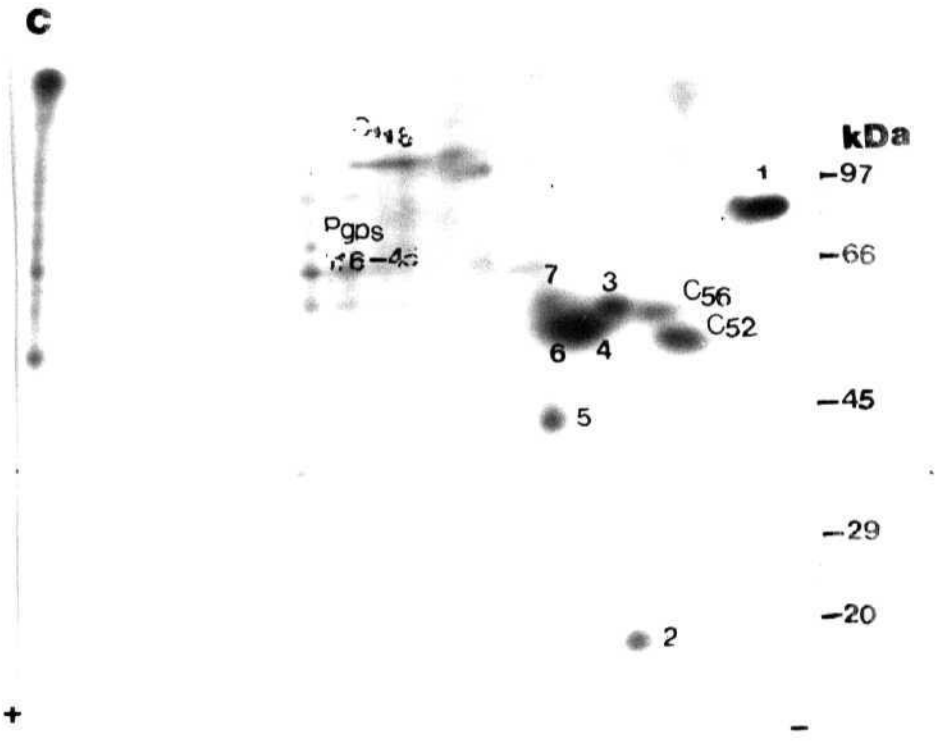
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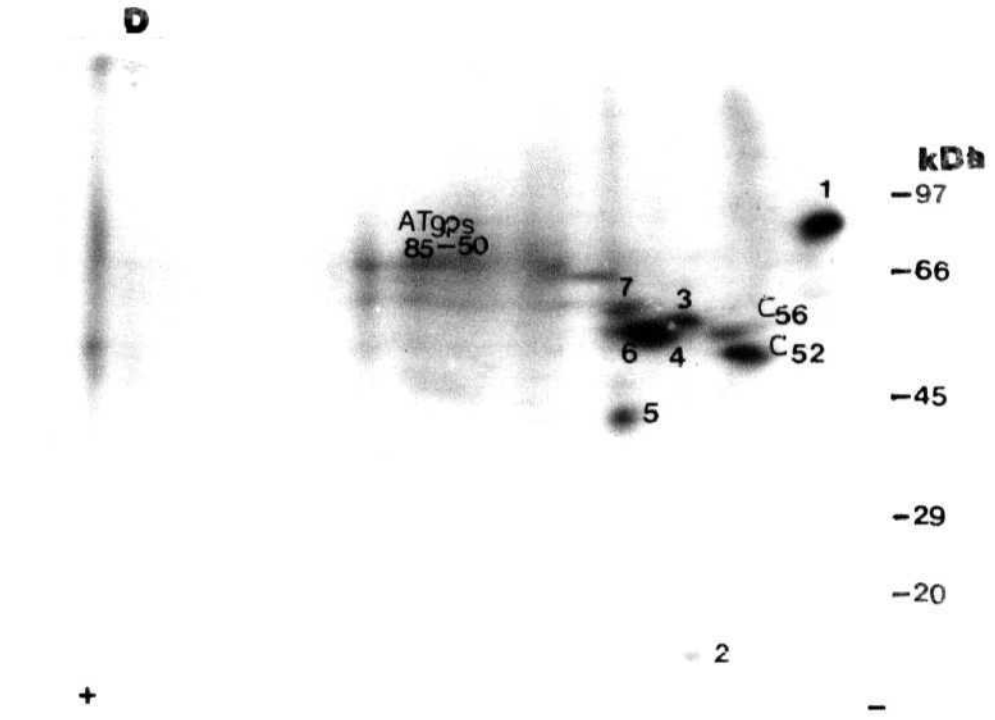
Fig. 2D: Autoradiograms of nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from rat brain control samples. After phosphorylation 100µgs of nuclear protein was loaded on 2DE gel. Calcium (100µM) and AA(10µM) were included in phosphorylation assays to stimulate PKC *y* mediated phosphorylation. Molecular weight is shown on the right side of the panel. Proteins numbered 1 to 8 are common, while proteins AT gps represent proteins specifically phosphorylated under the experimental conditions. The pI and M.W of these proteins are r;hown in Table I

Fig. 2E: Autoradiograms of nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from rat brain control samples. After phosphorylation 100µgs of nuclear protein was loaded on 2DE gel. Calcium(100µM) and AA(100µM) were included in phosphorylation assays to stimulate PKC a mediated phosphorylation. Molecular weight is shown on the right side of the panel. Proteins numbered 1 to 8 are common, while proteins AH gps represent proteins specifically phosphorylated under the experimental conditions. The pI and M.W of these proteins are shown in Table I


D
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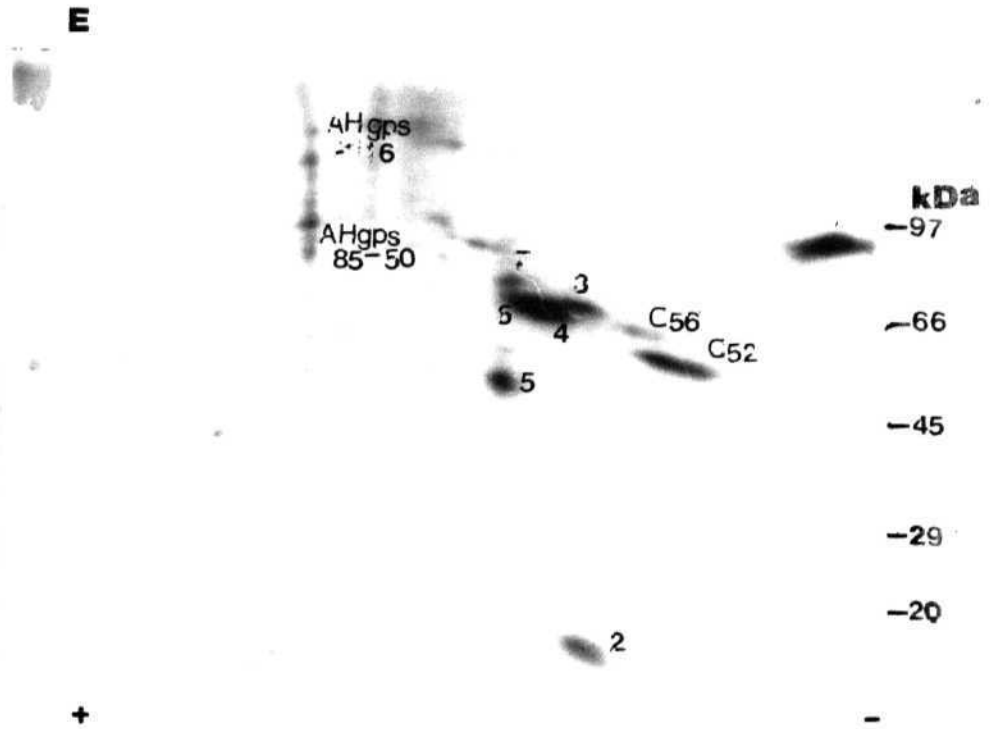
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C56
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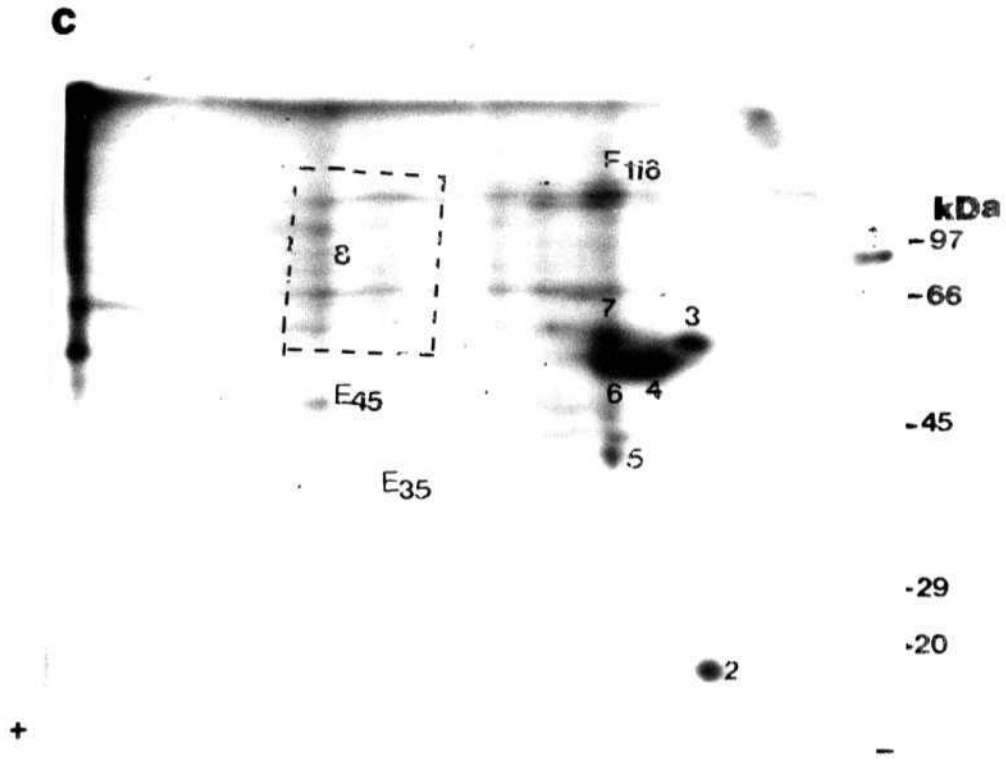
Fig. 3: Autoradiograms showing calcium independent phosphorylation patterns of brain nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from control (C) and chronic ethanol (E) treated rats. Nuclear protein (100μgs) was loaded on each gel. The proteins showing specific changes are shown in Table II


C
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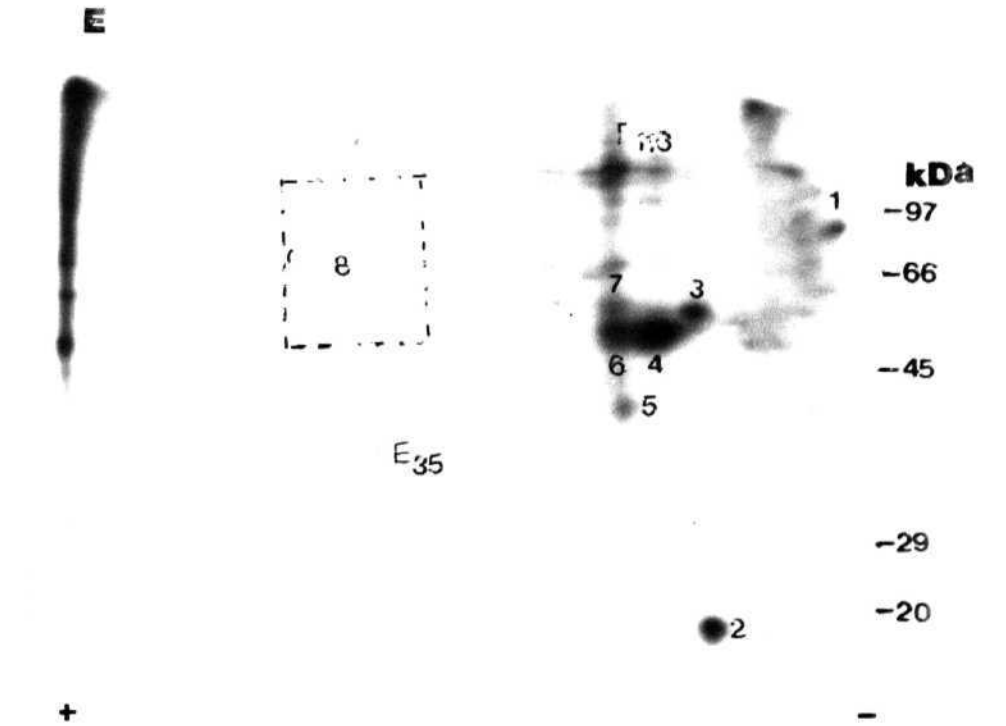
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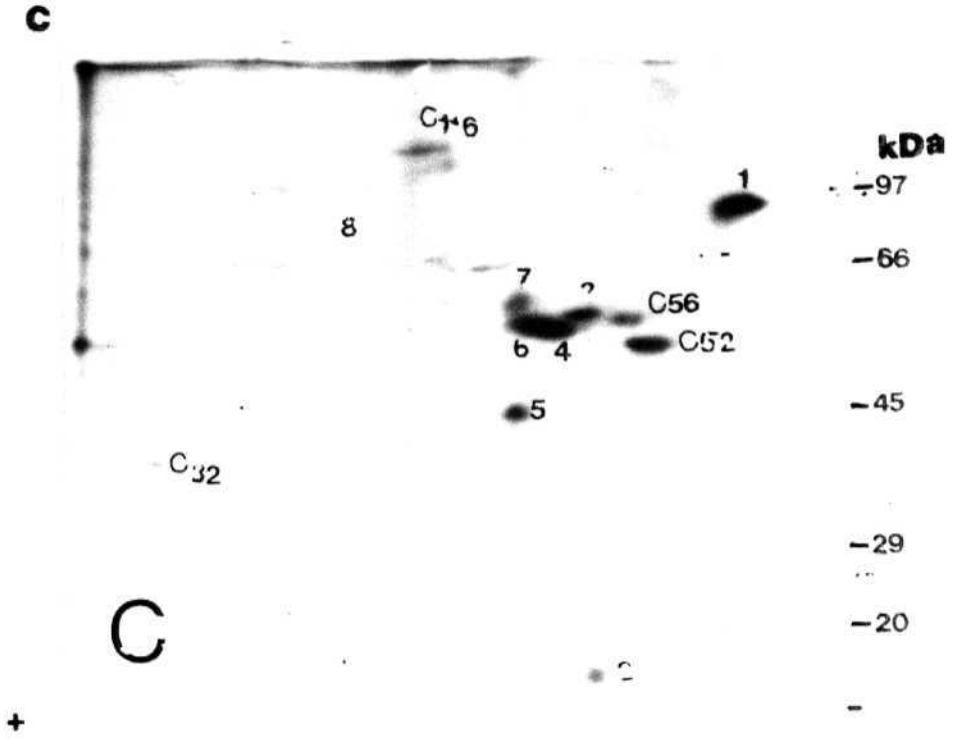
Fig. 4: Autoradiograms showing calcium dependent phosphorylation patterns of brain nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from control (C) and chronic ethanol (E) treated rats. Nuclear protein (100µgs) was loaded on each gel. The proteins showing specific changes are shown in Table II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+
-

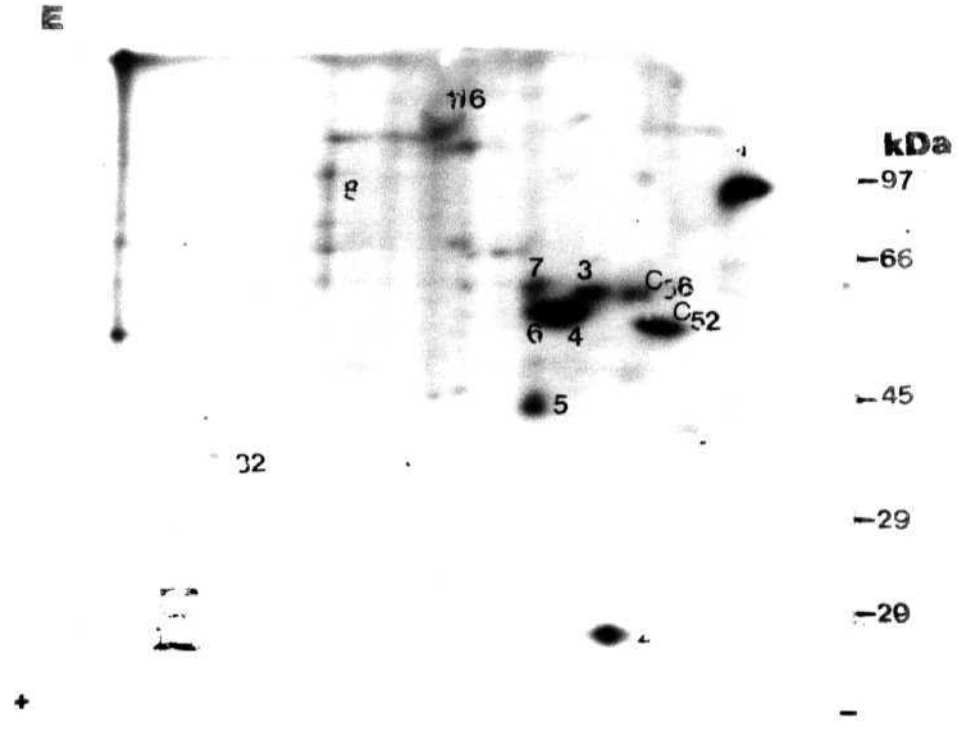
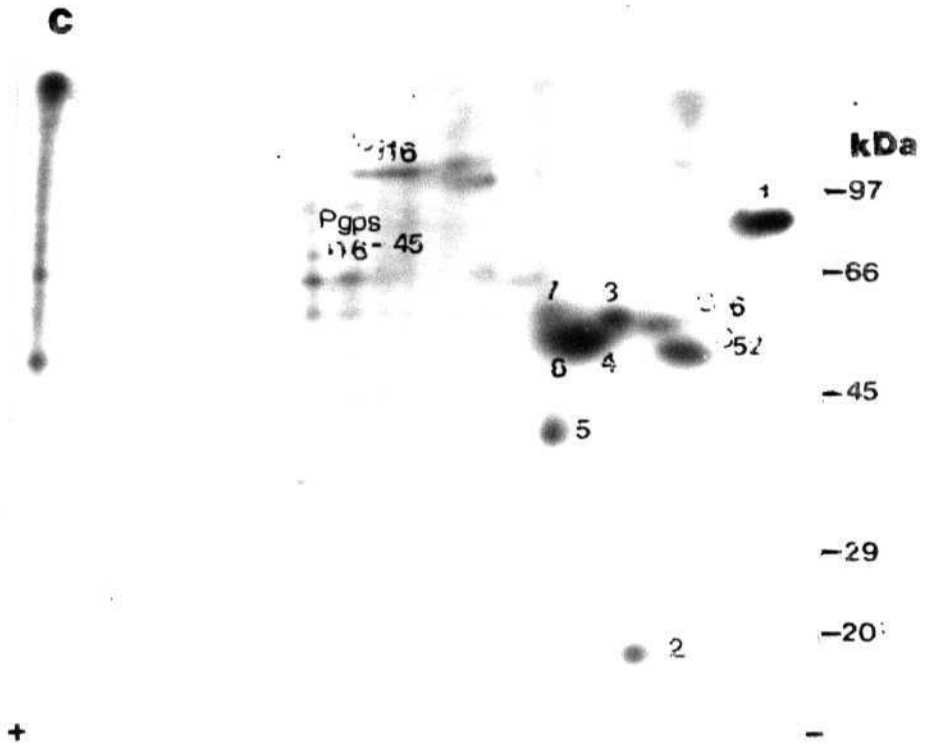

E
kDa
97
66
45
29
20
7
3
6
4
5
C56
C52
+
-

Fig. 5: Autoradiograms showing PKC dependent phosphorylation patterns of brain nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from control (C) and chronic ethanol (E) treated rats. Nuclear protein (100μgs) was loaded on each gel. The proteins showing specific changes are shown in Table IV


C
kDa
97
66
45
29
20
Pgps
1
2
3
4
5
6
+
-

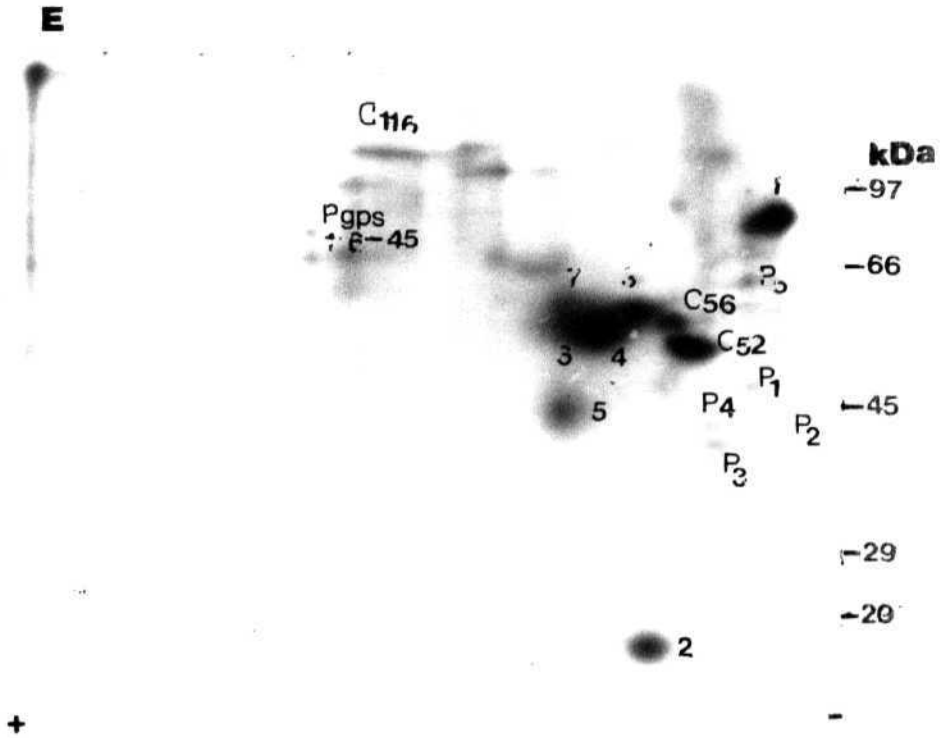
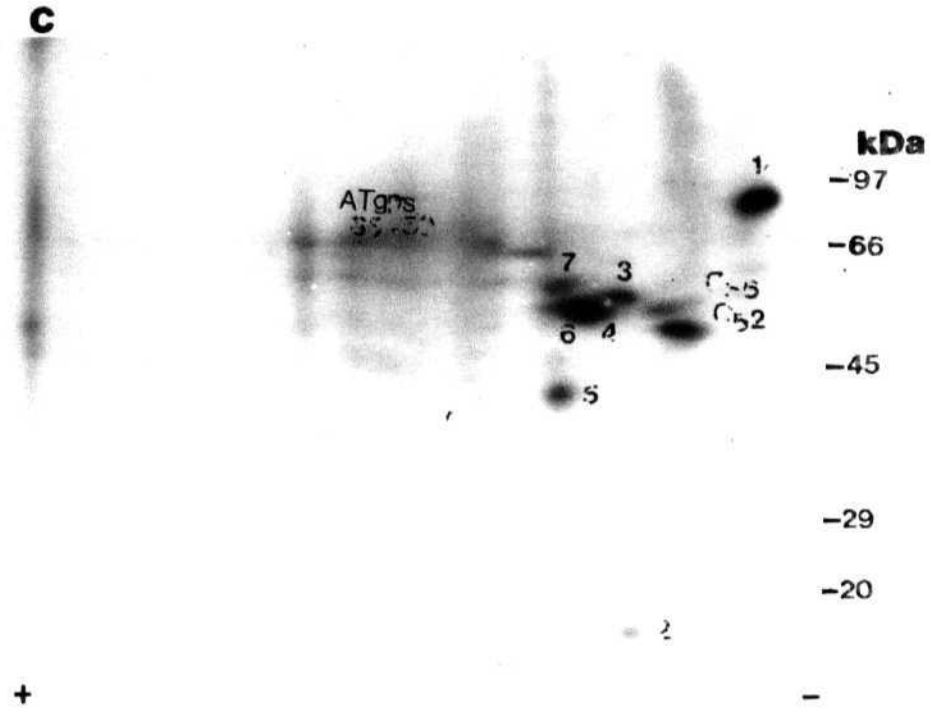
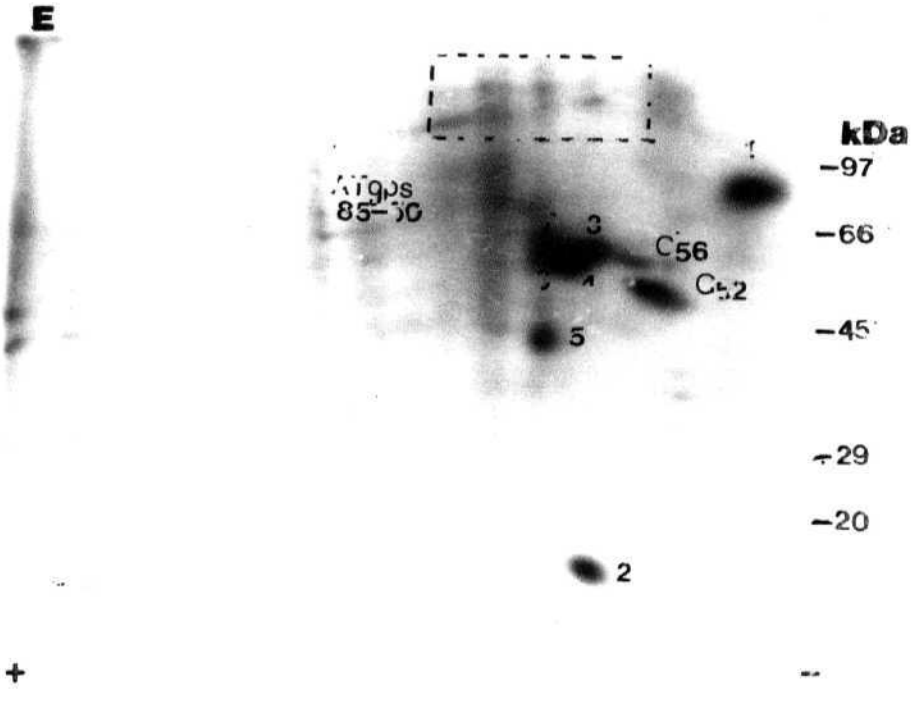

E
kDa
97
66
45
29
20
Pgps
C56
C52
P1
P2
P3
P4
P5
1
2
3
4
5
+
-

Fig. 6: Autoradiograms showing PKC y dependent phosphorylation patterns of brain nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from control (C) and chronic ethanol (E) treated rats. Nuclear protein (100μgs) was loaded on each gel. The proteins showing specific changes are shown in Table V


C
kDa
97
66
45
29
20
ATgps
7
3
6
4
5
1
C56
C52
+
-


E
kDa
97
66
45
29
20
ATgps
85-50
3
C56
C52
5
2
+
-

Fi ': Autoradiograms showing PKC a dependent phosphorylation patterns of brain nuclear phosphoproteins analyzed by two dimensional gel electrophoresis from control (C) and chronic ethanol (E) treated rats. Nuclear protein (100µgs) was loaded on each gel. Th proteins showing specific changes are shown in Table VI.

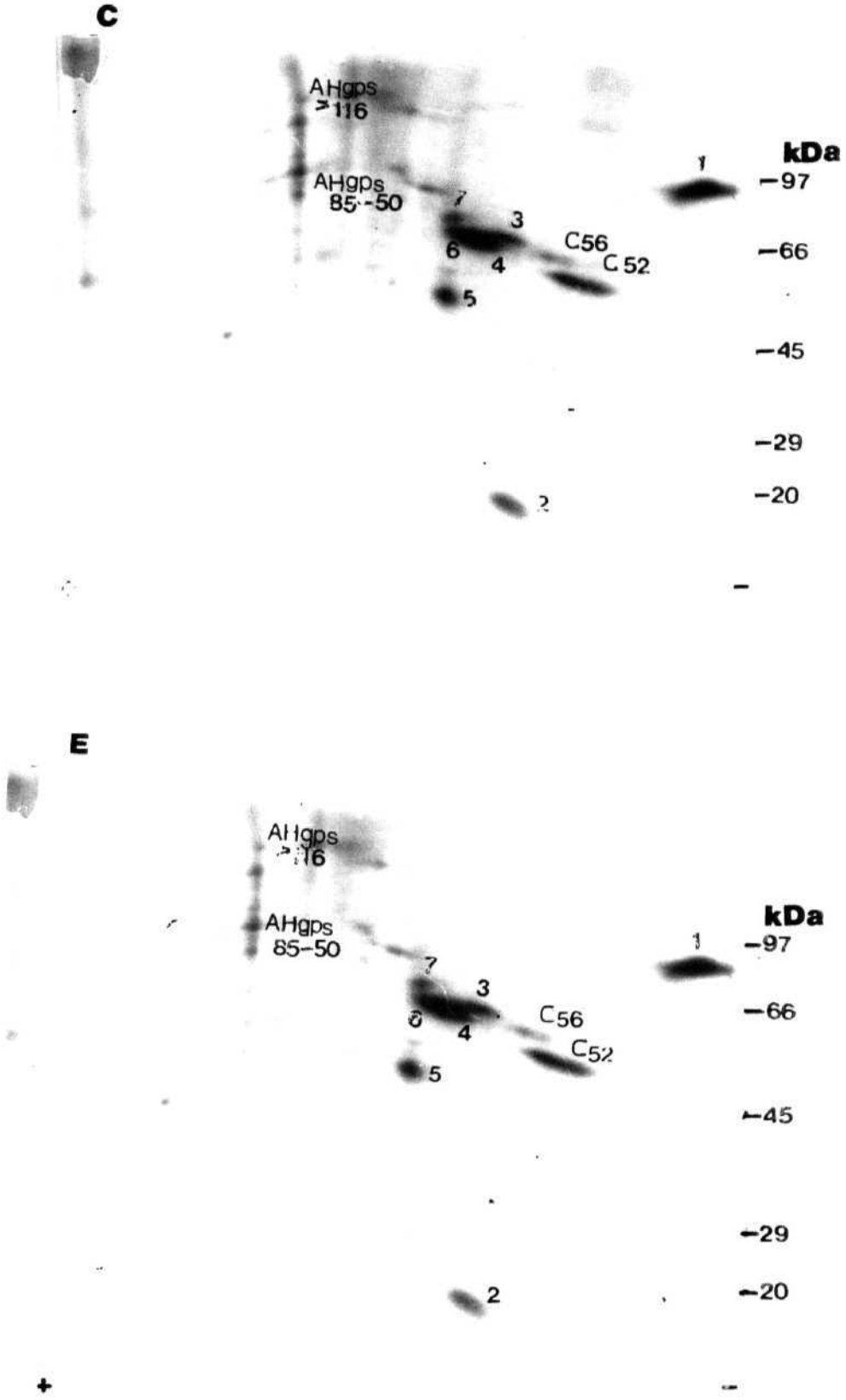

C
AHgps
>116
AHgps
85-50
kDa
-97
-66
-45
-29
-20
C56
C52
E
AHgps
>116
AHgps
85-50
kDa
-97
-66
-45
-29
-20
C56
C52
+
-

Fig. 8: Assay of brain nuclear kinases activity for the control (C) and ethanol treated (E) rats. The reaction mixture in a total volume (100µl) contain 10µg of nuclear protein. The reaction was allowed to proceed for 15mins at 30 ^{0}C and arrested by 10% TCA and 1% $Na_4P_2O_7$. The radioactivity in the TCA precipitate is expressed as CPM X 10^{-7} /mg protein. The data represented in each bar is mean ± SD for four individual experiments.

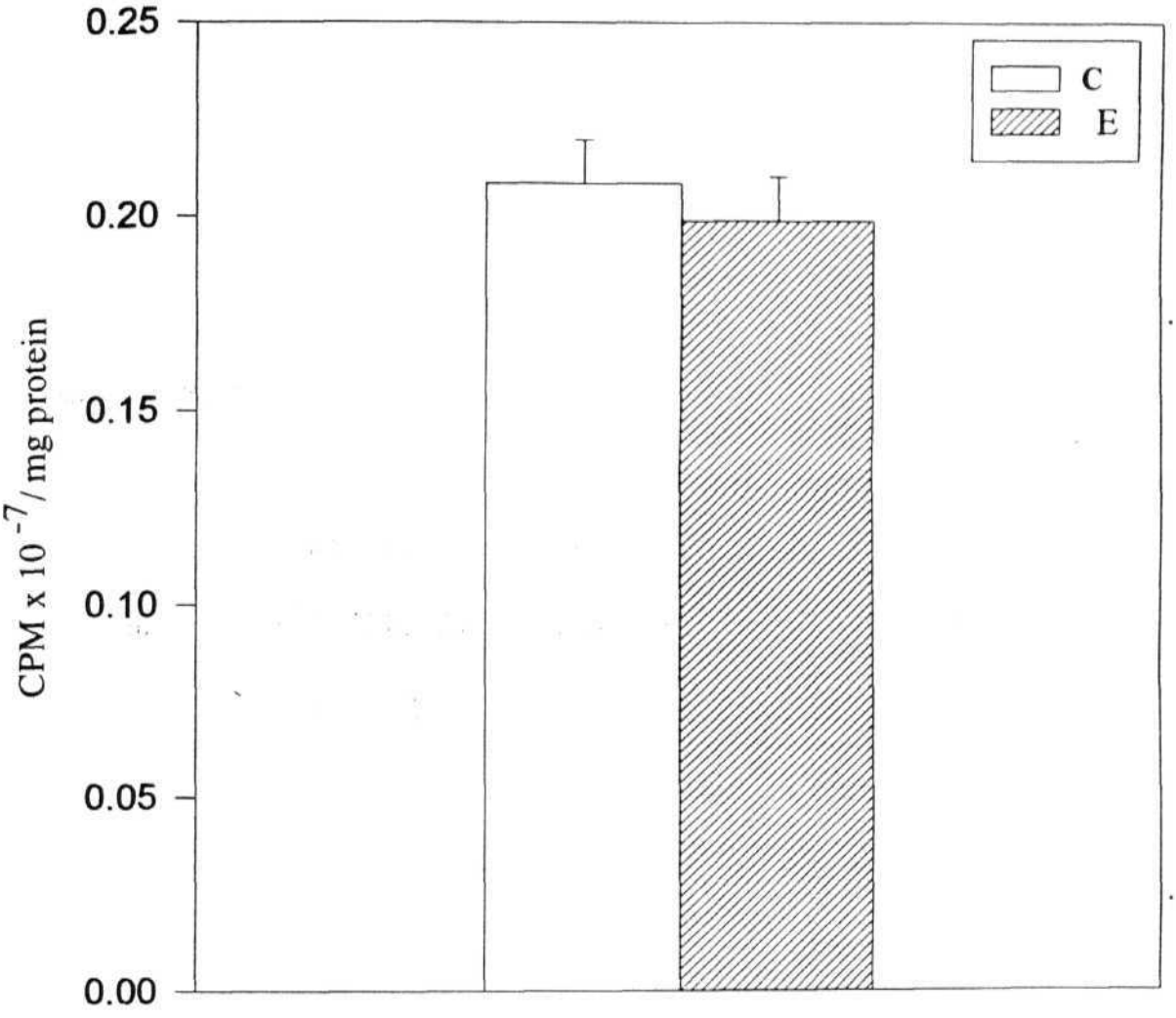

0.25
0.20
0.15
0.10
0.05
0.00
CPM x 10 -7 / mg protein
C
E

Fig. 9: Assay for casein kinases I and II in the nuclear samples of control(C) and ethanol (E) treated rat brain as a function of heparin concentration. The reaction conditions are same as described for fig. 8 excepting for the addition of heparin.

EFFECT OF HEPARIN ON CASEIN KINASE ACTIVITY

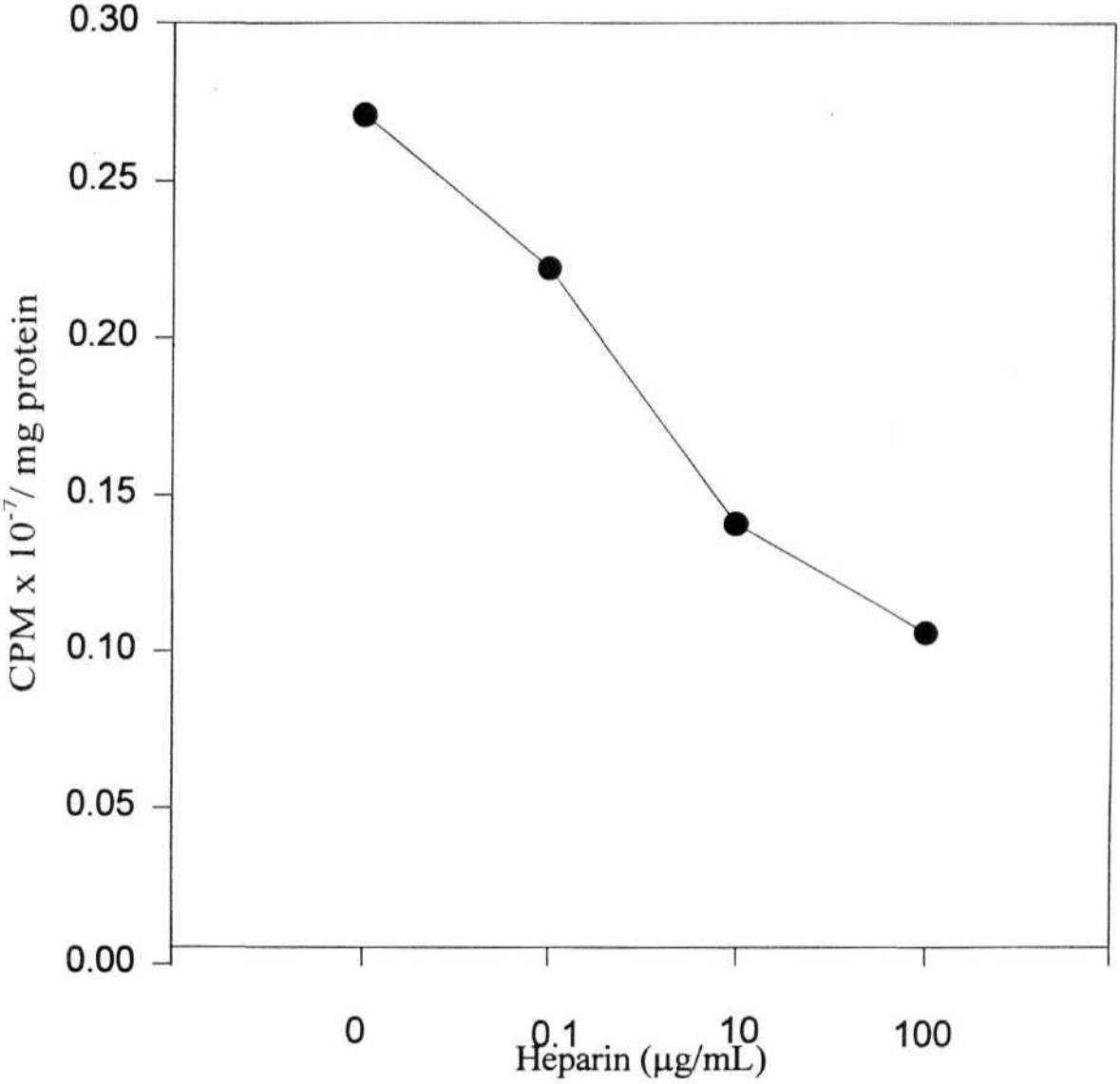


Fig. 10: Assay for casein kinase II in the nuclear samples of control (C) and ethanol (E) treated rat brain. Casein kinase II activity was determined by adding heparin (0.1μg/ml) to the reaction mixture (as described in fig.8). CK II activity was calculated by subtracting heparin insensitive incorporation from total kinase activity and expressed as CPM X 10^{-7} / mg protein. The data represented in each bar is a mean ± SD of four individual experiments.

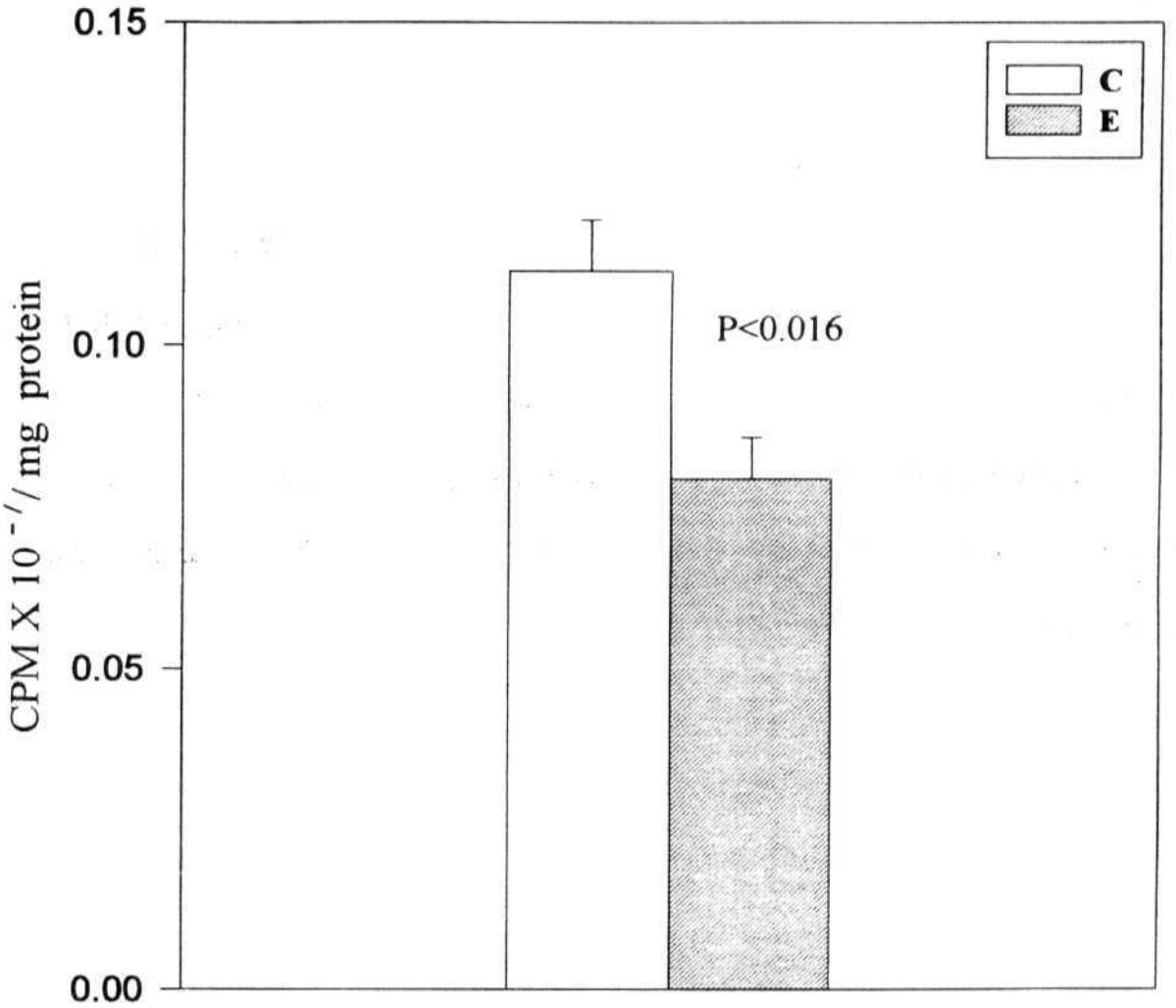

0.15
0.10
0.05
0.00
CPM X 10 -1 / mg protein
C
E
P<0.016

Fig. 11: Assay of casein kinase II in the nuclear samples of control rat brain to show the incorporation of GTP by casein kinase II. Cold GTP was included in the reaction mixture in increasing concentration from 5μM to 100μM. For further experiment 50μM GTP was used as it showed a plateau at this concentration.

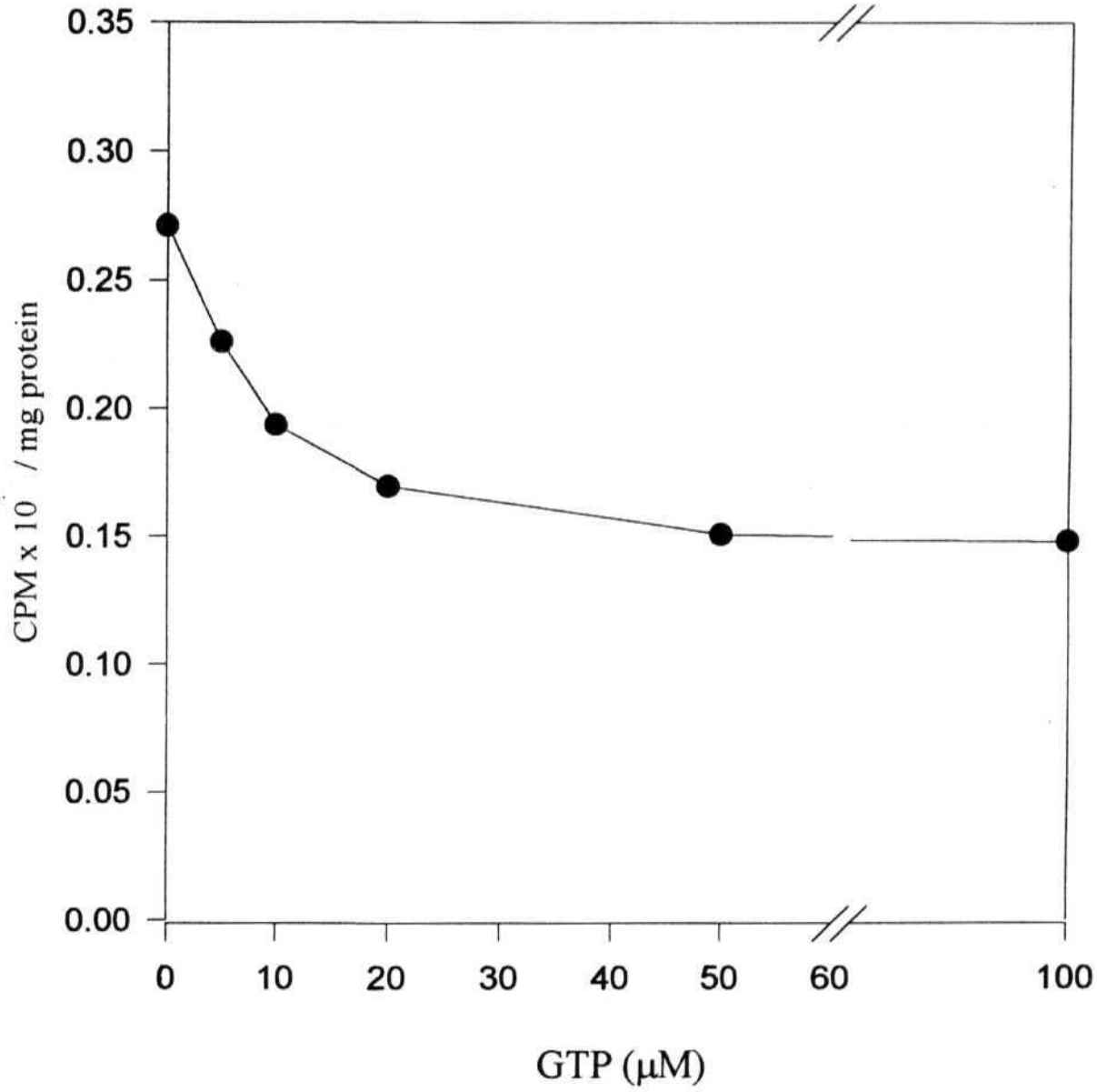

0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
CPM x 10 / mg protein
0
10
20
30
40
50
60
100
GTP (μM)

Fig. 12: Assay for casein kinase II activity in the nuclear samples of control (C) and ethanol (E) treated rat brain. Casein kinase II activity was determined by including GTP (50μM) in the reaction mixture. The data in each bar is a mean ± SD of four individual experiments

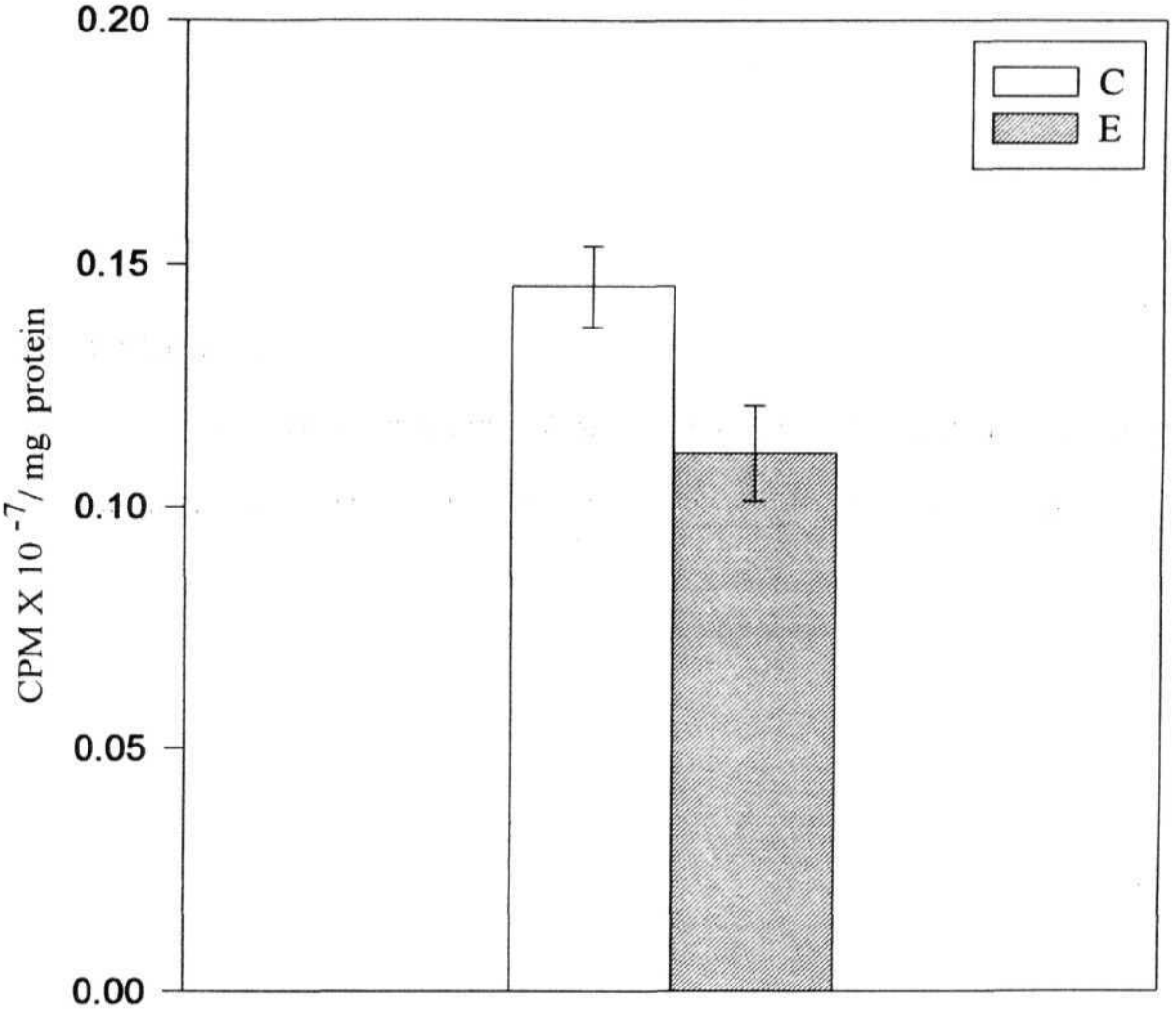

0.20
0.15
0.10
0.05
0.00
CPM X 10 -7/ mg protein
C
E

Fig. 13 : Identification of immuno reactive CK II in the nuclear samples of control (C) and ethanolic (E) treated rat brain. Nuclear proteins were electrophoresed by SDS-PAGE , transferred on to a nitrocellulose sheet and immunoprobed with polyclona) antibodies for CK II. The CK II is visualized by alkaline phosphatase-BCIP immuno detection system.

First two lanes 50 μgs of protein
Next two lanes 100 μgs of protein

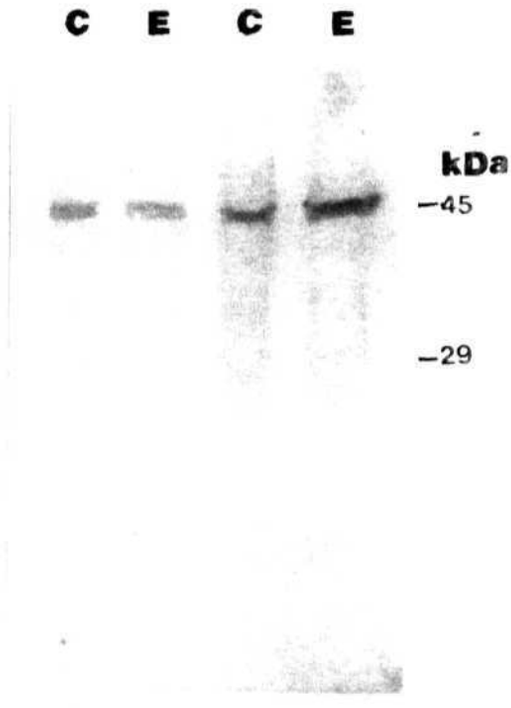

C
E
C
E
kDa
45
29

Fig. 14: Tyrosine kinase activity in the presence of vanadate (200μM) in the nuclei of control and ethanol treated rat brain. Tyrosine kinase activity was measured in the presence of exogenous substrate (poly glu-tyr) as described in methods.

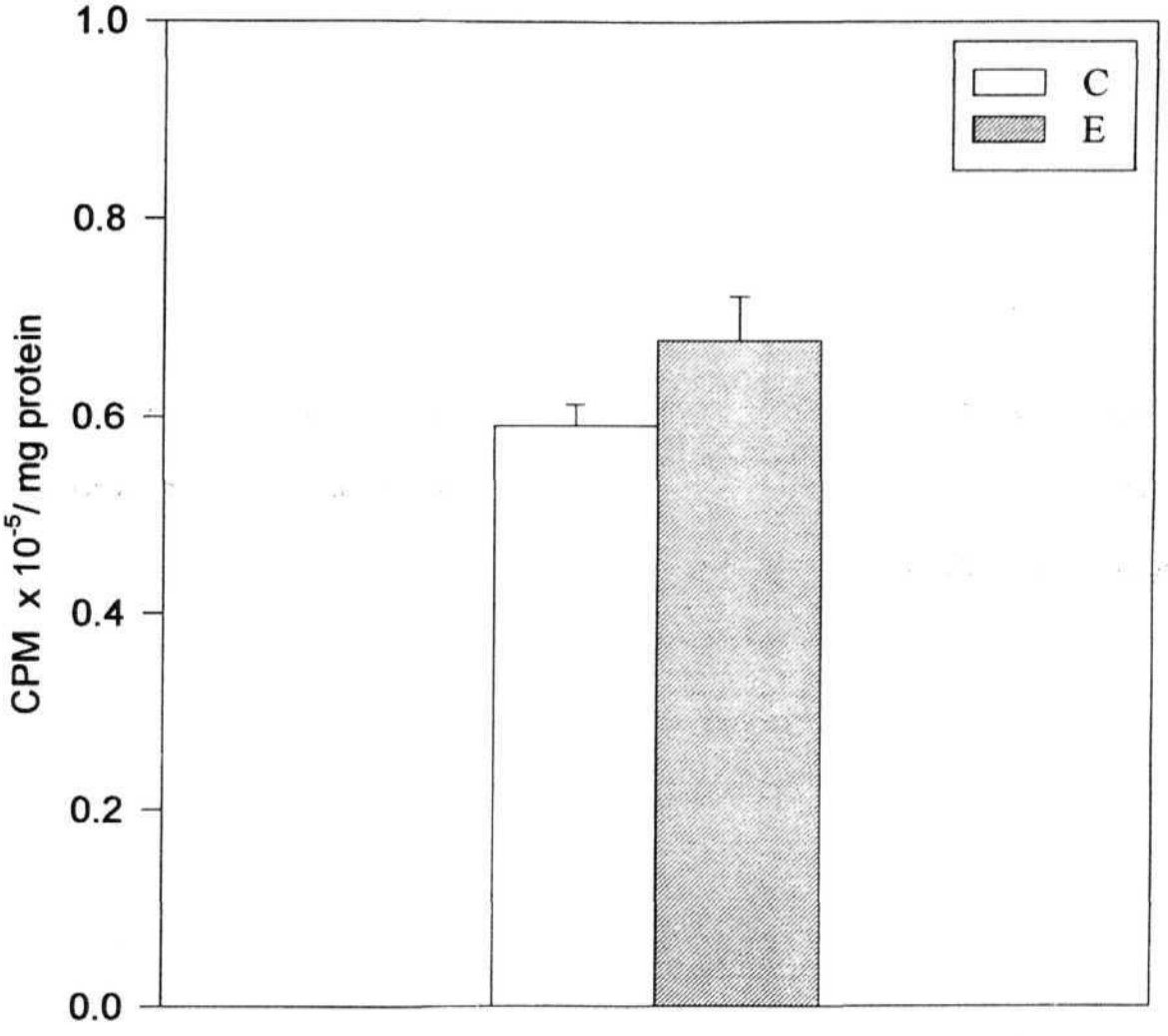

1.0
0.8
0.6
0.4
0.2
0.0
CPM x 10-5/ mg protein
C
E

Fig. 15: Tyrosine kinase activity in the absence of vanadate in the nuclei of control and ethanol treated rat brain. Tyrosine kinase activity was measured in the presence of exogenous substrate (poly glu-tyr) as described in methods.

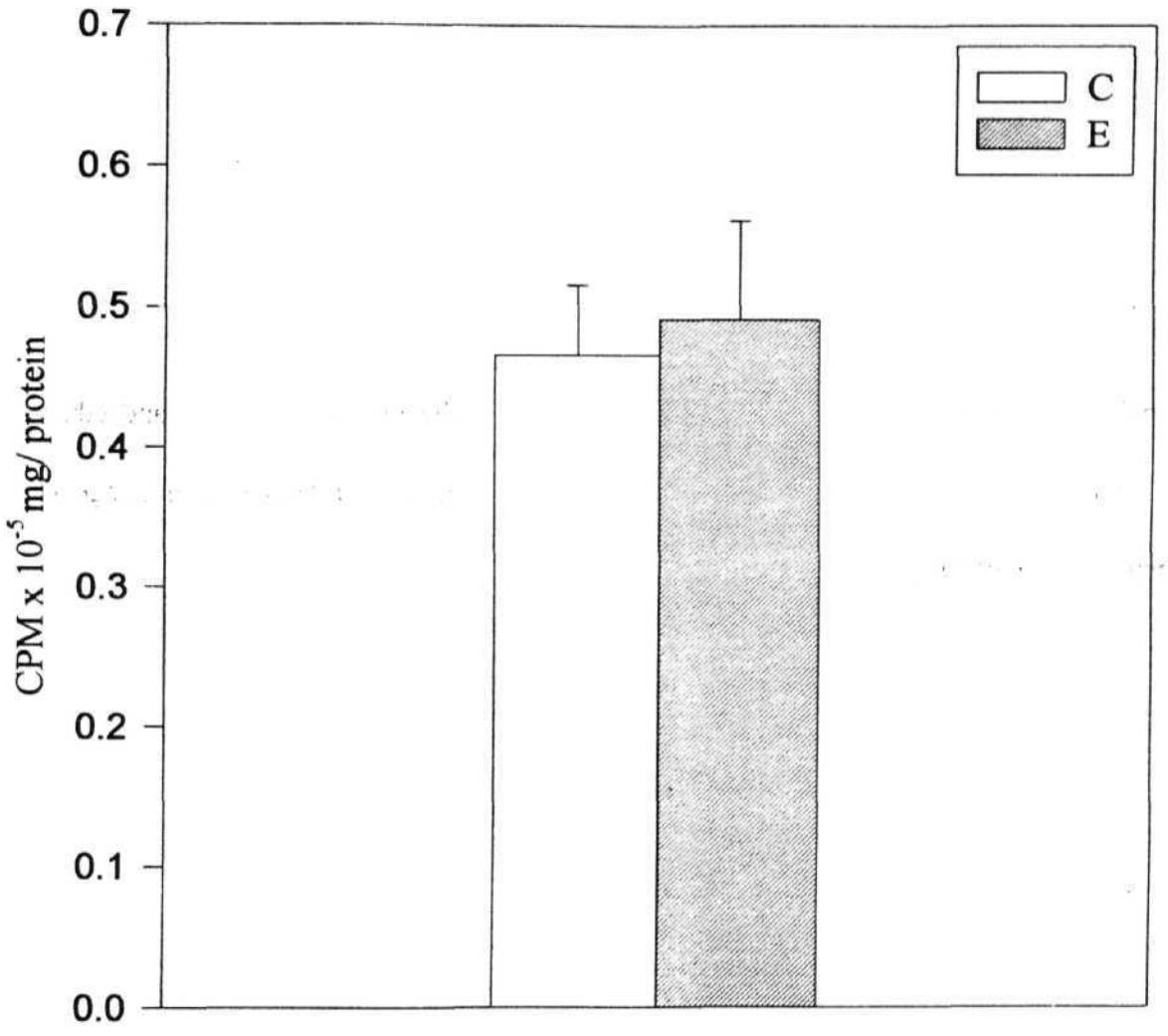

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CPM x 10-5 mg/ protein
C
E

Fig. 16 A : SDS-PAGE profile of nuclear proteins from control and ethanol treated rat brain.

Lane 1: Nuclear proteins from the brain of control rat brain

Lane 2: Nuclear proteins from the brain of ethanol treated rat brain

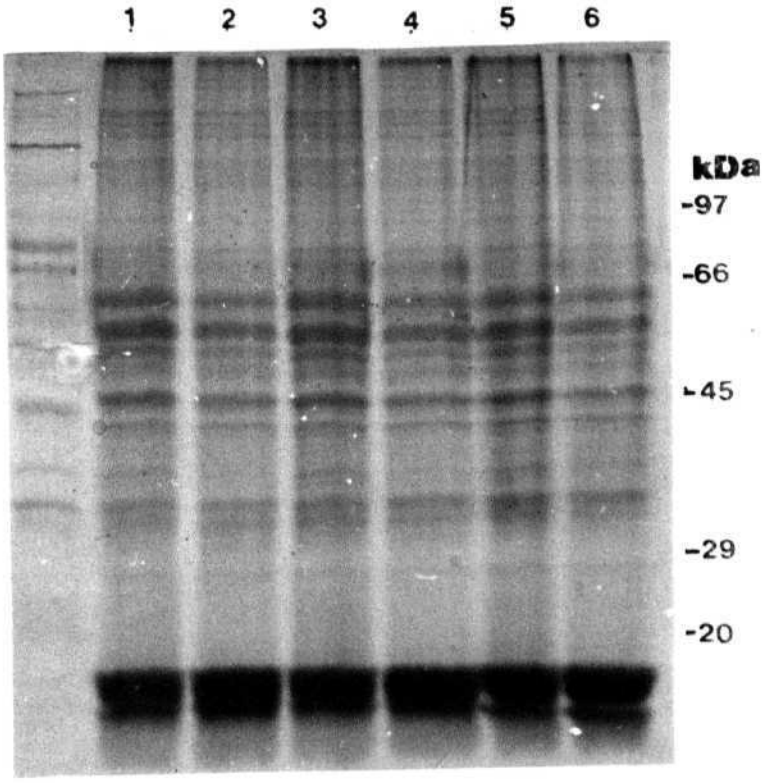
Lane 3: Nuclear proteins from the brain of control rat brain + ATP (100μM)

Lane 4: Nuclear proteins from the brain of ethanol treated rat brain + ATP (100μM)

Lane 5: Nuclear proteins from the brain of control rat brain + ATP (100μM) + Vanadate (200μM)

Lane 6: Nuclear proteins from the brain of ethanol treated rat brain + ATP (IOOjiM) + Vanadate (200μM)

Fig. 16 B: Immunoreactivity of phospho tyrosine containing proteins from the nuclei of control and ethanol treated rat brain. The proteins shown in Fig. 16A are transferred on to nitrocellulose sheet and probed with phosphotyrosine antibodies (PY20) and visualized by alkaline phosphatase- BCIP detection system. Number on the right side refer to molecular weight or the proteins


1
2
3
4
5
6
kDa
-97
-66
-45
-29
-20

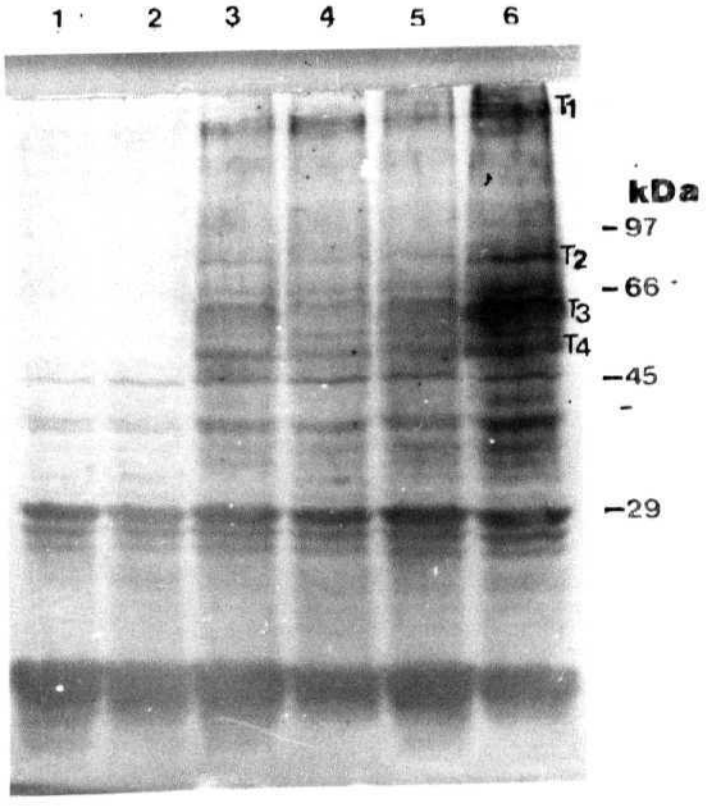

1
2
3
4
5
6
T1
T2
T3
T4
kDa
-97
-66
-45
-29

Fig. 17: Assay of nuclear CaM kinase II from rat brain as a function of ATP concentration. 3μCi was used in all subsequent experiments.

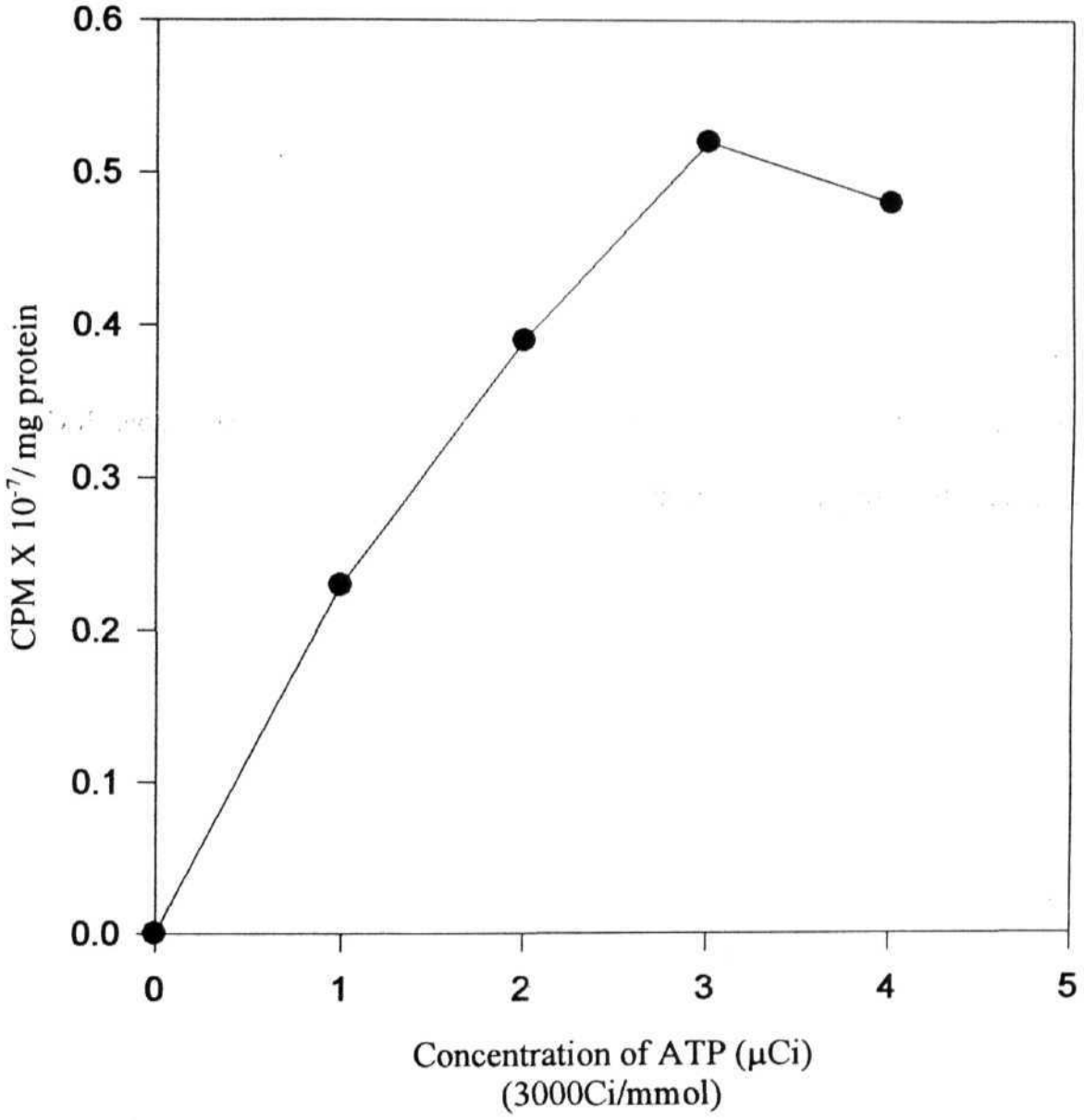

0.6
0.5
0.4
0.3
0.2
0.1
0.0
CPM X 10-7/ mg protein
0
1
2
3
4
5
Concentration of ATP (μCi)
(3000Ci/mmol)

Fig. 18: Assay of nuclear CaM kinase II from rat brain as a function of calmodulin concentration. The optimal concentration of calmodulin at 2μM was used for further studies

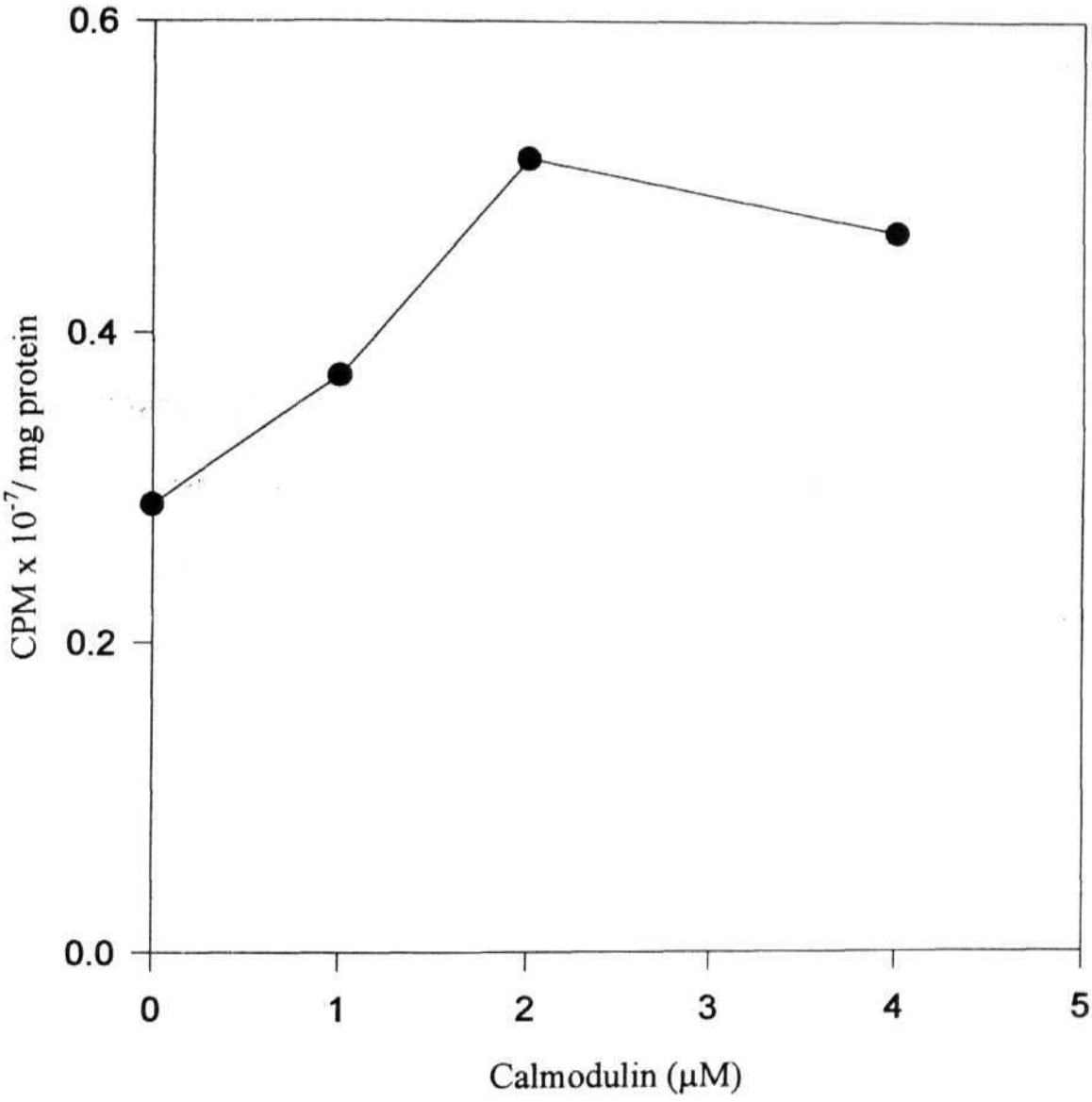

0.6
0.4
0.2
0.0
CPM x 10-7/ mg protein
0
1
2
3
4
5
Calmodulin (μM)

Fig. 19: Assay of nuclear CaM kinase II from rat brain as a function of calcium concentration. The concentration of calcium at 250 μM was used for further studies.

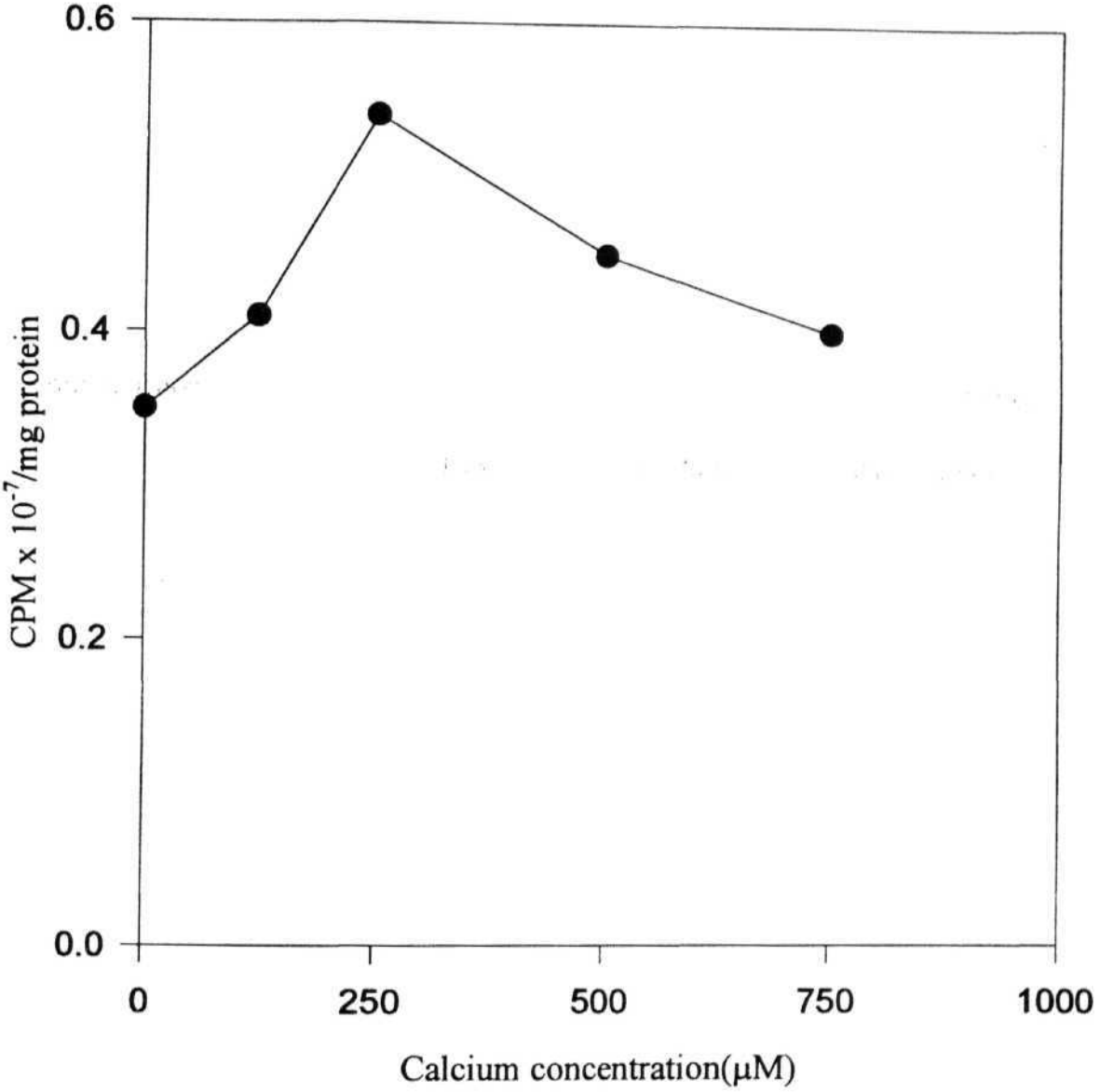

0.6
0.4
0.2
0.0
CPM x 10-7/mg protein
0
250
500
750
1000
Calcium concentration(μM)

Fig. 20: Assay of nuclear calcium calmodulin dependent kinase II from control (C) and ethanol (E) treated rat brain samples. The reaction mixture contains calcium (250μM), calmodulin (2μM), ATP (3μCi 3000Ci/mmole), protein (5μgs).

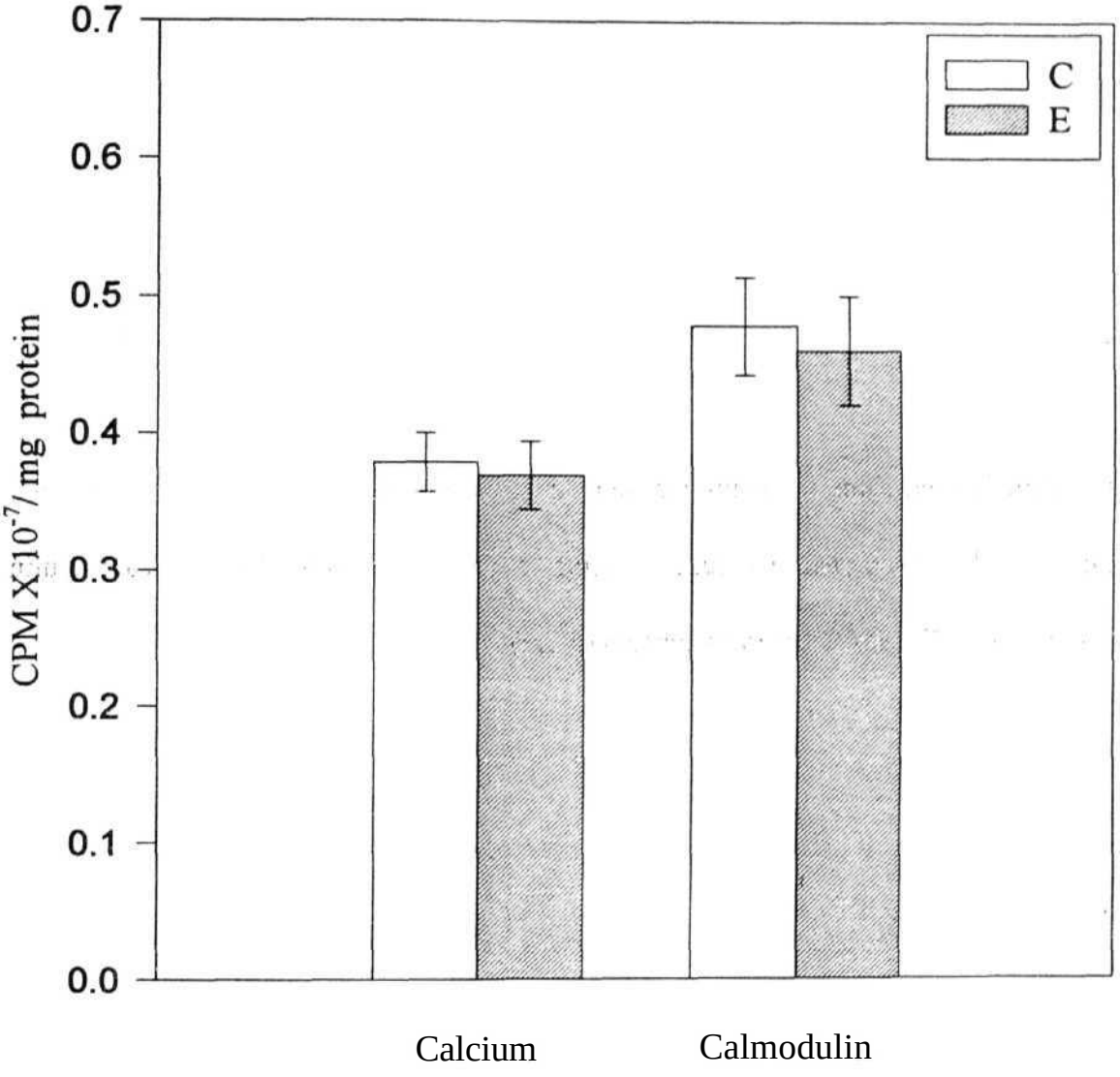

0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
CPM X10-7/ mg protein
C
E
Calcium
Calmodulin

Fig. 21 A : SDS-PAGE profile of nuclear proteins from control and ethanol treated rat brain.

Lane 1: Nuclear proteins from the brain of control rat brain + Calcium

Lane 2: Nuclear proteins from the brain of ethanol treated rat brain + Calcium

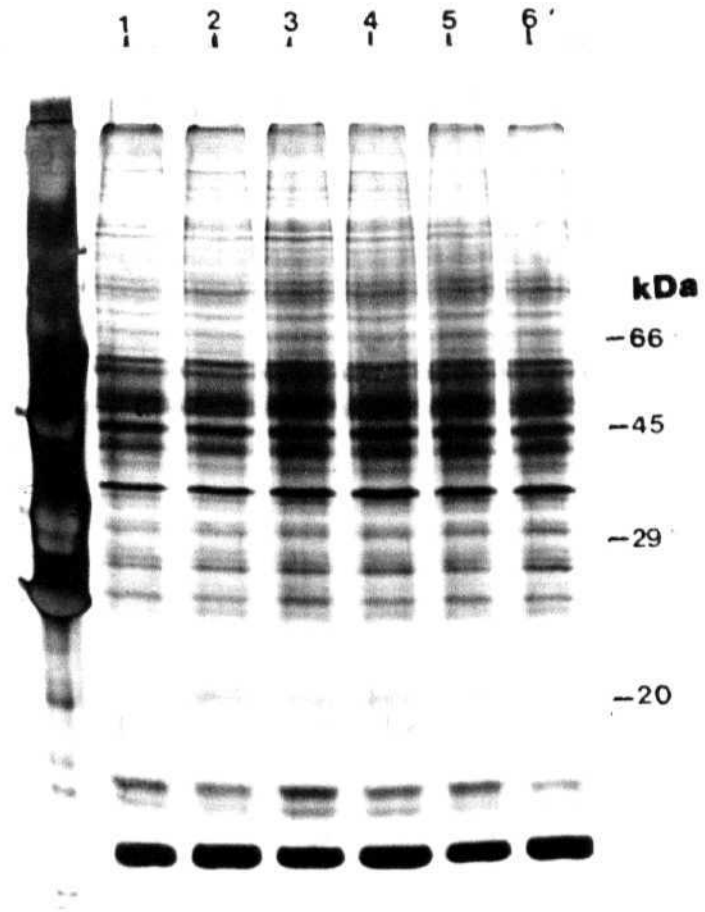
Lane 3: Nuclear proteins from the brain of control rat brain + Calmodulin

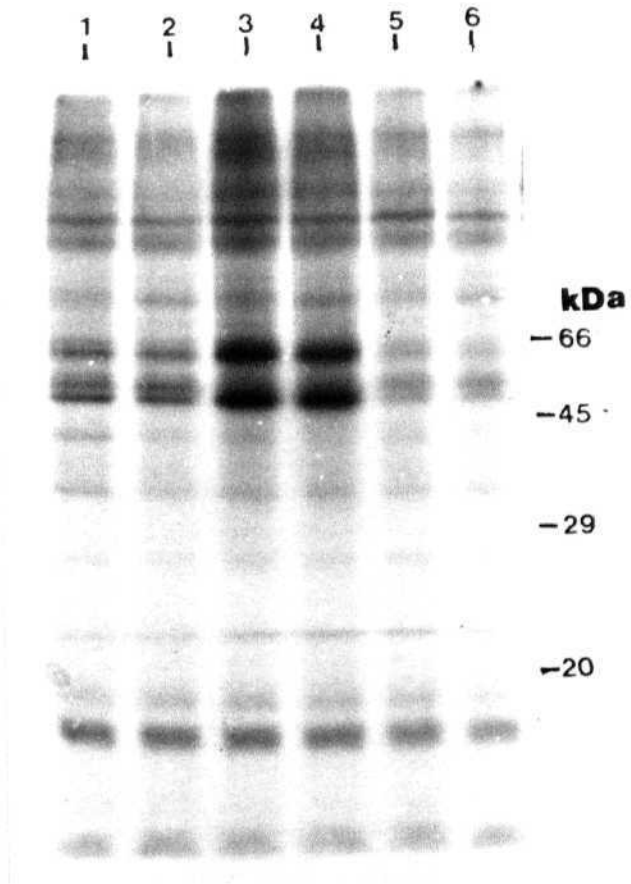
Lane 4: Nuclear proteins from the brain of ethanol treated rat brain + Calmodulin

Lane 5: Nuclear proteins from the brain of control rat brain + KN-62

Lane 6: Nuclear proteins from the brain of ethanol treated rat brain + KN-62

Fig. 21 B : Autoradiogram of calcium calmodulin dependent phosphorylation of nuclear proteins from the control and ethanol treated rat brain. The number on the right side refer to molecular weight of the proteins.


1
2
3
4
5
6
kDa
66
45
29
20


1
2
3
4
5
6
kDa
66
45
29
20

TABLES

TABLE I

Protein Number	Molecular Weight	Isoelectric Point (pI)	EGTA	Calcium	PS/DAG	AA(AT)	AA(AH)
1 •	90kDa	4.4	+++	+++++	+++++	+++++	+++++
2 *	18kDa	5.3	+++	+++	+++	4	+++
3	60kDa	5.4	+++	++4	+++	444	+++
4	55kDa	5.7	+++	+++	+++	444	444
5	40kDa	5.8	+++	++++	++++	4444	++++
6	55kDa	5.8	+++	+++	+++	+++	+++
7	60kDa	5.8	+++	+++	+++	+++	+++
8	116-50 kDa	6.1-6.6	+++	+++	+++++	+++	+++
E35 *	35 kDa	6.4	+++	-	-	-	-
E45 *	45 kDa	6.58	+++	-	-	-	-
E116 *	116kDa	5.9	+++	-	-	-	-
C32 *	32kDa	6.8		+++	-	-	-
C52	52kDa	5.0		+++	+++	+++	+++
C56	56kDa	5.2		+++	+++	+++	+++
C116	116kDa	6.1		+++	+++		
Pgps *	116-45 kDa	6.58-6.1			+++		
ATgps 85-50 *	85-50 kDa	5.6-6.58				++•+	
AHgps 85-50*	85-50 kDa	6.58-6.0					+++
AHgps>116	>116 kDa	6.58-6.0					+++

+++++ : Hyperphosphorylated
+++ : Phosphorylated
++ : Hypophosphorylated
- : Not phosphorylated
E : EGTA
C : Calcium
P : Phosphatidyl serine/diacylglycerol
AT : Arachidonic acid 10μM
AH : Arachidonic acid 100μM
• : Proteins affected

TABLE II

Protein Number	Molecular Weight	Isoelectric Point (pI)	Control	Expt.
1	90kDa	4.4	+++	+++++
2	18kDa	5.3	4+4	++4
3	60kDa	5.4	444	+++
4	55kDa	5.7	+++	+++
5	40kDa	5.8	+44	+++
6	55kDa	5.8	+++	+++
7	60kDa	5.8	+++	+++
8*	116-50kDa	6.6-6.1	444	-
E35	35kDa	6.46	+++	+++
E45*	45kDa	6.58	4++	-
E116	116kDa	5.9	+++	+++

+++ : Phosphorylated
- : Not Phosphorylated
E :EGTA
* : Protein(s) affected

TABLE III

Protein Number	Molecular Weight	Isoelectric Point(pI)	Control	Expt.
1	90kDa	4.4	+++	+++
2	18kDa	5.3	+++	+++
3	60kDa	5.4	+++	+++
4	55kDa	5.7	+++	+++
5	40kDa	5.8	++4	+++
6	55kDa	5.8	+++	+++
7	60kDa	5.8	+++	+++
8 *	116-50kDa	6.6-6.1	+++	+++++
C32	32kDa	6.8	+++	+++
C52	52kDa	5.0	+++	+++
C56	56kDa	5.2	+++	+++
C116	116kDa	6.1	+++	+++

+++ : Phosphorylated
+++++ : Hyper phosphorylated
* : Protein(s) affected
C : Calcium

TABLE IV

Protein Number	Molecular Weight	Isoelectric Point(pI)	Control	Expt.
1	90kDa	4.4	+++	+++
2	18kDa	5.3	+++	+++
3	60kDa	5.4	+++	+++
4	55kDa	5.7	+++	+++
5	40kDa	5.8	+++	+++
6	55kDa	5.8	+++	+++
7	60kDa	5.8	+++	+4-4
C52	52kDa	5.0	+++	+++
C56	56kDa	5.2	+++	+++
C116	116kDa	6.1	+++	+++
Pgps 116-45	116-45kDa	6.58-6.1	+++	4-4-4-
P1 *	50kDa	4.6	-	+++
P2 *	45kDa	4.4	-	+++
P3 *	40kDa	4.8	-	+++
P4 *	45kDa	4.8	-	4-4-4-
P5 *	66kDa	4.6	-	+++

+++ : Phosphorylated
- : Not phosphorylated
P : Phosphatidyl serine/Diacylglycerol
* : Proteins affected

TABLE V

Protein Number	Molecular Weight	Isoelectric Point(pI)	Control	Expt.
1	90kDa	4.4	+++	++4
2 *	18kDa	5.3	44+	+++++
3	60kDa	5.4	+++	+++
4	55kDa	5.7	+++	+++
5	40kDa	5.8	+++	+++
6	55kDa	5.8	+++	+++
7	60kDa	5.8	+++	+++
8	116-50kDa	6.6-6.1	+++	+++
C52	52kDa	5.0	+++	+++
C56	56kDa	5.2	+++	+++
ATgps85-50	85-50kDa	6.58-5.6	+++	++4
ATgps>116 *	>116kDa	6.25-5.6	-	+++

+++ : Phosphorylated
AT : Arachidonic acid 1 QuM
gps : group of proteins
* : Protein(s) affected in ethanolic condition

TABLE VI

Protein Number	Molecular Weight	Isoelectric Point(pI)	Control	Expt.
1	90kDa	4.4	+++	+++
2	18kDa	5.3	4-4-+	+++
3	60kDa	5.4	+++	+++
4	55kDa	5.7	+++	+++
5	40kDa	5.8	+++	+++
6	55kDa	5.8	+++	+++
7	60kDa	5.8	+++	+++
8	116-50kDa	6.6-6.1	+++	+++
C52	52kDa	5.0	+++	+++
C56	56kDa	5.2	+++	+++
AHgps85-50	85-50kDa	6.58-6.0	+++	+++
AHgps>116	>116kDa	6.58-6.0	+++	4-4-4-

+++ : Phosphorylated
AH : Arachidonic Acid 100μM
gps : group of proteins

TABLE VII

Protein Number	Molecular Weight	Control -ATP	Expt. -ATP	Control +ATP	Expt. +ATP	Control +Van	Expt. +Van
T1	206kDa	-	-	4	4	4	4-4-4
T2	90kDa	-	-	4	4	4	4 4 4
T3	60kDa	-	-	+	4	4	+++
T4	50kDa	-	-	+	4	+	+++

T : Tyrosine phosphorylated protein
- : Not phosphorylated
+ : Phosphorylated
+++ : Hyperphosphorylated
Van : Sodium Vanadate

DISCUSSION

Endogenous Phosphorylation of Nuclear Proteins

The experiments conducted to check the general pattern of nuclear protein phosphorylation in brain suggested that, at any given point of time there is a possibility for calcium independent and calcium dependent phosphorylations. A set of proteins such as E116, E45 and E35 are selectively phosphorylated in the absence of calcium (Fig. 2A), while the proteins such as C32, C52, C56 and C116 are phosphorylated in a calcium dependent manner (Fig.2B). Among the proteins that are phosphorylated in the presence of calcium, proteins # C32, C52, C56 and C116 appeared to be newly phosphorylated, while proteins *ti* 1 & 5 were fayperphosphorylated (Fig.2B). The calcium induced hyperphosphorylation of these proteins (# 1&5) might be due to the exposure of calcium regulated phosphorylatable sites or due to the phosphorylation of already available sites. Since phosphorylation of C32, C52, C56, and C116 is occurring immediately on calcium availability, these proteins obviously do not need prior phosphorylation but the very presence of calcium itself is enough to trigger the change.

The use of Phosphatidyl serine/Diacylglycerol in the reaction mixture and specific phosphorylation of a group of proteins (Pgps 50-116) under these conditions, clearly suggest, that these group of proteins must be serving as possible substrates of PKC (Fig. 2C). Arachidonic acid (AA) is known to activate PKC, independent of Phosphatidyl serine and diacylglycerol and earlier studies have shown that arachidonic acid can specifically stimulate PKC y and PKC a at a concentrations of 10μM and 100μM respectively (Huang 1989). The phosphorylation reactions mediated by PKC y and PKC a demonstrated that specific groups of proteins as shown in the results are

selectively phosphorylated in the nuclei. It is perhaps direct evidence that PKC γ and PKC a are localised in the nucleus and can be stimulated using agents like arachidonic acid. It also confirms that the isozymic pattern of PKC is evident even in the nucleus and implicates a wider horizon for the role of nuclear PKC's.

*Effect of Ethanol on Calcium **Independent** and Dependent Phosphorylation*

The influence of ethanol on calcium independent phosphorylation was studied by including EGTA in the reaction mixture. The general phosphorylation profile was similar to the control samples, excepting for the fact that a group of proteins # 8 and another protein E45 (Fig.3) were not phosphorylated, indicating a low activity of calcium independent kinases. Further when phosphorylation was performed in the presence of calcium, the same group of proteins (#.8), [which were not phosphorylated in the presence of EGTA], got hyperphosphorylated. These results suggest the possibility that the group of proteins (# 8), during ethanolic ingestion are highly acted upon by calcium dependent kinases, while calcium independent kinases fail to act on them.

Effect of ethanol on PKC mediated Phosphorylation

The nuclear PKC mediated phosphorylation during chronic ethanol exposure in brain led to the identification of a subset of proteins with a MW ranging from 40-66 kDa (Fig. 5). Ethanol induced increase in PKC activity might have resulted in the phosphorylation of these proteins in the nuclei. The activity of PKC appears to be affected by the redox state of intracellular environment (Kass et al., 1989). Earlier studies have shown that inhibition of PKC resulted in an increase in the intracellular glutathione content in neural cell lines (Francisco et al., 1992). Ethanol is reported to decrease the glutathione content and thereby disturb the redox state of the cell

(Wieland and Lauaterburg 1995). Hence an increased PKC activity might have resulted in the phosphorylation of these proteins. Since these proteins are specifically phosphorylated, their localization in the nuclei emphasises the fact that these proteins might regulate the expression of other subset of proteins in the nuclei such as cell cycle regulating proteins etc. Alternatively the phosphorylation status of these proteins might render them a capability to work as cis/trans transcriptional activators, which might facilitate the cell to make necessary adjustments in chronic ethanol exposure.

By using different concentrations of AA to look into the performance of PKC a and y forms in control and experimental samples, interesting results were obtained. Basing on the group of proteins phosphorylated as described in results, we could conclusively demonstrate that the y form of PKC, which is stimulated by 10μM concentration of AA is active in ethanolic samples. Earlier studies have shown that ethanol is capable of increasing the cytosolic PKC δ and c forms. This study is probably the first report to show the specific phosphorylation of nuclear proteins by PKC y in ethanolic condition. This further suggests there is an yet to be clearly identified interplay between the isoforms of PKC in ethanol toxicity to facilitate cellular adaptation to ethanol by making adjustment between the nuclear and cytosolic phosphorylation events. This could be still a point of ambiguity in the sense that the different isoforms of nuclear PKC's are not that clear, compared to cytosolic PKC isoforms. Under such conditions, two possibilities must alone account for the observed results in the present study. The PKC y form must have been activated because 10 μM of arachidonic acid is used. The second possibility is that the PKC β form might also be active in ethanolic condition. This inference is drawn as PKC β is stimulated by low AA level but only in the presence of calcium (Huang 1989). Since the

phosphorylation assays contained calcium. it is likely that either PKC β or PKC *y* might be responsible for the phosphorylation of proteins in (AT gps > 116) ethanol ingestion.

Nuclear Kinases

Casein Kinases

Casein kinases are multifunctional proteins with a potential to phosphorylate serine and threonine residues of proteins in the cytosol (Singh and Huang 1985), and nucleus (Rose et al., 1981) *in vitro* and *in vivo.* Phosphorylation by Casein kinase II is known to alter the biological activities of several important molecules such as Calmodulin (Meggio et al., 1987), DNA Topoisomerase I and II (Ackerman et al., 1985; Durban et al., 1985), RNA Polymerase I (Duceman et al., 1981) and II (Dahmus., 1981), mRNP particles (Rittschof. and Traugh 1982), HMG proteins (Inoue, et al., 1980). Nucleolin (Caizergues et al 1987), Nucleolar protein B (Chan et al., 1986), hsp90 (Dougherty, et al., 1987), and oncoproteins such as Myc (Bernhard et al, 1989), Myb, p53 (Meek et al., 1990) and several growth factors (Sommercorn et al. 1987). The eukaryotic casein kinase I and II are differentiated basing on their dependence in response to GTP. CK I can accept only ATP as phosphate donor, while CK II can accept both ATP and GTP as phosphate donors (Hathaway and Traugh 1982). Studies also have shown that CK I is a monomer while CK II is an oligomer containing a and β subunits of which a subunit posses the catalytic activity while the β subunit carries the regulatory domain. Like many other protein kinases, CK II is a transducer of signals and translates the signals into biochemical events at the target site. Several chemicals such as heparin, hyaluronic acid, poly phosphates and poly glutamates, poly glutamate-tyrosine and benzimidazole have been shown to inhibit

casein kinase II while polyamines and basic polypeptides arc found to stimulate its activity. CK II has also been shown to regulate its activity by autophosphorylation (Lorenzo. 1990).

The results from this study indicated that the CK 11 is possibly the target site during ethanol toxicity in brain. The inhibition of CK II to the extent of 30% indicates the decreasing enzyme efficiency in phosphorylating serine/threonine residues in phosphorylatable substrates. Though the physiological significance of this inhibition is difficult to derive specifically, the overall impact on the nuclear events cannot be ignored under these conditions. This reason stems from the fact, that a cursory look into the list of critical enzymes/factors in the nucleus like RNA Polymerase 1 and II, mRNP particles, DNA Topoisomerase 1 and II, HMG Proteins, etc are localised in the nucleus and are involved in various important steps of DNA and RNA metabolism. The decreased efficiency of CK II on any of these serine/threonine containing substrates can have an impinging impact on the whole nuclear events, ultimately affecting the gene expression. This logical conclusion is also supported from the fact that CK II activity has been known to be associated with nuclear matrix. Nuclear matrix being a dynamic structure in the gene expression and DNA replication, the phosphorylation of several nuclear matrix proteins might invariably require the catalysis by CK II (Sherif and Ahemed, 1994). CK II activity is decreased by 30% in the present study, while quantitatively the enzyme appears to be unaffected. One of the possible reasons for decreased activity of CK II could be the limitation of polyamine supply into the nucleus. Polyamines are known to stimulate CK II activity and the polyamine levels have been shown by other studies to decrease in alcoholic conditions (Janaka et al., 1993). Therefore, as such polyamines could be one of the limiting

factors in ethanolic condition which could be responsible for the decrease in the CK II activity noticed in the study. This study in which 30% decrease is noticed on alcohol treatment is only a tip of the iceberg and requires deeper investigation into the perspectives of CK II mediated phosphorylation and the cellular adaptation to chronic ethanol challenge.

The functional implications of the decrease of CK II activity after chronic alcoholic treatment can only be speculated, supported by the factual information available in the literature as to what happens to CK II in other pathological conditions of the central nervous system. A selective activation and preservation of CK II has been shown to be important for neuronal survival after cerebral ischemia (Hu and Wieloch 1993). Hence it is likely that the decreased CK II activity could be responsible for selective neuronal death observed in Purkinje cells of cerebellum following chronic ethanol treatment (Victor et al., 1959; Philip and Cragg 1984). Since neuronal death is implicated, it is essential to check and prove the relevance of decreased CK II activity in apoptotic cells of brain. Another supportive evidence comes from a study of human patients suffering from schizophrenia and Alzheimer's where CK II was shown to drastically decrease and it has been co-related well with the aberrations in the phosphorylation of specific structural proteins in schizophrenic and Alzheimer's patients (Aksenova et al., 1991). This clearly suggests that the CK II is intrinsically very important in the pathology of brain in Alzheimer's and Schizophrenics. CK II activity is important in the memory formation and retrieval and infact alcoholics suffer a memory deficit and cognitive impairment under excessive doses of alcoholic consumption (Parsons and Stevens1986). The memory loss is one of the central behavioural features of Alzheimer's and Schizophrenic patients. The cognitive

impairment in alcoholics could be related to the decrease in CK II activity. This argument can be further supported from the studies of Marlangue (Marlangue et al., 1991) who showed that CK II activity is important in LTP induction. Long Term Potentiation being a form of synaptic plasticity, has been well recognised as the physiological basis underlying the mechanisms of memory. Hence the CK II might be playing an important role in ethanol induced cognitive impairment.

Tyrosine Kinases

The earliest protein tyrosine kinases were found to be localized at membranes and coupled to receptors, mediating effector-receptor coupling in response to signal transduction. However, several tyrosine kinases were readily found to enter nucleus and phosphorylate nuclear resident proteins such as the β subunit of RNA polymerase II. Nuclear tyrosine kinases to participate in the regulation of transcription, cell cycle and in several other nuclear associated events. This study also demonstrated the presence of phosphotyrosine kinases as well as phosphotyrosine containing proteins in the nucleus of brain cells. Though several phospho proteins could be identified none of them showed significant change between control and ethanol treated groups. However, on inclusion of vanadate, a potent inhibitor of protein tyrosine phosphatase, a group of proteins get highly phosphorylated in ethanol treated samples. Although the function and identity of these proteins is still not known, the very stimulation of tyrosine dependent phosphorylation could have some relevance in the cellular functions. The results could also be interpreted by the fact that the endogenous protein tyrosine phosphatases levels in ethanolic samples must have been reasonably high enough so that in the absence of vanadate, tyrosine phosphorylation is masked by the continuous action of tyrosine phosphatase. The other possibility could be, the homeostatic balance

between the activities of protein tyrosine kinase and protein tyrosine phosphatase is altered in experimental samples in such a way that the protein tyrosine kinase activity exceeds protein tyrosine phosphatase activity resulting in the hyperphosphorylation of specific substrates noticed in this study. Though the physiological significance of these changes are still not clear, the presence of tyrosine containing proteins and protein tyrosine kinase activity suggests the ubiquitous nature of these proteins in a subcellular compartment like nuclei in the cells of central nervous system.

Calcium Calmodulin dependent Kinase II (CaM kinase II)

Nuclear CaM kinase activity was monitored in this study in control and ethanolic samples, mainly because of the following reasons. CaM kinase 11 activity has been noticed to alter in several other nervous system disorders such as Alzheimer's disease (Saitoh, et al., 1991), Ischemia (Taft et al., 1988), and Kindling (Goldenring et, al., 1986). CaM kinase II is known to phosphorylate GABA receptors at the membrane level. Further Ca^{++} channels are reported to be upregulated by ethanol for an effective cellular inflow of calcium. Since these events are altered, there is every possibility that the CaM kinases might get affected in ethanolic treatment. In this study it has been shown (Fig.4), when calcium is added a group of nuclear proteins get selectively phosphorylated in ethanolic condition, indicating calcium dependency for the phosphorylation of these proteins, which should be carried out by either CaM kinase II or PKC. In this study the results of CaM kinase II assays and phosphorylation profiles (Fig. 20, 21) clearly revealed that they are not involved while the addition of PS/DAG showed an increased phosphorylation (Fig.5). A logical conclusion of these observations rules out the possibility of the involvement CaM kinases while PKC

might play a dominant role in the nuclear protein phosphorylation in ethanol toxicity in brain.

In conclusion, this study has revealed the following salient aspects of nuclear protein phosphorylation as a consequence of chronic ethanol treatment in brain.

- The nuclear protein phosphorylation in brain showed a calcium dependency as well as calcium independency.
- Addition of PS/DAG in the phosphorylation assays and the response in ethanolic samples suggested the involvement of PKC.
- By using different concentrations of AA, PKC a and y activities were noticed in the nucleus. Chronic ethanol treatment appears to have a preferential activation of PKC y or β leading to a specific phosphorylation of a subset of proteins.

The calcium independent protein kinases i.e. casein kinases and tyrosine kinases showed the following trend. Casein kinase II which acts on ser/thr residues and which also can accept both ATP and GTP as phosphate donors appear to be the target site during ethanolic toxicity. The CK II enzyme activity was decreased by 30%, while quantitatively the enzyme showed no difference. The decrease in CK II activity might be fundamental for cognitive impairment in alcoholics besides its role in neuronal degeneration. Although there is no experimental evidence to establish a relationship between the decrease in CK II activity and decreased calcium independent phosphorylation in ethanol nuclei (Fig. 3), it is possible that these proteins might also be possible substrates of CK II. This conclusion can be drawn by the fact that some of these proteins are phosphorylated in the presence of calcium and Phosphatidyl serine and arachidonic acid.

The nuclear tyrosine kinases showed no difference between the control and the experimental samples. However, on inclusion of vanadate. a potent inhibitor of tyrosine phosphatase, specific proteins were phosphorylated in ethanolic samples suggesting a loss in the balance between protein tyrosine kinases and protein tyrosine phosphatases in ethanolic samples.

The calcium dependent assay for CaM kinase 11 activity revealed, that CaM II is not involved in the phosphorylation of nuclear protein in ethanolic condition, reinforcing the conclusion that PKC alone might be responsible in the calcium dependent nuclear protein phosphorylation in ethanol toxicity in brain.

A large number of nuclear proteins arc involved in protein phosphorylation and the nuclear kinases revealed a specific pattern in the phosphorylation mechanisms in ethanol toxicity. The calcium dependent phosphorylation appears to be least affected as CaM kinase is not affected while PKC appears to be involved to a limited extent. The nuclear PKC isozymes *y* and a appear to have an inter play in ethanol toxicity. On the other hand, in the calcium independent phosphorylation, CK II is affected, the balance between tyrosine kinases and tyrosine phosphatase is affected which implicates that the calcium independent phosphorylation is relatively more affected. Further, these deficiencies involving CK II and PKC might be contributing to the cognitive impairment in alcoholics due to their role in LTP. Since CK II is known to facilitate phosphorylation of PKC *in vitro* (Tominaga et al., 1991), it can not be ruled out that the synergistic balance between these two enzymes is lost leading to drastic change in LTP, which is crucial for cognitive functions.

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