DEDICATED
TO MY
PARENTS

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DECLARATION

I hereby declare that the work presented in this thesis entitled *"Effect of **Ethanol on Protein Kinases in Rat Brain: A Developmental Study"*** has been carried out by me under the supervision of **Dr. Mohan C. Vemuri** and this has not been submitted for any degree or diploma of any University.

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CERTIFICATE

This is to certify that **Mr. Mahadev. K** has carried out the work in the present thesis under my guidance for a full period prescribed under the Ph.D. ordinances of the University. I recommend his thesis entitled ***"Effect of Ethanol on Protein Kinases in Rat Brain: A Developmental Study"*** for submission for the award of the degree of ***Doctor of Philosophy*** of this University.

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Mahadev

ABBREVIATIONS

AA	: Arachidonic acid
AIP	: Autocamtide-2-related inhibitory peptide
ATP	: Adenosine 5'-Triphosphate
BCIP	: 5-bromo-4-chloro-3-indoyl Phosphate
$CaCl_2$	: Calcium Chloride
CaM	: Calmodulin
DAG	: Diacylglycerol
DTT	: Dithiothreitol
EDTA	: Ethylene diamine tetra acetic acid
EGTA	: Ethylene glycol-bis (β-aminoethyl ether)- N,N,N'N' tetra acetic acid
kDa	: Kilo dalton
	: micron
M	: Molar
uci	: 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
NBT	Nitroblue tetrazolium chloride
PAGE	Poly Acryiamidc Gel Electrophoresis
TBS	Tris buffered saline
TCA	Trichloroacetic acid
Poly(Glu_4 Tyr_1)	Poly Glutamate Tyrosine
PMSF	Phenyl Methyl Sulfonyl Fluoride
PS	Phosphatidyl serine
SDS	Sodium Dodecyl Sulfate
TBS	Tris Buffered Saline
Tris	Tris [hydroxymethyl] amino methane
V/V	Volume/Volume

Introduction

Ethanol is a neurotoxin and most commonly used as a drug, disinfectant and preservative. Ethanol is water and lipid soluble and is readily distributed into the cytoplasm and membranes of all cells in the body. The cytotoxic effects of ethanol are not entirely understood but certain possibilities are attributed. First, ethanol can exert cytotoxic effects through its lipid soluble properties and might display its biological effects by physical action as a denaturing agent of macromolecules. Second, the cytotoxic effects of ethanol are linked to its metabolic fate and most probably are mediated by oxygen-dependent generation of free radicals, which may directly react with vital cell constituents, or may be transformed into more reactive species. Third, the cytotoxic effects of ethanol may result from a combined influence of its physical, chemical and metabolic properties.

Effect of Ethanol on Central Nervous System

The complex effects of ethanol are primarily due to changes in the function of central nervous system as a result of direct ethanol action on cerebral tissue, since ethanol readily diffuses across the blood brain barrier. The immediate effects of ethanol ingestion are euphoria, sedation, intoxication and narcosis (Mello, 1968). Chronic alcohol abuse results in a variety of pathological effects, primarily damage to brain and liver, although other organs are not excluded. Clinical studies showed brain dysfunction, increased tolerance, physical dependence, encephalopathies, defects in learning, memory and conceptual abilities (Parson, 1977; Arendt et al., 1988; Charness et al., 1989). Addiction following alcohol consumption results in neuroadaptational changes within CNS involving neurochemical and neurophysiological process.

One of the most notable abnormalities in the brains of alcoholics is the presence of brain shrinkage'. 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 might be a result of increased calcium concentration (Arendt et al., 1983). Further, 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 to hippocampus and neocortex corrects both cholinergic deficits and memory abnormality implicating ethanol in the damage of cholinergic neurons (Arnedt et al., 1983). Furthermore, ethanol is also involved in the disruption of serotonergic and adrenergic pathways in amnestic alcoholics (Charness et al., 1989). Chronic ethanol treatment inhibited superoxide dismutase activity, there by increasing the vulnerability of the brain to free radical damage (Ledig et al., 1980 and Mandel et al., 1980). Studies from our laboratory and several others have showed the involvement of free radical induced damage to chromatin and proteins in brain and liver in chronic ethanol exposure (Mahadev and Vemuri, 1998). Recent findings have proposed that the alcohol induced neuronal death could be due to apoptosis (Freud, 1994).

Ethanol has been shown to affect the functioning of neurotransmitter receptors, ion channels 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 (LTP) 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 calcium concentration (Dildy and Leslie, 1989). Ethanol also inhibits the release of ^{3}H nonadrenaline from brain cortex (Fink and Gother, 1990) and endogenous dopamine from striatal slices (Woodward and Gonzales, 1990) in response to NMDA stimulation. Further studies using cultured cerebellar neurons have found that chronic ethanol treatment can significantly increase NMDA stimulated calcium influx (Iorio et al., 1992). Increase in NMDA receptor ion channels and NMDA mediated calcium flux are not the only changes that would sensitise neurons to excitotoxicity. Chronic ethanol treatment of cells in culture in vitro (Brennan et al., 1990; Messing et al., 1986) and in vivo in animals (Brennan 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; Machu et al., 1989) Furthermore, ethanol acutley has shown to be a potent inhibitor of the function of NMDA receptors in cerebellar granule neurons when they are grown in medium containing 25mM KCl, as well as in many other neuronal preparations (Hoffman et al., 1989, Hoffman, 1995). In a recent study ethanol induced the inhibition of NMDA receptor function and increase in intracellular Ca^{2+} concentration $(Ca^{2+})_i$ in cerebellar granule neurons grown in a medium containing 5 mM KCl and determined the effect of ethanol on apoptotic neuronal death in the absence and presence of NMDA (Bhave et al., 1997). In addition, chronic ethanol increases receptor stimulated production of nitric oxide which in turn expected to increase excitotoxicity (Fulton and Chandler, 1993). Thus, a variety of data suggest that chronic ethanol treatment may disrupt calcium homeostatic mechanisms and enhances excitotoxicity. Ethanol is also shown to interfere with glutamate metabolism due to a decrease in glutamate synthetase activity of astrocytes in culture leading to an increased sensitivity to withdrawal seizures by NMDA (Shanley and Peter, 1993). There was a significant decrease in glutamate decarboxylase activity of GABAergic neurons to death (Shanley and Peter, 1993). Chronic ethanol treatment decreased the efficiency of GABAergic transmission in synaptosomes (Morrow et al., 1990), and decreased GAB A 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). 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 1989). 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.

Effect of Ethanol on Developing Central Nervous System

Normal brain development depends on the coordinated patterns of a series of complex processes in which neurons and glial cells participate (Rakic, 1991). It has been shown that developing brain is highly vulnerable to the effects of ethanol. Ethanol produces a variety of disruptions in some of these developmental processes, including decreased neurogenesis, delayed and aberrant neuronal migration, anomalous morphological development (Miller, 1992), changes in the ontogeny of neurotransmitter synthesis (Druse, 1992), and neuronal depletion in several brain regions (West et al., 1986). Studies involving human and experimental animals provide evidence that alterations of glial development may contribute to the effects of alcohol on the developing brain (Phillips, 1994; Guerri and Renau-Piqouras, 1997). In vivo studies also showed that prenatal ethanol exposure to brain reduces the number of glial cells in cortex (Gressens et al., 1992; Miller, 1992), alters myelinogenesis (Lancaster et al., 1982; Phillips, 1994), induces abnormal morphology of radial and Bergamann glia (Shetty and Phillips, 1992; Miller and Robertson, 1993; Shetty et al., 1994), and decreases significantly the levels of astrocyte-specific marker glial fabrillary acidic protein (GFAP) in the brains of pups from ethanol-fed mothers (Saez et al., 1991).

Further, prenatal ethanol exposure induces major morphological changes in the brain. There are several studies from different laboratories that emphasize the susceptibility of the developing hippocampus to ethanol exposure. For example, the number of hippocampal pyramidal cells was reduced when ethanol was administered during development of brain (Barnes and Walker, 1981; Wigal and Amsel, 1990). The major reductions associated with prenatal ethanol exposure were found in the hippocampal CA1 region (Barnes and Walker, 1981). Early ethanol exposure produces a delayed appearance of multiple and complex synapses and multiple synaptic contacts on axonal terminals (Hoff, 1988). In the neocortex of brain, there is a reduction of neuronal cell numbers in all neocortical areas in rats exposed to ethanol during gestation (Miller and Potempa, 1990). Early ethanol exposure not only reduces the number of neocortical neurons, but also the size of their cell bodies in most cortical layers (Miller and Potempa,

1990). In addition, the onset of neurogenesis and the proliferation period are delayed in the neocortex of the ethanol exposed rats (Miller, 1989; Miller and Potempa, 1990). Areas corresponding to the motor and somatosensory cortices appear to be particularly sensitive to the effects of ethanol (Miller and Potempa, 1990; Miller and Dow-Edwards, 1988). The volume of the somatosensory cortex reduced by 33% in ethanol exposed rats, but glucose utilization is significantly reduced in both the motor and somatosensory cortices (Miller and Dow-Edwards, 1988). Impaired growth of the cerebellum has been associated with early exposure to high ethanol concentrations (Pierce, et al., 1989; Bauer-Moffett and Altman, 1975; 1977; Cragg and Phillips, 1985). The cerebellar purkinje cells are particularly affected by either prenatal ethanol exposure or early postnatal ethanol treatment (Baur-Moffett and Altman, 1975; 1977; Cragg and Phillips, 1985). Further, prenatal ethanol exposure reduces the number of the trigeminal nerve. This abnormality correlates with a delayed onset and cessation of neurogenesis (Miller and Muller, 1989). The volume of the granule cell layer of the olfactory bulb was also reduced during prenatal ethanol exposure (Nyquist-Battie and Gochee, 1985). The majority of abnormalities that were found in developing ethanol exposed animals were, a decreased content of neurotransmitters (eg. 5-HT, domapmine, norepinephrine, glycine, histamine), uptake sites (5-HT, dopamine), and certain classes of receptors (5-HT,, 5-HT_{1A}, D1, Kainate, NMDA, histamine) (Druse, 1992). These specific actions of ethanol are related to its ability to interact with neuronal membrane leading to changes in membrane fluidity, neurotransmitter action and signal transduction (Goldstein and Chin, 1981). The effects of ethanol on developing brain thus are shown to be deleterious (Abel, 1982; West and Pierce, 1986; Samson and Diaz, 1982; Kenndy, 1984; Pauli et al., 1995).

A particularly notable paradigm under the effects of ethanol on development includes foetal alcohol syndrome (FAS), which is an abnormal in utero growth of the foetus due to maternal alcohol consumption. FAS children are characterised by mental retardation, microcephaly, hyperactivity, intellectual deficits, decreased intelligent quotients, motor abnormalities and several other behavioural problems. All these changes contribute to devastating developmental deficits in the CNS of FAS children and

in FAS experimental animal models. (Druse, 1992; Druse, 1992; Manteuffel, 1996; Clarren and Smith; 1978).

The developmental cues in brain involve signal transduction events primarily protein phosphorylation, and these events are highly susceptible for the action of ethanol. The signalling pathways in the central nervous system involves different protein kinases such as Protein Kinase C (PKC), Ca^2/Calmodulin dependent protein kinase II (CaM kinase II), and Protein Tyrosine Kinases (PTKs), regulating diverse processes such as modulation of ion channel activity, neurotransmitter synthesis and release, receptor desensitisation, synaptic enhancement, short-term modulation of membrane excitability, long-term potentiation (LTP) etc.

Protein Phosphorylation in the Central Nervous System

The protein phosphorylation events in nervous tissue are more active than those in non-nervous tissue and play a prominent role in the regulation of nervous system functions. The first messengers or extracellular signals in the CNS include a variety of neurotransmitters and hormones, as well as the nerve impulse. These first messengers produce many of their biological responses by regulating the intracellular concentrations of cyclic AMP, cyclic GMP or calcium in specific target neurons; these three molecules can, therefore, be considered as second messengers in the signal transduction pathways. The actions of these second messengers on neuronal function are mediated by cylic AMP dependent, cylic GMP dependent and calcium dependent protein kinases. The brain contains one type of cylic AMP-dependent protein kinase and one type of cylic GMP-dependent protein kinase and multiple types of calcium dependent protein kinases which fall in to two subclasses. One subclass activated in conjugation with the calcium-binding protein calmodulin is referred to as calcium/calmodulin dependent protein kinase. The other subclass, activated in conjunction with phosphotidylserine and other lip
referred to as Calcium/phospholipid dependent protein kinase.

These protein kinases when activated result in the phosphorylation of substrate proteins leading to specific biological responses. Protein phosphorylation 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

gamma-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 remains constant. (2) By change in the activity of protein phosphatase while the activity of the protein kinases remaining unaltered. (3) Change in the activity of both protein kinase and phosphatase. (4) The phosphorylation state of a protein can also be affected by a conformational change in the protein, with no change in the kinases or phosphatases.

Calcium/phospholipid dependent Protein Kinase (PKC)

Protein kinase C (PKC) is a calcium-activated and phospholipid-dependent protein kinase originally isolated from rat brain by Nishizuka and colleagues and is distributed in variety of tissues (Nishizuka, 1984). It is an integral part in the cell signalling machinery. Receptor mediated hydrolysis of phosphatidylinositol is a common mechanism involved in transmembrane signalling through which inositol 1,4,5-triphosphate (IP3) and diacylglycerol are generated in the cell (Berridge and Irvine, 1984, Nishizuka, 1984). Diacylglycerol in the presence of calcium activates protein kinase C and IP3 stimulates calcium release from intracellular stores in the endoplasmic reticulum, thus initiating the reactions that change the biochemical state in the cell. Most of the key elements of this signalling mechanism have been found in the central nervous system (Nishizuka, 1984).

Biochemical and molecular cloning analysis has showed that this enzyme comprises a large family with multiple isoforms exhibiting individual characteristics and distinct patterns of tissue distribution. The biological significance of this heterogeneity has not been fully clarified (Nishizuka, 1995).

Members of the protein kinase C family consist of a single polypeptide, comprised of an N-terminal regulatory region (approximately 20-40 kDa) and a C-terminal catalytic region (approximately 45 kDa) (Fig.l). Cloning of the first isozymes in the mid - 1980s revealed conserved domains: C1-C4 (Coussens et al., 1986). The Cl domain contains a Cys-rich motif, duplicated in most isozymes, that forms the diacylglycerol/phorbol ester binding site (Bell and Burns, 1991). This domain is preceded by a C2 domain that

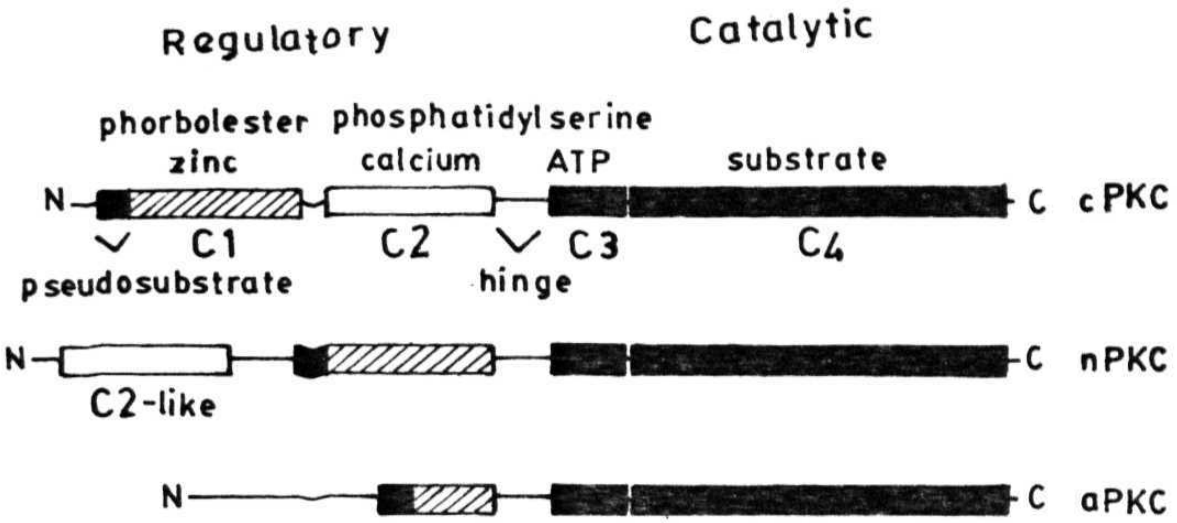


FIG. 1. Schematic representation of the primary structure of conventional (cPKC), novel (nPKC) and atypical (aPKC) protein kinase Cs. Indicated are the pseudosubstrate domain, C1 domain comprising one or two Cys-rich motifs, C2 domain in the regulatory half and the ATP binding lobe, C3 and C4 of the catalytic region. (From Newton A. C, 1995. J. Biol. Chem. 270: 28495-28498)

contains the recognition site for acidic lipids and, in some isozymes, the Ca^{24} binding site. The C3 and C4 domains form the ATP and substrate binding sites. The regulatory and catalytic halves are separated by a hinge region that is labile for proteolysis when the enzyme is membrane bound (Newton, 1993).

Eleven protein kinase C isozynies have been identified and classified into three groups based on their structure and cofactor regulation (Nishizuka, 1995). First discovered are the conventional protein kinase Cs (cPKCs): PKC-α, two alternatively spliced variants βI and βII, and y. These isozymes are regulated by Ca^{2+}; its C2 domain contains a putative Ca^{2+} binding site. The second type of protein kinases are novel protein kinases (nPKCs): 8, ε, η (L), 9, and μ. These isozymes are structurally similar to the cPKCs, but lack the C2 domain which contains Ca^{2+} binding site. These isozymes show DAG and phospholipid dependent but Ca^{2+} independent activity. The least understood isozymes are the atypical protein kinases (aPKCs): ζ and *X* (ι). These differ significantly in structure from the other two classes and they neither require Ca^{2+} nor DAG or phorbol esters (Nishizuka, 1995). These subspecies show structural

heterogeneity, differential distribution in mammalian CNS and differ from each other in enzymological properties, suggesting, each PKC subspecies presumably plays a distinct role in different cells (Nishizuka, 1995, 1988).

Protein Kinase C has been implicated in several cell functions (Kikkawa et al., 1989; Nishizuka et al., 1991), such as modulation of ion channels (Shearman et al., 1989), neurotransmitter release (Robinson 1992), receptor desensitisation (Huganir and Greengard 1990), synaptic enhancement (Nishizuka, 1986, Sposi et al., 1989), and short-term modulation of membrane excitability (Nishizuka, 1988) in the central nervous system. In cerebral cortex, hippocampus, amygdala and cerebellum, PKC along with other protein kinases, seems to play a key role in the long-term potentiation (LTP) (Linden and Routtenber, 1989) as well as use dependent enhancement or depression of synaptic transmission (Ito 1989, Kennedy, 1989).

Immunochemical distribution of protein kinase C isozymes in the CNS:

Immunocytochemical studies using antibodies specific for each PKC isozymes revealed the distinct distributions of a, βI, βII and γ isozymes in the rat brain and spinal cord. The detailed localisation of 5, e, ζ, and η PKC in the brain has remained unknown. The α-PKC immunoreactive cells were most abundant in the olfactory bulb, septo hippocampal nucleus, indusium griseum and CA2, CA3 regions of the hippocampus and the α-PKC immunoreactive neuropils were most intensely stained in the olfactory bulb, stratum radiatum of the hippocampus, septohippocampal nucleus, indusium griseum, island of Calleja, and intermediate part of the lateral septal nucleus (Ito et al., 1990). The largest number of βI-PKC immunoreactivity was generally weak in neuropils, with the exception of areas such as triangular septal nucleus, pontine nuclei, superficial layer of the superior colliculus and gray matter of the spinal cord (Hosoda et al., 1989). The βII-PKC immunoreative cells were abundant in the CA1 region of the hippocampus, caudata-putamen and neocortex. The βII-PKC immunoreactive neuropils were most intensely stained in the olfactory tubercle, caudata-putamen, accumbens nucleus, CA1 region of hippocampus and substantia nigra (Saito et al., 1989). In almost all brain regions, the βII-isozyme was more predominantly expressed than the βI-isozyme. Over 97% of the type II (PKC βI and βII) PKC was the piI-PKC, in the hippocampus and cerebral cortex. The

γ-PKC immunoreactive cells were abundant in the hippocampus, neocortex, thalamus, amygdaloid complex, cerebellum, cochlear nucleus and dorsal horn of the spinal cord. The intense immunoreactivity of γ-PKC was seen in the olfactory bulb, olfactory tubercle, anterior olfactory nucleus, molecular layer of the cerebellum and dorsal horn of the spinal cord (Saito et al., 1988). Most immunoreactivity of the four subspecies was seen in neurons, and little or none in glial cell.

Differential localisation of PKC subspecies has been most extensively studied in the mammalian cerebellar cortex (Ase et al., 1988). The Purkinje cell and the granular cell expressed a single subspecies, the y-PKC and the βII-PKC respectively. While multiple subspecies are co-expressed in the interneuron of the molecular layer, the a, βI and y-PKC are present in the basket cells and the a and y-PKC in the stellate cells. The immunohistochemical localisation of PKC isozymes in the cerebellum has also been demonstrated using isozyme-specific polyclonal antibodies (Huang et al., 1988), and also by using monoclonal antibodies (Hidaka et al., 1988; Mochly-Rosen et al., 1987). In the Purkinje cell the y-PKC is present through out the cytoplasm including, perikaryon, nucleus, dendrite, axon and axon terminal (Kitano et al., 1987). Electron microscopic studies showed that in the Purkinje cell bodies, the highest density of y-PKC is present in the plasma membrane and cytoplasm with a weaker density in the nucleoplasm.

The pyramidal cell of hippocampus was seen to contain the a, βII and y-PKC but no or little βI-PKC (Kose et al., 1990; Tanaka et al., 1988). By immunoblot analysis, however, a low amount of βI-PKC was detected in the hippocampus. The a and y-PKC were distributed throughout Ammon's horn (CA1 - CA3) with the highest concentration in the CA2 and CA1 respectively, and in the granule cells of dentate gyrus while intense immunoreactivity of βII-PKC was observed predominantly in the CA1 region of Ammon's horn. In situ hybridisation histochemistry showed co-expression of various protein kinases, such as PKC, cAMP dependent protein kinase (PKA) or Ca^{2+}/calmodulin dependent protein kinase II (CaM kinase II) in addition to PKC. The transcripts of the a, βII, y and ε-PKC were abundant in all pyramidal cells but no transcripts of pII-PKC were found in the CA2 region. The transcripts of two catalytic, Cα and Cp, and four regulatory subunits, RIα, RIβ, RIIα, RIIβ of PKA, were found in all pyramidal cells and

granule cells of the mouse hippocampal formation (Cadd and McKnight, 1989). The transcripts for CaM kinase II a, β (Burgin et al., 1990), and y were also found in all pyramidal and granule cells of rat hippocampal formation. Therefore, the pyramidal cells in the CA1 and CA3 express multiple kinds of protein kinases and these kinases might cross talk between the signal transduction pathways in the pyramidal cells.

Expression of protein kinase C mRNAs in the central nervous system:

The localisation of PKC subspecies has been studied by *in situ* hybridisation. Brandt et al., (1987) were the first to report localisation of the transcripts for βI and βII and y-PKCs in rat tissues. The transcripts βI and βII-PKC were prominent in the pyramidal cells of the hippocampal formation, except for the CA2 region, granule cells of the cerebellum and in the neocortex, basolateral amygdaloid, nucleus of the lateral olfactory tract and caudate-putamen. The mRNA of y-PKC was abundant in pyramidal and granule cells of the hippocampal formation, cells of the neocortex, pyramidal cells of the periform cortex and Purkinje cells in the cerebellum. Later Ito et al., (1990) demonstrated distribution of mRNA for other PKC isozymes. The mRNA for α-PKC was found throughout the brain but seemed less abundant than the other PKC isozymes. The highest amount of the a-PKC transcript was found in pyramidal cells of hippocampus, granule cells of the olfactory bulb, cells in the septohippocampal nucleus and indusium griseum. The mRNAs of e-PKC and ζ-PKC were also widely distributed throughout the brain, whereas the mRNA for 8-PKC concentrates in the thalamus, a region that participates in the relay of sensory information to the cortex. The mRNA for e-PKC is prominent in the olfactory bulb, olfactory tubercle, lateral septum and hippocampus. The η-PKC is the only one subspecies expressed more abundantly in the peripheral tissues than in the CNS (Osada et al., 1989). The r|-PKC is predominantly expressed in the skin and lung, but distribution of this subspecies in the brain is unknown. Although the immunocytochemical localization of δ, ε, and ζ-PKC isozymes has not been clarified, the genes for these subspecies appear to be expressed in different neurons and play individual roles in the CNS. The localization of PKC mRNA in the CNS obtained by *in situ* hybridisation histochemistry generally agrees with that of PKC containing neurons

determined by immunohistochemistry and represents neuronal populations that synthesize each PKC isoforms.

Differential expression of protein kinase C subspecies:

The individual role of each PKC subspecies was suggested by findings that the neurotransmitter is contained in neurons immunoreactive for each subspecies. With the following areas, each PKC subspecies was found in a different type of neuron and was co-localised with one or two neurotransmitters, hence that each PKC subspecies is involved in different neuronal functions in the restricted brain areas. Differential expression of each PKC subspecies was evident in the striatum, substantia nigra and cerebellar cortex.

The α-PKC immunoreactive neurons in the striatum were intrinsic cholinergic and most of the oc-PKC containing neurons in the substantia nigra were dopaminergic neurons. The pI-PKC containing neurons of the striatum were intrinsic GABAergic neurons with medium or large sized soma. Lesion studies revealed that most neurons expressed the pH and y-PKC in the striatum and send their exons to the substantia nigra. PKC-βII was present in the medium sized neurons with GABA immunoreactivity only in the colchicine treated striatum, the βII-PKC may be involved in the functions related to GABAergic striatonigral neuron. The neurotransmitter of y-PKC immunoreactive neurons is yet to be identified. There are reports that a, pI-PKC modulate the release of acetylcholine, dopamine and GABA neurotransmitters from striatal slices (Tanaka et al., 1986; Cubeddu et al., 1989; Chandler and Leslie, 1989). Furthermore, choline acetyltransferase is phosphorylated by PKC (Bruce and Hersh, 1989) and tyrosine hydroxylase is one of the substrates of PKC (Albert et al., 1984; Nishizuka, 1986; Onali and Olianas, 1987), thus the PKC may be involved in the regulation of synthesis of acetylcholine and dopamine.

Developmental expression of Protein Kinase C isozymes in CNS:

The differential expression of PKC isozymes has been demonstrated in the developing rat brain (Hashimoto et al., 1988; Yoshida et al., 1988; Huang et al., 1990; Sposi et al., 1989; Hirata et al., 1992). The **a** and pI-PKC are present at birth and progressively increase up to 2-3 weeks of age, while the βII and y-PKCs are very low at

birth and increase between 2-3 weeks of age. The βI-PKC was predominant in the brain stem at birth and was located in the perikarya, dendrites and growth-cone. Similarly, the α-PKC is also present in the growth-cone like structure in the early stage of development. These findings suggest that a and pi-PKC may be involved in specialised functions of growth-cones, e.g. neurite outgrowth, even at an early stage of brain development.

Substrate proteins of PKC:

The major substrates of PKC so far identified include: Myristoylated alanine rich c-kinase substrate (MARCKS) protein with an apparent molecular mass of 80-87 kDa (Albert et al., 1987; Erusalimsky et al., 1991), neuromodulin (GAP-43, Protein F1, B50, p57, pp46) (Baudier et al., 1989; Coggins and Zwiers, 1991), neurogranin (Represa et al., 1990; Baudier et al., 1991) and dephosphin/dynamin (Robinson, 1992, Liu et al., 1994). Neuromodulin and neurogranin are both calmodulin-binding phosphoproteins at the presynaptic membrane and postsynaptic level, respectively (Goslin et al., 1989; Represa et al., 1990). Neuromodulin has been reported to be related to nerve growth, neural plasticity, and modulation of neurotransmitter release (Meiri et al., 1988; Nelson et al., 1989; Dekker et al., 1989). Phosphorylation of this protein correlates highly with the degree of LTP (Akers and Routtenber, 1985). On the other hand, the cellular role of neurogranin, which was found exclusively in the rat forebrain (Represa et al., 1990), has not been clearly established and only found to be related to long-term potentiation in the CA1 region of the hippocampus (Klann et al., 1992).

Protein Kinase C and Diseases

The PKC activity was noted to be reduced in human autopsied brains obtained from patients with various neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease (Nishino et al., 1989; Kitamura et al., 1990). In tissues from patients with Parkinson's disease, phorbol ester binding showed a significant reduction in the substantia nigra (SN) (ca 60%) and striatum (ca 40%). Reduction in phorbol ester binding in the SN may be attributed to both the decrease in a-PKC in dopaminergic neurons and the decrease βII-PKC in the GABAergic nerve terminals due to transynaptic degeneration of striatal neurons. In the patients of Huntington's disease a marked reduction of phorbol ester binding in the striatum (ca 70%) was noticed. In these

patients, there are decreases in several neurotransmitters, including those of GABA, acetylcholine, and substance P present within the striatonigral pathway. In contrast, dopamine, glutamate and somatostatin are either not altered or are only slightly increased. Reduction of phorbol ester binding in the striatum from patients with Huntington's disease may possibly be related to decreases in the βII or γ-PKC rather than the βI-PKC and α-PKC

Calcium/Calmodulin-dependent protein kinase II (CaM kinase II)

Calcium calmodulin-dependent protein kinase II is a multifunctional serine/threonine protein kinase and is one of the most abundant protein kinases in mammalian brain. It is highly concentrated in cortical structures and hippocampus (up to 2% of the protein). It is localised on both sides of the synapse where events central to neurotransmission are likely to be directly regulated. Many of its substrates are involved in neuronal signalling. CaM kinase modulates both neurotransmitter release and neurotransmitter synthesis (Erondu and Kennedy, 1985).

Five isoforms of CaM kinase II from brain have been identified a, β, β', y and 8. The amino acid sequences of these isoforms have been deduced from their cDNA structures (Lin et al., 1987; Hanley et al., 1987; Bennett and Kennedy 1987; Bulleit et al., 1988; Tobimatsu et al., 1988; Tobimatsu and Fujisawa, 1989). Although these isoforms are encoded in four independent genes, the amino acid sequence is highly conserved. The a and p-isoforms are primarily expressed in brain (Lin et al., 1987; Bulleit et al., 1988) as the major components of brain CaM kinase II, whereas the y and δ-isoforms (~59/60 kDa) are expressed in various tissues (Tobimatsu and Fujisawa, 1989).

Biochemical characterisation has also been well studied for brain CaM kinase II. It has been demonstrated that CaM kinase holoenzyme isolated from rat forebrain has an apparent molecular weight of between 460 - 650 kDa and is composed of two distinct subunits with molecular masses of 50-54 kDa (α-subunit) and 58 /60 kDa (β/β'subunits). There are approximately nine a subunits and three p subunits per molecule of holoenzyme. Their cDNA sequences show that both subunits contain catalytic, regulatory (calmodulin-binding), and association domains (Bennet and Kennedy, 1987; Hanley et al., 1987; Lin et al., 1987). The subunits are the products of two highly homologous

transcription units, with the larger size of the β subunit due to the calmodulin-binding domain. The composition of a and p subunits in CaM kinase II depends on the region of brain and on the stage of development. The ratios of a to β subunits are reported to be ~ 3 - 4 : 1 and ~ 1 : 3 - 4 in the adult forebrain and cerebellum, respectively (McGuinness et al., 1985; Miller and Kennedy, 1985).

CaM kinase II plays a major role in the regulation of the synthesis and secretion of neurotransmitters, receptor function, structural modifications of cytoskeletal proteins, microtubule assembly/disassembly, axonal transport, and in long term potentiation in the brain (Yamauchi and Fujisawa, 1983; Colbran et al., 1989; Nairn et al., 1985; Soderling, 1990).

CaM kinase II can catalyse Ca^{2+}/CaM-dependent intramolecular auto-phosphorylation of both (a and p) subunits (Bennett et al., 1983; Goldenring et al., 1983; Schworer et al., 1985). Following this autophosphorylation, the soluble kinase is able to phosphorylate exogenous substrates (Lai et al., 1986; Lou et al., 1986 Miller and Kennedy, 1986) and to undergo additional autophosphorylation (Miller and Kennedy, 1986; Hashimoto et al., 1987) in the absence of calcium. Recent results have demonstrated that formation of the partially Ca^{2+}- independent form of CaM-kinase II is associated with autophosphorylation of Thr^{286} (Miller et al., 1988; Schworer et al., 1988; Thiel et al., 1988) of the a subunit. Thr^{286} is located close to adjacent calmodulin binding and inhibitory domains contained within a multiregulatory region of the kinase (residues 281-314 in the a-subunit) (Hanley et al., 1988; Colbran et al., 1988 Payne et al., 1988). The amino acid sequence of this region is highly conserved in the β/β' subunits (residues 282-315; Bulleit et al., 1988). Tryptic Phosphopeptide mapping demonstrates that Ca^{2+}-independent autophosphorylation occurs at sites largely distinct from those phosphorylated in the presence of Ca^{2+}/calmodulin (Lickteig et al., 1988; Patton et al., 1990; Lou and Schulman, 1989). The sites are located in peptides containing Thr^{105} and Ser^{314} in the calmodulin-binding domain (of a subunit) (Patton et al., 1990).

Regional distribution of CaM Kinase II in Brain

In adult rat forebrain, only about 20% of CaM kinase II is cytoplasmic (Kelly and Vernon, 1985; Hashimoto and Soderling, 1987) and is 20-50 fold more concentrated in

the brain than in non-neuronal tissues. There are striking regional and developmental variations in its concentration, isoform ratio, and regional and subcellular localisation, consistent with a highly regulated role in neuronal functions. Immunocytochemical studies with antibodies recognising CaM kinase subunits demonstrate high levels of kinase in regions of the telencephalon, including particularly hippocampus, cortex, amygdala, striatum, and lateral septum (Erondu and Kennedy, 1985; Fukunaga et al., 1988; Ouimet et al., 1984). CaM kinase expression is prominent chiefly in neurons. High level expression in particular neurons is striking in hippocampus, cerebral cortex, and cerebellar cortex. Within the hippocampus, the strongest immunoreactivity is observed in molecular and pyramidal cell layers and in the ectal and endal limbs of the dentate gyrus. In the neocortex, the pyramidal neurons of layer V and the dendrites of layer I are most prominently stained, and layer IV exhibits lowest staining. Glial cells exhibit no immunoreactivity with some CaM kinase antibodies (Erondu and Kennedy, 1985; Ouimet et al., 1984), and low immunoreactivity with a polyclonal antiserum that efficiently recognised a variety of non-neuronal isozymes (Fukunaga et al., 1988). CaM kinase has since been purified from cultured astrocytes, but was not found in cultured oligodendrocytes (Scholz et al., 1988). The level of expression in astrocytes is far below levels observed in neurons. CaM kinase localisation in human brain tissues was found to be similar to that the rat (Iwatsubo et al., 1991; Mckee et al., 1990). Hippocampal pyramidal neurons of CA1 and the subiculum were particularly intensely stained (Mckee et al., 1990).

Similar regional distribution has been described for the mRNAs that encode CaM kinase II. In situ hybridisation with probes specific to a or p (Lin et al., 1987; Burgin et al., 1990) subunits demonstrated high levels of a expression in hippocampal and cortical neurons, and high levels of p expression in the Purkinje and granule cells of the cerebellum. Each of the four isozymes (a, p, γ, 8) is expressed in brain, as shown by Northern blot analysis of message levels. a subunit is most abundant in forebrain, less in the brain stem or lower brain, and much less in cerebellum (Bulleit et al., 1988; Tobimatsu et al., 1988). β is highest in cerebellum, and somewhat less abundant in the lower brain and forebrain (Bulleit et al., 1988; Tobimatsu et al., 1988). CaM kinase II y

is present at highest levels in forebrain, with slightly lower levels in brain stem and cerebellum (Tobimatsu et al., 1988). Delta is most prominent in cerebellum, and slightly lower in forebrain and brain stem (Tobimatsu and Fujisawa, 1989).

Subcellular localization of CaM Kinase II

CaM kinase II is highly concentrated in neuronal cell bodies and dendritic processes (Fukunaga et al., 1988; Ouimet et al., 1984). Moderate levels are seen in nerve terminals and spines, and lower levels in the axons of the main fiber tracts. Nuclei are not stained or weakly stained ((Fukunaga et al., 1988; Ouimet et al., 1984). Biochemical fractionation demonstrate that CaM kinase is present in the cytosol, as well as in particulate fractions, including plasma membrane, post synaptic densities (PSD), cytoskeleton, synaptic vesicles, and the nucleus. (Schulman, 1988).

Developmental Regulation of CaM Kinase II

Developmental changes in CaM kinase expression have been well documented, with changes noted in total CaM kinase activity, subunit ratios, and subcellular distribution during neuronal development (Rostas, 1991). Early studies of the major PSD protein (a subunit) demonstrated a 20 fold increase in its level during development (Kelly and Cotman, 1981). Subsequent analyses have confirmed that a subunit levels are sharply regulated, with concentrations in rat forebrain rising linearly 10 fold between days 5-25 (Kelly and Vernon, 1985). CaM kinase β subunit is the dominant isozyme at birth and remains at a relatively stable level throughout development, although some decline is evident after the second week (Sahyoun et al., 1985). The large developmental increase in forebrain **a** subunit levels changes the overall ratio of α:β from less than one in the neonate to greater than two in the adult (Kelly et al., 1987). Interestingly, the increase in **a** subunit levels peaks near the end of the most active period of synaptogenesis (Kelly et al., 1987), and may be coincident with synaptic maturation (Rostas, 1991). Cerebellum maintains relatively low levels of **a** subunit throughout development, and therefore always contains kinase with β subunits exceed a subunits (Sahyoun et al., 1985). A cerebellar isoform with an α:β ratio of 1:8 is dominant at postnatal day 10, whereas one with an α:β ratio of 1:5 predominates at postnatal day 40

(Vallano, 1990). CaM kinase is approximately 75% soluble in the neonate and 75% particulate in the 90 day adult (Kelly and Vernon, 1985).

Protein Tyrosine Kinases (PTKs)

Tyrosine-specific protein kinases are a class of protein kinases that were originally shown to be involved in the regulation of cell transformation and cell proliferation and first identified as the protein products of the oncogenes of transforming retroviruses (Hunter and Cooper, 1985). Growth factor receptor kinases have been identified as a related class of tyrosine kinases. These include the epidermal growth factor receptor, the insulin receptor, the platelet-derived growth factor receptor, and the insulin-like growth factor receptor (Cohen et al., 1980; Kasuga et al., 1983; Heldin et al., 1983; Jacobs et al., 1983). It appears that the tyrosine-specific protein kinase activity intrinsic to these receptors and to the viral oncogene products mediates many of the effects of these proteins on cellular growth (Hunter and Cooper, 1985).

The adult brain contains high levels of protein tyrosine kinase (PTKs) activity and play a specific role in the regulation of neuronal function (Cotton and Brugge, 1983; Dasgupta et al., 1984). In the brain several cellular oncogene products having tyrosine kinase activity, include *c-yes* (Sudol et al., 1988), *c-fyn* (Viellette and Bolen, 1989), *elk* and *flk* (Letwin et al., 1988) *bek* (Kornbluth et al., 1988), *trk B* (Klein et al., 1989), and *c-srn* (Neer and Lok, 1985) and its neuron-specific homologue *c-src*$^{+}$ (Brugge et al., 1985), have been identified in nervous tissue. Tyrosine kinases are present at synapses in the adult brain and have been identified in synaptic vesicles, synaptic membranes, post-synaptic densities (PSDs), and the synaptosplasm (Ellis et al., 1988; Hirano et al., 1988; Pang et al., 1988). Several recent observations have suggested a role for tyrosine kinases in the regulation of synaptic function. Tyrosine phosphorylation has been shown to regulate the rate of desensitisation of the nicotinic acetylcholine receptor (Hopfield et al., 1988), and also implicated in the development of long-term potentiation in hippocampal slices (O'Dell et al., 1991). The consensus sequences for tyrosine phosphorylation in both the gamma-aminoburyric acid$_A$ and glycine receptors (Huganir and Greengard, 1990) has been identified. Phosphorylation of the synaptic vesicle glycoprotein, synaptophysin, on tyrosine residues have also been described (Pang et al., 1988; Bernekow et al., 1990).

The decline in the tyrosine kinase activity was observed during the brain development (Okada and Nakagawa, 1988). The expression of individual tyrosine kinases, however, may increase, decrease, or remain unchanged, depending on the particular enzyme and brain region analysed. Thus, the developmental expression of $pp60^{c\text{-}src}$ is differentially regulated in the striatum, hippocampus, and cerebellum of rat brain (Cartwright et al., 1988), and in the chick cerebellum *c-yes* kinase activity increases at a time when *c-src* kinase activity decreases (Sudol et al., 1988). Apart from the general observation that synaptosomcs prepared from the mature rat brain express less tyrosine kinase activity than growth cone preparations (Aubry and Maness, 1988; Cheng and Sayhown, 1990), altered protein tyrosine phosphorylation and decreased PTK activity was observed during Alzheimer's disease (Shapiro et al.,1991).

Protein phosphorylation in CNS diseases

Selective diseases of nervous system are 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, CaM kinase II and PKC activity was altered during complete cerebral ischemia (Hu 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 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

nfluence PKC activity like in the intracellular calcium (Daniell et al., 1987; Rabe and Weight. 1988; Davidson et al., 1988). 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 e 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^{2+} 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; Gordon et al., 1986). This heterologous desensitization is due to decrease in Gsa 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 50 kDa 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).

Scope of the Present Study

Ethanol is the most commonly used and abused drug, which affects every organ of the body. The mechanism by which it exerts its toxic effects, such as tissue damage secondary to cellular injury, carcinogenesis and teratogenesis are still unclear. Further, ethanol is considered as a neurotoxin as it causes neuronal degeneration resulting in loss of memory and cognitive impairments (Arendt et al., 1988). Normal brain development depends on coordinated complex events in which neurons and glial cells participate (Rakic, 1991). It has been shown that ethanol affects development by decreased neurogenesis, delayed and aberrant neuronal migration, anomalous morphological development (Miller, 1992), changes in the ontogeny of neurotransmitter synthesis (Druse, 1992), and neuronal depletion in several brain regions (West et al., 1990). These specific actions of ethanol are related to its ability to interact with neuronal membrane leading to changes in membrane fluidity, neurotransmitter action and signal transduction (Glodstein and Chin, 1981). The effects of ethanol on developing brain thus are shown to be deleterious (West et al., 1994).

Development of brain is made possible by diverse signal transducing pathways and these events are highly susceptible for the action of ethanol. The signalling pathways in the central nervous system involve different protein kinases which include Protein Kinase C (PKC), Ca^{2+} /Calmodulin dependent protein kinase II (CaM kinase II), and Protein Tyrosine Kinases (PTKs), regulating diverse processes. Several reports showed protein phosphorylation in developing rat brain using synaptosomcs, subcellular fractions and tissues (Salbego and Rodnight, 1996) in normal physiological conditions as well as using PKC activators like phorbol esters (Molina and Ashendel, 1991). Protein phosphorylation has been shown to be affected by chronic ethanol ingestion in rats (Babu et al., 1994). Long term incubation of cultured neuronal cells in the presence of ethanol resulted in the upregulation of calcium channels by PKC mediated protein phosphorylation (Messing et al., 1991). Further, phosphoinositide turnover has been shown to be disrupted in ethanol fed animal (Pietrazak et al., 1990) which can also modulate PKCs.

The involvement of PKC, CaM kinase II and protein tyrosine kinases in brain development, neuronal connectivity, maturation and in several forms of learning and memory including its role in LTP has been demonstrated (see reviews Nishizuka 1992, 1995, Tanaka and Saito, 1992, Linden and Routtenberg, 1989; Nairne et al., 1985; Malenka et al., 1989; Malinow et al., 1989; Gurd, 1997; Hell, 1997). Clearly all these neuronal functions are also affected in varying degrees of severity in chronic alcoholics (Samson and Diaz, 1996). However, reports on the effects of pre and postnatal ethanol exposure on protein kinases in developing brain are scanty and where available are varying drastically as both supportive and differing studies can be noticed in the literature, which is perhaps due to different methods used in such as mode of ethanol treatment, animal models, or tissue culture cells used etc. Animal models have shown ethanol induced microencephaly, neuronal impairment, decreased DNA and protein content in brain, which are directly related to growth impairment and neuronal maturation in brain (West and Pierce, 1986).

Therefore, a thorough study was undertaken in the present investigation to elucidate calcium dependent and independent protein phosphorylation events affected by pre and postnatal ethanol exposure in developing rat brain. This study provides an insight into the potential role of specific protein kinases and protein substrates in ethanol induced neurological dysfunction and fetal alcohol syndrome. The specific goals of this study are:

- Identification of calcium dependent protein kinases Protein Kinase C and CaM kinase II response during pre and postnatal ethanol exposed rat cerebral cortex development.
- Identification of calcium independent Protein Tyrosine Kinase response during pre and postnatal ethanol exposed rat cerebral cortex development.

General Methods

Animals and ethanol treatment

One month old male and female Wistar strain rats weighing 70±10 g were used in this study. Ethanol treatment of animals was carried out using standard procedures followed earlier (Babu and Vemuri, 1990; Fuches et al, 1987). The animals were housed and maintained at 12/12 h light/dark cycle; temperature 25 - 28°C in the animal house. They were divided into three groups (each group consisting of five animals, one male and four females) as control, isocaloric control and ethanol treated group. All the three groups had free access to standard commercial rat chow obtained from National Institute of Nutrition (NIN), India and were maintained as follows throughout the experimental schedule of two months. Control group of animals received basal diet and water, Isocaloric controls were fed basal diet plus isocaloric glucose-NaC1l which was calculated according to standard protocol (Koch et al., 1994) based on the final regimes consumed by the ethanol treated group. Ethanol treated group received basal diet and 10% (v/v) ethanol in water as their only drinking fluid in a bottle. The caloric percentage of ingredients of the final regimen (basal diet + alcohol) consumed by ethanol treated group was: alcohol - 13.8 %, carbohydrates - 61.2 %, fat - 7.0 %, protein - 18.0 %. Ethanol induced changes in food and fluid consumption pattern, blood alcohol concentration, body weight, and morphological changes in brain cell types have been described earlier from our laboratory (Haviryaji et al., 1992; Babu et al., 1994). During ethanol treatment, female rats were matched with male rats at appropriate times to get litters born to mothers intoxicated for 2 months before pregnancy. The offspring of alcohol intoxicated mothers also received alcohol till they were sacrificed. After 8, 30 and 90 postnatal days, animals were decapitated and brains were stored at -70°C till they were used.

General condition and behavioural symptoms of animals

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, as noticed in our earlier studies as well (Babu et al., 1994; Haviryaji et al., 1992). 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. The blood alcohol contents (BAC) as measured by ADH activity was 0.19%. Normal behavioral 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 was normal and the data agree with the earlier results of other laboratories (Klemn and Engen, 1978) as well as our laboratory (Babu et al., 1994; Haviryaji et al., 1992; Haviryaji and Vemuri, 1997).

The number of litters born to alcoholic mothers were less when compared to controls. Their body weight and size was also observed to be less over controls (data not shown). The general behaviour, standing and reaching for the food was slightly different from that of control pups. The animals also showed mild aggressive behaviour.

Subcellular fractionation of membrane and cytosol for the study of PKC, CaM Kinase II and Protein tyrosine kinases

The membrane and cytosolic fractions were prepared as described elsewhere (Souza and Ramirez, 1991) with minor modifications. Briefly, cerebral cortex from control and ethanol fed rats was homogenised in 5 volumes of ice-cold homogenizing buffer containing 20 mM Tris-HCl buffer (pH 7.4), 1mM dithiothreitol, 5 mM EGTA, 2 mM EDTA, 10% glycerol, 1 mM $MgCl_2$, 1 mM PMSF, 2 µg/ml of leupeptin and 2 jag/ml of aprotinin by Dounce homogeniser using pestle 'B' (Wheaton Scientific, USA) which allows disruption of tissue without disrupting the nuclei. The homogenate was centrifuged twice at 1000 x g. for 15 min, and pellets were discarded. The supernatant was further centrifuged at 27,000 g for 1 h at 4°C, and the supernatant was designated as the crude cytosolic extract. The resulting pellets were resuspended in homogenizing buffer containing 0.5% Triton X -100, incubated for 1 h at 4°C, and then recentrifuged at 27,000 g for 30 min. The supernatant from this centrifugation was designated as crude membrane extract. Protein content was determined by the method of Bradford (Bradford, 1976).

Perfusion of rats and tissue preparation for Immunohistochemical analysis of PKC and CaM Kinase II

Perfusion of rat for fixing the brain tissue

Perfusion was performed at 8, 30 and 90 days of age to control and ethanol treated group of rats. The animals were deeply anaesthetised with 50mg/kg (i.p.) pentobarbital. The rat was layed on its back and opened the thorax carefully to avoid excessive bleeding. Rib cage was cut carefully and the diaphragm was removed for exposing the heart, needle was inserted in the left ventricle carefully. The vascular system was perfused with phosphate-buffered saline (PBS; pH 7.4). An incision was made in the right atrium to flush the blood out of the whole body. Once the blood has been flushed out, a solution of freshly prepared 4% w/v paraformaldehyde in PBS was infused to fix the brain. A successful perfusion was performed by appreciable hardening of the animal's body. Whole brains were removed, post fixed overnight in 4 % paraformaldehyde and stored in Tris-buffered saline (TBS; 25mM Tris, pH 7.4, 150 mM NaCl) with 0.02% sodium azide at 4 ºC until used.

Tissue preparation for immunohistochemical analysis

At the level of hippocampus, coronal sections of 5 mm thick blocks were cut removed and dehydrated through increasing concentrations of ethanol (40, 70, 90 and 100 % 30 min each) and clearing was done using xylene (xylene : ethanol 1:1, xylene x 2, 30 min each). The tissue blocks were infiltrated with molten paraffin wax at 60 °C (xylene : paraffin wax 1:1 for 30 min, wax x 2 for 6 h each). Tissue blocks were sectioned using microtome and the coronal slices of 20 µm thick from both control and alcohol fed animals were mounted on gelatinised microscope slides. The slices were heat adhered at 40 °C for 48 hours.

Chapter—I

Chapter - I

Effect of Ethanol on Protein Kinase C

Prenatal ethanol exposure exerts deleterious effects on fetal growth and postnatal development. Fetal alcohol exposure (FAE) is characterised by developmental retardation of the CNS. FAE is a reliable animal model to study the Fetal Alcohol Syndrome (FAS) (Abel, 1982; Nelson and Taylor, 1987). In rat, neurogenesis and neuronal differentiation occur during the brain growth spurt and it is one of the most vulnerable periods for the developing brain (West and Pierce, 1986; Samson and Diaz, 1982). Exposure to ethanol during growth spurt resulted in decreased cell size and/or number and affected maturation process of the brain region receiving more alcohol (Kennedy, 1984). These specific actions of ethanol are related to its ability to interact with neuronal membrane leading to changes in membrane fluidity, neurotransmitter action and signal transduction (Goldstein and Chin, 1981). Infact, short periods of alcohol exposure during brain development caused long lasting impairment of spatial learning behaviour in rats (Pauli et al., 1995).

Protein Kinase C (PKC) has been implicated in several neuronal cell functions (Kikkawa et al., 1989; Nishizuka et al., 1991), such as modulation of ion channels (Shearman et al., 1989), neurotransmitter release (Robinson, 1992), receptor desensitisation (Huganir and Greengard, 1990), synaptic enhancement (Nishizuka, 1986, Sposi et al., 1989), and short-term modulation of membrane excitability (Nishizuka, 1988) in the central nervous system (CNS). In cerebral cortex, hippocampus, amygdala and cerebellum, PKC along with other protein kinases plays a key role in the long term potentiation (LTP) as well as use dependent enhancement or depression of synaptic transmission (Ito, 1989; Kennedy, 1989). Cloning and biochemical analysis revealed a multigene family of PKCs consisting three major classes: conventional PKCs (cPKCs) isoforms α, βI, βII and γ which are calcium dependent and activated by diacylglycerol (DAG), novel PKCs (nPKCs) 5, 6, η, 6 and μ are calcium independent and atypical PKCs (aPKCs) ζ and *X* (ι) isoforms, which neither require Ca^{2+} nor DAG (Nishizuka, 1995).

These subspecies show structural heterogeneity, differential distribution in mammalian CNS and differ from each other in enzymological properties, suggesting, each PKC subspecies presumably plays a distinct role in different cells (Nishizuka, 1995). The multiple isoforms of PKC pose a difficulty to discern the specific role of isoforms, as most cells express multiple isoforms. Molecular approaches employing identification and characterisation of in vivo substrates for PKC in CNS supported PKC activation requirement for neuronal functions in CNS (Albert et al., 1987; Coggins and Zwiers, 1991; Baudier et al., 1991; Liu and Sorm, 1990).

Several studies showed protein phosphorylation in developing rat brain using synaptosomcs, subcellular fractions and tissues (Salbego and Rodnight, 1989, 1996) in normal physiological conditions as well as using PKC activators like phorbol esters (Molina and Ashendel, 1991; Castagna et al., 1982). Ethanol exposure has been shown to affect several biochemical constituents which may ultimately influence PKC activity (Rabe and Weight, 1988; Davidson et al., 1988), for example a rise in intracellular Ca^{2+} after ethanol treatment (Daniell et al., 1987). Further, phosphoinositide turnover has been shown to be disrupted in ethanol - fed animal (Pietrzak et al., 1990) which can also modulate PKCs. The involvement of PKC in brain development, neuronal connectivity, maturation and in several forms of learning and memory including its role on LTP has been demonstrated (see reviews Nishizuka 1992, 1995; Tanaka and Saito, 1992; Linden and Routtenberg, 1989). Clearly, all these neuronal functions are also affected in varying degrees of severity, in FAS experimental animal models of chronic ethanol treatment and in chronic alcoholics (Samson and Diaz, 1996). The presence of various PKC isoforms, PKC substrates in brain and their involvement in overall development of brain prompted us to investigate the identification and characterisation of specific PKC isoforms and endogenous substrate proteins involved in ethanol induced changes in the development of cerebral cortex.

MATERIALS AND METHODS

Materials

Histone type HIS, Phenylmethylsulfonyl fluoride (PMSF), Dithiothreitol (DTT), Ethylene Glycol-bis tetraacetic acid (EGTA), Ethylenediaminetetraacetic acid (EDTA), leupeptin, aprotinin, Ultrapure ATP, Phosphotidylserine (PS), 1,2-diacylglycerol (DAG), Staurosporine and reagents for sodium dodecyl Sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE), 5-bromo-4-chloro-3-indoylphosphate / nitroblue tetrazolium chloride (BCIP/NBT) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Diethylaminoethyl-cellulose (DE-52) was from Whatman (Whatman BioSystems Ltd., England). [γ ^{32}P] ATP (3000 Ci/mmol) was from Board of Radiation and Isotope Technology (Mumbai, India). Phosphocellulose filter (P81) was obtained from Whatman (Canlab Corp., Mississauga, ON, Canada). Nitrocellulose sheets were from Schleicher and Schuell (Keene, NH, U.S.A.). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Rabbit polyclonal antibodies against PKC a, βI, pH, γ, 5, ε, η and ζ were generous gift from Prof. Yusuf A. Hannun, Duke University Medical Centre, Durham, USA.

Partial purification and assay of PKC

PKC was partially purified following the method of Huang and Huang (1991). The crude membrane and cytosolic extracts (~15 mg of protein) derived from control and ethanol treated rats were applied to a DE-52 column (0.6 X 3.5 cm) equilibrated with 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM EGTA, 0.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol (buffer A). The column was washed with 10 ml of buffer A, and bound PKC was eluted stepwise with 2.4 ml of buffer A containing 50 mM KC1, then 2.4 ml of buffer A containing 100 mM KC1, and finally 2.4 ml of buffer A containing 200 mM KC1. The active PKC-containing fractions were pooled, concentrated, made 20% in sucrose, and stored at –70°C.

The assay was performed in a final volume of 50 ul which contained 30 mM Tris-HCl buffer pH 7.4, 10 mM $MgCl_2$, 1mM DTT, 0.4 mM $CaCl_2$, 40 μg/ml of phosphotidylserine, 8 fig/ml of 1, 2 diacylglycerol, 1 mg/ml of histone type III-S as phosphate accepting substrate and enzyme preparation (1.5 μg of protein) from pooled

fractions of 100 and 200 mM KC1 eluales as described by Huang and Huang (1991). The reaction was started by the addition of 10 μM ATP [γ^{-32}P] ATP (1-2 μCi) (3000 Ci/mmol) followed by incubation at 30°C for 5 min. The samples were immediately sported on the 2 x 2 cm Watmann P-81 filter paper discs, washed three times with 75mM phosphoric acid and dried. The filters were kept in 5.0 ml of scintillation fluid and the radioactivity was measured. Enzyme activity for PKC was calculated by subtracting the activity of the enzyme determined in the absence of calcium and phospholipids from its activity in their presence. The activity levels of PKC are expressed as $pmol^{-1}min^{-1}\mu g$ protein.

Statistical analysis

PKC activity values are in mean ± SEM of multiple data acquisition. Statistical analyses were performed using the paired t-test, employing the Sigma plot software. A value of $P<0.05$ was considered significant.

Immunochemical** analysis of PKC **isoforms

For the immunochemical analysis of PKC isoforms, the membrane and cytosolic fractions were prepared as described earlier in the section subcellular fractionation of membrane and cytosol. Equal amount of protein (75ug/lane) was electrophoresed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % polyacrylamide gels as described by Laemmli (1970). Immunoblotting was performed by the method of Towbin et al., (1979). Proteins from the SDS-PAGE gels were transferred to 0.2 μm nitrocellulose using transblot apparatus (LKB, Sweden). Protein transfer was performed at 45 mA for 3 h in 25 mM Tris-HCl (pH 8.8), 192 mM glycine, 20% methanol buffer. After blotting, the membrane was washed with TBS and non-specific binding sites were blocked with 5% (w/v) fat free milk powder for 2 h.

The immunoblots were incubated overnight at 4°C in polyclonal antibodies directed against PKC a, βI, pH, *y,* 8, ε, η and ζ isoforms. Following three washes with TBS, blots were incubated with secondary antibody (goat anti-rabbit conjugated with alkaline phosphatase). After washing three times with TBS, immunoreactive PKC isoforms were visualised using 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium chloride (BCIP/NBT) chromogen.

Immunohistochemical analysis of PKC

Tissue slices were deparaffinized with xylene (5 min each) and rehydrated through decreasing concentrations of ethanol (xylene : ethanol 1:1, 100, 90, 70 and 40% ethanol, DD H_2O for 5 min each) the slices were placed in cold Tris-buffered saline (TBS pH 7.4). Slices were washed thoroughly (5 x 5 min) in TBS to remove all traces of xylene and ethanol. Sections were placed in TBS containing 0.5% Triton x 100 for 15 min at 40°C then rinsed in TBS (3 x 10 min) at room temperature. To limit the amount of non-specific binding, slices were incubated for 1 hr in 5% goat normal serum from the animal in which the corresponding secondary antibody was made. Sections were rinsed in TBS, incubated with polyclonal antibody directed against PKC ∝, βI, βII, y and 5 isoforms for 24 hrs at 40 °C using dilution 1:1000.

Following the incubation with primary antibody, sections were washed 3 x 10 min at room temperature in TBS. Brain slices not incubated in primary antibody were included as a negative control. Following three 10 min washes in TBS, slices were visualised using diaminobenzidine (0.5mg/ml; Sigma) and 0.02 % hydrogen peroxide. After a final wash in distilled water, sections were mounted on gelatinised slides and allowed to air dry overnight. Slides were dehydrated through a graded series of alcohol, coversliped with DPX mount, examined and photographed under a Nikon Labphot Microscope, Japan.

In Vitro phosphorylation of endogenous substrate proteins

Calcium independent phosphorylation

Calcium independent phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and alcohol fed rat cerebral cortex were carried out by following standard methods (Ali et al., 1988; Babu et al., 1994). Equal amount of protein (20 μg) from membrane and cytosolic fractions were used for the assay. The reaction was carried out in 50 μl of 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$ and 1 mM DTT and 100 μM EGTA to chelate calcium. The reaction was started by the addition of 10 μM ATP, [γ^{-32}P] ATP (2 μCi per assay) (3000 Ci/mmol) followed by incubation at 30°C for 1 min. The reaction was stopped by Laemmli sample buffer (0.125 M Tris-HCl (pH 6.8), 2% SDS, 10 % glycerol, 2% β-mercaptoethanol and 0.01% bromo

phenol blue) and the mixture was heat denatured for 2 min in boiling water. The proteins were separated by SDS-PAGE on 10% polyacrylamide gel as described by Leammli (1970). The gels were silver stained, dried, and exposed to X-ray film (Kodak) using intensifying screens (Kodak) at -70°C for 12 h. The exposed film was developed to detect the phosphorylated proteins.

Calcium dependent phosphorylation

The calcium dependent phosphorylation was performed exactly as described above, excepting that the EGTA in the reaction mixture was replaced with 0.4 mM calcium chloride.

Calcium/phospholipid dependent phosphorylation

PKC dependent endogenous phosphorylation was performed by using equal amounts of membrane and cytosolic protein (20 μg of protein) form control and ethanol fed rats. The reaction was carried out in 50 |il of 20 mM Tris-HCl buffer (pH 7.4), 10 mM $MgCl_2$, 1mM DTT, 0.4 mM $CaCl_2$, mixed micelles containing 10 μg/ml of phosphotidylserine, l|ig/ml of 1,2 diacylglycerol, 0.06% (vol/vol) Triton X-100 and in the presence or absence of staurosporine. The reaction was started by the addition of 10 μM ATP, [γ^{-32}P] ATP (2-4 μCi) (3000 Ci/mmol) followed by incubation at 30°C for 1 min. The reaction was stopped by Laemmli sample buffer (0.125 M Tris-HCl, pH 6.8), 2% (wt/vol) SDS, 10 % (vol/vol) glycerol, 2% (vol/vol) β-mercaptoethanol and 0.01% bromophenol blue) and the mixture was denatured at 100°C for 2 min. The proteins were separated by SDS-PAGE on 10% polyacrylamide gel as described by Laemmli (1970). The gels were silver stained, dried and exposed to X-ray film (Kodak) using intensifying screens (Kodak) at -70°C for 12 h. The exposed film was developed to detect the phosphorylated proteins.

Arachidonic acid dependent phosphorylation

The arachidonic acid dependent phosphorylation of endogenous proteins was performed exactly as described above, excepting that the calcium and phosphotidylserine/diacylglycerol in the reaction mixture was replaced with 100 μM arachidonic acid.

RESULTS

Protein Kinase C activity in control and ethanol treated rats

PKC activity was assayed for its involvement in the phosphorylation of endogenous substrate proteins of membrane and cytosol from cerebral cortex of control and ethanol fed rats. The PKC activity showed an increasing trend in the membrane and cytosolic samples during the development of rat cerebral cortex from 8 to 90 days of age. However, the membrane fraction retained relatively higher activity of PKC when compared to cytosolic fraction. Further, the PKC activity was found to be increased in ethanol treated rats, in membrane and cytosolic fractions (Fig. 2, 3). One striking difference in the activity of PKC was observed at the age of 8 days when compared to 30 and 90 days of age. The increase in PKC activity due to ethanol was more sharp and prominent at 8 days of development (in cytosol and membrane) than at 30 and 90 days of age.

*Protein Kinase C **isoforms** in control and ethanol treated rats*

Identification and characterisation of the involvement of specific PKC isoforms in control and ethanol fed animals was performed by immunoblotting with i so form specific antibodies (Fig.4). The PKC activity appears to be chiefly due to increased expression of PKC βI and βII isoforms as these isoforms are present abundantly in the cerebral cortex (Huang et al., 1987). The expression of cPKCs a, βI, βII, y, nPKCs 8, z, η and aPKCs ζ isoforms were detected in control and ethanol fed samples. All PKC isoforms in the control membrane samples showed an increased expression as a function of postnatal development. However, PKC - βI isoform was not detected in the cytosolic samples and other isoforms PKC - βII, y, 6, e and ζ showed increased expression with age. Membrane samples from ethanol treated rat cerebral cortex showed developmentally increased levels of βI and βII isoforms and decreased levels of PKC - S isoform over controls. In the cytosolic fraction, PKC a, y, e, η and ζ showed no changes, besides βI was not detected in control as well as in ethanol treated samples. Further, PKC - βII isoform in cytosolic fraction could not be noticed at the age of 8 and 30 days in control rats whereas its expression could be well observed in the progeny of ethanol fed rats. Calcium

independent PKC isoform 8, e and η could not be detected at 8 days in the cytosol of control and ethanol fed rats. The expression of 8 and e isoforms in membrane and cytosol was noticed at 30 and 90 days, of which, the isoform 8 showed slightly more expression in ethanol fed rats. PKC e and η could not be detected in samples from alcoholic rats excepting in very faint quantity in the immunoblots. Further, expression of atypical PKC ζ isoform was well noticed in control and experimental samples. At the age of 90 days its expression markedly increased in the cytosol of ethanol fed rats over control.

In general, the PKC isoforms βI, βII, y, 8, and ζ showed increased levels and appear to be involved during postnatal development of cerebral cortex. Prenatal and postnatal ethanol exposure increased membrane specific PKC isoforms βI, βII, while 8 isoform alone showed decreased levels (Fig. 5). In the cytosolic fraction, βII, 8 and ζ isoforms showed specific increase, while cytosolic a form showed a decrease at 90 days of age due to ethanol treatment (Fig. 6). Clearly, these studies show a selective increase of PKC βI and βII and decrease of 8 isoform. Though other nPKCs isoforms such as ε and η are also involved, their involvement appears to be nominal when compared basing on the sheer level of expression.

Effect of ethanol on Immunohistochemical localisation of Protein Kinase C

Changes in the expression and localisation of PKC in the cerebral cortex after prenatal ethanol exposure to rats was analysed by western blot and immunohistochemical methods. Immunoblot showed a single band at ~ 80 kDa position (Fig. 7). Different isoforms cannot be seen on the blot due to very narrow differences in their molecular weights. Western analysis showed an increased PKC immunoreactivity during postnatal development in controls as well as in ethanol exposed rat brain. The PKC levels gradually increased with age (8, 30 and 90 days) in control rats and the same pattern was observed in the ethanol treated rat brain. But in the ethanol treated rats, the expression of PKC was higher when compared to controls (Fig. 7). Changes in PKC expression were also analysed by immunohistochemical method. Figure eight show brain slices from representative control and prenatal ethanol treated rats. The level of PKC immunolocalisation was sharply increased during development in controls as well as in

ethanol treated animals. In cerebral cortex, immunoreactivity was found to be more in the cortical layers of ethanol treated rat brain particularly at the age of 90 days (Fig. 8).

Effect of ethanol on phosphorylation of endogenous substrate proteins

In vitro phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and ethanol exposed rat brain cerebral cortex was performed. The phosphorylation studies were performed using equal amount of protein (20μg) in the absence and presence of calcium. Further using specific stimulators and inhibitor for PKC, the involvement of specific substrate proteins during the course of postnatal development of brain under ethanol exposure was studied. Phosphorylated proteins were separated by SDS-PAGE (Fig. 9) and subjected to autoradiography. During such study several proteins have been noticed to show modification in the state of phosphorylation but we followed only prominent changes involved in selective proteins of CNS, ~~basing~~ on their relative abundance and their protein phosphorylation state.

Calcium independent phosphorylation of substrate proteins

Calcium independent phosphorylation was performed using EGTA as calcium chelator. Selective membrane and cytosolic proteins with molecular weight 87, 65, 60, 50, 43, 40, 36, 29 and 17 kDa showed altered protein phosphorylation during the postnatal development of brain (8, 30 and 90 days) in control as well as in alcohol fed rats (Fig. 10). The overall phosphorylation state of proteins under such condition was shown to be very high at the age of 8 days in alcohol fed rat brain cerebral cortex. The phosphorylation of substrate proteins from membrane fraction decreased during the postnatal development of brain from control and alcohol fed rat. Phosphorylation of substrate proteins from cytosolic fraction of alcoholic brain cerebral cortex did not show significant changes through out the development. In general, calcium independent phosphorylation of membrane samples both from control and alcoholic samples was less when compared to cytosolic fractions.

Calcium dependent phosphorylation of substrate proteins

Further to identify the calcium dependent phosphorylation of endogenous substrate proteins, the phosphorylation assay was performed in the presence of calcium, a second messenger in the signal transduction pathways which can stimulate calcium

dependent protein kinases (Fig. 11). Specific proteins with molecular weight 65, 50, 43, 40, 36, and 29 kDa showed hyperphosphorylation in the presence of calcium during the postnatal development of brain (8, 30 and 90 days) both in the control and alcohol fed rat cerebral cortex. The phosphorylation was shown to be high at the age of 8 days in alcoholic samples both in the membranes and cytosolic fractions. The 87 kDa protein did not show much phosphorylation in the presence of calcium and it is less when compared to calcium independent phosphorylation. The 65-kDa protein showed hyperphosphorylation in the alcohol treated samples in all the ages studied. However, in the cytosolic fraction, its phosphorylation decreased at the age of 30 days and again restored to normal stage at the age of 90 days. The other proteins 50, 43, 40, 36, and 29 kDa showed decreased phosphorylation after 8 days of brain development in the alcoholic samples of membrane as well as in cytosol. The 17 kDa protein did not show phosphorylation in the presence of calcium in alcohol treated membrane fraction at the age of 8 days where as it is well observed in the absence of calcium.

Calcium/phospholipid dependent phosphorylation of substrate proteins

The phosphorylation of endogenous substrate proteins of membrane and cytosol from the cerebral cortex of control and ethanol fed rats showed highly altered profiles of several proteins in the presence of Ca^{2+}/phospholipid (Fig. 12). Though many proteins showed modifications, analysis was restricted to proteins showing prominent changes in phosphorylation. In the presence of calcium and phospholipids, specific membrane and cytosolic proteins with 87, 65 60, 50, 43, 36, 29 and 17 kDa showed altered protein phosphorylation during postnatal development (8, 30 and 90 days) in control as well as in ethanol fed rats (Fig. 12). These proteins showed high phosphorylation selectively in the presence of calcium and PS/DAG, specific activators of PKC. However, in the presence of staurosporine, phosphorylation of these proteins decreased (Fig. 14), indicating the participation of these proteins as endogenous substrates for protein kinase C or/and serine/threonine dependent protein kinases, since staurosporine is not a selective inhibitor for PKC alone. In the membrane samples from control animals, these substrate proteins showed decreased phosphorylation during the development (Table I, II), where as in the progeny of prenatal ethanol fed rats, a different pattern of protein

phosphorylation was observed. Proteins with molecular weight 87 (Fig. 15), 65 (Fig. 16), 43 (Fig. 17) and 36 kDa (Fig. 18) showed increased phosphorylation in experimental membrane samples particularly at the age of 8 days where as the 17 kDa protein showed decreased phosphorylation in 8 day old experimental sample (Fig. 19). Cytosolic proteins with same molecular weight showed similar pattern of decreased phosphorylation in the control and experimental samples in all developmental stages studied. Proteins with molecular weight 87, 65, 60, 43 and 29 kDa showed increased phosphorylation at the age of 8 and 90 days whereas 50 and 36 kDa proteins showed increased phosphorylation only at the age of 8 days in the progeny of ethanol fed rats (Fig. 12). Irrespective of membrane or cytosol fractions, in the presence of staurosporine, the phosphorylation of all these substrate proteins decreased and the presence of PS/DAG increased their phosphorylation, which supports the dependency of these proteins on PKC mediated phosphorylation.

Arachidonic acid dependent phosphorylation of substrate proteins

In the presence of arachidonic acid, a specific stimulator of PKC βII i so form, protein phosphorylation was performed to identify the substrate proteins of membrane and cytosol fractions during development of ethanol exposed rat cerebral cortex (Fig. 13). During normal development of rat cerebral cortex the protein phosphorylation was not affected in the presence of arachidonic acid. However, in the ethanol exposed rat brain the phosphorylation status of the proteins increased drastically at the age of 8 days. Proteins with molecular weight 87, 65, 50, 43, 36, 29 and 17 kDa were highly phosphorylated both in the membrane and cytosolic fractions of ethanol fed rat cerebral cortex but in the later ages of development i.e. 30 and 90 days the phosphorylation of these proteins decreased. The 87-kDa protein was hyperphosphorylated at the age 90 days in cytosolic fraction of both control and ethanol fed rat cerebral cortex.

FIG. 2. Membrane associated PKC activity in control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. Membranes were prepared and assayed for PKC activity as described in Materials and Methods. Activity is expressed as picomoles of $PO4^{-3}$ incorporated into the exogenous histone^{-1} minute^{-1}microgram of protein. The data points are mean±SEM (bars) values of five separate experiments. $^{**}P<0.01$ as compared with the control.

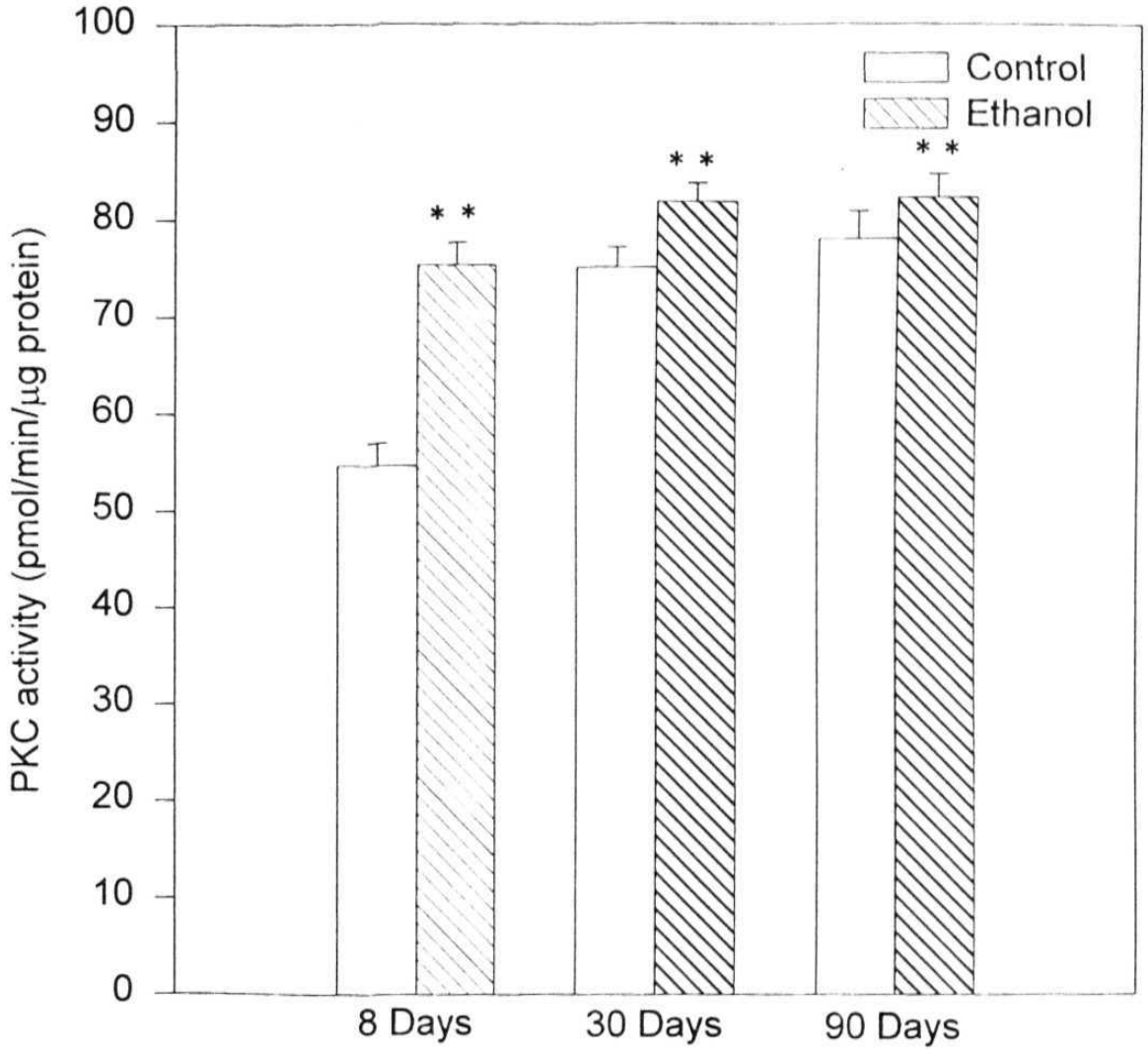


Fig. 2. Protein Kinase C activity in Membrane

FIG. 3. Cytosol associated PKC[1] activity in control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. Cytosol was prepared and assayed for PKC activity as described in Materials and Methods. Activity is expressed as picomoles of PO_4^- incorporated into the exogenous histone^{-1} minute" microgram of protein. The data points are mean±SEM (bars) values of five separate experiments. $P<0.05$ as compared with the control.

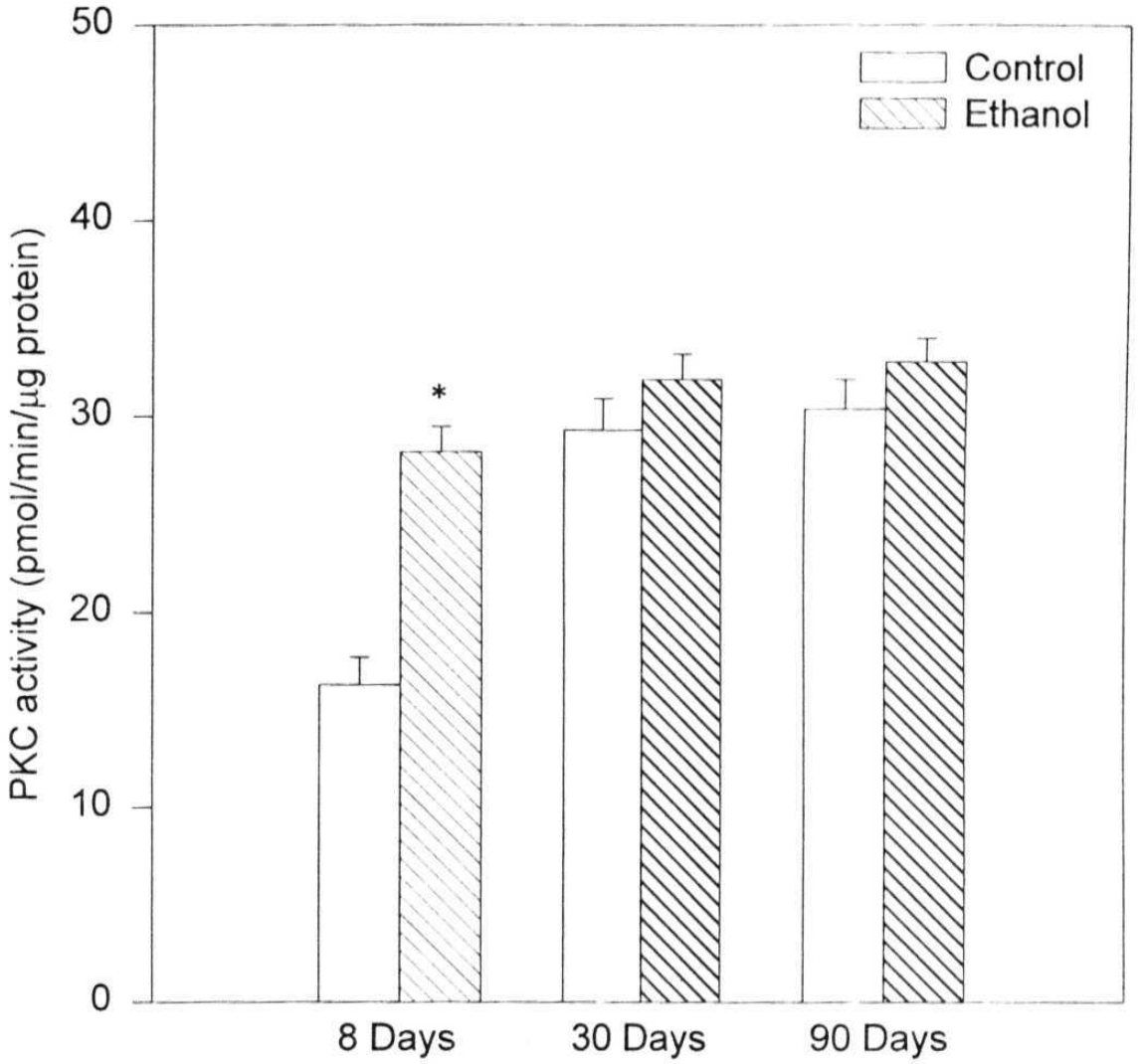


Fig. 3. Protein Kinase C activity in Cytosol

FIG. 4. Immunochemical analysis of PKC isoforms of membrane and cytosolic fractions from control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. Immunoblots were developed using isoform specific antibodies as described in Materials and Methods.

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC – Control cytosolic fraction; EC - Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

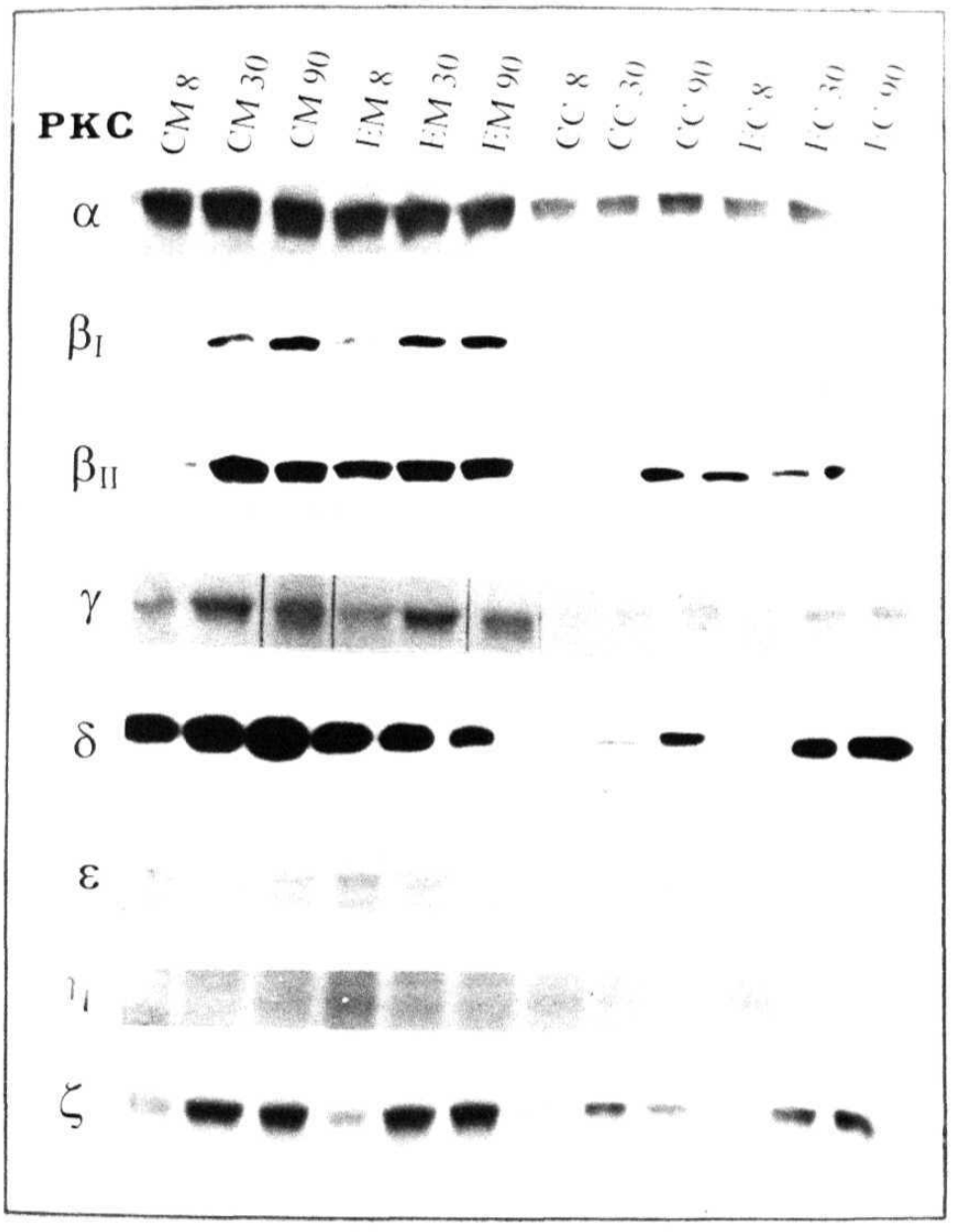

PKC
CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
FC 8
FC 30
FC 90
α
βI
βII
γ
δ
ε
η
ζ

Fig.4

FIG. 5. Changes in the immunoreactivities of membrane associated PKC isoforms from control (O) and ethanol treated (•) rat cerebral cortex at 8, 30 and 90 days of development. The immunoblots shown in fig. 3 were subjected to scanning densitometry and % changes were plotted as arbitrary units. Each data point is a mean ± SEM (bars) values of three separate experiments. *P<0.05

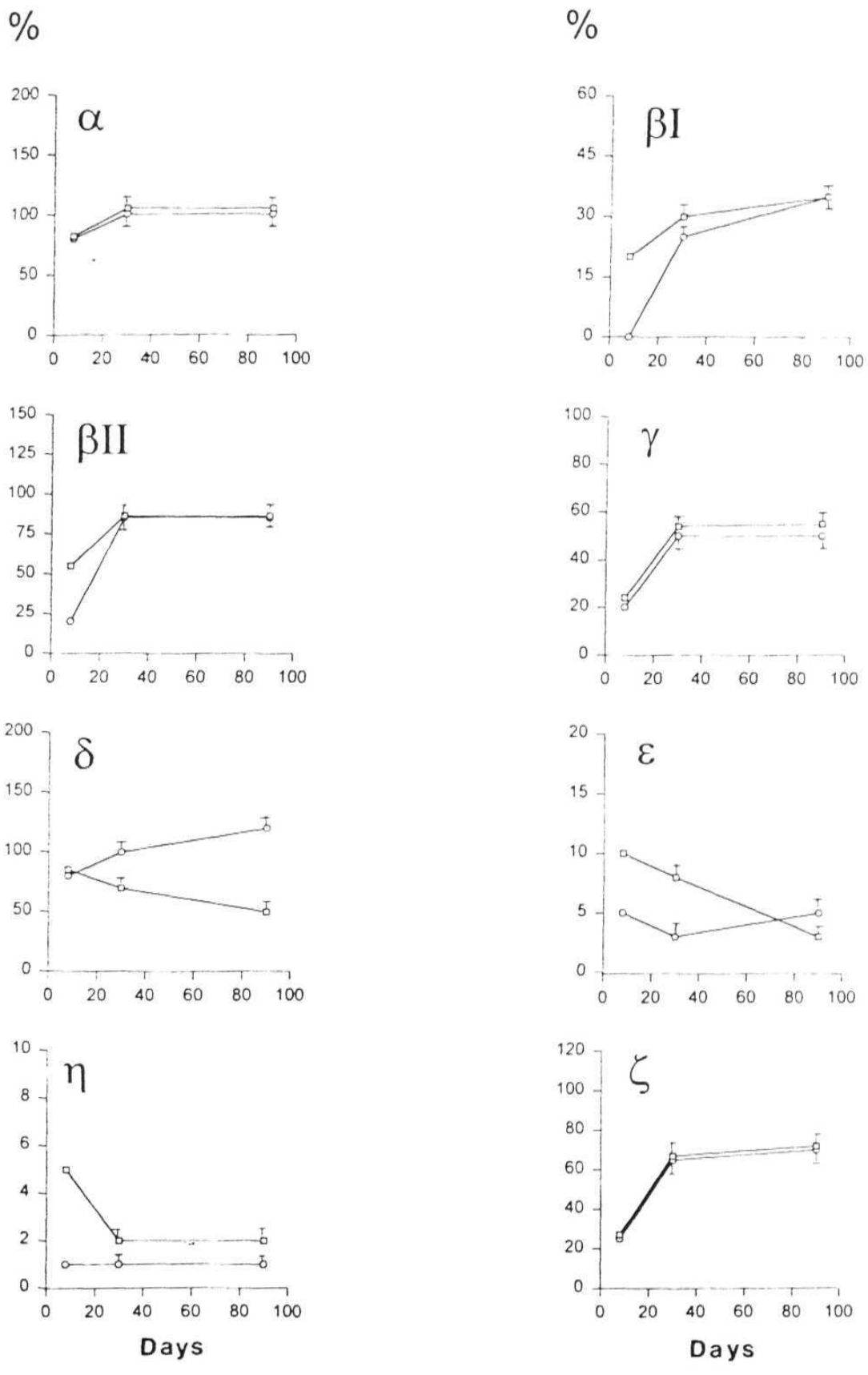


Fig.5.

FIG. 6. Changes in the immunoreactivities of cytosol associated PKC isoforms from control (O) and ethanol treated (Cl) rat cerebral cortex at 8, 30 and 90 days of development. The immunoblots shown in fig. 3 were subjected to scanning densitometry and % changes were plotted as arbitrary units. Each data point is a mean ± SEM (bars) values of three separate experiments. *P<0.05

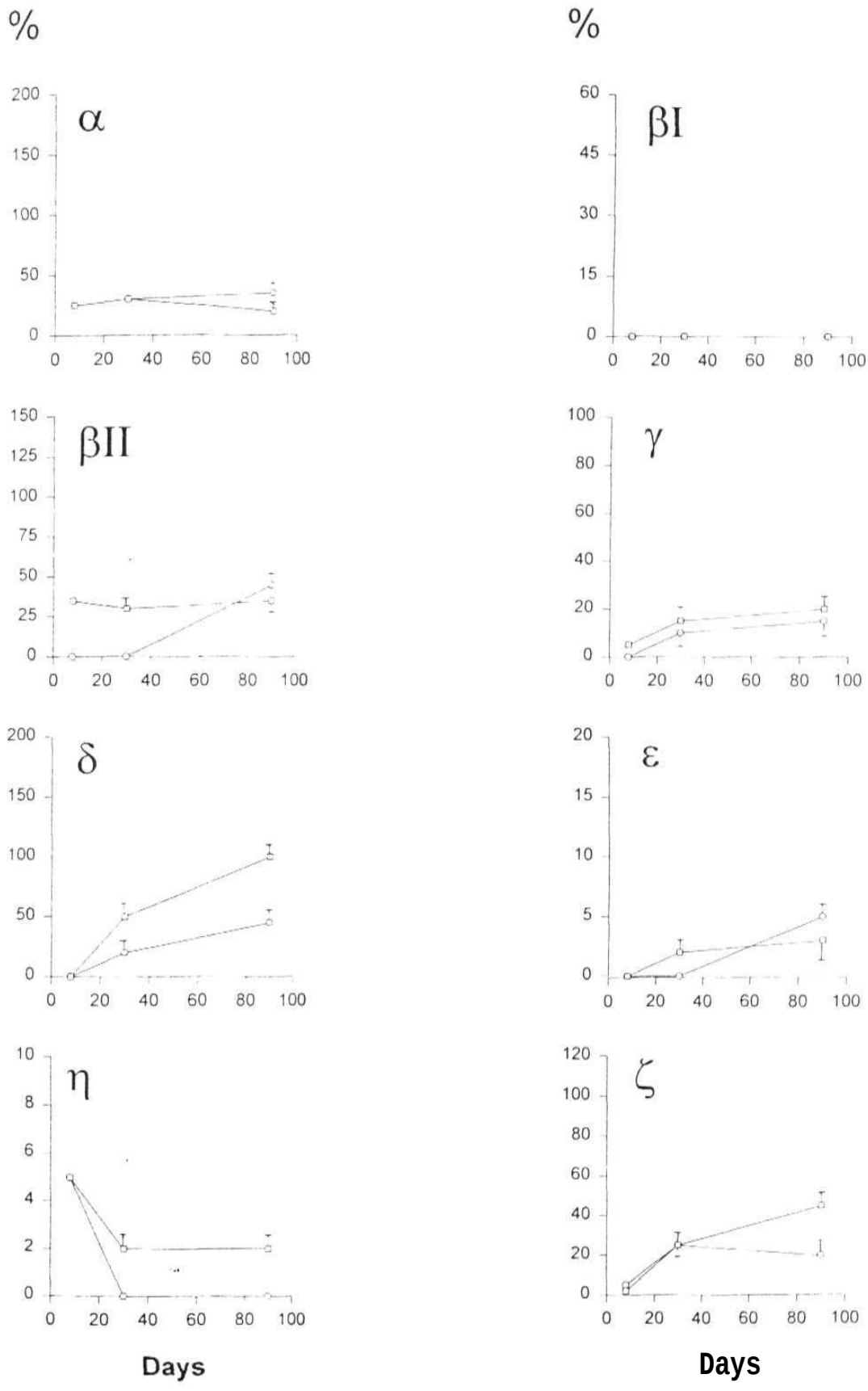

%
α
200
150
100
50
0
0 20 40 60 80 100
%
βI
60
45
30
15
0
0 20 40 60 80 100
βII
150
125
100
75
50
25
0
0 20 40 60 80 100
γ
100
80
60
40
20
0
0 20 40 60 80 100
δ
200
150
100
50
0
0 20 40 60 80 100
ε
20
15
10
5
0
0 20 40 60 80 100
η
10
8
6
4
2
0
0 20 40 60 80 100
Days
ζ
120
100
80
60
40
20
0
0 20 40 60 80 100
Days

Fig.6

FIG. 7. Immunochemical analysis of PKC in rat cerebral cortex from control and ethanol treated rat at 8, 30 and 90 days of development. Immunoblot was developed using polyclonal antibody against PKC which recognizes α, βI, βII, γ, 5 isoforms of PKC.

1. Control cerebral cortex 8 days
2. Control cerebral cortex 30 days
3. Control cerebral cortex 90 days
4. Ethanol treated cerebral cortex 8 days
5. Ethanol treated cerebral cortex 30 days
6. Ethanol treated cerebral cortex 90 days

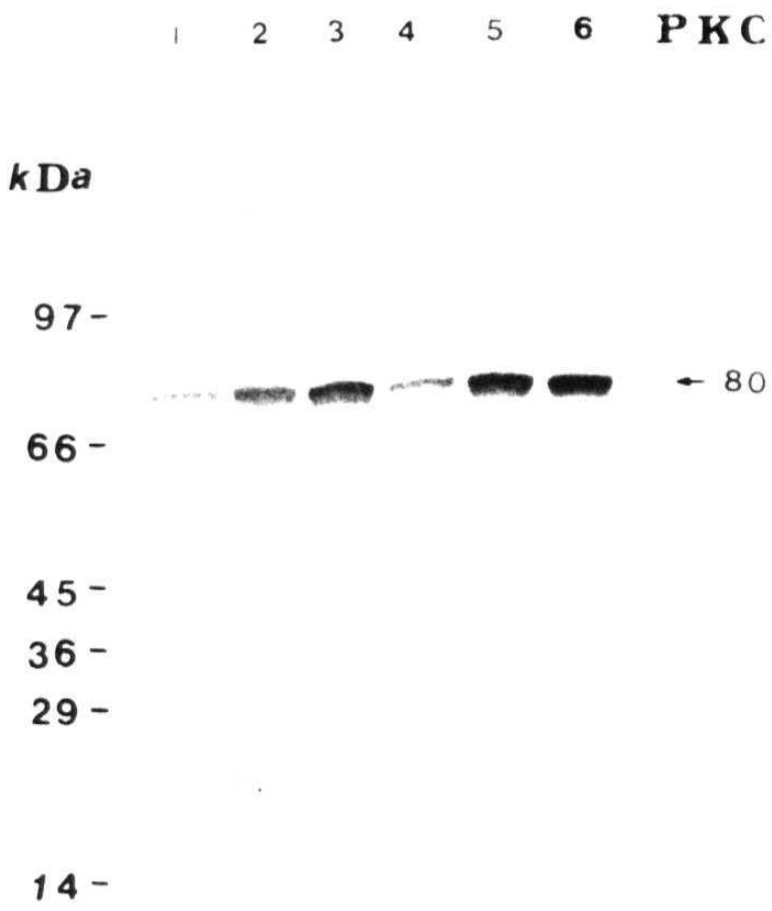
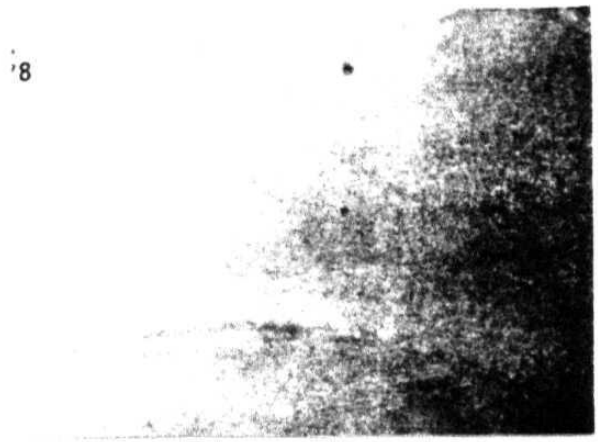


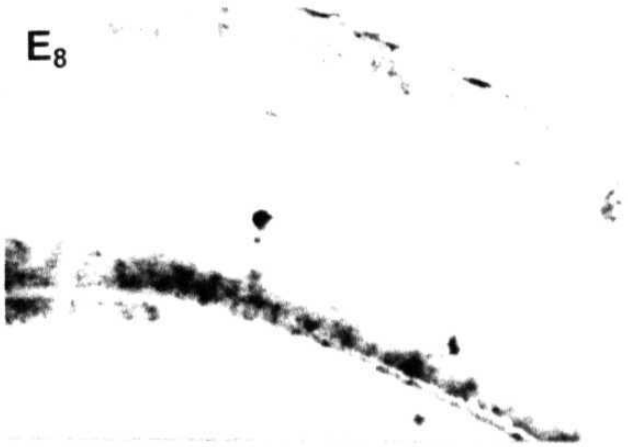
Fig. 7.

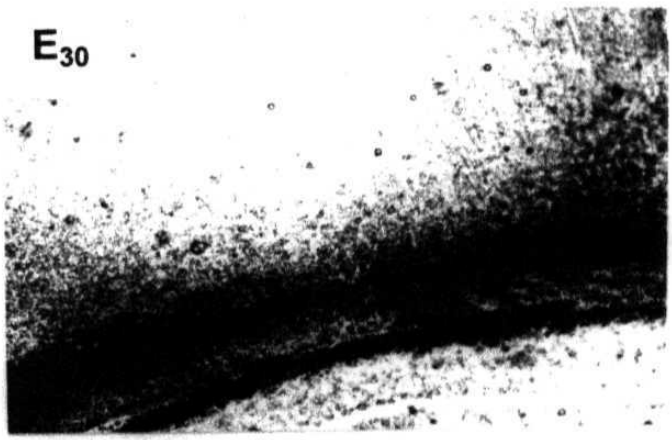
FIG. 8. PKC immunoreactivity in the rat cerebral cortex following exposure to ethanol. Coronol sections (20 μm) of rat brain were incubated with anti - PKC antibody. Water treated animals served as controls.

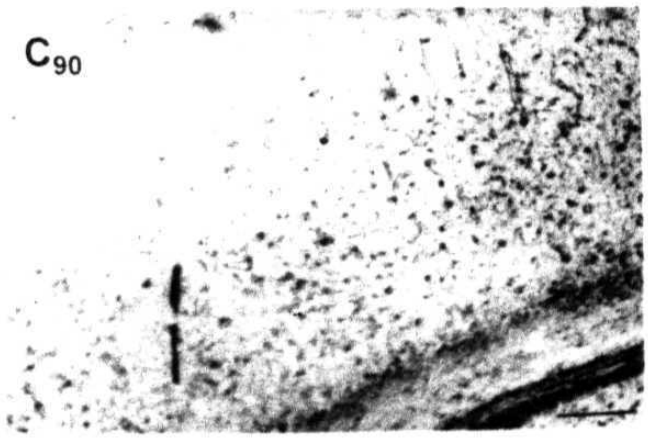
C_8, C_{30}, C90 immunoreactivity of PKC in control cerebral cortex seen at x 400 magnification.
E_8, E30, E_{90} increase in immunoreactivity of PKC in ethanol treated cerebral cortex seen at x 400 magnification.

C = Control
E = Ethanol treated









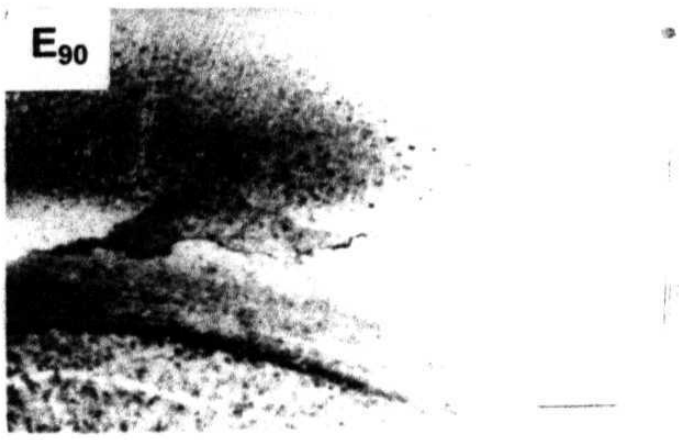


Fig.8

FIG. 9. SDS-PAGE profile of membrane and cytosolic proteins from control and ethanol treated rat brain.

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC = Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

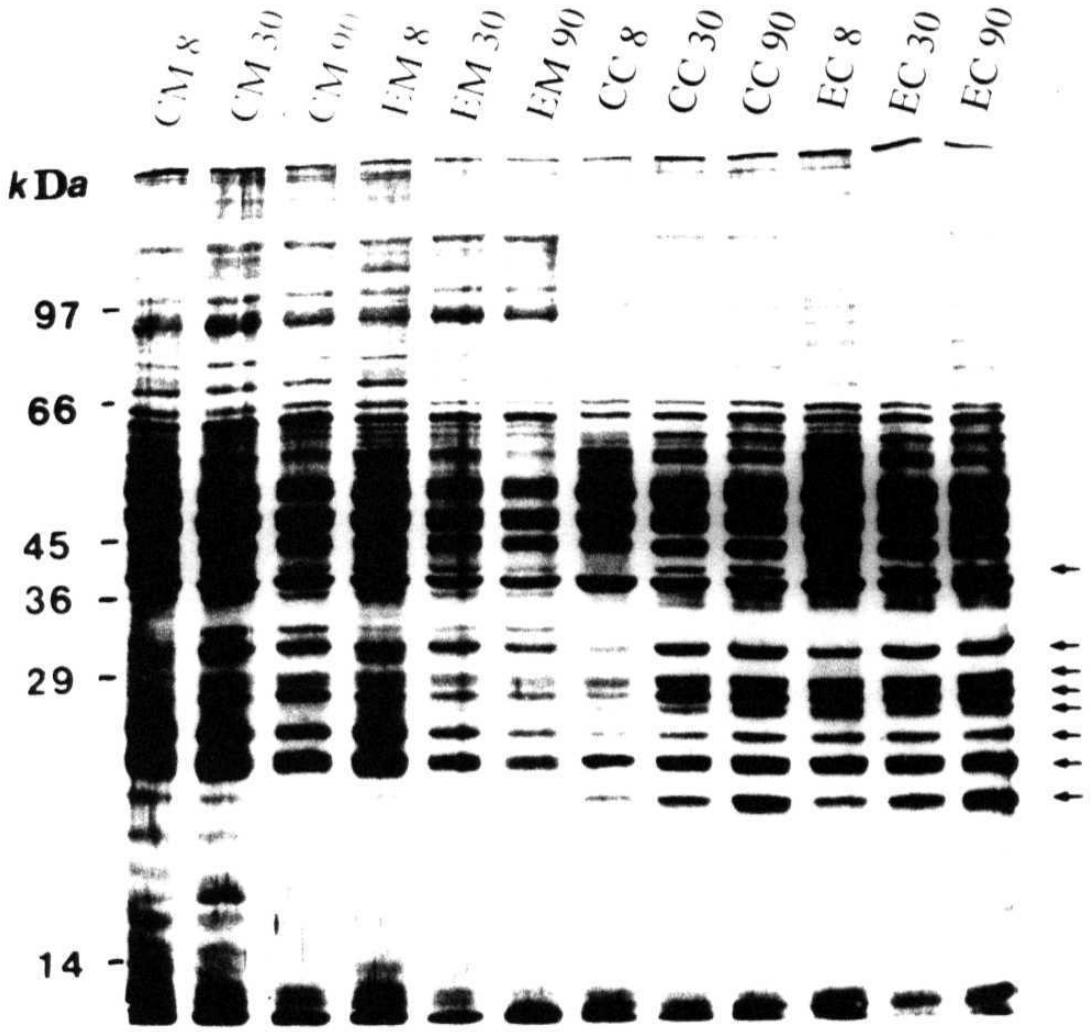

CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
EC 8
EC 30
EC 90
kDa
97
66
45
36
29
14

Fig. 9.

FIG. 10. In vitro phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. The in vitro phosphorylation assays were performed in the absence of Ca^{2+} as described in Materials and Methods. Samples were separated on 10% SDS-PAGE gels followed by autoradiography. The autoradiogram was obtained after exposure for 12 h at -70°C.

FIG. 11. In vitro phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. The in vitro phosphorylation assays were performed in the presence of Ca^{+} as described in Materials and Methods. Samples were separated on 10% SDS-PAGE gels followed by autoradiography. The autoradiogram was obtained after exposure for 12 h at -70°C.

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC = Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

Fig. 10.

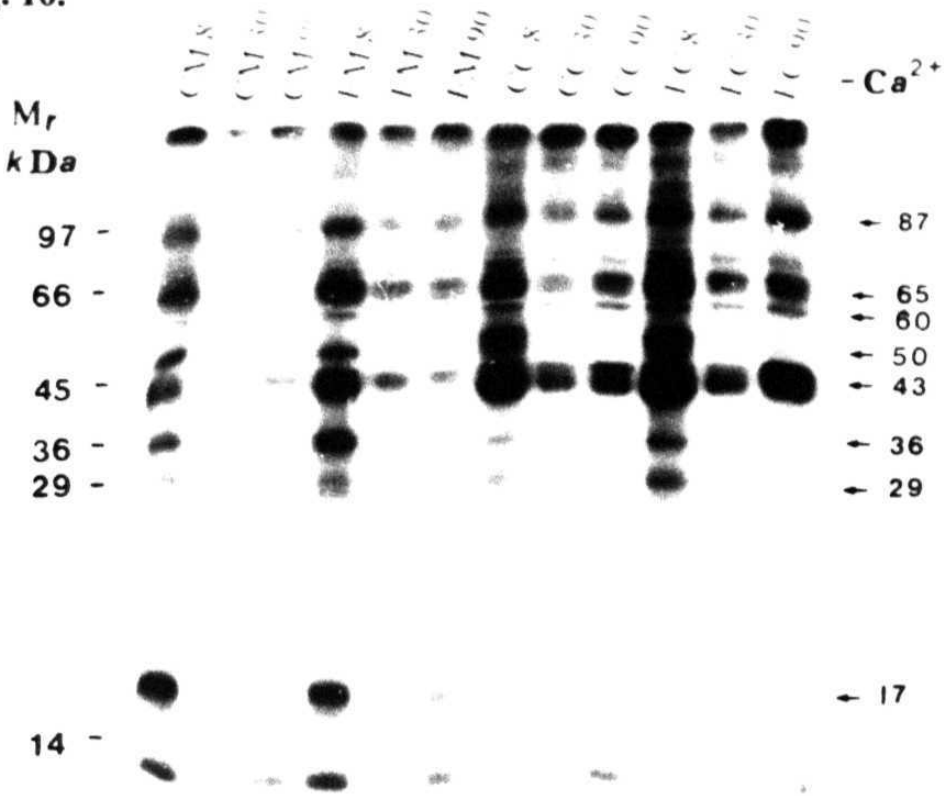

M_r
kDa
97
66
45
36
29
14
- Ca^{2+}
87
65
60
50
43
36
29
17

Fig. 11.

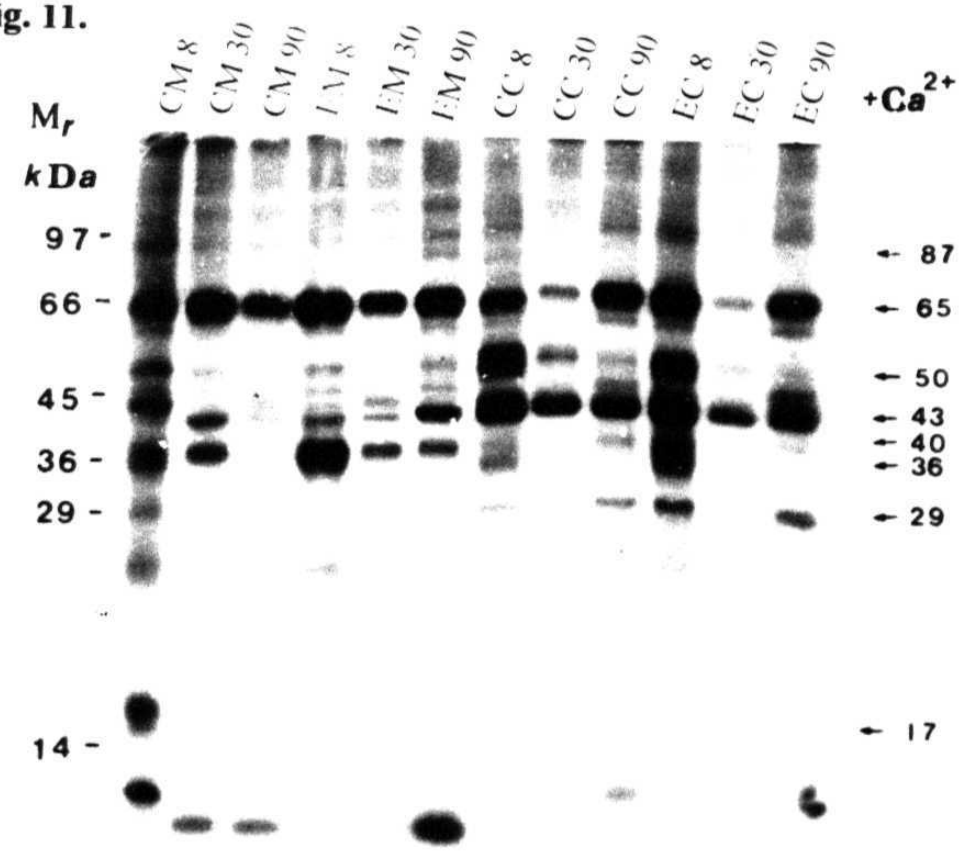

CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
EC 8
EC 30
EC 90
M_r
kDa
97
66
45
36
29
14
+ Ca^{2+}
87
65
50
43
40
36
29
17

FIG. 1.2. In vitro phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. The in vitro phosphorylation assays were performed in the presence of Ca^{+}/phosphotidylserine and diacylglycerol (PS+DAG) as described in Materials and Methods. Samples were separated on 10% SDS-PAGE gels followed by autoradiography. The autoradiogram was obtained after exposure for 12 h at - 70°C.

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC - Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

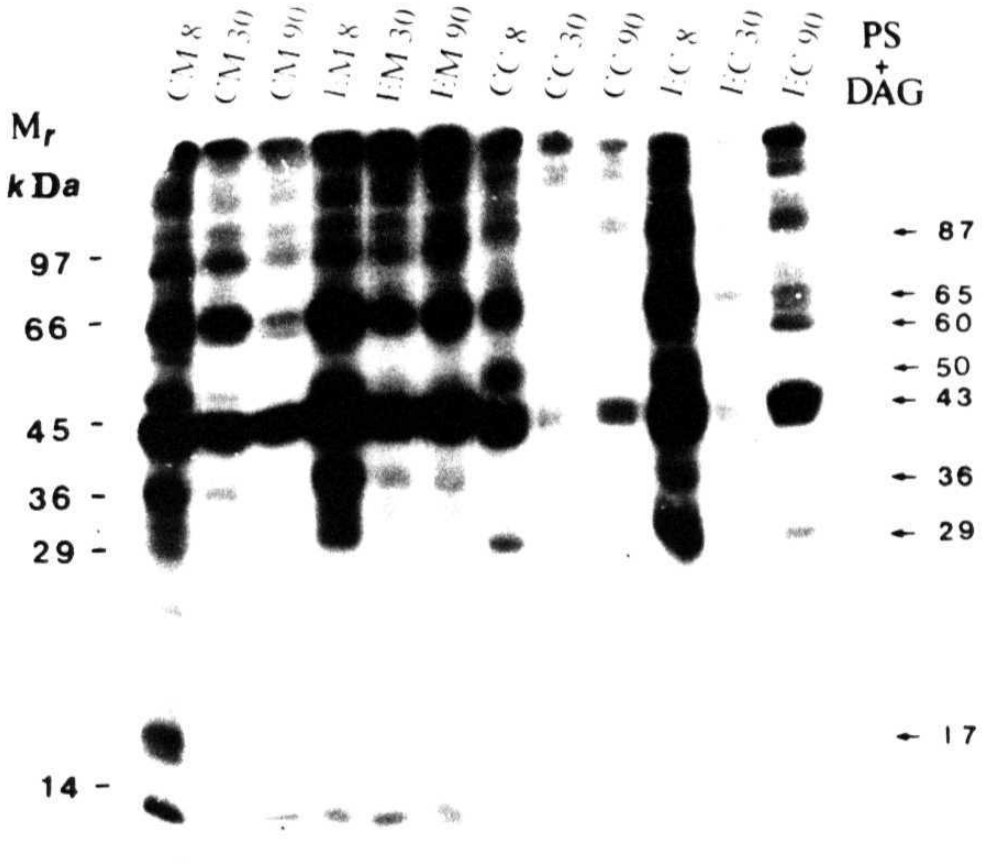

CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
EC 8
EC 30
EC 90
PS + DAG
Mr kDa
97
66
45
36
29
14
87
65
60
50
43
36
29
17

Fig. 12.

FIG. 13. In vitro phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. The in vitro phosphorylation assays were performed in the presence of Arachidonic acid as described in Materials and Methods. Samples were separated on 10% SDS-PAGE gels followed by autoradiography. The autoradiogram was obtained after exposure for 12 h at -70°C.

FIG. 14. In vitro phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. The in vitro phosphorylation assays were performed in the presence staurosporine (7 nM).

CM = Control membrane fraction; EM - Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC - Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

Fig. 13.

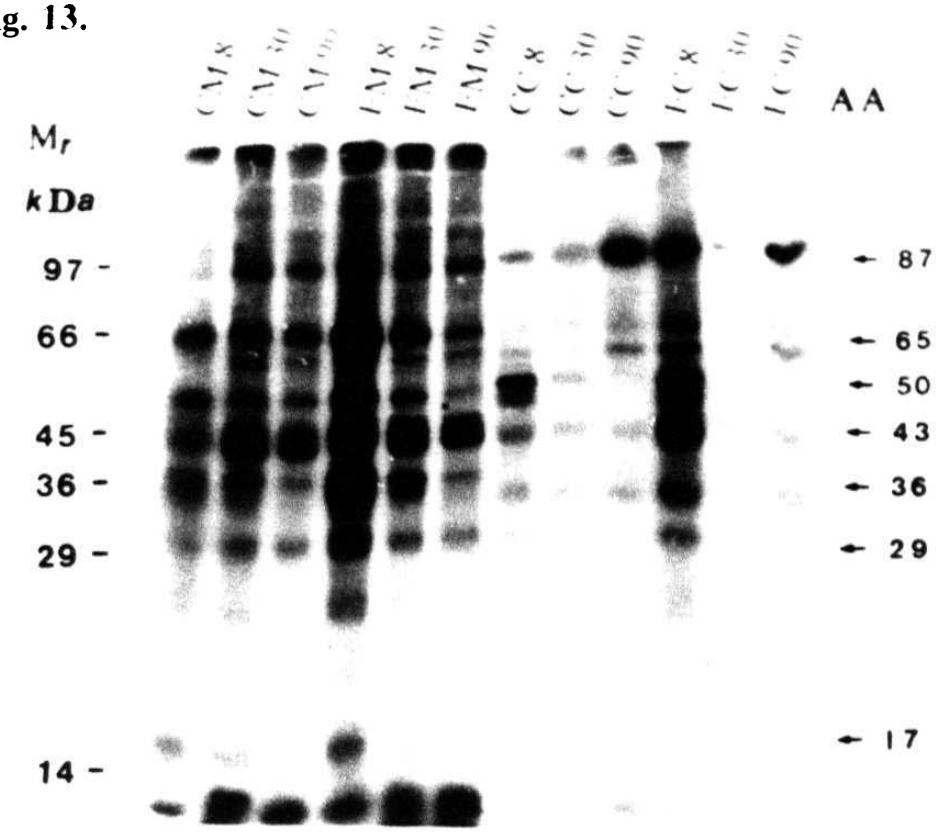

AA
Mr
kDa
97 -
66 -
45 -
36 -
29 -
14 -
87
65
50
43
36
29
17

Fig. 14.

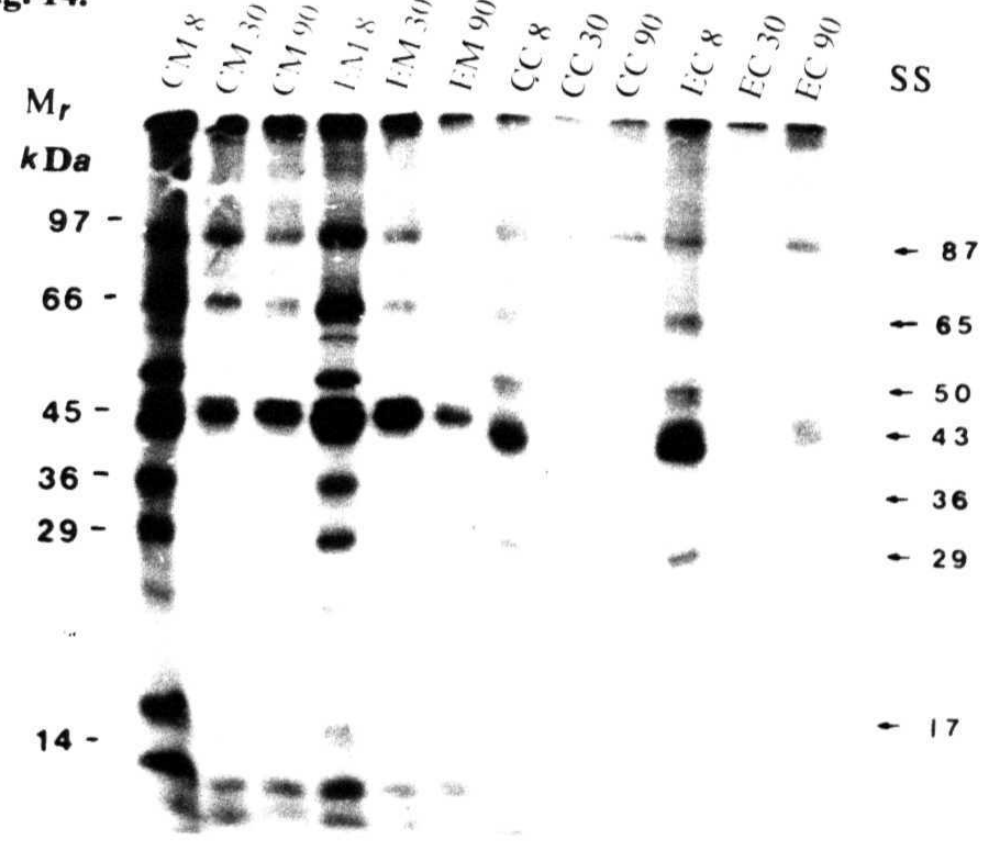

CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
EC 8
EC 30
EC 90
SS
Mr
kDa
97 -
66 -
45 -
36 -
29 -
14 -
87
65
50
43
36
29
17

FIG. 15. Phosphorylation of 87 kDa endogenous substrate protein in various conditions.

FIG. 16. Phosphorylation of 65 kDa endogenous substrate protein in various conditions.

$- Ca^{2+}$	=	Absence of Calcium
$+ Ca^{2+}$	=	Presence of Calcium
PS/DAG	=	Phosphotidylserine and diacylglycerol
AA	=	Arachidonic acid
SS	=	Staurosporine
CaM	=	Calmodulin
AIP	=	Autocamtide-2-related inhibitory peptide

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC = Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

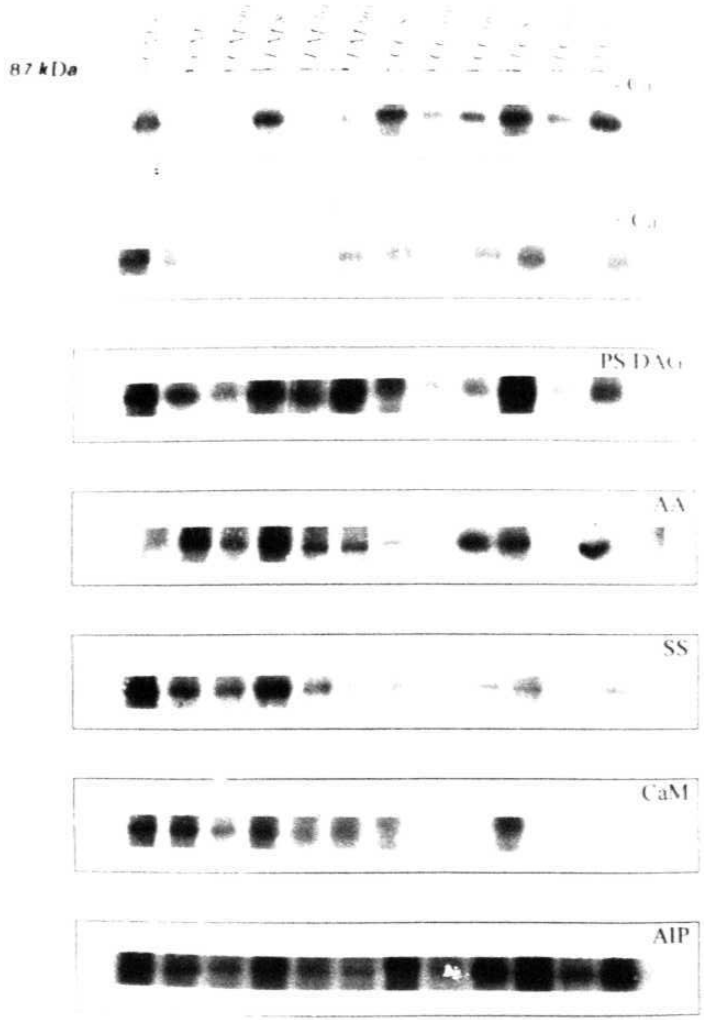


Fig. 15.

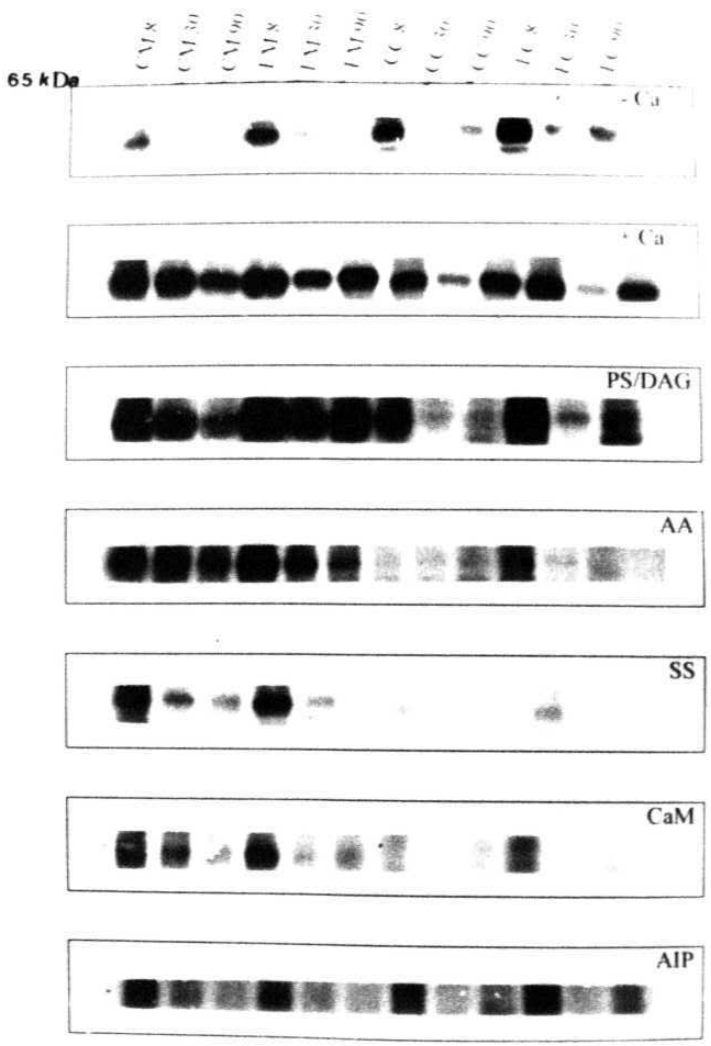


Fig. 16.

FIG. 17. Phosphorylation of 43 kDa endogenous substrate protein in various conditions.

FIG. **18.** Phosphorylation of 36 kDa endogenous substrate protein in various conditions.

- Ca	=	Absence of Calcium
+ Ca^{2+}	=	Presence of Calcium
PS/DAG	=	Phosphotidylserine and diacylglycerol
AA	=	Arachidonic acid
SS	=	Staurosporine
CaM	=	Calmodulin
AIP	=	Autocamtide-2-related inhibitory peptide

CM - Control membrane fraction; EM - Ethanol treated membrane fraction
CC - Control cytosolic fraction; EC = Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

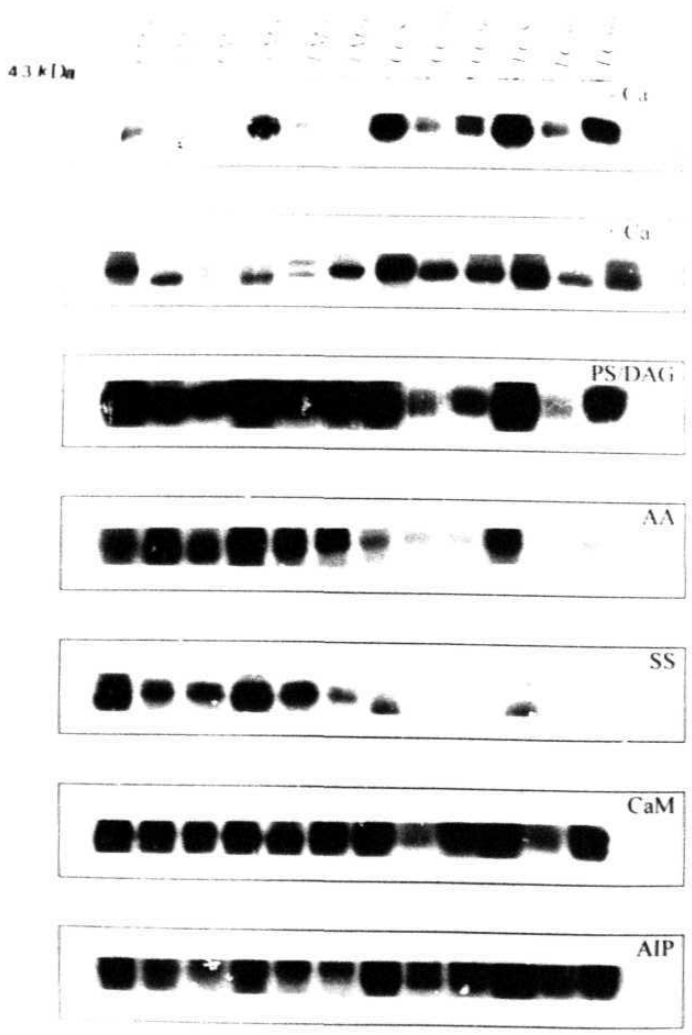


Fig. 17.

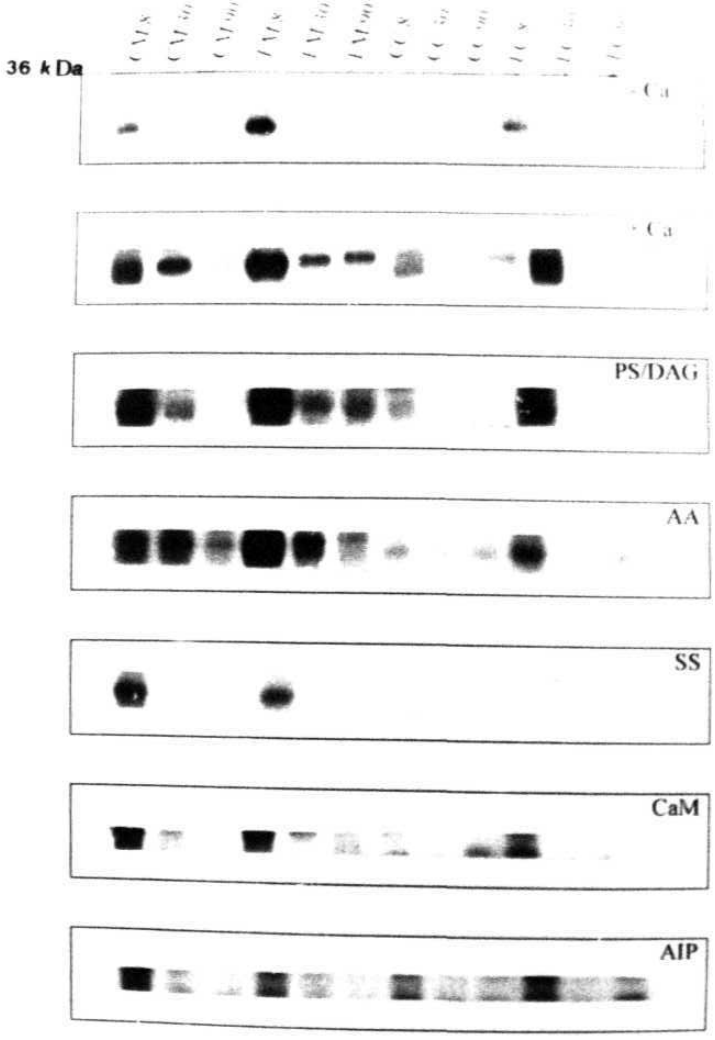


Fig. 18.

FIG. 19. Phosphorylation of 17 kDa endogenous substrate protein in various conditions.

- Ca	=	Absence of Calcium
+ Ca^{2+}	-	Presence of Calcium
PS/DAG	=	Phosphotidylserine and diacylglycerol
AA	=	Arachidonic acid
SS	=	Staurosporine
CaM	=	Calmodulin
AIP	=	Autocamtide-2-related inhibitory peptide

CM = Control membrane fraction; EM Ethanol treated membrane fraction
CC - Control cytosolic fraction; EC – Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

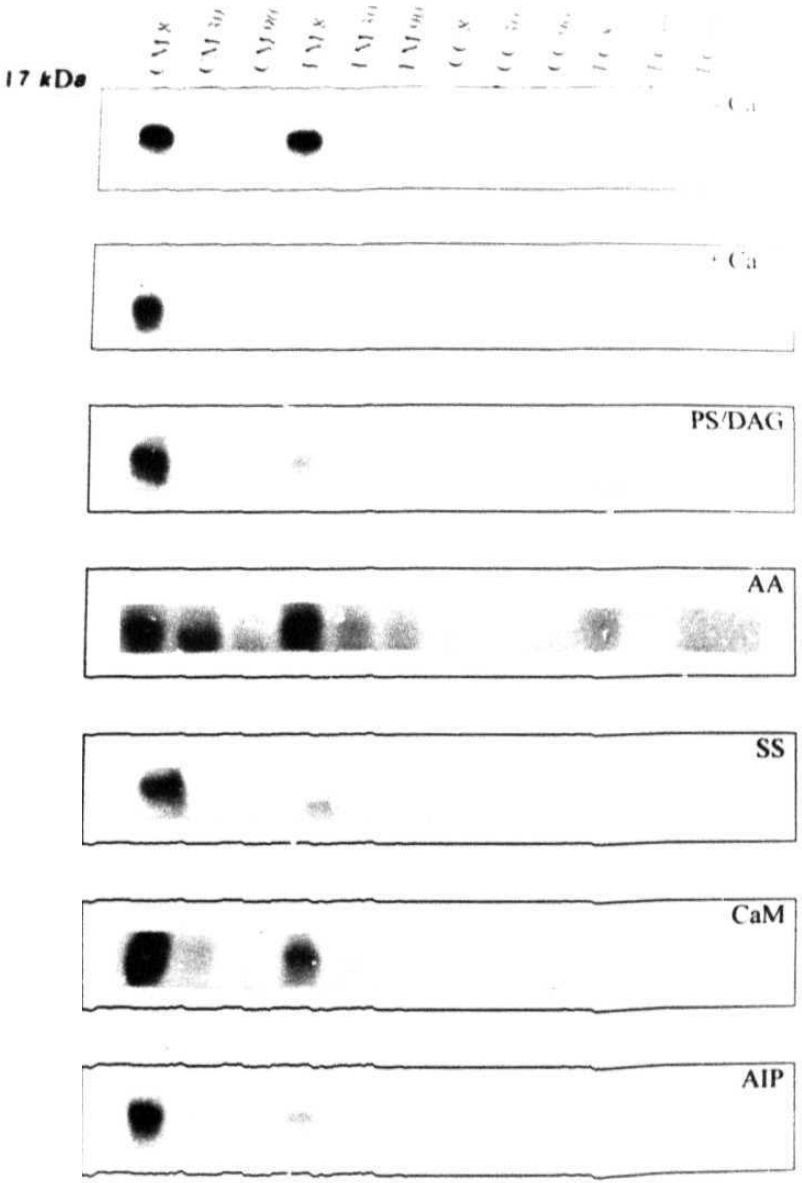


Fig. 19.

Table I. In vitro phosphorylation of endogenous substrate proteins of PKC from membrane fraction of control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development.

PS+DAG	CM 8	CM 30	CM 90	EM 8	EM 30	EM 90
87 kDa	+++	++	+	+++	+	+
65	++	+++	+	+++++	+++	+++
60	++	++	+	+	-	-
50	+++	+	-	+++	+	+
43	+++	+++	+++	+++++	+++	+++
36	++	+	-	++	+	+
29	+	-	-	++	-	-
17	+++	-	-	-	+	-

AA						
87 k Da	++	+	-	++	+	+
65	-	+	+	+++	++	+
50	++	+	+	+++	+	+
43	+	+	+	++-+-++	+	+
36	+	+	-	+++++	+	+
29	+	++		+++	+	+
17	++	+	-	+ +	-	-

Staurosporine						
87 kDa	++	+	+	+ + +	+	+
65	++	+	+	+ + +	+	-
60	+	-	-	+	-	-
50	+++	-	-	+ +	-	-
43	++	+	+	+ +		
36	+	-	-	+	-	-
29	++	-	-	++		-
17	+++	-	-	-	+	-

CM = Control Membrane fraction

EM = Ethanol treated Membrane fraction

Numbers indicate age in days

PS+DAG = Phosphotidylserine + Diacylglycerol
AA = Arachidonic acid

[+ indicates phosphorylated protein; more plus signs indicate still higher phosphorylation on a relative scale: - indicates absence of phosphorylation]

Proteins with specific M.Wt **are** shown in Figs: 12, 13 and 14

Table II. In vitro phosphorylation of endogenous substrate proteins of PKC from cytosolic fraction of control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development.

PS+DAG	CC 8	CC 30	CC 90	CE 8	CE 30	CE 90
87 kDa	+	+	+	++4	4	t 4
65	++	-	-	++++	+	+
60	-	-	+	+	-	4 4
50	+++	-	-	++++	-	-
43	++++	+	+4	++++++	4	+ + + 4
36	-	-	-	++	-	-
29	+	-	-	++ +	-	4
17	-	-	-	-	-	-

AA						
87 kDa	+	4	++	+++		+ + +
65	-	-	-	+	-	4
50	++	-	-	+++	-	-
43	+	+	+	+ ++++	-	-
36	++	4	+	+ t	-	
29	+	+	+	+ t	-	-
17	-	-	-	-	-	-

Staurosporine						
87 kDa	+	-	+		4	+
65	+	-	-	+	-	-
60	-	-	-	-	-	-
50	+	-	-	+	-	-
43	++	-	-	+ +	4	+
36	-	-	-	4	-	-
29	+	-	-	4	-	4
17	-	-	-	-	-	-

CC = Control Cytosolic fraction

EC = Ethanol treated Cytosolic fraction

Numbers indicate age in days

PS+DAG = Phosphotidylserine + Diacylglycerol
AA = Arachidonic acid

[+ indicates phosphorylated protein; more plus signs indicate still higher phosphorylation on a relative scale: - indicates absence of phosphorylation]

Proteins with specific M.Wt are shown in Figs: 12, 13 and 14

DISCUSSION

The main goal of this study was to determine the PKC isoforms involved and discern their role in the development of cerebral cortex during prenatal ethanol exposure. The overall PKC activity, both membrane and cytosol associated showed an increasing trend as a function of development. Present data on developmental changes in PKC is consistent with those reported in the literature (Hashimoto et al., 1988; Yoshida et al., 1988; Sposi et al., 1989; Hirata et al., 1992). Ethanol treatment induced an increase in the PKC activity in all the developmental stages. However, the extent of increase was high in early age groups when compared to the later age groups. This marked increase at a young age suggests that the early age of development could be a unique focus for the role of PKC in ethanol induced developmental defects. Further, most of the enzyme activity was present in the membrane fraction while cytosol fraction showed a restricted distribution.

Since multiple PKC isoforms have been suggested to be present in cerebral cortex, we investigated the isoform specific distribution of PKC in cerebral cortex and variation in their levels due to pre- and postnatal ethanol treatment. The PKC isoforms exhibited differential distribution in cytosolic and membrane fraction. The PKC isoforms βI, βII showed a preferential association with membrane and activated during ethanol treatment. Conversely, the PKC 5 isoform showed a decreased level in the membrane fraction and declined gradually as a function of age. In general, the PKC isoforms appear to be associated with membrane fraction irrespective of the signal due to ethanol treatment. The only deviation noticed in the study includes the association of ft isoform, which appears to be translocated from membranes to cytosol at the later age groups namely 30 and 90 days. The data indicates a clear correlation between the increased PKC activity and a parallel increase in the levels of specific PKC isoforms in the development of ethanol induced changes in the cerebral cortex at a very early age. The increase in specific PKC iso'forms such as βI and βII in the early age groups indicated a possibility of a crucial role that they might be playing at this developmental stage. It has been shown that phorbol esters activate several PKC isoforms (Kizaki et al., 1989; Mailhos et al., 1994; Radford, 1994) and the expression of PKC βI and βII has been correlated with the

susceptibility of the cells to stress-activated apoptosis (Knox et al., 1993; MacFarlane and Manzel, 1994; Pongracz et al., 1994; Emoto et al., 1995; Lu et al., 1997). This view can be further strengthened by the logical fate of decreased levels of PKC δ isoform, since the 6 isoform has been noticed to decline. Proteolytic degradation of PKC 6 was shown (Villa et al., 1997) in cells undergoing stress-activated apoptosis mediated by I CH like proteases termed caspases (Porter et al., 1997). It is likely that the cellular responses might really involve an interplay among these specific PKC isoforms and a clear cut cross-talk between them might drive the cell towards dysfunctional apoptosis. Since apoptosis is a hallmark for decrease in cell number, this could be the possible mechanism underlying the microencephaly during fetal alcohol syndrome.

In an earlier study using neuronal cell line PC 12, Messing et al., (1991) have shown that chronic exposure to ethanol increases PKC 6 and e isoforms where a selective over expression of PKC ε was found responsible for promoting ethanol induced neurite out growth. This data however can not be comparable with the results obtained in this study due to a variety of variations such as in vitro and in vivo, the mode of ethanol treatment, the dose and duration. To date there is no complete analysis of all PKC isoforms in the cerebral cortex as a function of ethanol treatment during pre and postnatal development. Thus, we believe that the present study is a first report of its kind in the analysis of PKC isoforms under in vivo conditions of pre and postnatal ethanol exposure.

The PKC ζ isoform has been implicated in the maintenance of long term potentiation (Sacktor et al., 1993). Though an increased level of PKC ζ in the membranes as a function of development has been noticed in this study, ethanol seems to have exerting no influence on membrane PKC ζ isoform. On the contrary, a moderate increase of PKC ζ has been noticed in the cytosolic fraction by ethanol. Considering the differences in the levels of membrane and cytosolic PKC ζ isoform, the change in cytosolic PKC ζ appears to be negligible and might not involve ethanol mediated translocation.

Immonohistochemical studies using polyclonal antibody to PKC (mostly recognizing a, βI, βII, γ **and** 5) in cerebral cortex showed developmental expression of PKC immunoreactivity (Fig.8). The PKC expression noticed in our study agrees with the

previous reports (Hashimoto 1988; Yoshida 1988; Huang 1990; Sposi 1989; Hirata 1992). In cerebral cortex the immunoreactivity was found to be more in the cortical layers of ethanol treated rat brain particularly at the age of 90 days. The increased expression of PKC may affect the neurotransmission and signal processing in specific neurons as these neurons participate in the release of neurotransmitter. A role for each individual PKC isozyme was suggested by findings that the neurotransmitter is contained in neurons immunoreactive for each isozyme. PKC a, (31 modulate the release of acetylcholine, dopamine and GABA neurotransmitters from striatal slices (Tanaka et al., 1986; Cubedelu et al., 1989; Chandler and Leslie, 1989). The PKC-βII is involved in the functions related to GABAergic neurons; while neurotransmitter of PKC-γ immunoreative neurons are yet to be identified. Long term administration of ethanol to rats causes reduction in choline acetyltrransferase and a slight loss of neurons in the nucleus basalis (Arendt et a., 1988). Furthermore, ethanol is also involved in the disruption of serotonergic and adrenergic pathways in amnestic alcoholis (Charness et al., 1989). All these possibilites indicate a direct role for each of the isozyme and the data from our study, eventhough is derived using polyclonal antibody also portends such a situation. Further studies using specific monoclonals for each of the isozyme are required to confirm this possibility.

To evaluate further, the role of PKC isoforms, we attempted to identify the substrate proteins which are phosphorylated by PKC, since several lines of evidence suggested increased intracellular Ca^{2+} in ethanol treated brain cell types (Dancill et al., 1987; Rabe and Weight, 1988; Davidson et al., 1988). The major consequence of Ca^{2+} influx is activation of several protein kinases involved in the regulation of development during pre and postnatal ethanol exposure. In this study in the presence of PKC stimulators such as Ca^{2+}/phospholipids and arachidonic acid, specific proteins of molecular mass 87, 65, 60, 50, 43, 36, 29 and 17 kDa were phosphorylated in membrane and cytosolic fractions (Fig. 12, 13). In the presence of staurosporine, an inhibitor of PKC! enzyme, phosphorylation of these proteins decreased (Fig. 14), suggesting PKC mediated phosphorylation of these substrates (Table I). Proteins in the membranes and cytosol with molecular mass 87, 65, 50, 43 and 36 kDa showed a decreased trend of phosphorylation

with age and, in contrast, there was an increase in the ethanol fed animals. Considering their molecular mass, phospholipid dependent phosphorylation, their presence in CNS and their inhibitory response in the presence of staurosporine, it is likely that the 87 kDa protein could be myristoylated alanine rich c-kinase substrate (MARCKS) protein (Albert et al., 1987; Erusalimsky et al., 1991), a major PKC substrate involved in the temporal development and differentiation of oligodendrocyte precursor cells (Deloulme et al., 1992). The 65 kDa protein in this study could be synaptophysin, considering its preferential association with the membranes (Gomez-Peurtas et al., 1994) besides its apparent molecular weight of 65 kDa in SDS-PAGE gels. The synaptophysin has been shown to be a possible substrate for PKC and undergoes a transient dephosphorylation following depolarisation of synaptosomcs in low Ca^{2+} influx conditions (Gomez-Peurtas et al., 1991). In our study, besides its association with the membranes, it showed a staurosporine induced decrease in phosphorylation indicating it as a substrate for PKC possibly playing a regulatory role in neuronal function during ethanol exposure. In this study, we also noticed PKC dependent phosphorylation of two candidate substrates with apparent molecular weights of 50 and 43 kDa. We presume, this could be a neuronal specific, calmodulin binding phosphoprotein B-50 (also referred as neuromodulin, P-57, GAP-43, F1) (Benowitz and Routtenberg, 1997; Cammarota et al., 1997; Heemskerk et al., 1991; Houbre et al., 1991; Baudier et al., 1989). These have been implicated as substrates for Ca^{2+}/phospholipid dependent PKC which is involved in the neuronal modulation by many transmitter systems. Considering their molecular weight and PKC dependency it is conceivable that these substrates belong to GAP-43 and the phosphorylation of these proteins has been shown to be linked with nerve terminal sprouting and long term potentiation (Linden and Routtenberg, 1989). Further two phosphoproteins with molecular weight 36 and 29 kDa showed PKC dependent phosphorylation. However, we could not trace their identity with comparable candidate proteins existing in the nervous system that might be playing a role in cerebral cortex-development and in ethanol exposure. Conversely, another protein with a molecular weight of 17 kDa was shown to be highly phosphorylated in the control membrane samples at 8 days of age which on ethanol exposure fails to get phosphorylated. This

protein appears to be neurogranin which is actually a calmodulin binding phosphoprotcin at the postsynaptic membrane (Represa et al., 1990; Watson et al., 1990; Baudier et al., 1991). Though studies have shown neurogranin exclusively in rat forebrain, the cellular role of this protein and its relation to LTP has not been yet clearly established (Represa et al., 1990; Klann et al., 1992). Though this study shows phosphorylation of specific substrate proteins mediated by PKC, their response to ethanol remains to be investigated by way of individual characterisation and further analysis by western blots using antibody specific probes for the possible substrate candidates.

Taken together, this study indicates a possibility that the pre-and postnatal ethanol exposure leads to PKC activation in general at all developmental stages, although the activation is more profound in young age. This study shows that the ethanol induced PKC activation is associated with more than one type of PKC isoform, as PKC -βI, -βII to a major extent and PKC - ε and -η to lesser extent seem to be involved. The data also suggests the degradation or decreased levels of PKC 6 following ethanol treatment, which has an apparent role in dysfunctional apoptosis. Finally, this study also qualitatively analyses the possible putative protein candidates serving as substrates for PKC in cerebral cortex under ethanol exposure. However, it still remains to be investigated which one of these substrates is selectively acted up on by specific PKC isoforms. Further investigation of a relationship among specific substrates and the interplay by PKC isoforms in neuronal regulation might give an insight into the PKC mediated molecular mechanisms involved in ethanol induced CNS dysfunction and FAS.

Chapter—II

Chapter - II

Effect of Ethanol on CaM Kinase II

Calcium/calmodulin (CaM) dependent protein kinase II (CaM kinase II) is a multifunctional serine/threonine protein kinase and is one of the most abundant protein kinases in mammalian brain (Colbran et al., 1989; Rennet, 1983; Goldstein, 1983; Kennedy and Greengard, 1981; Yamauchi and Fujisawa, 1983). It phosphorylates a variety of substrate proteins including synapsin I (Bennet et al., 1983; McGuiness et al., 1985), glycogen synthase (Ahmad et al., 1982; Payne et al., 1983; Woodgett et al., 1983), microtuble-associated protein-2 (Schulman, 1984), tyrosine hydroxylase (Yamauchi and Fujisawa, 1981; Vulliet, 1984) and smooth muscle myosine light chain (Fukunaga et al., 1989). It plays an important role in CNS functions, such as synthesis and secretion of neurotransmitters (Greengard et al., 1993), regulation of receptors and ionic channels (Kitamura et al., 1993; McGlade-McCulloh et al., 1993), structural modifications of cytoskeletal proteins, microtubule assembly/disassembly, axonal transport, selective gene expression, long term potentiation and most notably in specific forms of learning and memory in mice (Yamauchi and Fujisawa, 1983; Colbran et al., 1989; Nairn et al., 1985; Soderling, 1990; Morgan and Curran, 1988; Sheng and Greenberg, 1991; Bading et al., 1993; Bach et al., 1995). It is enriched in neurons and constitutes 1-2% of the total protein content of the hippocampus and 40% of the postsynaptic density (PSD) proteins (Kennedy and Greengard, 1981; Kennedy, 1993).

Ethanol exposure has been shown to affect neuronal activity due to derangement in the neurotransmitter function, ion channel conductivity, RNA metabolism, protein synthesis and signal transduction (see review Diamond and Gordon, 1997; Haviryaji and Vemuri, 1997; Mahadev and Vemuri, 1998). Most of these activities are regulated by protein phosphorylation and dephosphorylation events. Further, a rise in intracellular Ca^{+} (Daniell et al., 1987) was observed after ethanol treatment which results in the alteration of various Ca^{2+} mediated reactions primarily PKC and CaM kinase II activities. Changes in endogenous protein phosphorylation, PKC activity and its isoforms βI, pH and 8 levels due to pre and postnatal ethanol exposure to rat brain have already been described (Mahadev and Vemuri, 1998). In this part, we examine the role of calcium and calmodulin dependent protein kinase II in pre- and postnatal ethanol exposed rat brain. The involvement of

CaM kinase II in brain development, neuronal connectivity, maturation and in several forms of learning and memory including its role in LTP has been demonstrated (Melenka et al., 1989; Malinow et al., 1989). These functions are also affected to considerable degree in ethanol treatment model of FAS and chronic alcoholics (Samson and Diaz, 1996). Abundant presence of CaM kinase II, its substrates, their involvement in brain development, LTP formation and its possible impact in chronic ethanol challenge, prompted us to investigate the activity of CaM kinase II, identify the endogenous substrate proteins involved in pre- and postnatal ethanol induced changes in the cerebral cortex.

MATERIALS AND METHODS

Materials

Phenylmethylsulfonyl fluoride (PMSF), Dithiothreitol (DTT), reagents for sodium dodecyl Sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE), 5-bromo-4-chloro-3-indoylphosphate / nitroblue tetrazolium chloride (BCIP/NBT) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [y $^{-32}$P] ATP (3000 Ci/mmol) was from Board of Radiation and Isotope Technology (Mumbai, India). Phosphocellulose filter (P81) was obtained from Whatman (Canlab Corp., Mississauga, ON, Canada). Nitrocellulose sheets were from Schleicher and Schuell (Keene, NH, U.S.A.). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Monoclonal antibody against CaM Kinase II a subunit was purchased Boehringer Mannheim, Germany.

Assay of CaM kinase II Activity

CaM kinase II activity was measured in the membrane and cytosolic fractions of rat brain cerebral cortex from control and pre- and postnatal alcohol fed animals using syntide-2 as substrate (Fukunaga et al., 1989). The assay was performed in 50 µl reaction mixture containing 50 mM FIEPES (pH 7.5), 10mM magnesium acetate, 1mM DTT, 0.4 mM $CaCl_2$, 2 u,M calmodulin and 40µM syntide-2. The reaction was started by the addition of 50 µM ATP [γ^{-32}P] ATP (1-2 µCi) (3000 Ci/mmol) followed by incubation at 30°C for 5 min. The reaction was stopped by adding 10 µl of 0.4 M EDTA and were immediately spotted on 2 x 2 cm discs of Whatmann P-81 filter paper, washed three times with 75 mM phosphoric acid and air dried. The filters were placed in 5.0 ml of scintillation fluid and ^{32}Pincorporation was measured in a scintillation counter (Beckman). CaM kinase II 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 syntide-2 and expressed in pmol/min/mg protein.

Statistical analysis

CaM kinase II activity values are in mean ± SEM of five individual experiments. Statistical analyses were performed using the paired t-test, employing the Sigma plot software. A value of $P<0.05$ was considered significant.

Western analysis of CaM kinase II

At the end of experimental period, the brains were removed from both control and ethanol treated rats and homogenized in 10 mM Tris-HCl (pH 7.4), containing 3 mM $MgCl_2$, 100 µM leupeptin, 100µM aprotinin and 100µM phenylmethylsulfonyl fluoride. Equal amount of protein (75 µg/lane) was electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10 % polyacrylamide gels as described by Laemmli (1970). Immunoblotting was performed by the method of Towbin et al., (1979). Proteins from the SDS-PAGE gels were transferred to 0.2 µm nitrocellulose using transblot apparatus (LKB, Sweden). Protein transfer was performed at 45 mA for 3 h in 25 mM Tris-HCl (pH 8.8), 192 mM glycine, 20% methanol buffer. After blotting, the membrane was washed with TBS and non-specific binding sites were blocked with 5% (w/v) non fat milk powder for 2 h.

The immunoblots were incubated overnight at 4°C with monoclonal antibodies directed against CaM Kinase II a subunit (50 kDa). Following three times wash with TBS, blots were incubated with secondary antibody (goat anti-mouse conjugated with alkaline phosphatase). After washing three times with TBS, immunoreactive CaM kinase II was visualized using 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium chloride (BCIP/NBT) chromogen system (Sigma). Protein bands were analysed using scanning densitometry.

Immunohistochemical analysis of CaM kinase II

Tissue slices were deparaffinized with xylene and rehydrated through decreasing concentrations of ethanol and finally the slices were placed in cold Tris-buffered saline (TBS pH 7.4). Slices were washed thoroughly (5 x 5 min) in TBS to remove all traces of xylene and ethanol. Sections were placed in TBS containing 0.5% Triton x 100 for 15 min at 40 °C, then rinsed in TBS (3 x 10 min) at room

temperature. To limit the amount of non-specific binding, slices were incubated for 1 hr in 5% goat normal serum from the animal in which the corresponding secondary antibody was made. Sections were rinsed in TBS, incubated with monoclonal antibody directed against CaM kinase II a subunit for 24 hrs at 40 °C using the concentration of 10 μg/ml.

Following the incubation with primary antibody, sections were washed 3\10 min at room temperature in TBS. Brain slices not incubated in primary antibody were included as a negative control. Following three 10 min washes in THS, slices were visualized using diaminobenzidine (0.5mg/ml; Sigma) and 0.02 % hydrogen peroxide. After a final wash in distilled water, sections were mounted on gelatinized slides and allowed to air dry overnight. Slides were dehydrated through a graded series of alcohol, coverslipped with DPX mount, examined and photographed under a Nikon Labphot Microscope, Japan.

CaM kinase II dependent in vitro endogenous protein phosphorylation

CaM kinase II dependent endogenous protein phosphorylation was performed (Fukunaga et al., 1989). The reaction was carried out in 50 ul of 50 mM HEPES (pH 7.4), 10 mM magnesium acetate, 1mM DTT, 0.4 mM $CaCl_2$ and 2 uM calmodulin, 20 mg of protein from cytosol or membrane fraction and in the presence (1 μM) and absence of autocamtide-2-related inhibitory peptide (AIP), specific inhibitor of CaM kinase II (Ishida et al., 1995). (A generous gift from Prof. Hitoshi Fujisawa, Dept. of Biochem. Asahikawa Medical College, Asahikawa, Japan). The reaction was started by the addition of 10 uM ATP, [γ^{-32}P] ATP (2-4 uCi) (3000 Ci/mmol) followed by incubation at 30°C for 5 min. The reaction was stopped by Laemmli sample buffer [0.125 M Tris-HCl (pH 6.8), 2% (wt/vol) SDS, 10 % (vol/vol) glycerol, 2% (vol/vol) β-mercaptoethanol and 0.01% bromophenol blue] and heat denatured at 100°C for 2 min. The proteins were separated by SDS-PAGE on 10% polyacrylamide gel (Laemmli 1970), silver stained, dried and exposed to X-ray film (Kodak) using intensifying screens (Kodak) at -70°C for 12 h. The exposed film was developed to detect the phosphorylated proteins.

RESULTS

Effect of ethanol exposure on CaM kinase II activity

CaM kinase II activity was assayed for its involvement in the phosphorylation of endogenous substrate proteins of membrane and cytosol from cerebral cortex of control and ethanol fed rat brain. The CaM kinase II activity showed an increasing trend in the membrane and cytosolic fractions during the course of postnatal development of rat brain from 8 to 90 days of age. These results agree with existing reports on CaM kinase II behaviour in development (Kelly and Vermon, 1985). Further, the CaM kinase II activity was found increased in ethanol treated rats both in the membrane and cytosol over controls (Fig. 20, 21). The overall activity of CaM kinase II at the age of 8 days in the membrane and cytosolic fraction of control and ethanol fed rats was low but increased gradually in the later ages. At the age of 90 days the activity increased approximately two folds in both membrane and cytosolic fractions. There was no difference in the CaM kinase II activity between control and isocaloric control group of rats. Hence in all further experiments comparison is carried out only between control and ethanol treated groups.

Effect of ethanol exposure on CaM kinase II enzyme

To assess CaM kinase II activation, we relied on western analysis to examine the levels of CaM kinase II. CaM kinase II en/.yme levels were detected in membrane and cytosolic fractions of brain from control and ethanol exposed rats using specific monoclonal antibody to CaM kinase II a subunit (Fig. 22, 23). The increased CaM kinase II activity in experimental samples appears to be due to increased levels of the enzyme. The levels of CaM kinase II gradually increased as a function of postnatal development from 8 to 90 days of age both in the membrane and cytosolic fractions. The enzyme quantity is very low at the age of 8 days in the membrane and cytosolic fractions, whereas it increases to 4-5 fold in the later age i.e. 30 and 90 days. Ethanol treatment also increased the levels of CaM kinase II a subunit over controls at the age of 8, 30 and 90 days in membrane fractions. However, in cytosolic fraction CaM kinase II showed a different trend. In the cytosolic fraction from control rats the enzyme levels were elevated only at 30 days of development. While in 8 days as well as 90 days, the enzyme levels were very low. The ethanol treatment in this fraction did not alter the trend but increased the levels of enzyme at 30 and 90 days. In

general, the CaM kinase II increased activity and its levels appear to be involved during pre- and postnatal development of rat brain cerebral cortex and found vulnerable for the action of ethanol.

Immunohistochemical localization of CaM kinase II during ethanol treatment

Changes in the expression and localization of CaM Kinase II in the brain after prenatal ethanol exposure to rats was analyzed by western blot and immunohistochemical methods. Immunoblot showed a single band at ~ 50 kDa position. Western analysis showed an increased CaM Kinase II immunoreactivity during postnatal development in controls as well as in ethanol exposed rat brain. The CaM kinase II levels gradually increased with age (8, 30 and 90 days) in control rats and the same pattern was observed in the ethanol treated rat brain. However, in the ethanol treated rats, CaM kinase II expression was higher compared to controls (Fig. 22, 23). Changes in CaM kinase II expression were analyzed by immunohistochemical method. Figure (24) shows brain slices from representative control and ethanol treated rats. The level of CaM kinase II immunolocalisation was sharply increased during development in controls as well as in ethanol treated animals. In cerebral cortex, immunoreactivity was found to be more in the cortical layers of ethanol treated rat brain particularly at the age of 90 days (Fig. 24).

Calcium/Calmodulin dependent phosphorylation of substrate proteins

In vitro phosphorylation assays were performed in the presence of calcium and calmodulin to identify calcium/calmodulin dependent phosphorylation of endogenous substrate proteins of membrane and cytosol fractions from control and pre- and postnatal ethanol fed rat cerebral cortex (Fig. 25) to identify CaM kinase II targeted proteins. Selective proteins such as 87, 65, 60, 50, 43, 40, 36 and 29 kDa were found phosphorylated under these experimental conditions in all the ages of development. However, in the presence of AIP, phosphorylation status of these proteins is not altered, excepting for two proteins with molecular weight 50 and 40 kDa. This suggests CaM kinase II is mediating the phosphorylation of only these two selective substrates (Fig. 26). Phosphorylation of all the proteins noticed could be because of other calcium dependent protein kinases such as PKC (Mahadev and Vemuri, 1998). The 50 and 40 kDa proteins showed decreased phosphorylation during the course of development in membrane samples of control and alcohol fed rat brain. The phosphorylation of 50 kDa protein decreased at the age of 30 days in membrane samples from alcoholic brain compared to controls. The 40-kDa protein in the control

membrane samples showed decreased phosphorylation during the postnatal development of rat cerebral cortex (Fig. 27, 28). However, ethanol exposure increased the phosphorylation of 40-kDa protein at 90 days. Cytosolic fractions showed no change in the state of phosphorylation of these two proteins. Phosphorylation of these proteins was decreased specifically in the presence of AIP, suggesting their involvement as substrate proteins for CaM kinase II enzyme during prenatal alcohol exposure to rat brain cerebral cortex.

FIG, 20. CaM-kinase II activity in the membrane fraction of cerebral cortex. After pre- and postnatal exposure of ethanol to rats, cerebral cortex was dissected out at different time intervals of 8, 30 and 90 days. The crude membrane fraction was prepared and the kinase activity was measured as described in Materials and Methods. Kinase activity is expressed in pmol/min/mg protein. Data are mean ± SEM (bars) values from three independent experiments. Values significantly different from the control are indicated: $p<0.05$

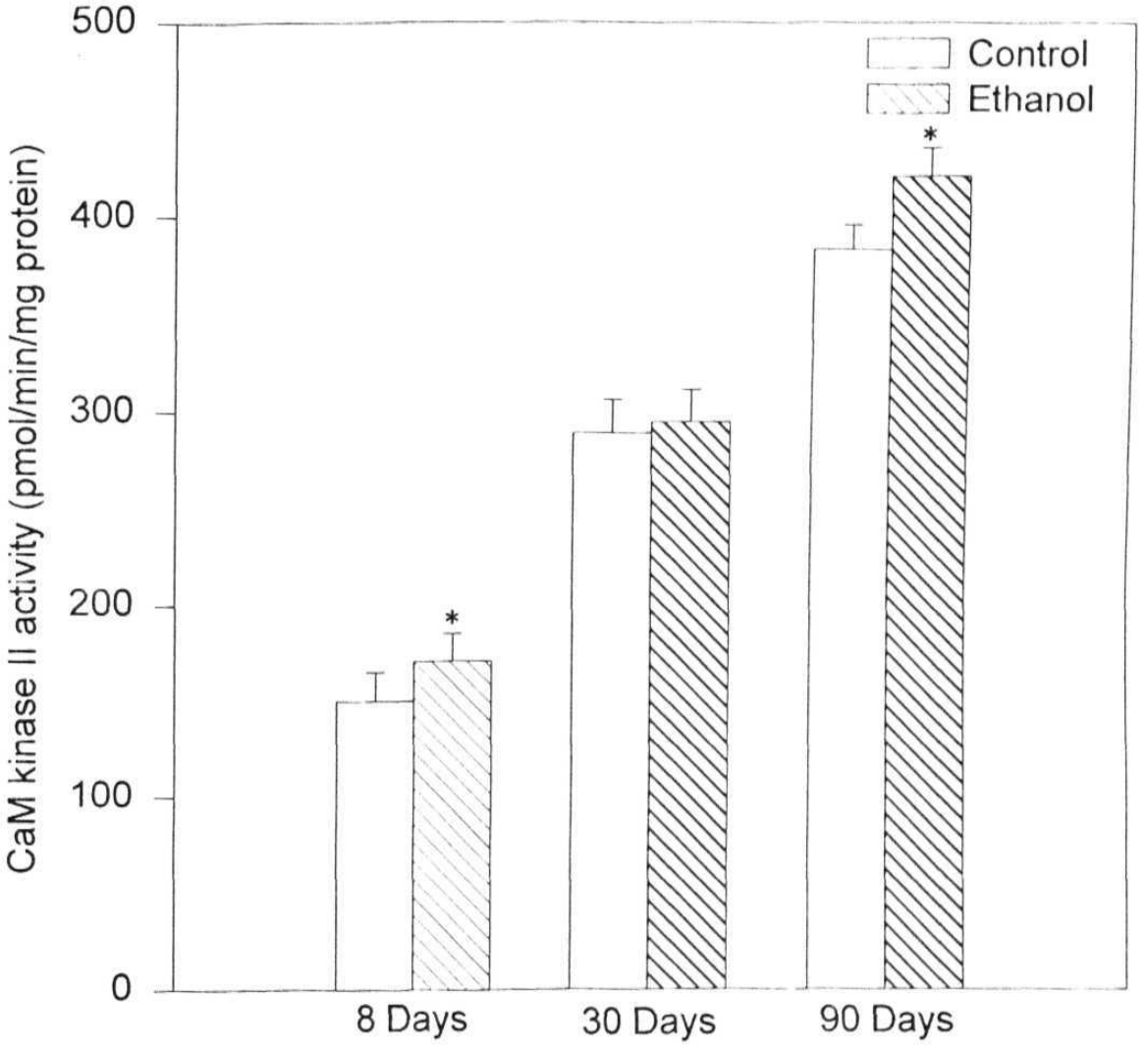


Fig. 20. CaM kinase II activity in Membrane

FIG. 21. CaM-kinase II activity in the cytosolic fraction of cerebral cortex. After pre- and postnatal exposure of ethanol to rats, cerebral cortex was dissected out at different time intervals of 8, 30 and 90 days. The crude cytosolic fraction was prepared and the kinase activity was measured as described in Materials and Methods. Kinase activity is expressed in pmol/min/mg protein. Data are mean ± SEM (bars) values from three independent experiments. Values significantly different from the control are indicated: $p<0.05$

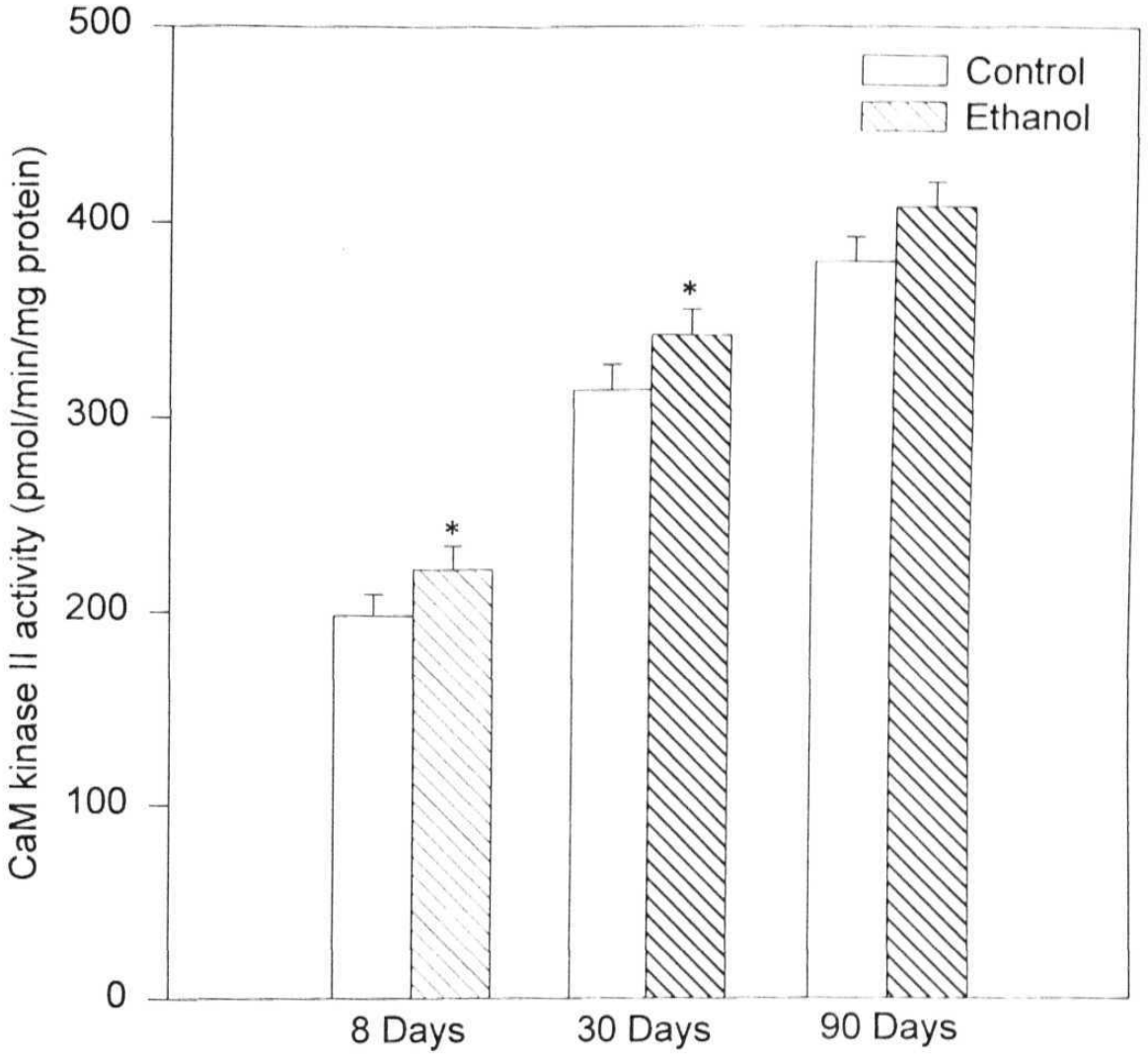


Fig. 21. CaM kinase II activity in cytosol

FIG. 22. Immunoblot analysis of CaM kinase II in membrane and cytosolic fractions of cerebral cortex from control and ethanol exposed rat. Equal amount of protein (75 jig/lane) was electrophoresed by SDS-PAGE, protein was transferred on nitro -cellulose membrane and was probed with CaM kinase II a subunit primary antibody as described in Materials and Methods. The data are representative of three independent experiments with similar results.

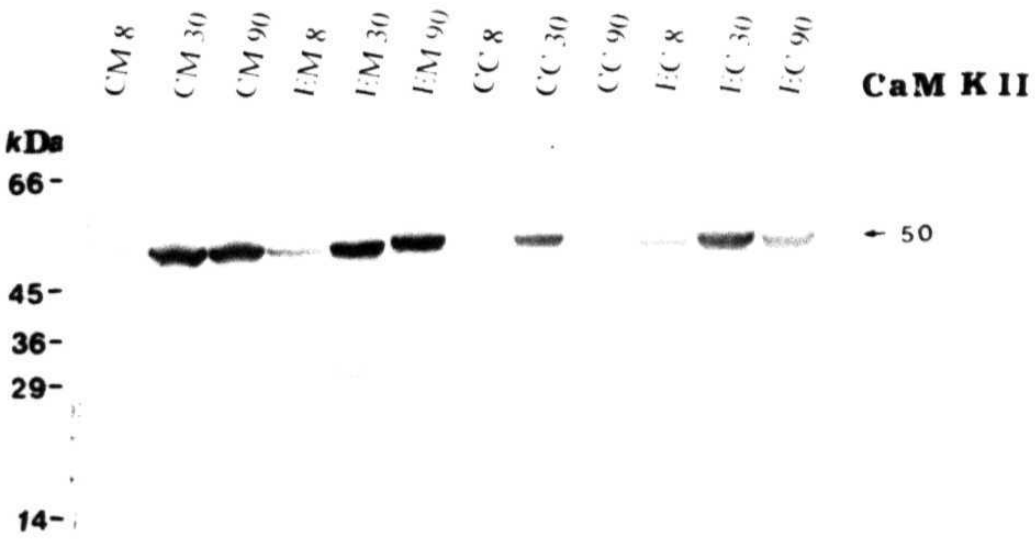

CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
EC 8
EC 30
EC 90
CaM K II
kDa
66-
45-
36-
29-
14-
50

Fig. 22.

FIG. 23. Quantification of CaM kinase II immunoblot (fig 22). Values are mean ± SEM (bars) of triplicate experiments. The values for CM8 and CC8 are considered as 100% for relative quantification.

On x-axis, CM = Control membrane fraction; EM = Ethanol treated membrane fraction CC = Control cytosolic fraction; EC = Ethanol treated cytosolic fraction. The numbers within bars indicate the postnatal days.

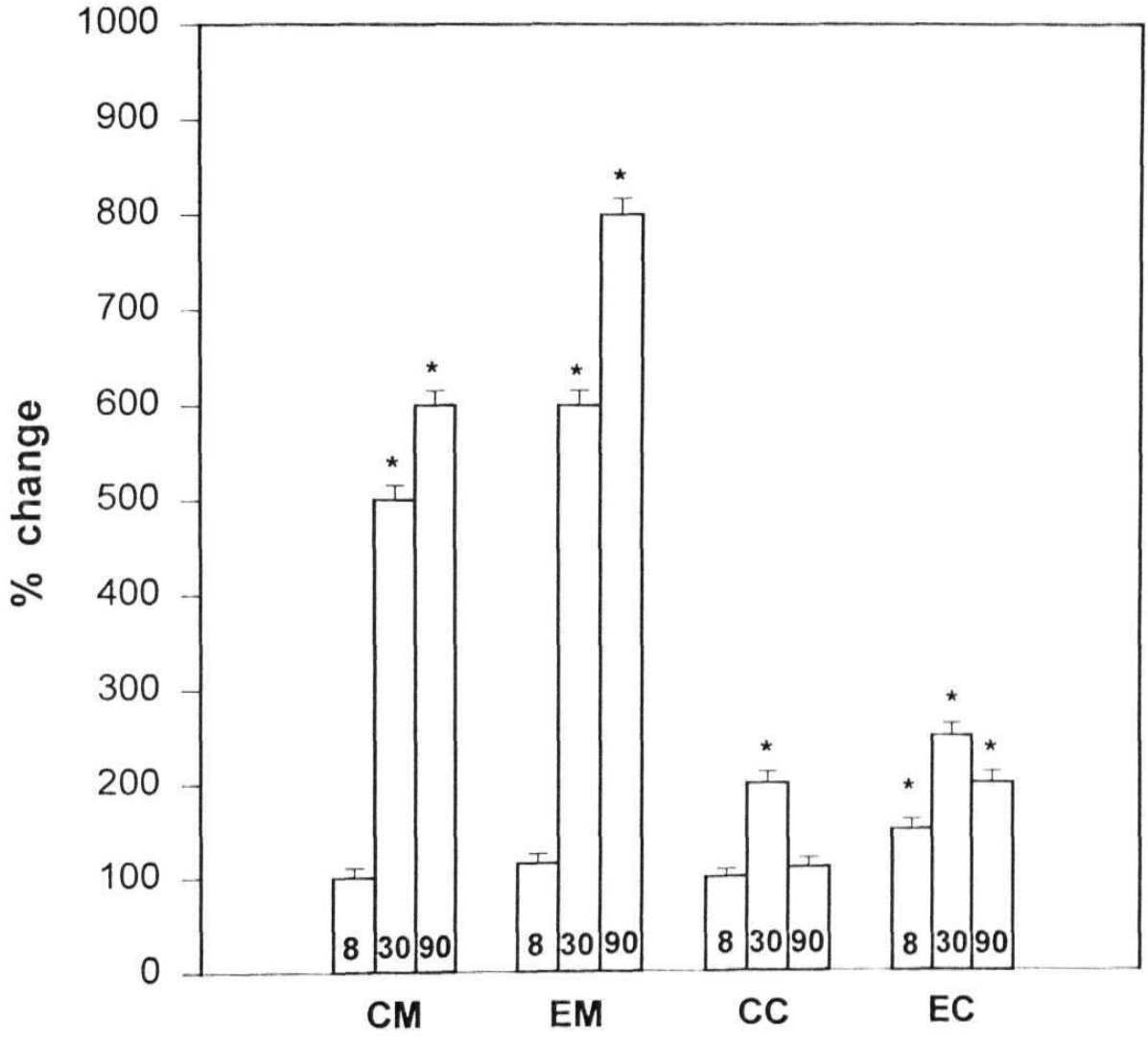

1000
900
800
700
600
500
400
300
200
100
0
% change
8
30
90
CM
EM
CC
EC

Fig. 23.

FIG. 24. CaM kinase II immunoreactivity in the rat cerebral cortex following exposure to ethanol. Coronol sections (20 μm) of rat brain were incubated with anti - CaM kinase II antibody. Water treated animals served as controls.

C_8, C30, C_{90} immunoreactivity of CaM kinase II in control cerebral cortex seen at x 400 magnification.
E_8, E30, E_{90} increase in immunoreactivity of CaM kinase II in ethanol treated cerebral cortex seen at x 400 magnification.

C = Control
E = Ethanol treated

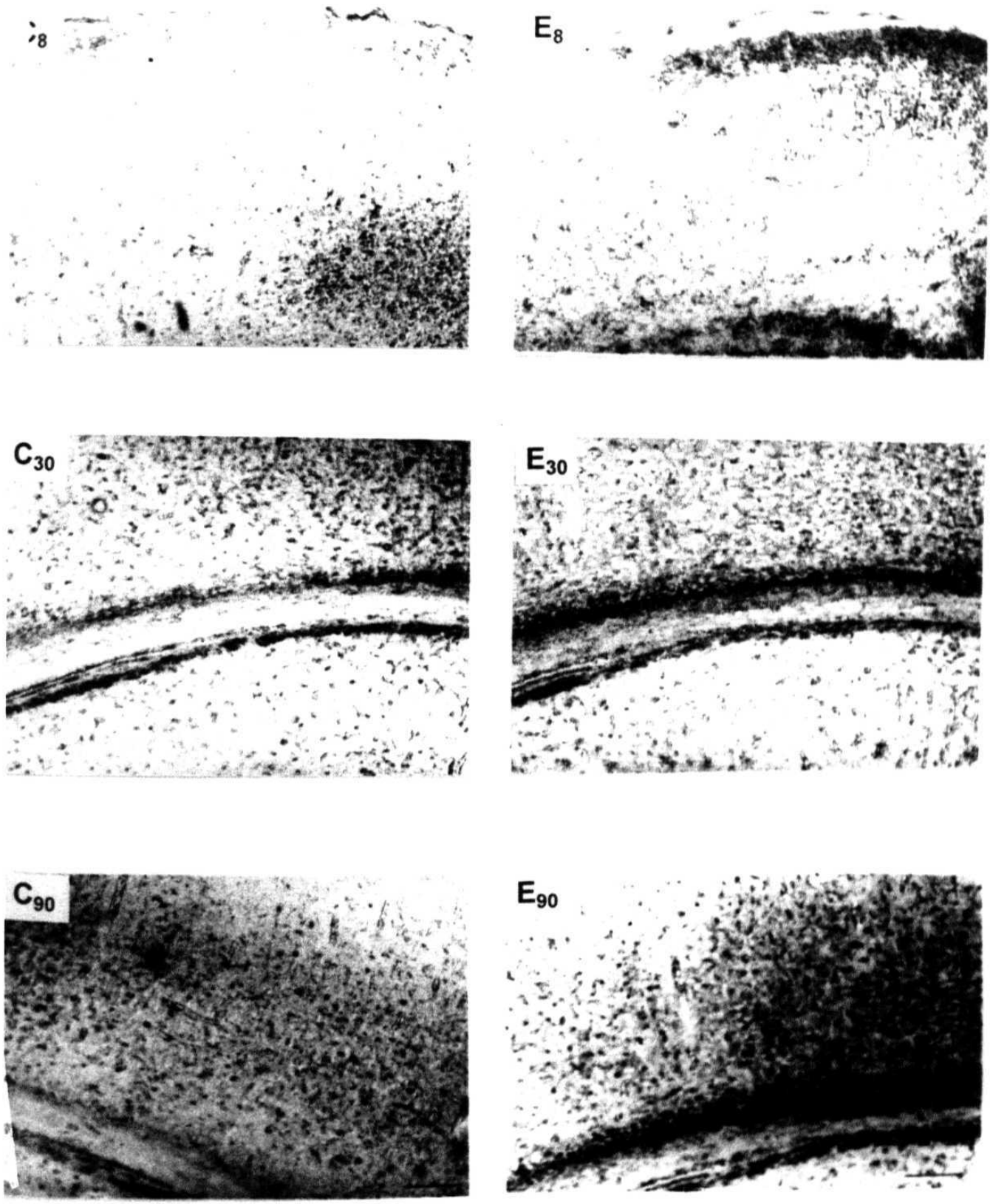


Fig.24

FIG. 25. In vitro phosphorylation of endogenous substrate proteins of membrane and cytosolic fractions from control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. The in vitro phosphorylation assays were performed in the presence of Ca^{+}/calmodulin as described in Materials and Methods. Samples were separated on 10% SDS-PAGE gels followed by autoradiography. The autoradiogram was obtained after exposure for 12 h at -70°C. The data are representative of three independent experiments with similar results.

FIG.26.A11 conditions and analysis same as described in the legend for fig. 25, excepting that in addition to all constituents in phosphorylation assays, autocamtide-2-related inhibitory peptide (AIP) (1 μM) is also included along with calcium and calmodulin. The data are representative of three independent experiments with similar results.

CM – Control membrane fraction; KM = Ethanol treated membrane fraction
CC - Control cytosolic fraction; EC - Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

Fig. 25.

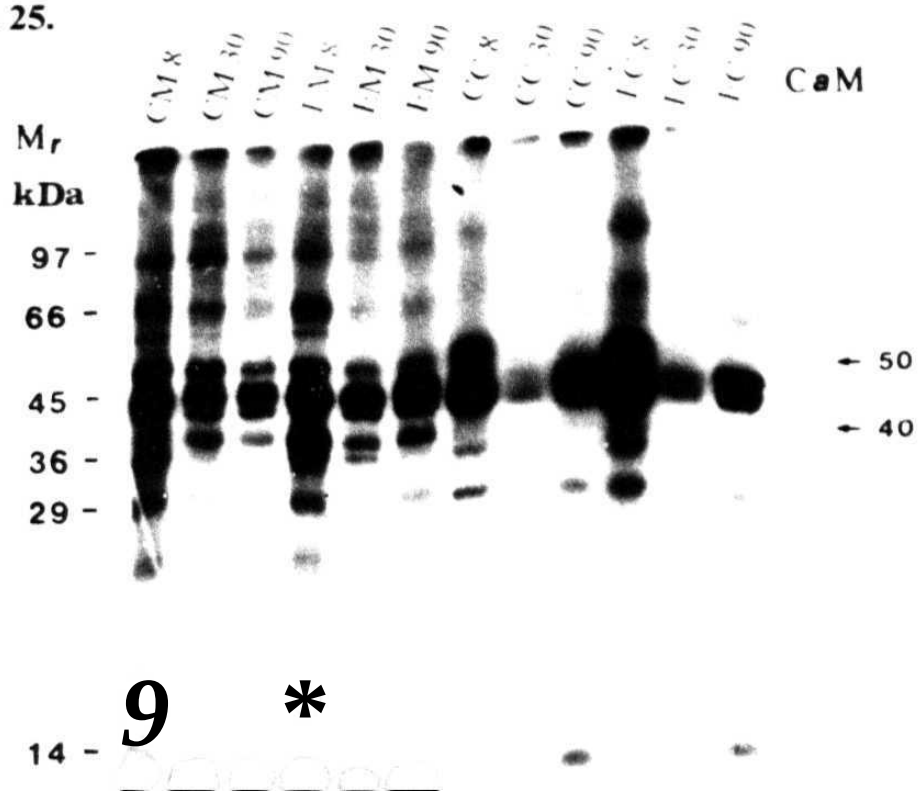

CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
EC 8
EC 30
EC 90
CaM
Mr
kDa
97
66
45
36
29
14
50
40

Fig. 26.

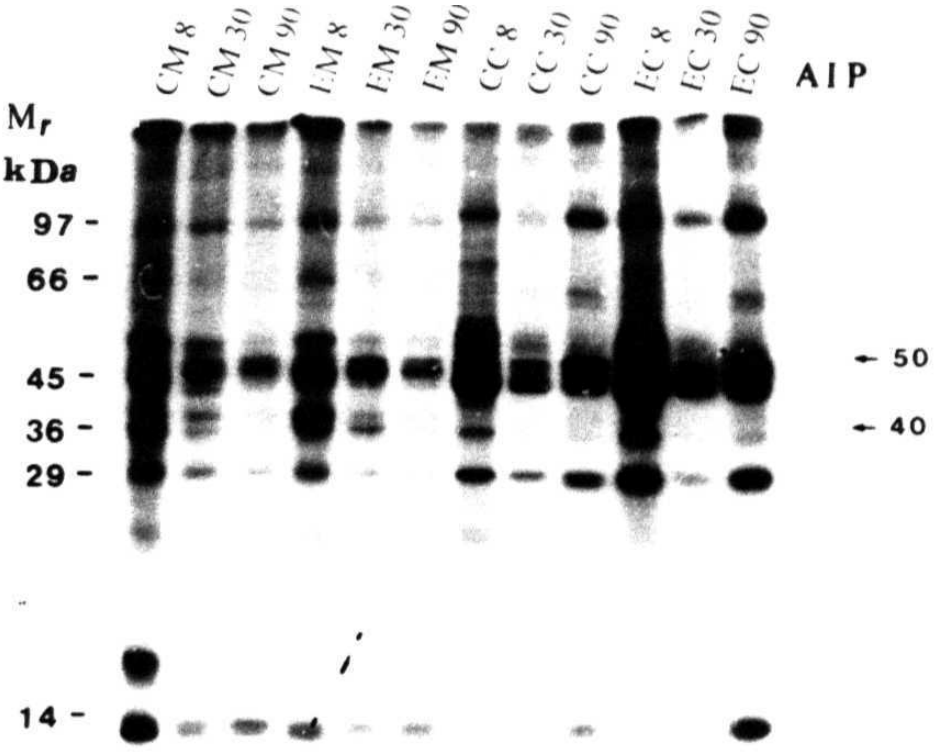

CM 8
CM 30
CM 90
EM 8
EM 30
EM 90
CC 8
CC 30
CC 90
EC 8
EC 30
EC 90
AIP
Mr
kDa
97
66
45
36
29
14
50
40

FIG. 27. Phosphorylation of 50 kDa endogenous substrate protein in various conditions.

FIG. 28. Phosphorylation of 40 kDa endogenous substrate protein in various conditions.

$- Ca^{2+}$	-	Absence of Calcium
$+ Ca^{2+}$	=	Presence of Calcium
PS/DAG	=	Phosphotidylserine and diacylglycerol
AA	=	Arachidonic acid
SS	-	Staurosporine
CaM	=	Calmodulin
AIP	-	Autocamtide-2-related inhibitory peptide

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC = Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

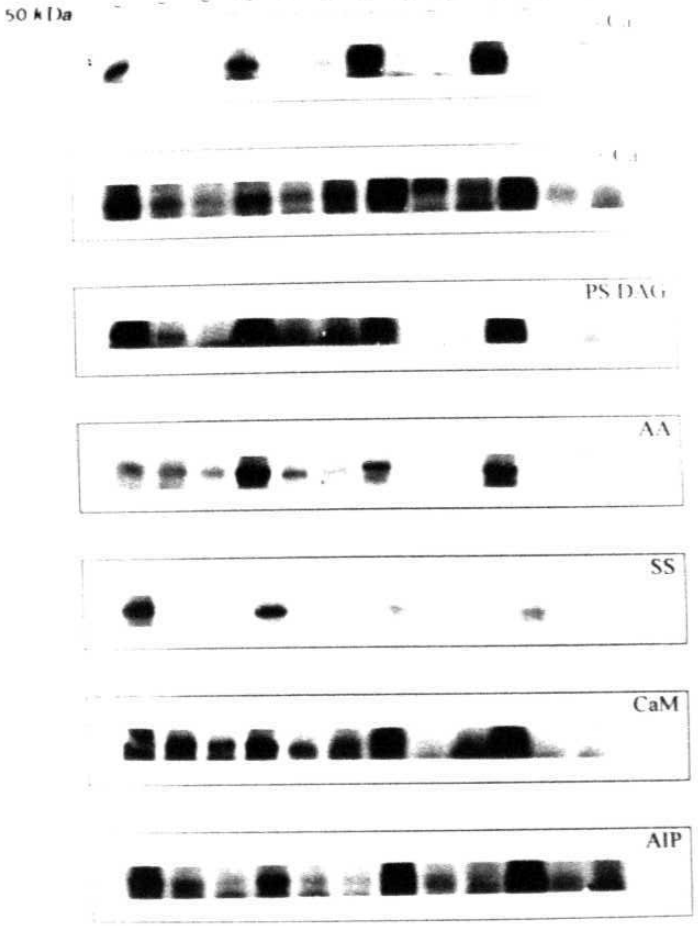


Fig. 27.

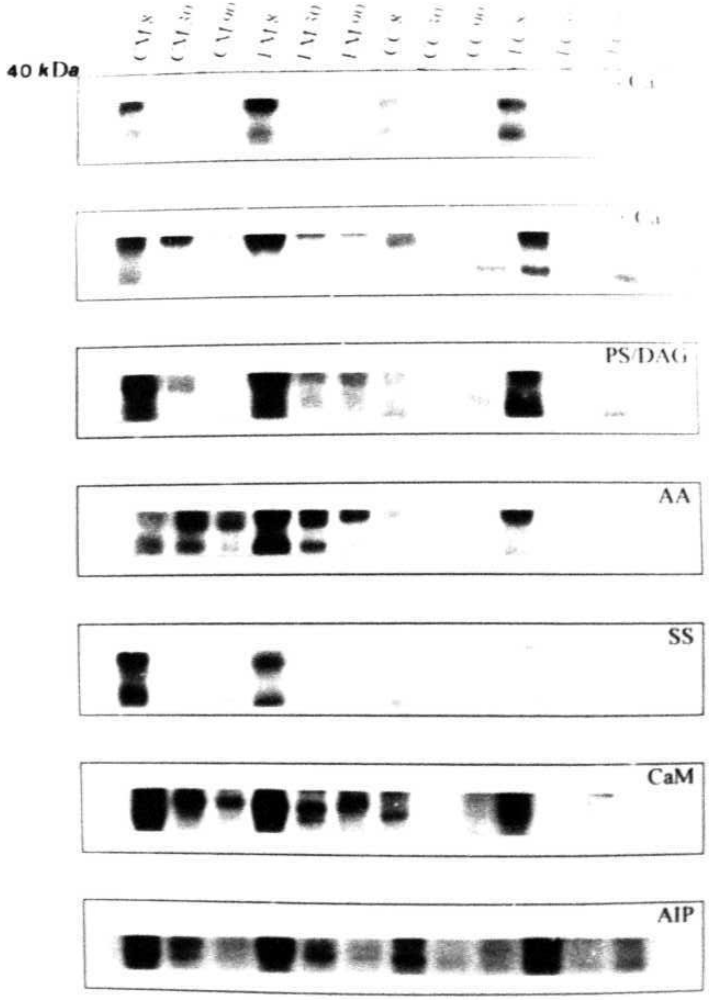


Fig. 28.

DISCUSSION

The results of the present study demonstrate that both pre- and postnatal ethanol exposures modulates membrane and cytosolic CaM kinase II activity as well as quantitatively in terms of its levels. The developmental pattern of this enzyme also establishes that its activity and levels are selectively promoted by ethanol in specific periods of development.

Five isoforms of CaM kinase II from brain have been so far reported α, β, β, y and 8 in which a/p ratio is 4:1 in cerebral cortex. The α and p isoforms are primarily expressed in brain (Lin et al., 1987; Bulleit et a., 1988) as major components of brain CaM kinase II, while y and 8 isoforms (~59-60 kDa) arc expressed in other tissues (Tobimatsu and Fujisawa, 1989; Bennett et al., 1983; Goldering et al., 198.1; Schwores et al., 1985). Both a and β subunits arc autophosphorylated in the presence of Ca^{2+} and calmodulin (Lin et al., 1987; llanley et al., 1987; Tobimatsu and Fujisawa, 1989, Hanson and Schulman, 1992; Rostas and Dunkley, 1992). The present data on developmental expression of CaM kinase II α-subunit is consistent with the reported literature (Kelly and Vermon, 1985). The results also agree with changes noticed in total CaM kinase II activity and subcellular distribution during brain development (Kelly and Vermon, 1985; Rostas, 1991).

Pre- and postnatal ethanol exposure induced an increase in the CaM kinase II activity in all the developmental stages. The increase in CaM kinase II activity can be attributed to increased influx of Ca * during chronic ethanol treatment (Daniell et al., 1987) or increased expression of CaM kinase II enzyme levels. Increased levels of CaM kinase II in membranes as well as in cytosolic fractions of ethanol exposed rat cerebral cortex was noticed in this study. Increased Ca^{2+} influx can activate the calcium sensitive mechanisms. Similar to PKC and CaM kinase II activation, increased calcium influx can also activate calpains, which are calcium activated neutral proteases, causing neuronal death (Choi and Rothman, 1990). Calpains mediate proteolysis of CaM kinase II (Kwiatowski and King, 1989; Rich et al., 1990) as well as PKC isoforms (Kishimoto et al., 1989; Cressman et al., 1995). CaM kinase II is a known substrate for calpain *in vitro* and chronic ethanol exposure has been reported to decrease the activity of calpain (DePetrillo, 1997). The increased CaM kinase II activity noticed in this study could be a result of such a decrease in calpain mediated proteolysis of CaM kinase II, which results in elevated levels of CaM kinase

II. The increased CaM kinase II activity during pre- and postnatal ethanol exposure could have possible implication in LTP that in turn could be involved in ethanol induced cognitive impairment. Immunohistochemical analysis for CaM kinase II distribution in cerebral cortex showed complementary results to western blot trend as for the development is concerned. The same developmental trend was also observed in ethanol treatment, although slightly elevated grain density of CaM Kinase II immunoreactivity can be noticed in sections from ethanol treated rat brain. Ethanol treatment particularly at 30 and 90 days of development showed a conspicuous increase in CaM kinase II reactivity.

To evaluate further, the role of CaM kinase II, we attempted to identify the target substrate proteins that are phosphorylated by CaM kinase II. In the presence of Ca^{2+}, calmodulin (CaM kinase II stimulators) and AIP selective proteins of molecular weight 50 and 40 kDa were phosphorylated which appear to be specific target proteins for CaM kinase II. The 50 and 40 kDa proteins showed decreased phosphorylation during the course of development in membrane samples of control and ethanol exposed rat cerebral cortex. CaM kinase II can undergo autophosphorylation (Miller et al., 1988). Autophosphorylated form of soluble kinase is able to phosphorylate exogenous substrates (Lai et al., 1986, Lou et al., 1986) and undergoes additional autophosphorylation in the absence of calcium (Miller and Kennedy, 1986; Hashimoto et al., 1987). The Ca^{2+} independent form of CaM kinase II is associated with autophosphorylation of Thr286 ' of the α subunit (Miller et al. 1988; Schwores et al., 1988; Thiel et al., 1988; Fukunaga et al., 1989, 1992) and full autophosphorylation depresses the enzyme activity (Hashimoto et al., 1987; Lou and Schulman, 1989). The phosphorylation of 50 kDa protein decreased at the age of 30 days in membrane samples from cthanolic brain over controls. Considering their molecular weight, its presence in brain, it is likely that the 50 kDa protein could be CaM kinase II itself which gets autophosphorylated in presence of calcium and calmodulin (Miller et al, 1988). Once the enzyme is phosphorylated, it becomes calcium independent and renders its activity even in the absence of Ca^{2+} (Miller et al., 1988). In the cytosol we did not observe any change in the state of phosphorylation of 50-kDa protein. In this study, we also noticed CaM kinase II dependent phosphorylation of 40-kDa substrate. However, we could not trace its identity with

comparable proteins existing in the nervous system that might he playing a role in the cerebral cortex development and in ethanol exposure.

In summary, these results establish that CaM kinase II activity and levels increased in membrane and cytosolic fractions following pre- and postnatal ethanol exposure. The increased activity of CaM kinase II could be partly due to increased levels of the enzyme itself. The finding that calpain activity is low in chronic ethanol treated cells could also be involved as calpains use CaM kinase II as substrate. The decreased calpain activity might result in elevated levels of CaM kinase II. This study further showed specific endogenous protein phosphorylation by elevated CaM kinase II, two proteins, 50 and 40 kDa. The 50 kDa protein appears to be autophosphorylated a subunit of the CaM kinase II. Increased CaM kinase II activity and levels, as shown in this study, can affect LTP and may contribute to the neuronal injury and cognitive impairment associated with ethanol neurotoxicity.

Chapter - III

Chapter-III

Effect of Ethanol on Protein Tyrosine Kinases

Protein tyrosine phosphorylation plays an important role in the transduction of extracellular signals. A protein tyrosine kinase (PTK) localized in plasma membrane and coupled to receptor mediates the binding of extracellular factors. Besides, several cytoplasmic kinases enter nucleus and play a major role in the transcriptional regulation and cell cycle control (Hunter and Cooper, 1985; Hunter, 19%). Several PTKs also have been implicated in diseases such as cancer (Boutin, 1994), Alzheimer's disease (Shapiro et al., 1991) and oncogenic activation of transforming retroviruses (Hunter and Cooper, 1985). The presence of high levels of PTK activity in adult brain (Cotton and Brugge, 1983), suggest that PTKs might play specific role in the regulation of neuronal functions. The neuronal and synaptic PTK activity has been further shown to be involved in the modification of synaptic activity particularly depolarization, induction of long term potentiation (LTP) and long term depression (LTD) and ischemia (Gurd, 1997). The high levels of PTK activity in postmitotic neurons resulted in an altered tyrosine phosphorylation in the synaptic proteins (Dasgupta et al., 1994; Cotton and Brugge, 1983). The tyrosine phosphorylation of the synaptic proteins lead to a short term effect due to phosphorylation of acetylcholine, NMDA and GABAA receptors besides K^+ and Ca^{2+} channels (Gurd,1997). The long term effects of tyrosine phosphorylation have been shown to be particularly essential for the induction of LTP, LTD and ischemia (Gurd, 1997).

The presence of high PTK activity in adult brain and due to the role of tyrosine phosphorylation in neuronal functions such as LTP and LTD which form the crucial events for the memory in neuronal circuitry, studies were carried out on tyrosine specific phosphorylation in rat CNS using a tyrosine containing synthetic peptide as exogenous substrate. The synthetic polymer poly(Glu4 Tyr_1) used in this study has also been shown as an effective substrate for various tyrosine specific protein kinases (Braun et al., 1984). Using this substrate the PTK activity was determined for its subcellular distribution as well as for its role in ethanol treated animals as a function of development.

MATERIALS AND METHODS

Materials

Sodium orthovanadate, poly (Glu_4 Tyn). Phenylmethylsulfonyl fluoride (PMSF), Dithiothreitol (DTT), reagents for sodium dodecyl sulfate (SOS) - polyacrylamide gel electrophoresis (PAGE), 5-bromo-4-chloro-3-indoylphosphate / nitroblue tetrazolium chloride (BCIP/NBT) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ ^{32}P] ATP (3000 Ci/mmol) was from Board of Radiation and Isotope Technology (Mumbai, India). Phosphocellulose filter (P81) was obtained from Whatman (Canlab Corp., Mississauga, ON, Canada). Nitrocellulose sheets were from Schleicher and Schuell (Keene, NH, U.S.A.). All other chemicals were of analytical grade and obtained from standard commercial suppliers. Monoclonal antibody (PY20) against phosphotyrosine was a generous gift from Dr. Nagaraju, USA.

Assay of Protein tyrosine Kinases (PTKs) Activity

The endogenous protein tyrosine kinases activity was determined in the membrane and cytosolic fractions from control and alcohol fed rat cerebral cortex using synthetic peptide poly (Glu_4Tyr_1). Phosphorylation of the synthetic substrate was carried out by the procedure described (Trembley et al., 1994). The reaction mixture consisted of 10mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 50 µg poly (Glu_4Tyr_1), 10 uM ATP, [γ^{-32}] ATP (1 µCi per sample) (3000Ci/mmol), 200µM sodium orthovanadate and 10µg protein from membrane and cytosolic fraction. Phosphorylation was performed for 10 min at 30^{0}C and stopped by spotting the sample on Whatman No 1 (2 x2 cms) filter papers and precipitating the protein with 10%TCA/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 filters was measured in a liquid scintillation spectrometer. Specific tyrosine kinase activity was calculated by subtracting the incorporation of labeled phosphate into the endogenous protein from the total activity measured in the presence of endogenous protein and Poly(Glu_4Tyr_1) and expressed in pmol/min/mg protein.

Detection of Tyrosine Phosphorylated Endogenous Proteins

Endogenous phosphorylation of membrane and cytosolic proteins was carried out as described above except that the poly(Glu_4Tyr_1) was omitted and unlabelled ATP (100µM) was used instead of labeled ATP. The reaction was terminated by

adding sample buffer (2X) (Laemmli, 1970) and western blot was performed using anti-phosphotyrosine monoclonal antibodies (PY20) to detect the phosphorylated tyrosine residues in proteins.

*Western analysis **for** the detection of phosphotyrosine proteins*

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 (0.45μm) using TGSM buffer (25mM Tris, 192mM Glycine, 01% SDS and 20% Methanol) as electrode buffer at 0.8mA/ cm2 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 TBS and blots were incubated over night at 4°C on a shaker. The antibody solution was removed and the blots were washed thrice with TBS, followed by incubation with alkaline phosphatase coupled secondary antibodies for 2 hrs. Finally, the nitrocellulose sheet was washed thrice with TBS and visualized using 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium chloride (BCIP/NBT) chromogen system.

RESULTS

*Effect of ethanol treatment on protein tyrosine kinase **(PTK)** enzyme*

Phosphorylation of the synthetic peptide substrate poly ($Glu_4 Tyr_1$) was used to measure the endogenous protein tyrosine kinase activity in the membrane and cytosolic fractions of control and alcohol fed rat brain cerebral cortex (Fig. 29, 30). The activity was measured in the presence and absence of sodium orthovanadate, a specific protein tyrosine phosphatase inhibitor. During the course of development PTK activity decreased both in the membrane and cytosolic fractions from 8 to 90 days of age. Maximum activity was shown to be associated at the age of 8 days and was gradually declined in the later ages (30 and 90 days) of postnatal development and this data agrees with the previously reported literature (Cudmore and Gurd, 1991). The PTK activity in the alcohol exposed rat cerebral cortex was decreased when compared to control in all the ages of postnatal development (8, 30 and 90 days) in membrane as well as in cytosolic fractions. In the presence of sodium orthovanadate the PTK activity was increased in the membrane and cytosolic fractions of control and alcohol samples. However, the enzyme showed a decreased

activity over development only in the absence of orthovanadate. This trend is abolished once orthovanadate is included in the assay even though it contributes a overall significant increase in the PTK activity. Since orthovanadate is a potent inhibitor of tyrosine phosphatase, it is likely that the balance between tyrosine phosphorylation and dephosphorylation is involved at this cellular context.

Effect of ethanol treatment on phosphorylation of endogenous substrate proteins of protein tyrosine kinases

To examine the endogenous substrate proteins of tyrosine specific protein kinases, the membrane and cytosolic fractions of control and ethanol fed rat cerebral cortex were incubated under phosphorylating conditions with unlabelled ATP and analyzed by immunoblotting technique using monoclonal antibody specific for phosphotyrosine residues (Fig. 31, 32). The phosphotyrosine proteins with molecular weight 114, 70, 36, 34, 32, 20 and 14 kDa in the rat brain cerebral cortex membrane fraction were detected both in the control and alcohol fed rat brain during the course of postnatal development from 8 to 90 days of age. However, a higher level of immunoreactivity of these proteins was found in the alcoholic fractions when compared to control fractions particularly at the age of 30 and 90 days of age. Two phosphotyrosine proteins with molecular weight 40 and 38 kDa showed decreased immunoreactivity at the age of 90 days in the cytosolic fraction of alcohol fed rat brain. Several other phosphotyrosine proteins were also detected in the membrane and cytosolic fractions of both control and alcohol fed rat brain cerebral cortex but the comparison was restricted only to the phosphotyrosine proteins showing a clear change during the course of development and prenatal alcohol exposure.

FIG. 29. Membrane associated PTK activity in control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. Membrane was prepared and assayed for PTK activity in absence and presence of vanadate as described in Materials and Methods. Activity is expressed as picomoles of $PO4^{"3}$ incorporated into the exogenous histone" minute" milligram of protein. The data points are mean±SEM (bars) values of five separate experiments. $P<0.05$ as compared with the control.

CM = Control membrane fraction
EM = Ethanol treated cytosolic fraction

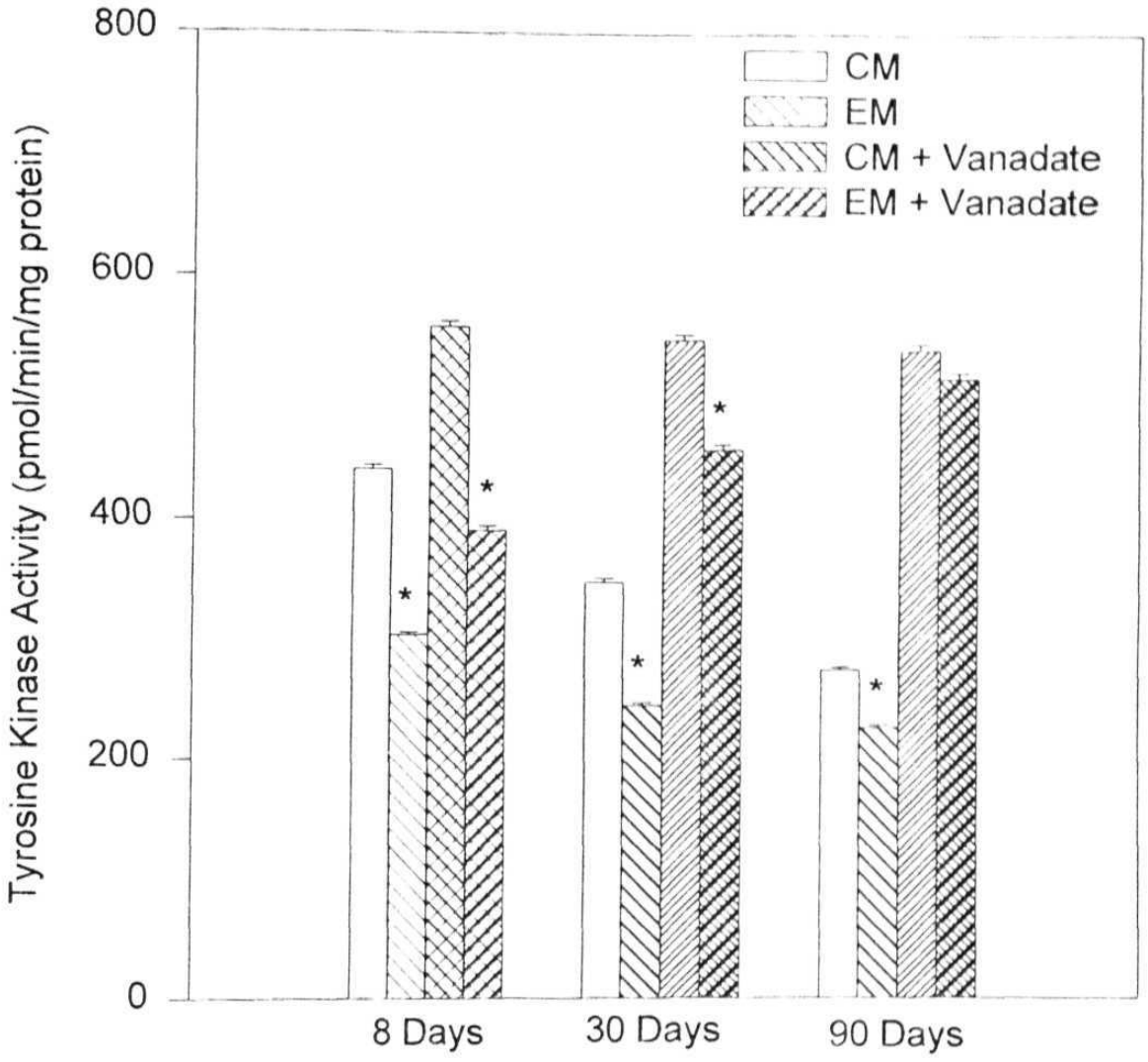


Fig. 29. Protein Tyrosine Kinase activity in Membrane

FIG. 30. Cytosol associated PTK activity in control and ethanol treated rat cerebral cortex at 8, 30 and 90 days of development. Cytosol was prepared and assayed for PTK activity in absence and presence of vanadate as described in Materials and Methods. Activity is expressed as picomoles of PO4" incorporated into the exogenous histone^{-1}minute^{-1}milligram of protein. The data points are mean±SEM (bars) values of five separate experiments. $P<0.05$ as compared with the control.

CM = Control membrane fraction
EM = FAhanol treated cytosolic fraction

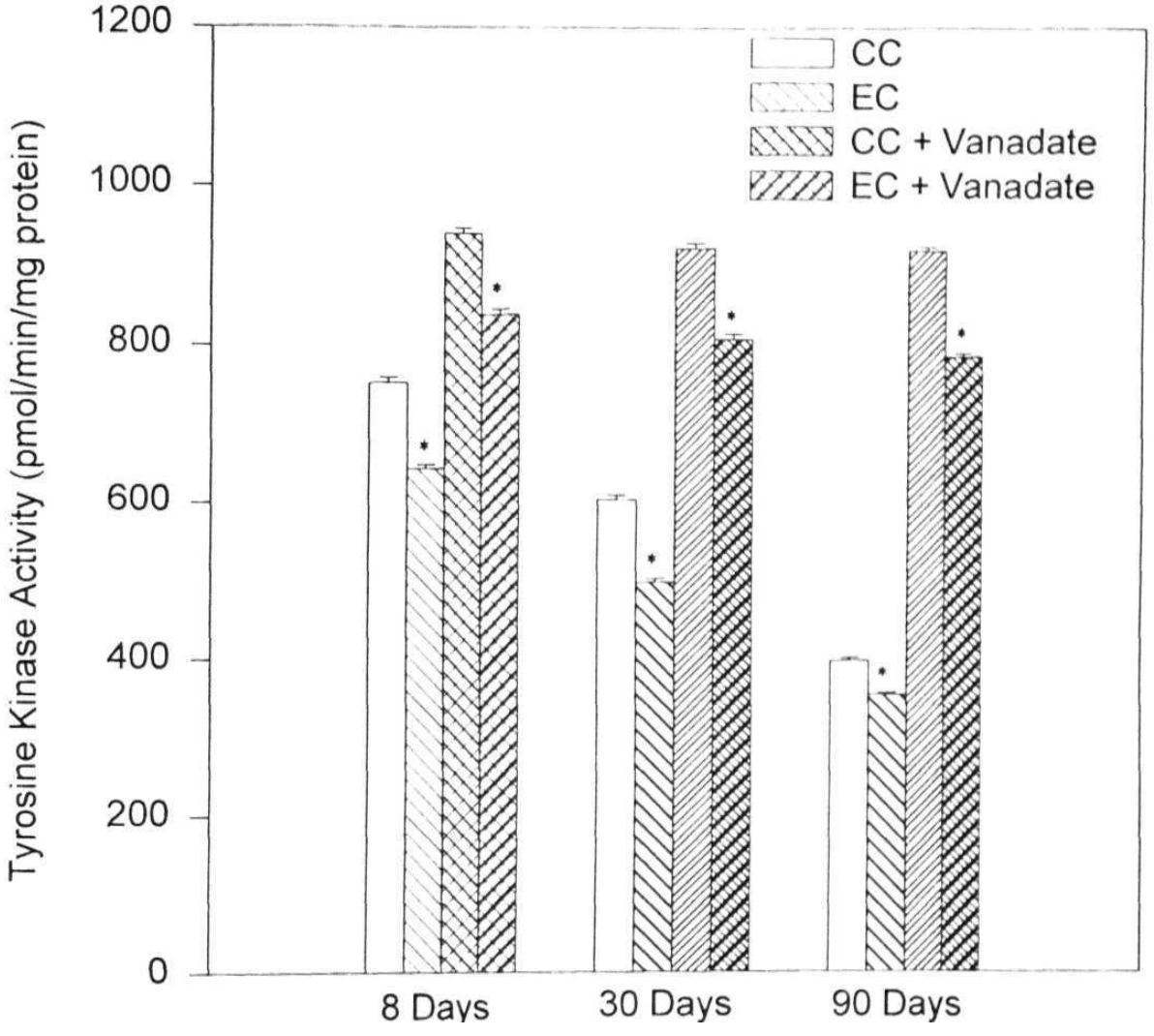


Fig. 30. Protein Tyrosine Kinase activity in cytosol

FIG. 31. Immunoreactivity of phospho tyrosine containing proteins in the absence of vanadate from membrane and cytosolic fractions of control and ethanol treated rat cerebral cortex. The blot was probed with phosphotyrosine antibodies (PY20) and visualized by alkaline phosphatase-BCIP/NBT detection system.

FIG. 32. Immunoreactivity of phospho tyrosine containing proteins in the presence of vanadate from membrane and cytosolic fractions of control and ethanol treated rat cerebral cortex. The blot was probed with phosphotyrosine antibodies (PY20) and visualized by alkaline phosphatase-BCIP/NBT detection system.

CM = Control membrane fraction; EM = Ethanol treated membrane fraction
CC = Control cytosolic fraction; EC = Ethanol treated cytosolic fraction
The numbers 8, 30 or 90 suffixed to CM, EM, CC or EC indicate the age in postnatal days.

Fig. 31

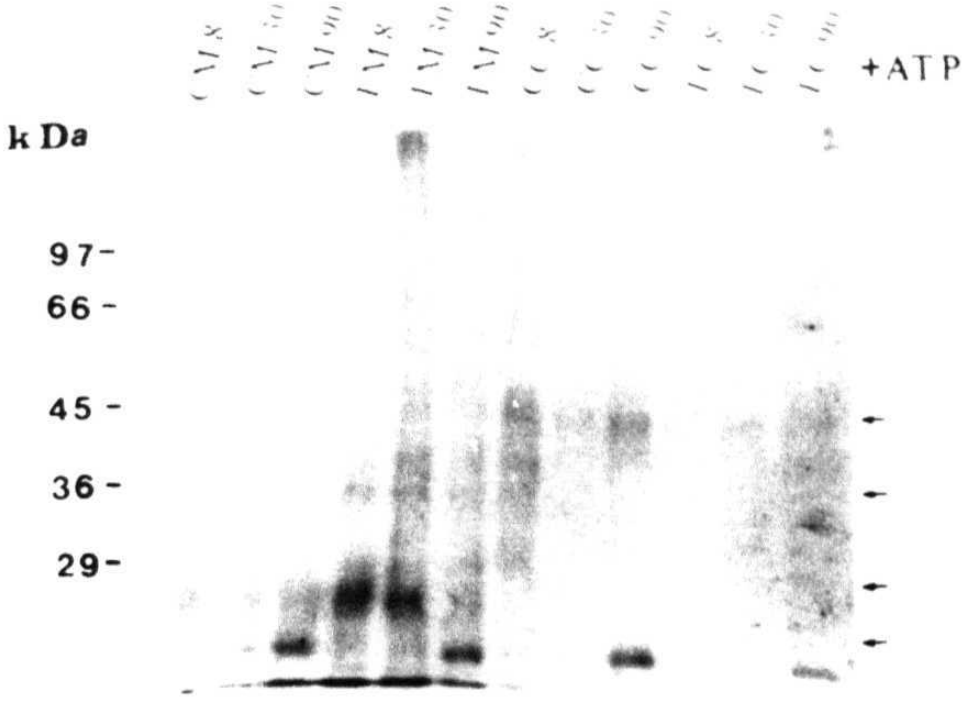
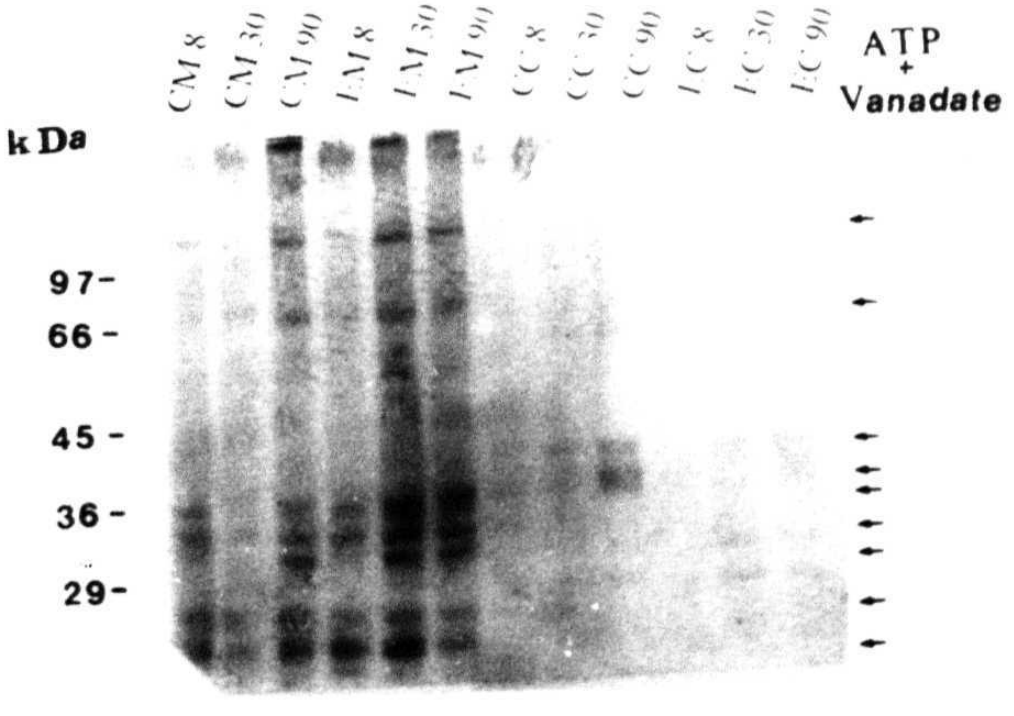

+ATP
k Da
97-
66-
45-
36-
29-

Fig. 32.


CM 8
CM 30
CM 90
LM 8
LM 30
LM 90
CC 8
CC 30
CC 90
LC 8
LC 30
LC 90
ATP
+
Vanadate
k Da
97-
66-
45-
36-
29-

DISCUSSION

In the present study, the subcellular fractions of membrane and cytosol showed considerable levels of protein tyrosine phosphorylation based on the PTK assay. The cytosolic fraction relatively had higher activity compare to membrane. In most tissues including brain, the increase in the cytosolic tyrosine kinase activity coincides with the decrease in membrane associated tyrosine kinase activity which is in agreement with the previous data (Pamela, 1991). An overall gradual decrease in the tyrosine phosphorylation was noticed during postnatal development of cerebral cortex. The low levels of tyrosine kinase activity during development could be due to a decrease in the enzyme synthesis or due to a postranslational modification which causes a decrease in enzyme specific activity. If these two possibilities are excluded, the development of cerebral cortex might involve the synthesis of a specific PTK inhibitor (Swarup et al., 1983; Okada and Nakagawa, 1988). Inspite of decreased tyrosine phosphorylation in adult cerebral cortex (90 days), it showed considerable activity that could still account for the endogenous phosphorylation of tyrosine residues.

The inclusion of vanadate in both control and ethanol treated samples of membrane and cytosol not only relieved the inhibition of PTK activity but shooted it up almost two fold. The outburst in the PTK activity in the presence of vanadate could be attributed partially to the potent inhibition of tyrosine phosphatase activity by vanadate. This would explain the inhibition of tyrosine phosphatase activity, which might promote PTK activity so that protein substrates would remain phosphorylated on tyrosine residues for relatively longer duration i.e. as long as the vanadate concentration prevails. The data from this study suggest that the PTK activity is modulated during development and is amenable for the action of agents like tyrosine phosphatase inhibitors. The addition of vanadate showed an increase in tyrosine kinase activity both in the control and ethanol treated group irrespective of different developmental stages studied, indicating the modulation of protein tyrosine kinase activity directly or indirectly by the protein tyrosine phosphatases. However, tyrosine kinase activity in ethanol treated group remained always lesser than the control group. One plausible explanation seems apparent in this case. Earlier studies have shown that the brain may contain endogenous inhibitors of tyrosine kinases (Pamela, 1991) and further in a recent study it was demonstrated that the induction

and higher levels of *pag* protein, a known physiological inhibitor of nuclear c-*abl* tyrosine kinase using compounds inducing oxidative stress (Prosperi et al., 1998). Therefore, it is quite tempting to speculate that certain endogenous inhibitors of tyrosine kinase in the cytosol too might be induced that could serve to down regulate the tyrosine kinase activity. Further, ethanol has been shown to induce oxidative stress reactions in brain through its lipophilic and free radical generating properties (Renis et al., 1996). The consequence of protein phosphorylation both in control and ethanol treated samples are noticed in such a way that tyrosine phosphorylation events are tightly controlled in development as they show gradual decrease.

To examine the major substrates of PTKs, the membrane and cytosolic fractions from control and ethanol treated samples from various developmental stages were incubated under phosphorylation conditions using unlabeled ATP and analyzed by immunoblotting techniques by monoclonal antibody specific to phosphotyrosine. The tyrosine phosphorylation of endogenous membrane substrate proteins showed selective proteins of less than 29 kDa in ethanol treated samples from 8 and 30 days. However, inclusion of vanadate along with ATP lighted up considerably significant number of tyrosine phosphorylated proteins in the membrane fraction. The cytosolic fractions did not show significant changes.

The presence of high levels of protein tyrosine kinase and their endogenous substrates in membrane and cytosolic fractions is consistent with tyrosine phosphorylation being involved in the neuronal activities like neurotransmitter release, modulation of neurotransmitter receptors and ion channels (llirano et al., 1988). Further, the vanadate-induced increase in the PTK activity could possibly underlie potent protein tyrosine phosphatase (PTPs) inhibition. These data could also be interpreted in such a way that the endogenous protein tyrosine phosphatase levels in ethanolic samples must have been reasonably high enough so that in the absence of vanadate tyrosine phosphorylation is masked by the high activities of tyrosine phosphatase. In turn all these data points to a possibility of an altered homeostatic balance between the activities of PTKs and PTPs that requires further detailed analysis.

Conclusions

CONCLUSIONS

In conclusion, this study has revealed the following salient features of protein kinases with respect to pre and postnatal ethanol exposure to rat cerebral cortex development.

1. Protein kinase C activity was increased both in the membrane and cytosol during pre- and postnatal ethanol exposure to rat cerebral cortex.
2. Increase in PKC activity both in cytosol and membrane due to ethanol was more sharp and prominent at early stages than at later stages of development.
3. Early age of development could be a unique focus for the role *of* PKC in ethanol induced developmental defects.
4. PKC βI and βII isoforms showed increased expression while PKC δ isoform showed decreased expression in membrane samples of ethanol exposed rat cerebral cortex.
5. Immunohistochemical studies showed increased levels of PKC in cerebral cortex, over development as well as in ethanol exposed rat brain.
6. Specific membrane and cytosolic endogenous proteins showed altered protein phosphorylation during postnatal development as well as in ethanol exposed rat cerebral cortex and these were identified as PKC dependent endogenous substrate proteins in the brain.
7. CaM kinase II activity increased during ethanol exposure in membrane as well as in cytosol over development.
8. CaM kinase II a-subunit expression was increased during the course of development as well as in ethanol exposed membrane and cytosolic fractions.
9. Specific substrate proteins showed increased phosphorylation in ethanol exposed rat cerabral cortex.
10. Among calcium dependent protein phosphorylation - PKC mediated phosphorylation seems to be more affected than the CaM kinase II phosphorylation during pre- and postnatal ethanol exposure to rat brain. The effect is more prominent at the early stage of development.

11. Tyrosine kinase activity decreased during ethanol exposure in membrane as well as cytosol over development, but in the presence of vanadate the activity was increased, indicating the balance between protein tyrosine kinase and protein tyrosine phosphatases might be lost during ethanol exposure.
12. Increased phosphorylation of specific phospho-tyrosine protein was observed in the presence of vanadate in membrane at the age of 30 and 90 days during ethanol exposure.

In summary, this study clearly showed alterations in the protein phosphorylation, changes in the expression and activities of protein kinases in alcohol exposed rat brain. Considering the importance of different protein kinases as an integral part of the signal transduction cascade and in particular, the PKC, CaM kinase II and tyrosine kinase enzymes in a number of brain functions, perturbations in these enzymes might likely be responsible for many of the chronic effects of ethanol on the brain and this could possibly underlie the mechanism contributing to Fetal Alcohol Syndrome (FAS). Since protein phosphorylation is a strategic event involved in neuronal development, ethanol induced changes in these protein phosphorylation events mediated by specific kinases could be directly responsible for the neurotoxic effects of ethanol contributing to memory loss and cognitive impairment in chronic alcoholics and further explains the neuronal degeneration.

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