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). In fact, 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 $\alpha$, $\beta I$, $\beta II$ and $\gamma$ which are calcium dependent and activated by diacylglycerol (DAG), novel PKCs (nPKCs) 5, 6, $\eta$, 6 and $\mu$ are calcium independent and atypical PKCs (aPKCs) $\zeta$ and $X$ (i) isoforms, which neither require $\text{Ca}^{2+}$ 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 synaptosomes, 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-32P] 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, 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 μl which contained 30 mM Tris-HCl buffer pH 7.4, 10 mM MgCl2, 1 mM DTT, 0.4 mM CaCl2, 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 KCl eluates as described by Huang and Huang (1991). The reaction was started by the addition of 10 µM ATP [γ-32P] ATP (1-2 μCi) (3000 Ci/mmol) followed by incubation at 30°C for 5 min. The samples were immediately spotted 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·min⁻¹·µg 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.

Immunoochemical analysis of PKC isoforms

For the immunoochemical 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 (75µg/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 α, βI, pH, γ, 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 H₂O 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 α, βI, βII, γ 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 MgCl₂ and 1 mM DTT and 100 μM EGTA to chelate calcium. The reaction was started by the addition of 10 μM ATP, [γ-³²P] ATP (2 uCi per assay) (3000 Ci/mmoll) 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 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.

**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 μl of 20 mM Tris-HCl buffer (pH 7.4), 10 mM MgCl₂, 1mM DTT, 0.4 mM CaCl₂, mixed micelles containing 10 μg/ml of phosphotidylserine, 1μg/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-³²P] 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 isoform 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 α, βI, βII, γ, nPKCs γ, δ, 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, γ, δ, ε 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 α, γ, δ, 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, γ, 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 e 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 iso 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 PO$_4^{3-}$ incorporated into the exogenous histone$^4$ 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\textsuperscript{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 \textsuperscript{32}P incorporated into the exogenous histone\textsuperscript{1} minute\textsuperscript{1} microgram of protein. The data points are mean±SEM (bars) values of five separate experiments. \textit{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.
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
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. Coronal sections (20 μm) of rat brain were incubated with anti-PKC antibody. Water treated animals served as controls.

C₈, C₃₀, C₉₀ immunoreactivity of PKC in control cerebral cortex seen at x 400 magnification.

E₈, E₃₀, E₉₀ increase in immunoreactivity of PKC in ethanol treated cerebral cortex seen at x 400 magnification.

C = Control
E = Ethanol treated
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.
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\(^{\circ}\)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\(^{2+}\) 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\(^{\circ}\)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. 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\(^{2+}\)/phosphatidylycerine 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; FM = 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. 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.** 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. 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. 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.

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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.

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**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 \( \beta I \), \( \beta III \) 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 \( \alpha \) isoform, which appears to be translocated from membranes to cytosol at the later age groups namely 30 and 90 days. The data indicate 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 isoforms such as \( \beta I \) and \( \beta III \) 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 \( \beta I \) and \( \beta III \) 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 PC12, Messing et al., (1991) have shown that chronic exposure to ethanol increases PKC 6 and e isoforms where a selective over expression of PKC ζ 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 α, βI modulate the release of acetylcholine, dopamine and GABA neurotransmitters from striatal slices (Tanaka et al., 1986; Cubedulu et al., 1989; Chandler and Leslie, 1989). The PKC-βII is involved in the functions related to GABAergic neurons; while neurotransmitter of PKC-γ immunoreactive neurons are yet to be identified. Long term administration of ethanol to rats causes reduction in choline acetyltransferase and a slight loss of neurons in the nucleus basalis (Arendt et al., 1988). Furthermore, ethanol is also involved in the disruption of serotonergic and adrenergic pathways in amnestic alcoholics (Charness et al., 1989). All these possibilities 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\(\gamma\) 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 synaptosomes in low Ca\textsuperscript{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; Hecmskerk et al., 1991; Houbre et al., 1991; Baudier et al., 1989). These have been implicated as substrates for Ca\textsuperscript{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 phosphoprotein 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 -γ to lesser extent seem to be involved. The data also suggests the degradation or decreased levels of PKC δ 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.