# Differential translocation of protein kinase C isozymes in rats characterized by a chronic lack of LTP induction and cognitive impairment

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**Abstract The translocation of protein kinase C isozymes was investigated in an animal model of cognitive deficit and lack of induction of long-term potentiation (LTP). In MAM rats, presynaptic α, β, ε PKC showed enhanced translocation, while postsynaptic γ PKC displayed decreased translocation when compared to control levels. This imbalance of PKC isozyme translocation between the pre- and post-synaptic compartment might therefore represent a possible molecular cause for the lack of synaptic plasticity observed in these animals.**

*Key words:* Protein kinase C; Translocation; Synaptic plasticity

## 1. Introduction

The $Ca^{2+}$/phospholipid-dependent protein kinase (PKC) is a serine/threonine kinase highly expressed in the brain where it has been implicated in a variety of neuronal functions among which synaptic plasticity is one of the most widely studied [1]. At the cellular level the involvement of PKC in plasticity at both pre- and post-synaptic sites has been investigated using either in vitro [2–5] or in vivo approaches [6–8]. PKC activation has been reported to modulate $Ca^{2+}$-dependent neurotransmitter release in nerve terminals [9,10] and to phosphorylate neurotransmitter receptors and ion channels in post-synaptic compartment [11,12], thus influencing synaptic efficacy. Since PKC exists as a family of closely related enzymes ($Ca^{2+}$-dependent, α, β and γ; $Ca^{2+}$-independent ε, δ; and atypical η, ζ, ι, λ), showing differential expression at cellular and subcellular level [1], the effects observed in the different neuronal compartments might be mediated by individually acting isozymes. In particular it is known that: (i) the α isoform is widely expressed in the intermediate layer of cortex, in pyramidal cells and interneurones of hippocampus at the periphery of perikarya and in perinuclear areas; (ii) the β isoform is localized mainly in glutamatergic hippocampal neurons and in cortical pyramidal cells both pre- and post-synaptically; (iii) the neuron specific γ isoform is localized in dendrites of pyramidal cells of both cortex and hippocampus, and in the granule cells of dentate gyrus; (iv) the ε isoform is localized in the nerve terminals of neocortex and of hippocampal pyramidal cells, mossy fibers, Schaffer collaterals and perforanth pathway.

In order to examine the contribution of PKC isoforms localized in different neuronal compartments in synaptic plasticity events, we have utilized an animal model characterized by cognitive deficit and lack of LTP induction in hippocampal slices. These animals have been obtained by prenatal exposure, at the 15th day of uterine life, to methylazoxymethanol (MAM), an antiproliferative agent showing neuroepithelial selectivity [13]. As a consequence of the treatment, these animals show a marked cellular ablation in the intermediate layers of the cortex and in the cornu ammonis (CA) of the hippocampus in agreement with the neurogenetic gradient in rodents [14]. In adulthood, MAM rats are characterized by a marked alteration of synaptic plasticity revealed both as alterations in the inducibility of LTP in the CA1 region of hippocampus [15] and, at behavioural level, as impairment of learning and memory processes [16,17]. We have previously described an increased membrane-bound PKC concentration in the pre-synaptic compartment of the hypoplastic regions in MAM rats, while there was no difference in the total concentration of the kinase [8].

We now show that all the PKC isoforms present in the presynapse show increased translocation, whereas the γ isoform, selectively present in the post-synapses, is consistently less translocated. These data underline the important role of the compartmentalization of the different PKC isozymes in modulating synaptic plasticity.

## 2. Materials and methods

### 2.1. Animal treatment

Sprague Dawley pregnant rats were obtained from Charles River (Calco, Italy) and housed in separate cages. On gestational day 15 the rats received a single intraperitoneal injection of either 25 mg/kg MAM acetate (Sigma, St. Louis, USA) diluted in sterile saline, or of the vehicle alone. Litters were born on day 22 or 23 of gestation. On the 21st day after birth, pups were weaned and housed under standard conditions with water and food ad libitum. At 2 months of age animals were killed by decapitation and brain areas, i.e. cortex and hippocampus, rapidly dissected, weighed and processed.

### 2.2. Subcellular fractionation and immunostaining

Purified synaptosomes from cortex and hippocampus of control and treated animals were prepared according to Dunkley et al. [18]. Synaptosomes prepared were resuspended in 0.5 ml of lysis buffer containing: 20 mM Tris-HCl pH 7.5, 1 mM DTT, 2 mM EGTA, 10 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF). The lysed suspension was transferred into Beckman tubes and centrifuged at 100000×*g* for 60 min. The resultant supernatant was processed as the cytosolic fraction. The pellet was suspended in lysis

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*Abbreviations:* CA, cornu ammonis; LTP, long-term potentiation; MAM, methylazoxymethanol; NMDA, *N*-methyl-D-aspartate; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride


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buffer containing 0.1% Triton X-100 for 45 min at 4°C. The centrifugation step was repeated and the supernatant represented the membrane fraction. No residual PKC immunoreactivity was found in the pellet obtained after this step.

Polyclonal antibody against α, β, γ and ε isozymes (Boheringer-Mannheim, dilution 1:1000) were utilized to analyze SDS-PAGE separation of total, cytosolic and particulate synaptosomal fractions. Blotting on nitrocellulose paper was performed in a running buffer containing 0.025 M Tris-HCl, 0.192 M glycine, 20% methanol, pH 8.3 at 60 mA for 15 h. Immunostaining was performed as previously described [8] and revealed using enhanced chemiluminescence (ECL Amersham, Italy).

Membrane and soluble fractions of brain area homogenates were prepared using the following method: brain areas were pottered glass/Teflon at 700 rpm, centrifuged at $3000 \times g$ and supernatant (S1) was collected and transferred into Beckman tubes and centrifuged at $100\,000 \times g$ for 60 min. The resultant supernatant (S2) was processed as the cytosolic fraction. The pellet was suspended in lysis buffer containing 0.1% Triton X-100 for 45 min at 4°C. The centrifugation step was repeated and the supernatant represented the membrane fraction. No residual PKC immunoreactivity was found in the pellet obtained after this step.

*2.3. Data analysis*

Western blots were subjected to densitometric analysis, performed on a computerized image analysis system using a program (NIH-Image) developed by Dr. Wayne Rasband (NIH, Bethesda, USA). Five different experiments were tested for statistical significance with a Student's *t*-test; differences are expressed as mean ± standard error (S.E.M.). Coefficient of variation was 2.25 and 2.55% for the homogenate and synaptosome experiments, respectively.

## 3. Results

To investigate whether a selective PKC isozyme might be differentially regulated in its translocation in nerve terminals of MAM rats, we performed Western blot experiments with specific antibodies to evaluate the concentration of membrane-bound α, β and ε PKC isozymes in cortical and hippocampal synaptosomes. Fig. 1 shows the densitometric analysis of 5 independent experiments performed on membrane fractions of cortical synaptosomes of both experimental groups; statistically significant increases in α PKC to 145 ± 16.3%, of β PKC to 133 ± 4.5% and of ε PKC to 148 ± 12.6% of control levels were observed in MAM rats (Student's *t*-test; MAM rat vs. control $P < 0.001$). Similar results were obtained in hippocampus where α PKC was increased up to 144 ± 14.1%, β PKC to 138 ± 13.0% and ε PKC 124 ± 11.9%. Representative Western blots of PKC isoforms are reported in the insets over the corresponding isoform bar.

The modification of membrane-bound γ PKC was studied in the particulate fraction obtained from homogenates of cortex and hippocampus of control and MAM rats, since it is present in neither glial cells nor nerve terminals. Fig. 2 shows densitometric analysis of 5 independent Western blots and immunostaining with a specific antibody raised against γ PKC (see inset) in the particulate fraction obtained from hippocampal homogenates from both experimental groups; statistically significant decreases to 65 ± 5.2% of control levels was observed in MAM rats. Similar results (82 ± 4.3%) were obtained in the cortex.

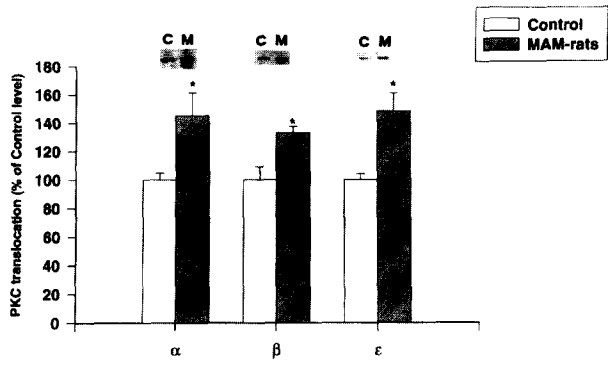


Fig. 1. Quantitation of Western blots of membrane-bound α, β and ε PKC in cortical synaptosomes from control and MAM rats. Insets show typical Western blot analysis for each isozyme. Data expressed as % of control rat levels. Means ± S.E.M. (n = 5). $^*P < 0.01$ MAM rat vs. control (Student's *t*-test).

To ascertain that the differences in membrane translocation observed in different PKC isozymes in MAM rats were not due to alterations in their relative content, the concentration of α, β, ε and γ PKC were measured. Table 1 summarizes data obtained in 5 independent experiments; no statistical differences were observed between control and MAM rats. The results of this set of experiments suggest that the relative amount of PKC subtypes is not changed in MAM rats in either the homogenates or the synaptosomes obtained from both areas. Furthermore, γ PKC was undetectable in synaptosomes, confirming previous reports indicating a selective post-synaptic localization of this isozyme in the forebrain.

## 4. Discussion

The role of different PKC isozymes in modulating synaptic plasticity has not been fully established. We report in this paper that the PKC isozymes present in nerve terminals of the hippocampus and cortex show increased translocation in an animal model characterized by a lack of LTP and cognitive deficit [15–17]. On the other hand, γ PKC, which is exclusively localized in the post-synaptic compartment, demonstrates a significant decrease in its translocation to the membrane compartment. These changes are not associated with changes in the total concentrations of the isozymes.

We have previously shown that MAM treatment induces, in the offspring, a marked hypoplasia of both cortex and hippocampus. This neuronal ablation results in increased PKC translocation and concomitant increased phosphorylation of B-50/GAP-43 [8], a specific PKC substrate localized in nerve terminals. Interestingly, glutamate release in these animals is

Table 1
PKC isozyme concentration in cortex and hippocampus of MAM rats

| Samples | α PKC | β PKC | γ PKC | ε PKC |
|---|---|---|---|---|
| Cortical homogenates | 93.1 ± 12.5 | 100.6 ± 11.9 | 98.9 ± 6.9 | 92.3 ± 12.1 |
| Hippocampal homogenates | 90.7 ± 8.3 | 101.7 ± 11.8 | 104.7 ± 11.7 | 93.5 ± 12.3 |
| Cortical synaptosomes | 106.3 ± 13.4 | 107.3 ± 8.0 | n.d. | 107.8 ± 8.7 |
| Hippocampal synaptosomes | 96.9 ± 2.8 | 103.8 ± 6.6 | n.d. | 105.7 ± 10.6 |

Data expressed as % of control rat levels. Means ± S.E.M. ($n$=5). n.d., not detected.

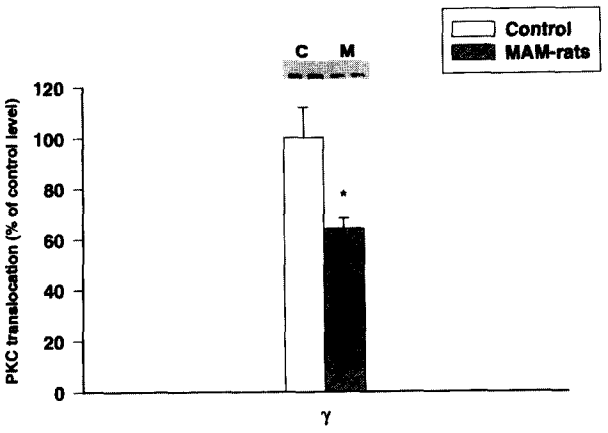


Fig. 2. Quantitation of Western blots of membrane-bound γ PKC in hippocampal homogenates from control and MAM rats. Insets show typical Western blot analysis for γ PKC. Data expressed as % of control rat levels. Means ± S.E.M. ($n = 5$). $^*P < 0.01$ MAM rat vs. control (Student's $t$-test).

enhanced [19], in keeping with the hypothesis that pre-synaptic PKC activation modulates neurotransmitter release [9,10].

The finding that all presynaptic isozymes tested revealed a normal concentration but greater translocation in cortex and hippocampus of MAM rats compared to controls indicates that in the MAM rat, the hypothetical mechanism(s) leading to increased translocation, such as increased presynaptic metabotropic or noradrenaline receptor stimulation, is not isozyme-specific, but common to all PKC isoforms, suggesting the existence of a general presynaptic hyperactivation of PKC that might be implied in different synaptic functions.

To evaluate the degree of translocation of PKC in the post-synaptic compartment, we have exploited the notion that γ PKC is exclusively localized in the soma and dendrites, but not in nerve terminals [1], as also found in this study by Western blot analysis (Table 1). In MAM rats, this isoform results in consistently reduced association to the membranes (Fig. 2), suggesting its reduced activity. Whether other PKC isoforms, also localized post-synaptically, show reduced translocation is technically difficult to ascertain in an in vivo system.

Despite this, the observation that γ PKC shows reduced translocation in MAM rats is relevant in consideration of the important function of this isozyme in mediating both LTP induction [6] and spatial discrimination learning in mice [20].

In conclusion, our data suggest that PKC isozymes are involved in processes modulating synaptic plasticity in vivo and that changes in their activity occur in the opposite manner depending on their pre- or post-synaptic localization, at least in the animal model here described. Moreover, MAM rats will help us in elucidating the role of PKC substrates in pre- and post-synaptic events associated with activity-dependent synaptic plasticity.

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