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Research report

Isozyme specific changes in the expression of protein kinase C isozyme (α - ζ) genes in the hippocampus of rats induced by kindling epileptogenesis

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Abstract

The transcript levels of the protein kinase C (PKC) isoform genes during the development of a kindled epileptogenic focus, elicited by stimulation of Schaffer collateral/commissural fibres in the CA1 area of the rat hippocampus, were compared with the expression levels in control animals using a semi-quantitative in situ hybridization approach. In the hippocampus of control animals, the levels of PKC- α - ζ transcripts showed a gene-specific expression pattern and significant differences in expression level were observed between the neurons of CA1, CA3 and fascia dentata. In the early stages of kindling epileptogenesis, i.e. following 6 and 14 afterdischarges, specific changes in the expression levels of PKC- β , - ϵ , and - ζ but not of PKC- α , - γ , and - δ were found. PKC- β expression was decreased in CA1, while the PKC- ϵ and - ζ specific hybridization signals were increased in CA1, CA3 and fascia dentata. In fully kindled animals, that had experienced 10 generalized seizures, most expression levels tended to return to control values. One month after the last seizure no significant alterations were encountered. These results indicate an involvement of specific PKC-isoform gene expression in the induction of an epileptogenic focus, but not in the maintenance of the long-lasting kindled state.

Keywords: PKC; Kindling; Epilepsy; In situ hybridization; Gene expression; mRNA; Plasticity

1. Introduction

The kindling model of epileptogenesis is a model to study the induction and expression of a focus for epileptiform activity. Kindling epileptogenesis is effectuated by electrical tetanic stimulation, repeated at regular intervals, of a specific brain site [5]. In the course of kindling, the afterdischarges triggered by the stimulation become progressively longer and are accompanied by convulsive behaviour of increasing severity [25]. The enhanced susceptibility for the generation and manifestation of epileptiform activity in the kindled animal is persistent for a long time [5].

The acquisition of the kindled state by Schaffer collateral/commissural fibre pathway stimulation is accompanied by a substantial increase in excitability of the local hippocampal CA1 network which is manifested by a re-

duction of paired-pulse inhibition of local evoked field potentials [13,36] and a diminished sensitivity of the pyramidal neurons for the inhibitory action of γ -aminobutyric acid (GABA) [11]. However, the reduction of GABAergic inhibition is associated with only a small reduction in the binding of GABA_A-receptor ligands [32], and is even paralleled by an unexpected increase of the expression levels of genes that encode for the subunits of the GABA_A-receptor [9]. These confounding results led us to consider the possibility that the diminished GABA_A-receptor function in kindled tissue may be a consequence of modifications in the mechanisms that regulate the activity of this receptor, particularly phosphorylation conditions. Since GABA_A-receptors contain several consensus sequences for phosphorylation by protein kinase C (PKC) and the phosphorylation of recombinant GABA_A-receptors by PKC results in the reduction of GABA-activated currents, it has been suggested that PKC activity is an important determinant for the GABA_A-mediated response in vivo [16,18].

PKC is a family of structurally and functionally closely related serine/threonine kinases that were originally thought to be activated by the second messengers Ca²⁺

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and diacylglycerol resulting in a translocation to membranes where the activity is expressed [29–31]. The PKC family can be subdivided in three groups that differ in terms of requirement for activation, distribution in mammalian tissue and structure: (i) the classical cPKC group (α , β I, β II, γ), activated by Ca^{2+} in the presence of phospholipids and diacylglycerol; (ii) the novel nPKC isoforms (δ , ϵ , η , θ) that lack the C_2 domain and can be activated in a Ca^{2+} -independent manner; and (iii) the atypical aPKC isoforms (ζ , λ) that have only one zinc finger-motif and do not require diacylglycerol, phorbol esters or Ca^{2+} for their activity [29–31].

Biochemical assays have revealed that in hippocampal kindled rats, the activity of membrane-associated fraction of PKC, is modified [1,3,15,20,24]. However, no attempts were made until now to differentiate between the activity of the various PKC isoforms. The aim of the present study was to investigate the potential involvement of changes in the PKC system induced by kindling stimulations by assessing the transcript levels of the different PKC isoforms expressed in the brain (α , $\beta\gamma$, ϵ , δ and ζ). Evidence is presented for a differential regulation of PKC isozyme expression in the hippocampal subregions and for specific changes in the expression of PKC- β , - ϵ and - ζ in the course of kindling induction but not at long-term.

2. Material and methods

2.1. *In situ* hybridization

Animals were deeply anaesthetised with ether and sacrificed by decapitation. The brain was rapidly removed and frozen on powdered dry ice and, wrapped in aluminium foil, and stored at -70°C . Coronal cryosections ($12\ \mu\text{m}$) were cut, thaw-mounted onto poly-L-lysine coated slides, and dried at room temperature. Sections were fixed for 5 min in 4% paraformaldehyde, washed in phosphate-buffered saline, dehydrated in 70% ethanol and stored in 95% ethanol at 4°C until use. Prior to hybridization, sections were removed from the ethanol storage boxes and air-dried. For this study, sections at a level of around -3.0 mm caudal to the bregma were selected, a region which is approximately 0.6 mm caudal with respect to the position of the stimulation electrode. *In situ* hybridization was carried out as described in detail previously [10,33]. Briefly; isozyme specific 35-mer oligodeoxynucleotides were 3' end-labelled with [^{35}S]dATP (N.E.N., 1200–1500 Ci/mmol) using terminal deoxynucleotidyl transferase (Life Technologies). The labeled probe was purified over a Sephadex G-25 spin column. The success of the purification of the probe from non-incorporated nucleotides was checked and the specific activity of the eluate was determined.

The probe was diluted to $1\ \text{pg/ml}$ (700–1300 cpm/pg) in a hybridization mix containing 50% formamide, $0.6\ \text{M}$

NaCl, $0.06\ \text{M}$ sodium citrate ($4\times\text{SSC}$), $100\ \mu\text{g/ml}$ polyadenylic acid, $25\ \text{mM}$ natriumphosphate, $1\ \text{mM}$ pyrophosphate, $5\times\text{Denhardt's}$, $200\ \mu\text{g/ml}$ sheared salmon sperm DNA and 10% dextran sulphate. Sections were incubated with this hybridization solution overnight at 37°C . Sections were rinsed several times in $1\times\text{SSC}$ at 20°C , and subsequently washed for 20 min at a stringency of $1\times\text{SSC}$ at 55°C . Sections were dehydrated and exposed to Kodak XAR-5 film. The total exposure time was optimized, using test slides, for the different probes to obtain an approximately comparable density of the autoradiograms allowing an equally accurate densitometric analysis.

The sequences of the six antisense oligonucleotides for the PKC isozymes were: PKC- α 5'-CGGGGCCAGCT-TGGCTTTCTCGAATTCTG CCTG-3'; PKC- β 5'-CCT-TGGTACCTTGGCCAATCTTGGCTCTCTCAAAC-3'; PKC- γ 5'-GAATGGG AGAGGAAGAGGGGCCATC-CGCACTCTC-3'; PKC- δ 5'-AGACAGCTGTCTTC TTCTCGAATCCCTGGTATATT-3'; PKC- ϵ 5'-TAGACGACGAGGCTCGGTG CTCCTCTCCTCG-GTTG-3'; PKC- ζ 5'-GTCTTGGTGGCCAGCATCC-CTCTCTGG CTGCTTGG-3'. The probes were obtained from Life Technologies and the sequences of the probes were based on published cDNA sequences [14,21–23]. On Northern blots of total RNA from rat forebrain, the probes identified transcript sizes that agreed with those reported earlier [2,22,34]. The β -probe recognized both the β -I and the β -II alternative splice forms of the PKC- β gene. When a 50-fold excess of unlabeled probe was added to the hybridization mix no hybridization signal was detected.

2.2. Densitometric analysis

The densitometric analysis of the obtained autoradiograms was described previously [10]. In short, the ipsilateral (left hemisphere) and the contralateral hippocampal areas within the autoradiogram were scanned and digitized. Of the three mounted sections only one was used for analysis since the variation between animals was found to be larger than the negligible variation between different sections of one animal (data not shown). All stored images were assigned a code. Quantification was carried out by an observer who had no knowledge of the treatment that the animals under study had been subjected to. The codes were broken only after completion of the quantification. Using a fixed sequence of grey level thresholds and erosion/dilation steps, we constructed a sample window over the pyramidal and granular cell layer. Subsequently, the mean extinction value (absorbance) for the different selected regions was calculated. The linearity of the relation between the determined extinction value and the actual amount of probe specifically hybridized with the complementary mRNA sequence present in the section and also the reproducibility of the quantification procedure, were described before [10].

2.3. Kindling procedure

Male Wistar rats (200–225 g, Harlan, Netherlands) were used. Stainless-steel, trimel insulated, electrodes were placed in the CA1 area of the left dorsal hippocampus of rats under pentobarbital anaesthesia. The stimulation bundle was placed in the Schaffer-collateral/commissural fiber pathway and the recording bundle was positioned in stratum radiatum of CA1. The details of this procedure were described previously [13]. After 2 weeks of recovery, the rats were connected to a stimulation/recording device to enable the delivery of kindling stimulations consisting of a train of 50Hz pulses of 1–2 s duration at an intensity of 200–300 μ A and to carry out local electroencephalographic (EEG) recordings.

2.4. Experimental design

A group of 61 implanted animals was divided into a non-stimulated control group ($n = 24$) and a group ($n = 37$) that received, twice daily, kindling stimulations at an intensity supra-threshold for the induction of an afterdischarge [5]. The animals of the control group were handled throughout the experimental period in a way comparable to the kindled rats but did not receive tetanic stimulations.

The behavioural and EEG characteristics of kindling development, initiated by tetanic stimulations to the Schaffer collateral/commissural fibers in the dorsal hippocampus, have been presented before [9,13]. The expression of PKC mRNAs was studied in four groups of animals that were sacrificed at different stages of kindling. Two groups were studied in the acquisition phase of kindling epileptogenesis, characterized by the steady increase of afterdischarge duration: (i) the 6-AD group ($n = 8$) sacrificed after the 6th tetanic stimulation which triggered afterdischarges lasting 36 ± 8 s (mean $\pm$ S.E.M.) and (ii) the 14-AD group ($n = 8$) after the 14th afterdischarge, lasting 60 ± 12 s. The animals of these groups were fixed 24 h after the session. The two other groups were fixed after kindling was fully established as evidenced by the occurrence of generalized seizures: (iii) the fully-kindled group ($n = 8$) sacrificed 24 h after the 31th afterdischarge, lasting 111 ± 6 s, (total 10 ± 1 class 5 seizures [25]). Two small additional groups were sacrificed at 2 h ($n = 3$) and 72 h ($n = 2$) after the last convulsion. The persistent changes in kindled tissue were studied in the (iv) long-term group ($n = 8$); these animals were kindled to the same degree as the fully kindled animals (mean total number of 31 afterdischarges lasting 91 ± 13 s and resulting in 9 ± 1 class 5 seizures) but they were killed 28 days after the last seizure. The rats of the control group were divided over the different stimulated groups and their brains were fixed at the same time as the latter.

2.5. Statistical analysis

Statistical comparison was carried out for each analyzed hippocampal region (CA1, CA3, fascia dentata) indepen-

dently. The paired Student's *t*-test revealed no statistical differences between ipsi- and contralateral hemisphere in any of the control and kindled groups studied and further analysis was, therefore, carried out on the mean extinction value of the two hemispheres. The variation in measured extinction values was comparable in control and kindled groups; S.E.M. represented typically between 3 and 9% of the mean extinction value.

For the statistical analysis of the kindling induced alterations of PKC isoform mRNA expression, the following statistical analysis of the determined extinction values was carried out. First, the control animals fixed along the 6-AD, 14-AD and fully kindled groups were compared by a one way ANOVA and no significant differences were found between these control groups ($F_{2,13}$; $P > 0.05$). Second, for further analysis the control animals were pooled into one group ($n = 16$) and the extinction values of the 6-AD, 14-AD and the fully kindled groups were analyzed with a one-way ANOVA followed by a post-hoc test comparing the kindled groups with the control group using the Student's *t*-test. For PKC-isoform β , a small but significant decrease in expression was observed in all hippocampal areas of the long-term control group in comparison to the controls sacrificed 4–6 weeks earlier (-7%). For PKC- ζ , the expression was increased in the long-term control group ($+20\%$), indicating age-dependent effects. Therefore, the long-term kindled group was compared only with the control group of rats fixed at the same time.

To facilitate the presentation of the changes found in the different kindled groups, we determined the percentual changes of the extinction values in the kindled groups in comparison to the control groups by normalizing the mean extinction of the kindled group using the mean of control group, set at 100%, as reference level. The estimated differences from the control value and the outcome of the statistical analysis are presented in Fig. 3 for the pyramidal cell layer of CA1 and CA3 region and the granular cell layer of the fascia dentata.

In order to gain insight in the relative abundance of the different PKC transcripts within the hippocampus in control animals, the obtained extinction values were corrected for the differences in specific activity of the probe used for the in situ hybridization and for the different film exposure durations [9]. For each probe, the correction factor for CA1, CA3 and fascia dentata was identical.

3. Results

3.1. Expression pattern of PKC-isozymes in coronal sections

In coronal sections at the level of the hippocampus, the distribution of the six PKC isoform mRNAs is unique for each species of PKC transcripts. The observed hybridization patterns are illustrated in Fig. 1. The PKC- α expres-

sion was highly abundant in the hippocampus, low levels were detected in the piriform cortex whereas the neocortical, thalamic and hypothalamic areas did not present clear expression. PKC- β transcripts were observed in the neocortex, hippocampus, habenular nucleus, caudate putamen, and amygdaloid nuclei. PKC- β mRNA levels in the CA1 neurons was prominent in comparison to CA3 or fascia dentata. Close examination of the autoradiograms also showed that the CA2 pyramidal neurons, at the border between CA1 and CA3 are without detectable levels of PKC- β transcripts. The antisense PKC- β probe recognized both splice variants β I and β II, but in view of the reported absence of PKC- β I immunoreactivity in contrast to a strong PKC- β II immunoreactivity in the rat hippocampus, it may be assumed that the observed hybridization signals reflect mainly the distribution of PKC- β II transcripts [6,28,31]. The expression of PKC- γ was prominent in the hippocampus but detectable levels were also present in neocortex, piriform cortex, amygdala and thalamus. The distribution of PKC- δ mRNA was restricted to the thalamus. Even prolonged exposure of the sections did not reveal detectable amounts of PKC- δ hybridization product in any other region. For PKC- ϵ , the expression was limited to the hippocampus with low expression levels in the piriform cortex. PKC- ζ hybridization revealed a widespread, but low-abundant, expression in all brain regions present in the coronal sections.

The densitometric analysis of stratum pyramidale and

Fig. 2. Comparison of the PKC isoform mRNA expression levels $\pm$ S.D. (control animals; $n = 16$) in the three analyzed subfields of the hippocampus. Values were obtained by correcting the measured extinction value for variations in exposure time and in specific activity of the probe corrected for the isotopic decay over the exposure period. n.d.e.l., non detectable expression level.

stratum granulosum in the autoradiograms allowed an analysis whether the expression of PKC genes is differentially regulated in the neurons of CA1, CA3 and the fascia dentata of the hippocampus. For this analysis, the densitometric data of the pooled group of control animals ($n = 16$) fixed along with the 6-AD, 14-AD and fully kindled groups was used. The probes used in our study were of the same length, had a similar GC content, and were hybridized under identical conditions. The different signal

Fig. 1. PKC- α , - β , - γ , - δ , - ϵ and - ζ mRNA distribution in coronal sections of control rats. The ipsilateral, electrode implanted, hemisphere is on the left side. Exposure times of the hybridized sections were chosen to allow equally accurate quantification of hippocampal regions. Note the different patterns of expression in hippocampus, thalamic and hypothalamic regions. Bar = 5.0 mm.

intensities, therefore, likely reflect the relative amounts of mRNA present for each isozyme in the tissue. The expression patterns observed in this group of control animals were highly reproducible and, consequently, highly significant differences of the extinction values were found between CA1, CA3 and fascia dentata for all isoforms ($P < 0.0001$; paired Student's *t*-test). When the extinction values were corrected for the differences in specific activity and in exposure duration, a comparison of the relative mRNA levels for the different PKC-isoforms was allowed. The outcome of this correction and the differences in expression levels between CA1, CA3 and fascia dentata is presented in Fig. 2. The most noticeable differences were: (i) the abundant expression of PKC- α in CA3 in comparison to CA1 and fascia dentata; (ii) the high level of PKC- β expression in CA1 and (iii) the high expression of PKC- γ in the pyramidal neurons in comparison to the granular neurons of the fascia dentata. PKC- α was the most abundant transcript of the PKC-isoform gene family. PKC- β , PKC- γ and PKC- ϵ were expressed at approximately equal levels and PKC- ζ was the least abundant.

Fig. 3. Relative changes in mean extinction values of the in situ hybridization autoradiograms of PKC isoforms in the different kindled groups (% $\pm$ S.E.M.). The mean extinction value obtained in the control group was used as reference and set at 100%. Statistical comparisons with respect to controls were carried out directly on the assessed extinction values using a one-way ANOVA, followed by a Student's *t*-test of the different kindled groups vs. the controls. * $P < 0.05$; ** $P < 0.025$; *** $P < 0.010$.

3.2. Kindling induced changes in PKC-isozymes expression

The relative changes in mean extinction in the kindled groups as compared to the values of the controls are presented in Fig. 3. Significant changes induced by the kindling stimulations were found for PKC- β , PKC- ϵ and PKC- ζ expression while no significant alterations were found for PKC- α , PKC- γ and PKC- δ mRNA levels. One way ANOVA revealed significant changes for PKC- β expression in CA1 ($F_{3,34} = 7.77$; $P < 0.0004$) but not in CA3 or fascia dentata. In all three kindled groups studied at a time interval of 24 h after the stimulation, the PKC- β expression was significantly decreased in CA1 by 10–22%. The levels of PKC- ϵ mRNA were significantly changed in CA3 ($F_{3,34} = 3.24$; $P < 0.034$) and fascia dentata ($F_{3,34} = 3.55$; $P < 0.025$). In these areas, the expression of PKC- ϵ was increased by 16–21% in the 6-AD and 14-AD groups. In fully kindled animals, the PKC- ϵ expression was significantly enhanced by 16% in the granular neurons only. The PKC- ϵ hybridization signals in CA1 showed also a tendency to increase but this alteration was not significantly different from controls. PKC- ζ mRNA was increased in all hippocampal regions (CA1 $F_{3,31} = 4.11$; $P < 0.015$; CA3 $F_{3,31} = 3.00$; $P < 0.046$; fascia dentata $F_{3,31} = 6.61$; $P < 0.0014$). Levels were elevated in all areas in the 6-AD group but after 14 afterdischarges a significant increase was found in the fascia dentata only, and in the fully kindled group no significant alterations were found for this PKC- ζ . Moreover, in the few animals sacrificed at a time interval of 2 h ($n = 3$) or 72 h ($n = 2$) after the last generalized convulsion, no clear changes in the expression were observed. No significant changes were found in the long-term group, studied 4 weeks after the last seizure.

4. Discussion

Using semi-quantitative in situ hybridization techniques, the expression levels of six different PKC-isoforms were studied in control rats and compared with those in kindled animals that were sacrificed at different stages in the course of kindling epileptogenesis and at long-term after the establishment of a kindled focus.

The distribution of the PKC mRNA patterns are in good agreement with the patterns reported [4,8,19,31,34,35] and with the distribution of the PKC proteins as visualized by immunohistochemistry [8,19,31]. When the extinction values were corrected for differences in specific activity of the labeled oligonucleotides and differences of exposure duration, it was shown that the expression of the PKC isoforms is subject to a gene-specific and a regionally differential regulation.

The main outcome of this study is that Schaffer collateral kindling is associated with specific alterations in the expression of PKC- β , - ϵ and - ζ but not of PKC- α , - γ ,

and δ mRNA in the hippocampal formation. The PKC- β isoform was down-regulated (in CA1), whereas PKC- ϵ (in CA3 and fascia dentata) and PKC- ζ (in all hippocampal areas) were up-regulated. This implicates that the calcium-activated forms of PKC are either unchanged or decreased while the calcium-independent forms are enhanced [30]. At present, the functional consequences of these heterogeneous set of alterations, f.i. on GABA_A-receptor mediated function, are difficult to interpret since only limited information is available concerning the precise functional role of the different PKC isoforms in terms of cellular localization, endogenous target proteins or activation routes [16–18,30]. PKC activity has been implicated in the induction and maintenance of long-term potentiation (LTP) [7]. A transient translocation of all PKC isozymes, 15 s after LTP induction via Schaffer collaterals in CA1 area, is followed by a persistent increase of a constitutively active kinase, generated by the proteolytic cleavage of the regulatory and catalytic domain of PKC- ζ [26]. The enhanced level of PKC- ζ mRNA found in this study may sustain a similar persistent activation in the early stages of kindling development. The location of PKC- ϵ in nerve terminals of hippocampal neurons suggests a role in presynaptic processes like exocytosis and consequently the observed enhanced levels of PKC- ϵ mRNA in kindled animals may maintain the enhanced neurotransmitter release in kindled tissue [12,27].

The presence of specific alterations, particularly during the stages of progressive kindling epileptogenesis, shows that paroxysmal activity, triggered by repeated tetanic stimulations, rapidly leads to alterations in the gene expression of specific PKC-isoforms. However, in the fully kindled state the PKC expression had returned to baseline levels in most areas and at long-term after the last seizure no significant changes were found, suggesting that the changes in PKC transcript levels are primarily associated with the acquisition of the kindled state and may not be essential for the maintenance of the enhanced excitability.

Biochemical assays of PKC activity in tissue of kindled rats have been carried out only under circumstances of maximal stimulated PKC activity by addition of phospholipids and Ca²⁺, and a detailed analysis of the activity of the different isoforms is not yet available. The biochemical studies on the PKC activity in the hippocampus of kindled rats revealed that, 16 weeks after the last seizure, the PKC activity of the membrane-bound fraction was enhanced by 24–33% while the cytosolic fraction did not differ [1,15]. This alteration in the membrane-associated PKC activity was only significant in the stimulated hemisphere [1]. When the PKCs were separated into three fractions on hydroxyapatite columns, an increased activity associated with the β - and the γ -fractions was found at 1 and 4 weeks after the last seizure [20]. In contrast, Osonoe et al. [24] reported one day after the last amygdala kindled seizure, a decreased PKC activity which was normalized after 2 days and after two weeks. Based on the available information, it

is difficult to correlate the unilateral persistent enhanced membrane-associated PKC activity with the bilateral changes at the mRNA level, although the results of the study by Osonoe et al. [24] are compatible with those of the present report. To clarify these issues it is necessary to investigate whether the changed PKC-expression of different isoforms is closely followed by parallel changes at the protein level, as can be studied by quantitative Western blotting or immunocytochemistry.

In conclusion, Schaffer collateral kindling of the rat hippocampus leads to isoform specific alterations in the expression pattern of PKC genes that are particularly associated with the early stages of kindling acquisition. Further studies are needed to elucidate the possible functional consequences of the changes in the expression of PKC- β , ϵ and ζ in relation to the excitability of the local neuronal circuits and the process of kindling epileptogenesis.

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