PH.D. THESES

CHANGES IN CALMODULIN GENE EXPRESSION IN THE RAT BRAIN DURING ONTOGENETIC DEVELOPMENT AND IN EXPERIMENTAL CONDITIONS

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INTRODUCTION

The calcium metal ion (Ca^{2+}) plays an uniquely important role in the physiology of higher organisms, and is involved in the regulation of many cellular processes ranging from transcription and neurotransmitter release to muscle contraction and cell survival. The intracellular concentration of free Ca^{2+} is tightly controlled and usually very low in the cytosol (0.1 lM), whereas the extracellular concentration of Ca^{2+} is roughly 10 000-fold higher (1 mM). Various stimuli can trigger the opening of calcium channels that generate local intracellular Ca^{2+} waves. The approximately 100-fold increase in free Ca^{2+} concentration upon stimulation of a cell allows Ca^{2+}-binding proteins to bind Ca^{2+} ions. Several hundred Ca^{2+}-binding proteins have been identified and most of them share a common Ca^{2+} binding motif. This motif comprises about 30 amino acids and consists of a helix-loop-helix: it is commonly called the EF-hand motif. One of the most important proteins that belong to the EF-hand protein family is calmodulin (CaM).

CaM is a small, acidic, heat-stable, monomeric protein abundant in all eukaryotic cells. CaM participates in the regulation of numerous essential cellular pathways, including motility and cell cycle progression. CaM is also necessary for specialized functions such as neurite outgrowth, neurotransmitter synthesis and release as well as signal transduction events and gene expression pattern regulation. CaM is a principal transducer of the calcium signal in

mammalian cells, therefore its not surprising that the CaM protein is highly abundant in the central nervous system, primarily in neurons, although reasonable amounts of CaM can be found in glial cells as well.

The 148-amino acid sequence of the CaM protein is highly conserved, and is encoded by multiple genes in vertebrates. The three CaM genes transcribe eight major mRNA species of different sizes by means of alternative polyadenylation, i.e. 4.2 kb, 4.1 kb, 1.7 kb and 1.0 kb for CaM I, 1.4 kb for CaM II, and 2.3 kb, 1.9 kb and 0.9 kb for CaM III. The coding regions of the three genes are identical, but differences in their 3' and 5' non-coding regions suggest a possibility for differential regulation of the genes.

A number of studies dealt with the determination of the expression patterns and the regulation of CaM mRNA content. Unfortunately, the majority of these studies focused on the expression of a particular CaM gene only, instead of all three CaM genes, and did not provide sufficient data on the expression of the CaM genes in the different intracellular compartments of the cell. Recent studies have revealed that the members of the CaM gene family differentially modulate the precise temporal and spatial regulation of the CaM protein content. Cellular mechanisms that regulate differential gene expression patterns under physiological conditions, and changes in gene expression patterns following various impacts, are not yet known, nor is the significance of the proportion of the diverse mRNAs.

AIMS

CaM interacts with a large and diverse array of targets specifically in a temporally and spatially regulated manner. The cell must regulate either the availability of CaM, its targets, or a combination of both. CaM is an intensely studied modulator and protein regulator of many catalytic reactions in the cell. Interestingly, many of these reactions reveal an apparent paradox of requiring CaM in reactions of opposing effects. Alternatively, the cell may control the temporal and spatial positioning of the mRNA instead of the protein, by mRNA translocation and local synthesis of the CaM, if necessary. With the aim of a better understanding the multiple levels of regulation of CaM we have carried out a series of investigations regarding the CaM gene expression patterns during development and in the adult rat under physiological and experimental conditions.

Our aims were:

1. Determining the *in vivo* expression patterns of the members of the CaM gene family during embryonic and early postnatal development. In these experiments, by utilizing radioactive *in situ* hybridization and high-resolution nuclear emulsion autoradiography, we demonstrated the gene expression patterns on a cellular level. We investigated whether the results of the quantitative tissue-specific expression in the developing rat brain is represented on a subcellular level. We also

investigated the possibility involving the targeting of CaM mRNAs to the relevant subcellular compartments such as dendritic and/or axonal processes.

2. Pattern analyses of the gene expression of the CaM gene family in the rat brain stem by quantitative and qualitative *in situ* hybridization. A comparative *in situ* hybridization study utilizing specific [^{35}S]cRNA probes complementary to CaM mRNAs has been performed in rat brain stem sections, with detection by quantitative film autoradiography. We investigated cellular CaM mRNA patterns utilizing qualitative color *in situ* hybridization.

3. Quantitative pattern analyses of the gene expression of the three CaM genes in the rostral and caudal parts of the rat trigeminal nuclei. We investigated whether differential intranuclear expression patterns can be found within these nuclei.

4. Quantitative analysis of the adult rat brain region-specific CaM mRNA patterns during chronic μ opioid receptor agonist ligand administration, withdrawal and antagonist ligand administration.

Methods and Results

1. Analysis of the CaM mRNA expression patterns during rat brain development

Male Sprague–Dawley embryos at embryonic day 19 (E19) and pups of different postnatal ages (1 day, PD1; 5 days, PD5; 10 days, PD10; 15 days, PD15; or 20 days, PD20) were killed by decapitation, and their brains were quickly removed, embedded in Cryomatrix embedding medium and frozen immediately at -70°C. Serial coronal cryostat sections (20 μm) were cut. On these slides radioactive *in situ* hybridization was carried out with CaM gene-specific [^{35}S]cRNA probes. To achieve high resolution, representative brain sections were dipped into Hypercoat nuclear emulsion and exposed for 10–30 days. Developed emulsion-coated tissue sections were examined in a microscope equipped for bright- and darkfield illuminations. The selected brain areas were the cerebral cortex, the cerebellar cortex and the hippocampus. All these areas develop laminar structures wherein the strict structural organization is suitable for the determination of a particular neuronal cell type (i.e. pyramidal cells of the cerebral cortex and the hippocampus, the granular cells of the dentate gyrus, or the Purkinje cells of the cerebellar cortex) or the different subcellular compartments of the neuron (i.e. dendrite rich molecular layers, or white matter structures containing mainly axonal tracts). In agreement with the results of the quantitative studies we observed the

strongest decrease of the CaM II mRNA content within the time frame examined. Our results indicate 1) an evident differential expression pattern of the multiple CaM genes in both tissue and cellular levels, 2) an intensive CaM gene expression detected during early postnatal development, and 3) that a large population of CaM mRNAs is targeted to the dendritic compartment in a gene-specific manner, and the level of the dendritic CaM mRNA population decreased during the development.

It seems well established that the changes in the CaM expression coincide with those coding its major target proteins. The apparently irregular expression profiles observed for each CaM gene in the cerebellum as a whole might be a result of a unique utilization of the CaM binding proteins in this region, especially during synaptogenesis.

2. Pattern analysis of the gene expression of the CaM gene family in the rat brain stem by quantitative and qualitative *in situ* hybridization

Coronal cryostat sections from the brain stem were cut, hybridized separately with antisense [^{35}S]cRNA probes specific for CaM I, CaM II, or CaM III mRNAs and exposed to autoradiographic film. Film autoradiographic images were analyzed by computer-assisted microdensitometry. Specific gray-scale values were calculated by subtracting the nonspecific values resulting from the hybridization

of the respective sense cRNA probes from those of the values of the antisense [^{35}S]cRNA probes. Our present aim was to determine the tissue-specific CaM gene expression in the brain stem. With the respect of the rostrocaudal extent of the trigeminal nuclei and the precise somatotopic organization of the sensory information we examined the distribution of the CaM mRNAs along the rostrocaudal axis within each nucleus of the trigeminal system.

Several lines of evidence – established by our workgroup – point to the differential neuronal expression of the three CaM genes. A thorough quantitative comparison of the mRNA levels corresponding to the three CaM genes in the brain under physiological conditions clarified the detailed regional distribution and the real proportions of these transcripts. However, microscopic observations of the CaM gene expression patterns concerning the rat brain stem areas had not yet been presented. Therefore, we performed qualitative, color *in situ* hybridization in the attempt to examine the CaM mRNA levels in the cells of the brain stem nuclei. Color *in situ* hybridization was based on utilizing sections hybridized with digoxigenin-labeled cRNA probes complementary to the multiple CaM mRNAs. Our experiments revealed 1) differential CaM gene expression in the nuclei of the rat brain stem, 2) that the CaM II mRNA content was generally the less abundant, and the highest differences in specific optical densities measured between the three CaM genes was more than two fold, 3) that the CaM gene-specific transcripts displayed a

clear differential distribution along the rostrocaudal axis and 4) our observations on the cellular level revealed that the low density of the CaM II mRNA population is due to a lack of expression of this gene by most of the cells.

3. Quantitative analysis of the brain region-specific CaM mRNA patterns during chronic opioid ligand administration

Male Sprague–Dawley rats (200–250 g) maintained under standard housing conditions were used. Animals were made dependent on morphine by a series of subcutaneous injections of morphine hydrochloride administered twice a day. Another group of animals received a 10 mg/kg dose of naltrexone after morphine treatment in order to develop withdrawal symptoms. Control animals were handled simultaneously by saline injections. In another set of experiments rats were treated for 5 days with the antagonist naltrexone with the same dose regimen used for morphine.

Morphine and its derivatives are the most often used opiate ligands as therapeutic drugs. Its powerful analgesic effects and the unfortunate side effects associated with it are the reasons for the comprehensive research on the cellular mechanisms underlying these effects. The MOR is the major receptor mediating the analgesic and rewarding effects of morphine. Most research to date has confirmed it's coupling predominantly with the G_i/G_o, the subunits of which are thought to be the major mediators of opioid action. Opioids

have been shown to open K^+ channels and close Ca^{2+} channels. This notwithstanding, there is also pharmacological evidence consistent with certain opioid receptor population coupling to G_s. All these research data indicate employment of multiple cellular signal mechanisms during chronic morphine stimulation. Since some of these cascade mechanisms need the involvement of CaM at several points, as well as cascade mechanisms induced by chronic opioid antagonist (naltrexone), we investigated the changes in the expression patterns of the three CaM genes during chronic morphine treatment, withdrawal and chronic naltrexone treatment. Our results demonstrate 1) a differential CaM gene expression regulation in distinct brain regions during opioid involvement, 2) that the cerebellar cortex – even thought it has very low opioid receptor density – displays the utmost significant changes in CaM gene expression, 3) in some cases diverse parts of a brain region containing different cell types or cellular domains gave the same response to opioid stimulation.

Conclusion

In agreement with the pivotal role of CaM in the nervous system, the multiple mRNAs corresponding to the three bona fide CaM genes are widely and differentially expressed in the rat brain during development, and in the adult rat brain under physiological conditions and during chronic stimuli. Our results indicate that the principle (multiple CaM genes — multiple CaM transcripts — one CaM protein) gives a necessity of exploring gene expression regulation considering all three CaM genes, and studies that focus on only one of the transcripts do not provide sufficient data. Moreover, with notable rostrocaudal extent and precise somatotopic organization of certain nuclei, it is expedient to broaden the investigation on to different parts of the nuclei. Investigations on the cellular level revealed that even different compartments of a neuron contain different proportions of CaM mRNAs, which presumably contribute differentially to the actual CaM protein levels. In different parts of the neurons or the brain, different stimuli might act in a unique manner on each CaM gene, resulting in shifts in the amounts and proportions of CaM mRNAs and finally in the amount of CaM protein on local demand.

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