CALMODULIN GENE EXPRESSION IN CENTRAL NERVOUS SYSTEM AREAS OF LOW CALMODULIN mRNA ABUNDANCE

PH.D. THESIS

BEATRIX KOVACS

THESIS ADVISOR: KAROLY GULYA, PH.D., D.SC.

DEPARTMENT OF ZOOLOGY AND CELL BIOLOGY
FACULTY OF SCIENCE
UNIVERSITY OF SZEGED

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Abstract

The calmodulin (CaM) protein plays a complex role in the regulation of the different cytoplasmic processes in general, and also (because of its paramount role in the neuronal signalization processes) in the nervous system as a whole in particular. While this protein is often present in medium-to-high concentrations in the nervous tissue, there are other regions (the spinal cord and the retina) that contain CaM only in minute quantities, and the amounts of the different CaM transcripts in these regions are also low. Our knowledge on the CaM gene expression in the different cell types of the spinal cord and the retina is sparse, mainly in consequence of their low CaM mRNA contents. Thus, during the initial stage of our research, we developed a sensitive color in situ hybridization method, employing a slightly alkaline pH (pH 8.0) in the hybridization mixture, which is sensitive enough to detect low-abundance transcripts in areas of the central nervous system (the white matter areas of the spinal cord and the retina) that may also have high lipid contents (the white matter areas of the spinal cord). The method was then tested for the detection of the different CaM gene transcripts in two central nervous system regions, the spinal cord and the retina, where these mRNAs are present in unusually low concentrations. The detection of CaM mRNAs in the spinal cord was hindered not only by the low abundance of these transcripts, but additionally by the high lipid content; our method was successfully employed here too.

On the basis of their morphology, we detected two distinct cell types that express CaM in the white matter area of the rat spinal cord. The medium-sized, astrocyte-like cells residing mainly in the dorsal column of the white matter displayed differential CaM expression: the CaM I mRNA content was most abundant, followed by the CaM III and CaM II mRNA contents in these cells. The CaM gene expression of the oligodendrocytes –in both the dorsal and the lateral columns – was less differentiated, although the CaM I mRNA content was slightly higher than that of the others. Our results indicate that 1) the CaM expression profile of the spinal cord is richer and more complex than previously thought on the basis of conventional radioactive in situ hybridization techniques, and 2) when a method that is sufficiently sensitive was used, more cell types could be

demonstrated to express CaM mRNAs; thus, in spite of their lower CaM expression and their lipid-rich environment, glial cells could also be visualized.

The neural retina is a tissue with low lipid content, where the conventional in situ hybridization techniques with relatively low sensitivity did not detect any CaM-expressing cells, in spite of the fact that the presence of the protein was previously demonstrated by immunohistochemical techniques. Our sensitive in situ hybridization technique revealed the presence of CaM mRNA populations in the adult rat retina, and we concluded that the expression levels of the different CaM genes were almost identical. In fact, the uniquely similar layer-specific retinal distribution pattern of these transcripts is the first such example for the different CaM genes in the central nervous system. Although the layer-specific distributions of these genes are strikingly similar, there are major differences in CaM expression within the different retinal layers. The strongest signals for all CaM mRNAs were demonstrated in the ganglion cell layer and the inner nuclear layer. Similarly intermediate signal intensities for all CaM genes were detected in the inner and outer plexiform layers, within the vicinity of the outer limiting membrane and in the pigment epithelium. Very low specific signals were characteristic in the outer nuclear layer and the photoreceptor inner segment layer, while no specific hybridization signal was observed in the photoreceptor outer segment layer.

In summary, our sensitive in situ hybridization technique was able to demonstrate the presence of low-abundance CaM transcripts in two central nervous system areas: the white matter of the rat spinal cord and the neural retinal of the adult rat. Our results contribute to a better understanding of the functional role(s) of the CaM protein and also of the CaM gene expression in these regions.

Introduction

Hybridization methods that are based on duplex formation between nucleic acids have become commonplace not only in basic and applied biomedical research, but also in everyday clinical practice. The ribo- and deoxyriboprobes labeled with radioactive nucleotides, chromophores or antibodies are suitable for the detection of extracted or

native tissue nucleic acid contents with high sensitivity and cellular or subcellular resolution. In recent years, detection systems based on riboprobes have quickly become the preferred choice among users because of their high specificity, convenient use in a wide concentration range of the target mRNAs and easy quantitability.

A number of factors influence the detection of tissue nucleic acid content by riboprobes. The high lipid content in myelin-rich regions of the nervous system can be a hindrance to the detection of mRNAs, as they decrease the penetration of the probe. Moreover, mRNA populations that are present in low abundance or in conformations that are inaccessible for the probe can also decrease the amounts of detectable mRNAs or the sensitivity of the protocol. In recent years, several in situ hybridization techniques have been developed to increase the amounts of detectable mRNAs or to sensitize the method for the detection of mRNA populations present in the tissue in low abundances. While methods such as lipid removal, (partial) enzyme digestion with proteases, tissue heating (e.g. microwave irradiation or autoclaving), tyramide signal amplification or in situ hybridization at slightly alkaline pH are used universally, certain means of tissue preparation, such as the use of frozen sections, have not benefited equally from these procedures. We have developed a highly sensitive in situ hybridization method that is suitable for the detection of low-abundance mRNA populations in both lipid-poor (the retina) and lipid-rich (the white matter of the spinal cord) regions of the central nervous system.

CaM is a multifunctional intracellular calcium ion (Ca^{2+})-receptor protein that is present in every eukaryotic cell and is involved in the regulation of numerous cellular functions. There are two Ca^{2+}-binding sites on each of its two domains. The binding of Ca^{2+} elicits the conformational change that is necessary for the interaction with the target proteins. CaM is abundant in the nervous tissue, where it is present mostly in the neuronal perikaryon, the nucleus and the dendrites; the latter contain the protein in characteristic granules. Although one of the main roles of CaM, displayed via the activation of its target proteins, is the synthesis of certain neurotransmitters, there are a number of other neuronal processes that are regulated by this protein, such as neurite outgrowth, the development

and maintenance of LDT and LTP, or microtubule (dis)assembly. Its role is also important in the maintenance of Ca^{2+} homeostasis and Ca^{2+} currents. Through the activation of different kinases, CaM regulates a number of processes in the cell nucleus: by activating different transcriptional factors and RNA-binding proteins, it plays an important role in the regulation of the cell cycle, the cell division and the gene expression. Although the Ca^{2+}/CaM complex lacks enzyme activity, it functions by activating more than 20 target enzymes, such as the cyclic (3', 5')-nucleotide phosphodiesterases, the adenylyl cyclases, nitric oxide synthase and the protein kinases. Perhaps the most important target enzymes are CaM kinase II and calcineurin.

At least 3 distinct bona fide CaM genes and 4 pseudogenes have been described in the rat. The different genuine CaM genes (CaM I, II and III) transcribe at least 7 mRNA species by alternative polyadenylation. Although encoded by multiple genes, the protein products have identical amino acid sequences. The CaM I transcripts are 4.0, 1.7 and 0.8 kb long, the lone CaM II transcript is 1.4 kb long, and the CaM III transcripts are 2.3, 1.9 and 0.9 kb long. There are major differences within the 5'- and 3'-untranslated regions of the different CaM genes; the different transcripts contain regulatory elements in different numbers and combinations. These short sequences are responsible for the unique regulation of these CaM genes, which not only display differential ontogenetic activity, but also undergo differentially targeted subcellular transport.

Wide expressions of the three CaM genes have been described in the developing and adult rat central nervous systems. Earlier studies in our laboratory contributed to a better understanding of the CaM gene expression in the adult rat brain. Strong CaM expression is characteristic of the cortical and hippocampal pyramidal cells, the hypothalamic magnocellular neurosecretory cells, the Purkinje cells, the neurons in the deep cerebellar nuclei, the motoneurons in the ventral horn of the spinal cord, the mitral cells of the olfactory bulb, and, in general, all larger neurons of the cortex, midbrain, basal nuclei and spinal cord. The CaM gene expression is considerably lower in the interneurons, and frequently undetectable in the glial cells. The amount of mRNA transcribed from the different CaM genes is much smaller in areas where there are few

neurons, e.g. the molecular layers of the hippocampus, cerebral and cerebellar cortices, and it is minimal in the white matter areas, such as the corpus callosum. A low CaM mRNA content is characteristic of the spinal cord white matter areas and the retina, where mRNA populations are extremely difficult to detect with conventional in situ hybridization techniques.

Earlier studies by our laboratory shed light not only on the widespread and differential distribution of the CaM transcripts under normal (physiological) conditions, but also on the characteristics of the CaM gene expression under certain experimental conditions, during early ontogenetic development or in certain in vitro systems, and additionally provided data on the differential intracellular CaM mRNA targeting.

Since most of the CaM-expressing cells in the central nervous system are neurons, CaM has been mentioned in connection with glial functions only rarely. Its presence in glial cells is clear from the numerous cellular functions mediated by CaM that are characteristic of glial cells. While the CaM level is generally low in these cells, certain pathophysiological or experimental conditions are known to increase the amount of this protein. The observation that CaM is present in glial cells under physiological conditions was not obvious for two reasons: 1) the glial CaM expression is low, and 2) the currently available in situ hybridization techniques are not sensitive enough for the detection of low-abundance transcripts, especially in areas with high lipid contents (the white matter of the spinal cord) or in connective tissues where the probe penetration can be hindered.

The presence of CaM has already been demonstrated in the vertebrate retina, with varying levels in the different retinal layers. CaM immunoreactivity has been observed in the ganglion, amacrine, horizontal and bipolar cells, and in the fibers emanating from the amacrine and ganglion cells of the inner plexiform layer of the rodent retina. The rod cells, however, have a very low CaM content across the vertebrate phylum. Interestingly, no data are available from in situ hybridization studies on the distribution of CaM mRNA populations and the regulation of their genes in the rodent retina. Since CaM is a key protein that modulates a number of target proteins involved in signaling processes in general, one might have expected a clearer picture to have emerged for CaM-related

phenomena in signaling processes in vision in particular, especially in light of the abundant data available for other Ca^{2+}-binding proteins or a number of CaM-dependent proteins in the retina, e.g. the Ca^{2+}/CaM-dependent adenylyl cyclase, the cGMP-gated cation channel, the Ca^{2+}/CaM-dependent protein kinases I, II and IV, and the G-protein-coupled receptor kinases.

After an initial survey of CaM gene expression in the adult rat retina, using conventional in situ hybridization techniques, we concluded that the lack of data regarding the CaM gene expression in this tissue is probably due to its generally low expression level, and to the relative insensitivity of most current in situ hybridization protocols for low-abundance mRNA populations. Thus, the objective of the present study was to localize and differentiate CaM mRNA populations transcribed from the multiple CaM genes in the mature rat retina by using our sensitive color in situ hybridization method, modified after Basyuk et al. (2000), employing a slightly alkaline hybridization solution (pH 8.0) to increase the sensitivity, with CaM gene-specific digoxigenin (DIG)-labeled cRNA probes in paraffin-embedded sections.

Specific aims

The goals of these studies were to develop a sensitive in situ hybridization method that can be used to detect CaM transcripts with low abundance, and to localize CaM mRNAs with high resolution in CaM mRNA-poor regions of the central nervous system (the white matter of the spinal cord and the retina). Our specific aims were as follows:

1) To compare the sensitivities of the conventionally used in situ hybridization methods in a CaM-poor and lipid-rich region of the central nervous system of the rat,
2) To optimize our sensitive color in situ hybridization method, using a slightly alkaline hybridization solution (pH 8.0) for the detection of low-abundance CaM transcripts,
3) To localize mRNA populations transcribed from the three different CaM genes in the white matter of the adult rat lumbar spinal cord,

4) To demonstrate the presence and localize mRNA populations transcribed from the three different CaM genes in the adult rat neural retina.

Materials and methods

The experimental procedures were carried out in strict compliance with the European Communities Council Directive (86/609/EEC), and followed the Hungarian legislation requirements (XXVIII/1998 and 243/1998) regarding the care and use of laboratory animals, and they complied with the directives of the University of Szeged on this subject. Paraffin-embedded and cryostat sections of lumbar spinal cord and retinal tissues of adult (200-220g) male Sprague-Dawley rats were used in these experiments. In situ hybridization was performed by using ^{35}S- or DIG-labeled antisense cRNA probes specific for each of the CaM genes; sense probes were used to detect nonspecific hybridization.

We developed an in situ hybridization method, using DIG-cRNA and employing a slightly alkaline hybridization solution (pH 8.0) in order to increase the sensitivity, especially in lipid-rich nervous tissue with a low-abundance CaM mRNA content (Kovacs, 2003; Kovacs and Gulya, 2001), and compared its sensitivity with those of the available in situ hybridization techniques (Szigeti et al., 2003). We successfully demonstrated the presence of different CaM-expressing cells in the white matter of the adult rat spinal cord (Kovacs and Gulya, 2002; Palfi et al., 2002), and also CaM mRNA populations in the adult rat neural retina (Kovacs and Gulya 2003). The retinal CaM expression was semiquantitatively analyzed by means of computer-assisted microdensitometry.

Results

CaM I gene-specific DIG-cRNA probes were used in those experiments that were designed to sensitize in situ hybridization. Among the in situ hybridization protocols tested, the one that used a lower triton X-100 concentration (0.1%) coupled with chloroform treatment proved to be more effective in detecting low-abundance CaM I

mRNAs than that with a high triton X-100 concentration (1.0%). Although both treatment regimens were able to detect CaM-expressing motoneurons in equal numbers in the ventral horn of the spinal cord, the number of labeled interneurons was higher when the lower triton X-100 concentration was used. Neither treatment with triton X-100 was able to detect CaM-expressing cells in the white matter of the spinal cord. In situ hybridization at a slightly alkaline pH (pH 8.0) resulted in a very low background and a large number of labeled cells, in both the gray matter and the white matter. Since tissue preservation was excellent when this protocol was used, further experiments were performed with hybridization solution adjusted to slightly alkaline pH (pH 8.0). With this protocol, we were able to detect low amounts of CaM transcripts in tissues that were either lipid-rich (the white matter of the spinal cord) or lipid-poor (the retina).

The conventional in situ hybridization techniques, at neutral pH, did not detect any cellular elements expressing CaM II mRNAs in the white matter area of the spinal cord. Heavy labeling could be seen only in the gray matter, where the motoneurons in the ventral horn and interneurons in the dorsal horn expressed CaM II mRNAs. When our sensitive color in situ hybridization technique employing a DIG-labeled riboprobe in a hybridization solution adjusted to low alkaline pH (pH 8.0) was used, the widespread distribution of CaM II mRNA-expressing cells was revealed throughout the white matter area of the spinal cord. Hybridization at neutral pH resulted in CaM II-expressing moto- and interneurons in the gray matter, but CaM II mRNAs could not be detected in the white matter. Hybridization at slightly alkaline pH, however, resulted in the detection of a number of CaM II mRNA-expressing cells in both the gray matter and the white matter. Two cell types with differential CaM expression could be discriminated. The first type was present predominantly in the dorsal column of the white matter, with differential CaM gene expression. Here, the somata of the medium-sized astrocyte-like cells were heavily labeled, with their surroundings too considerably labeled. The CaM I mRNA content was most abundant, followed by the CaM III and CaM II mRNA contents in the dorsal column. While the CaM I and CaM III mRNA populations clearly highlighted the

cells, sometimes together with their main processes, the CaM II mRNAs were present only in the somata.

Smaller, rounded cells, that were probably oligodendrocytes, occasionally with elongated processes running radially to a distance of 10-20 μm, and that were apparent throughout the white matter area of the spinal cord, also expressed CaM mRNAs. In these oligodendrocyte-like cells, the CaM II and CaM III genes exhibited slightly lower levels of expression that that of the CaM I gene. The myelin sheet formed by oligodendrocyte processes around the axons of the motoneurons was clearly labeled by CaM I and CaM III gene-specific probes. The DIG-labeled sense probes resulted in labeling comparable only to the background level.

In situ hybridization with DIG-labeled riboprobes specific for each CaM gene at neutral pH did not detect any specifically hybridized CaM mRNA species in the adult rat neural retina. However, when the hybridization was carried out at a slightly alkaline pH (pH 8.0), a widespread distribution of CaM mRNAs was revealed throughout the retina. Unlike the situation in the central nervous system, all CaM genes expressed their transcripts in similarly low amounts and with very similar distributions.

The ganglion cell layer contained high amounts of mRNAs for all CaM genes, where the majority of the label could be localized to the perinuclear region of the ganglion cells. Similarly high amounts of CaM mRNAs were also present in the inner nuclear layer, where the cell bodies of the horizontal, bipolar, amacrine and Muller cells reside. Interestingly, within the inner nuclear layer, the heaviest labeling was detected in the inner sublamina, where mostly the somata of the amacrine cells could be localized. It is noteworthy that, in the adult rat retina, this sublamina contained the highest amount of CaM mRNAs for all CaM genes where the hybridization signal was predominantly perikaryonal, while the remainder of the inner nuclear layer displayed mostly diffuse labeling, as the cell bodies were only occasionally labeled. Intermediate and, at the same time, homogeneously distributed signal intensities within the particular layers were detected in the inner and outer plexiform layers, in the retinal pigment epithelium and in the vicinity of the outer limiting membrane. Very low signal intensities were

characteristic for all CaM genes in the outer nuclear layer, where the nuclei of rods and cones reside. Interestingly, the photoreceptor inner segment layer displayed a very low signal intensity. However, no specific signal for any CaM mRNA population was detected in the photoreceptor outer segment layer when the signal value of the sense probe in this layer was subtracted from that of the antisense probe. A very low nonspecific hybridization signal intensity, not associated with any recognizable cellular constituent of the retina, was detected when sense DIG-CaM cRNA was hybridized to the tissue sections. In summary, a differential, layer-specific mRNA distribution pattern, similar for all three CaM genes, was detected in the adult rat retina.

Conclusions

The main results of our studies were as follows:

1) By comparing the parameters of the different in situ hybridization methods (microwave heating and lipid removal of the tissue, and change of the pH of the hybridization solution), we concluded that hybridization at a slightly alkaline pH (pH 8.0) detects CaM mRNA populations with high sensitivity in tissues with high lipid contents or where they exist only in low abundance (Kovacs, 2003; Szigeti et al., 2003).
2) We optimized our sensitive in situ hybridization method for the detection of low-abundance CaM transcripts in the white matter of the spinal cord (Kovacs and Gulya, 2001, 2002) and the retina (Kovacs and Gulya, 2003) of the adult rat.
3) With the use of our sensitive in situ hybridization method, we successfully detected two glial cell types (astrocytes and oligodendrocytes) in the white matter areas of the rat lumbar spinal cord that differentially express CaM genes (Kovacs and Gulya, 2001, 2002; Palfi et al., 2002; page 2832 and Fig. 2). Our results indicate that the CaM gene expression profile of the spinal cord is richer and more complex than previously thought. Further, primarily functional gene expression studies on the CaM positive cells in the white matter (the astrocytes and the oligodendrocytes)

may shed light on the roles of this protein in glial–neuron interactions, signalization and myelinization processes.

4) The amount of CaM transcripts in the adult rat retina is low. With the use of our sensitive in situ hybridization technique, we described the distributions of these mRNA populations in adult rat under physiological conditions (Kovacs and Gulya, 2003). The uniquely similar expression pattern of a layer-specific distribution for all CaM genes was observed. The different intensities of the CaM gene expression in the different retinal layers suggest a direct role for CaM in visual signaling processes. Although the functional significance of the different CaM genes in visual signaling processes remains unclear at present, we have provided an anatomical basis for further analysis of the functions and expression patterns of these genes in the neural retina under normal and pathologic conditions.

Own publications used for the dissertation

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