

THESIS

REGULATION OF CALMODULIN GENE EXPRESSION IN THE RAT BRAIN STEM– MEDULLA REGION AFTER INDUCTION OF CHRONIC OROFACIAL SKIN INFLAMMATION AND SUBSEQUENT STEROID TREATMENT

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Introduction

Dithranol (anthralin) is one of the most widely used and effective, albeit empirical, topical treatments for patients with psoriasis. The molecular basis of its mode of action is still unknown, but it is probably related to the redox activity leading to the production of free radicals, including oxygen radicals. A recent study showed that dithranol accumulated in keratinocyte mitochondria (McGill et al., 2005), induced structural damage, and interfered with the redox status of the endogenous ubiquinone pool at the level of the ubisemiquinone anion. These events lead to increased generation of reactive oxygen species and eventually resulted in apoptosis.

There are some indications that either or both epidermal proliferation and/or keratinization and cutaneous inflammation may be crucial in the antipsoriatic effect of dithranol; indeed, the often serious inflammation and the irritative response of the perilesional or uninvolved skin are the most serious limitations to its use. The extent of such inflammation on the peripheral nerves has been successfully demonstrated immunohistochemically by the use of the ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1; gene aliases: pan-neuronal marker protein gene product (PGP) 9.5, ubiquitin thiolesterase, PARK5), a cytosolic deubiquitinating enzyme that is highly expressed both in humans and in animals. UCH-L1 immunoreactivity was decreased in the oral mucosa of patients with chronic inflammation caused by oral lichen planus, after the intradermal injection of capsaicin, in leprosy skin and around the sweat glands in palmoplantar pustulosis. In animals, the expression of UCH-L1 was absent in necrotic areas of the colon of rats treated with 2,4,6-trinitrobenzenesulfonic acid to induce experimental colitis, and its immunoreactivity was decreased in rats with nasosinusitis infected artificially with *Staphylococcus* bacteria. Interestingly, psoriasis, an inflammatory skin disorder often treated with dithranol, was reported to be accompanied by either decreased or unchanged UCH-L1 immunoreactivity in the affected skin. In light of these conflicting results, we investigated whether the inflammation elicited by dithranol would affect the UCH-L1 immunoreactivity of the cutaneous nociceptive sensory fibers of the rat orofacial skin.

Deep and cutaneous orofacial tissue inflammation or painful temporomandibular disorders often elicit prolonged neuronal activation in the trigeminal nociceptive pathways that can lead to chronic pain, and they are therefore of paramount clinical importance. A large number of data are available on the molecular, cellular and system-wide mechanisms involved in the development of, or the amelioration of pain related to,

inflammation in the central nervous system (CNS). Nociceptive and pain-related information, mediated via different intracellular signalization processes, is transmitted and regulated at different levels of the CNS; from the point of view of our studies, the most important ones are those that shed light on the roles played by calmodulin (CaM) and its target proteins in the responses of the nervous tissue to the inflammatory stimuli.

CaM, a ubiquitous, multifunctional cytoplasmic calcium (Ca^{2+}) receptor protein encoded by three different genes in mammals, is especially abundant in the mammalian central nervous system. Its regional distribution and expression pattern in the developing and adult rodent brain have been well documented. The expression patterns corresponding to the three CaM genes displayed a widely differential distribution for the CaM gene-specific mRNA populations throughout the brain and spinal cord.

As CaM exerts its biological action through its target proteins that are involved in a number of cellular regulator processes, it is not surprising that immunohistochemical and in situ hybridization studies have demonstrated that CaM immunoreactivity or CaM gene-specific transcripts are often colocalized with those of the target enzymes of CaM within the same neuronal structures, not only in general, but in the brain stem-medulla region in particular. However, in spite of the number of neuronal functions associated with midbrain–brain stem structures and regulated by CaM-dependent mechanisms, there are only sporadic data on the distribution of CaM protein in this brain region. Since CaM plays an important role in regulating a key target protein, Ca^{2+} /CaM kinase II, in a number of neuronal functions related to the trigeminal system, including inflammation, neuropathic pain and nerve injury, a precise, high-resolution mapping of the CaM gene expression could promote a deeper understanding of the functioning of the trigeminal system in health and disease. In this study, involving quantitative in situ hybridization analysis through the use of CaM gene-specific [^{35}S]-labeled cRNA probes, we also report on the differences in CaM gene expression patterns seen along the rostrocaudal axis within each nucleus of the trigeminal system.

Understanding the distribution of CaM expressing cells in normal, physiological situation helped us to detect and understand the changes in the regulation of CaM gene expression patterns after chronic peripheral skin, in this case orofacial, inflammation. We thus set up experiments to map the changes in CaM gene expression patterns associated with chronic dithranol and subsequent corticosteroid treatments in the trigeminal system of the adult rat.

Specific aims

Our objective was to document the histological, immunohistological and Western blot characteristics of the inflamed orofacial skin after chronic dithranol treatment and corticosteroid reversal, and to localize and differentiate CaM mRNA populations transcribed from the multiple CaM genes in the different parts of the rat trigeminal system after chronic inflammation of the infraorbital region of the orofacial skin by means of hybridization of transcript-specific riboprobes labeled with [³⁵S]UTP.

Specifically, our aims were as follows:

- 1) To investigate the histochemical characteristics of the inflammation elicited by chronic dithranol treatment alone or in combination with corticosteroids, and of the UCH-L1 immunoreactivity of the cutaneous nociceptive sensory fibers of the rat orofacial skin after such treatment.
- 2) To localize, differentiate and semiquantitatively analyze in detail the different CaM mRNA populations transcribed from the multiple CaM genes in the adult normal rat midbrain–brain stem region,
- 3) and the possible rostrocaudal gradient by gene-specific [³⁵S]UTP- or DIG-labeled cRNA probes.
- 4) To localize and differentiate CaM mRNA populations transcribed from the multiple CaM genes in two prominent nuclei of the rat trigeminal system, namely the motor trigeminal (Mo5) and the principal sensory trigeminal (Pr5) nuclei, after chronic inflammation and reversal by corticosteroids of the infraorbital region of the orofacial skin by means of quantitative in situ hybridization.

Results

Dithranol has been used to treat psoriasis for decades. Although its beneficial effect may involve the induction of cutaneous inflammation, and inflammation often leads to damages in nerve fibers in the periphery or, consequently, in the central nervous system as well, these alterations, especially those that elicit neuronal adaptation or regulation of neuronal gene expression, are not well documented. Therefore, we investigated the effects of chronic dithranol treatment and subsequent corticosteroid treatment 1) on the immunohistochemical and Western blot characteristics of the cutaneous nerve fibers in the rat skin, where the epidermal nerve fiber component ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) was used as target antigen, and 2) on the regulation of

calmodulin (CaM) gene expression on the different components of the rat trigeminal system in the midbrain–brain stem region; we used in situ hybridization techniques to detect gene-specific [³⁵S]- and digoxigenin-labeled cRNA probes complementary to the multiple CaM mRNAs. The results of radioactive and color in situ hybridization histochemistries were analyzed with (semi)quantitative image processing techniques.

Chronic dithranol treatment for 5 days resulted a complete loss of UCH-L1 immunoreactivity. Topical application of corticosteroid onto the inflamed skin for 5 days reversed this effect: the UCH-L1 immunoreactivity was almost completely restored. Steroid treatment for 5 days did not change the appearance of the UCH-L1-immunoreactive nerve fibers. These findings were supported by Western blot analyses. We concluded that dithranol, incidentally similarly to psoriasis, causes inflammation and abolishes UCH-L1 immunoreactivity in the rat orofacial skin in a corticosteroid-reversible manner. This phenomenon may be due to the ability of dithranol to cause oxidative damage to the UCH-L1 protein, and to the antioxidant activity of the corticosteroids countering this effect.

The different CaM genes were widely expressed throughout the midbrain–brain stem region with moderate intensities in control animals. In spite of the similar general outline, significant differences in the distributions of the multiple CaM mRNA species were found in certain areas. In general, the CaM III mRNAs were most abundant, followed by the CaM I and CaM II mRNA populations. Most of the transcripts was found in the neuronal somata comprising the medullar nuclei, while much less label was detected in the neuropil. The CaM III mRNAs were more than 2.5 times more abundant than the CaM II mRNAs in the nucleus of the trapezoid body, and more than 2 times more abundant in the motor trigeminal nucleus, the principal sensory trigeminal nucleus and the olivary nucleus. The CaM III mRNAs were less dominant in the medial lemniscus, the inferior colliculus and the pontine reticular nucleus than those of the other CaM gene-specific transcripts. The CaM mRNA levels were low to moderate, without significant differences, in the mesencephalic trigeminal nucleus (Me5). The differential control of the expression of the CaM genes may thus contribute to the regulation of the multiple neuronal functions linked to this complex brain region and regulated by different CaM-dependent mechanisms via its target proteins.

The quantitative analysis of the expression patterns of the three CaM genes showed that significant differences in the amounts of the transcripts of some CaM genes were present between the rostral and caudal parts of the individual nuclei of the trigeminal

system in control animals. In most cases, the CaM gene-specific transcripts displayed a clear differential distribution along the rostrocaudal axis: they were more abundant in the rostral parts of these nuclei. For example, the levels of mRNAs transcribed from each of the CaM I, II and III genes were significantly higher in the rostral part of the principal sensory trigeminal nucleus (Pr5), while the rostral part of the motor trigeminal nucleus (Mo5) exhibited an elevated amount of transcripts for the CaM I gene only. Interestingly, the CaM II mRNAs were most abundant in the caudal part of the Me5. Moreover, the largest difference between any of the CaM gene-specific transcript contents of the rostral and caudal parts was found for those of the CaM II gene in the Pr5. Here, the intranuclear difference was about 50%, the rostral part being the richer in CaM II mRNAs. Our results draw attention to the possible causal relation between the differences in the neuronal circuitry of the rostral and caudal parts of these nuclei and their differential CaM gene expression. This somatotopy may have important functional implications.

The cutaneous and mucosal surfaces in the infraorbital region around the whisker pad are innervated by the maxillary division of the afferent fibers of the trigeminal nerve, while certain ganglion cells project to the Pr5. In turn, some of the neurons in the Pr5 project to the Mo5, whose neurons do not innervate the infraorbital skin. We analyzed the CaM gene expression in these nuclei after dithranol-induced inflammation and subsequent treatment with corticosteroid in the infraorbital skin. CaM gene-specific mRNA populations were detected through quantitative image analysis of the distribution of CaM gene-specific riboprobes in brain stem cryostat sections of control rats and rats chronically treated with dithranol, corticosteroid or both. These nuclei displayed a differentially altered CaM gene expression in response to the treatments. While the CaM I and II mRNA contents were increased, the CaM III transcripts remained unaltered after chronic dithranol treatment in the Mo5. In the Pr5, however, the CaM mRNA contents were either unchanged (CaM I and III) or increased (CaM II). Subsequent corticosteroid treatment reversed the stimulatory effects of dithranol on the expression of all the CaM genes in the Mo5, but was without significant effects on the CaM I and II genes, or even increased the CaM III mRNA contents in the Pr5. Corticosteroid treatment alone was either ineffective or decreased the levels of CaM mRNAs in these nuclei. These data suggest that peripheral noxae of dermal origin may result in a trans-synaptically acting differential regulation of the multiple CaM genes in the brain.

Conclusions

Our results draw attention to a possible causal relation between the differences in afferent and efferent neuronal connections (and consequently in their presumably segregated functions) of the rostral and caudal parts of the trigeminal nuclei and their differential CaM gene expression. In light of the above data, our present findings can be interpreted as trans-synaptic regulatory responses of the CaM gene expression to two opposing effects: the effect of dithranol in degrading proteins that include CaM and the deubiquitinating protein UCH-L1 itself, via its production of free radicals and oxygen radicals, which result in a differentially altered CaM gene expression; and the modulatory (generally inhibitory) effect of the corticosteroids on the CaM gene expression, resulting in a decreased transcription of the CaM genes. These data provide evidence that chronic inflammation and subsequent corticosteroid treatment can influence the neuronal CaM gene expression through multiple synaptic contacts. Long-lasting changes elicited in the periphery by chronic noxious stimuli (e.g. inflammatory processes or their treatment with steroids) through the peripheral nerves could elicit alterations in, and therefore influence, the CNS functions. These stressors may be important components in the regulatory mechanisms of neuronal gene expression as they could result in transneuronal plasticity, where compensatory increases or decreases in the multiple CaM genes occur in the Pr5 and Mo5.

Collectively, these findings suggest that the differential regulation of the CaM genes in these projection sites are ipsilaterally and trans-synaptically modulated, perhaps via multiple signaling pathways. However, the precise molecular mechanisms underlying this often opposing regulation are unknown.

Summary of the findings

- 1) Chronic dithranol treatment of the orofacial skin resulted in a complete loss of the UCH-L1 immunoreactivity in the cutaneous nerves. Subsequent steroid treatment restored UCH-L1 immunoreactivity in these structures. These findings were supported by Western blot analyses.
- 2) In situ hybridization of radioactive cRNA probes specific for CaM I, II and III genes revealed a specific and unique distribution of their transcripts in this brain area. In general, the rank order of abundance in the nuclei of the midbrain–brain stem region investigated was CaM III > CaM I > CaM II. Color in situ hybridization demonstrated many, primarily medium-sized multipolar neurons in the medullar nuclei.

- 3) For some CaM genes, significant differences in the amounts of their transcripts were found between the rostral and caudal parts of the individual nuclei of the trigeminal system. In most cases, the CaM gene-specific transcripts were more abundant in the rostral parts of the nuclei, while the rostral part of the motor trigeminal nucleus displayed an elevated amount of transcripts for the CaM I gene only.
- 4) Chronic dithranol treatment of the infraorbital skin selectively affected the CaM gene expression in selected parts of this brain region: an area-dependent and gene-specific differential regulation of CaM gene expression was demonstrated in two nuclei of the trigeminal system, the Mo5 and the Pr5 of the rat, as evidenced by quantitative in situ hybridization. Moreover, the CaM gene expression was, in general, sensitive to corticosteroid treatment, as it differentially lowered the amounts of the CaM mRNA populations after chronic dithranol treatment.

List of publications directly related to the thesis:

- Bakota L., Orojan I., Gulya K. (2005) Intranuclear differences in calmodulin gene expression in the trigeminal nuclei of the rat. *Acta Biol. Szeged.* 49:9-14.
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- Orojan I., Bakota, L., Szigeti C., Varszegi S., Gulya K. (2005) Orofacial skin inflammation upregulates calmodulin gene expression in the trigeminal nuclei of the rat. 3rd International Workshop for the Study of Itch, Heidelberg (poster No. 8) *Acta Derm. Venereol.* 85:477.

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