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Effect of vinpocetine on retrograde axoplasmic transport

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Summary

Vinpocetine, a derivate of vincamine, is widely used in the clinical pharmacotherapy of cerebral circulatory diseases. Herewith we report on a novel effect of vinpocetine: inhibition of retrograde axoplasmic transport of nerve growth factor (NGF) in the peripheral nerve. Blockade of retrograde transport of NGF results in transganglionic degenerative atrophy (TDA) in the segmentally related ipsilateral superficial spinal dorsal horn, which is characterized by depletion of the marker enzymes fluoride-resistant acid phosphatase (FRAP) and thiamine monophosphatase (TMP). At the same time, pain-related neuropeptides such as substance P (SP) and calcitonin gene-related peptide (CGRP), are depleted from lamina I–III from the segmentally related, ipsilateral Rolando substance of the spinal cord. On the basis of these experiments it is suggested that vinpocetine may result in a locally restricted decrease of nociception, that might be useful in clinical treatment of intractable pain. Pilot self-experiments support this assumption.

Introduction

Central terminals of primary nociceptive neurons, terminating in lamina I and II of the dorsal horn in the form of scallopped terminals, contain pain-related neuropeptides (substance P (SP) and calcitonin gene-related peptide (CGRP)), and the marker enzymes fluoride-resistant acid phosphatase (FRAP) and thiamine monophosphatase or TMP (Knyihar, 1971; Knyihar-Csillik et al., 1986). Pain is known to be the central representation of nociception transmitted to and perceived by the cerebral cortex (Wall, 1995; Knyihar and Csillik, 2006).

In a series of publications (Knyihar and Csillik, 1976; Csillik et al., 1977, 1985, 2003; Knyihar-Csillik

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and Csillik, 1981; Csillik and Knyihar-Csillik, 1986), it has been shown that blockade of retrograde axoplasmic transport in a sensory nerve results in transganglionic degenerative atrophy (TDA) of central terminals of the affected primary nociceptive neurons. Accordingly, transcutaneous iontophoresis of vincristine was successfully performed in order to alleviate chronic, neuropathic pain deriving from postherpetic neuralgia, toxic polyneuropathies and terminal oncological states (Csillik et al., 1982; Knyihar-Csillik et al., 1982; Szucs et al., 1984; Csillik and Knyihar-Csillik, 1986; Tajti et al., 1989; Rossano et al., 1989). However, this treatment has been discontinued for fear of unforeseen hepatotoxic side effects, that might arise from the usage of vincristine and vinblastine, known to be microtubule inhibitors.

Therefore, our interests was directed toward another vinca alkaloid, vinpocetine. In this paper, light- and electron microscopic effects of perineurally applied vinpocetine, upon the marker enzymes (FRAP and TMP), and pain-related neuropeptides (SP and CGRP) will be described in the ipsilateral, segmentally related upper dorsal horn. The effect of vinpocetine on the retrograde axoplasmic transport of nerve growth factor (NGF) in a peripheral nerve will be reported on the basis of radiochemical studies. On this basis it is suggested that transcutaneous iontophoresis of vinpocetine might be promising to the pain clinician, aiming to alleviate chronic, neuropathic pain.

Material and methods

Investigations were performed on 32 young adult male albino rats (*Rattus norvegicus albus*), Wistar strain, 200–250 g body weight. Care of the animals complied with the guidelines of the Hungarian Ministry of Welfare; experiments were carried out in accordance with the European Communities Council Directive (November 24, 1986; 86/609/EEC) and the Guidelines for Ethics in Animal Experiments of the Szeged University, Albert Szent-Györgyi Medical School.

For histochemical and immunohistochemical investigations, animals were anesthesized with 4% chloral hydrate i.p. and subjected to transcardial perfusion with a formaldehyde solution containing picric acid (Zamboni and De Martino, 1965) to which glutaraldehyde was added. The spinal cord was excised and the distribution of FRAP and TMP was studied in 15 μm frozen cross-sections, according to the technique described earlier (Knyihar-Csillik et al., 1982, 1986; Csillik et al., 1986). SP and CGRP immunoreactivity was visualized by means of the techniques described by Csillik et al. (2003).

In experiments aiming to study the effect of vinpocetine on retrograde transport, a slab of Gelita tampon (Braun, Melsungen, Germany) measuring $5 \times 5 \times 20$ mm, was soaked in 10^{-6}, 10^{-7} or 10^{-8} M vinpocetine (Sigma, catalog number V 6383) dissolved in physiological saline. The slab was applied around the sciatic nerve in the form of a loose cuff, in five animals in each group. Contralaterally, the sciatic nerve was surrounded by a similar Gelita tampon cuff, soaked in physiological saline. Seven days after the application of the cuffs, the animals were killed with an overdose of chloral hydrate, and subjected to transcardial fixation as described above.

For electron microscopic studies, FRAP and TMP were visualized in vibratome sections of the spinal cord (50 μm) as described above; however, the last step of the reaction, i.e. sulfide treatment, was omitted. The samples were embedded in Durcupan ACM and sectioned on a Reichert Ultrotome. Thin sections, silver interference color, were stained with lead citrate and studied with a Zeiss Opton 902 electron microscope (Oberkochen, Germany).

The effect of vinpocetine upon retrograde axoplasmic transport of NGF was studied after injection of 600 ng (50 μCi) ^{125}I-labelled NGF-β under the skin of the hind paw. NGF prepared from the murine submaxillary gland was obtained from Sigma (St. Louis, MO, USA); ^{125}I labeling was performed according to the technique described by Rohrer and Barde (1981). Radioactivity of 0.5 cm long portions of the sciatic nerve, the corresponding dorsal root ganglia and dorsal roots was determined in a Berthold 8F Gammascint apparatus. The left sciatic nerves of the rats, seven animals in each group were surrounded by Gelita tampon cuffs, soaked in 10^{-6}, 10^{-7} or 10^{-8} M vinpocetine, dissolved in physiological saline. The contralateral sciatic nerve was surrounded by a Gelita tampon cuff, soaked in physiological saline.

The *intensity* of the histochemical reaction was measured by densitometry of the slides, performed by digitalizing microscopic pictures obtained by histochemistry (FRAP, TMPase) or immunocytochemistry (SP or CGRP) with a SPOT RT Slider CCD camera (1600×1200 pixels, 8 bits) attached to a Nikon Eclipse E600 microscope using a $\times 16$ front lens and a $\times 10$ eyepiece. The captured images were analyzed by ImageProPlus v4.5 morphometric software (Media Cybernetics, Silver Sring, MD, USA). Areas of interest were rectangular areas measuring 50×200 μm. Fifteen rectangular fields

were analyzed per animal; each field was chosen randomly in different sections in a blinded manner. Gray values of the selected areas were obtained using a ScionNIH image analysis software. The images were captured directly from the microscopic slides, using a 5 × objective lens by means of a black and white camera Cohu CCD and displayed on a computer monitor. The software automatically assigned the average gray value of the screen pixels corresponding to the outlined area; a value of 0 indicated a white pixel and 225 indicated a black pixel.

Results

Perineurally applied vinpocetine (Fig. 1), at a concentration of 10^{-6} M, caused disappearance of FRAP and TMP from the ipsilateral, segmentally related Rolando substance (lamina II) of the dorsal horn, within 7 days after application. At the same time, there was no alteration seen contralaterally (Fig. 2). If applied at a concentration of 10^{-7} or 10^{-8} M, perineurally applied vinpocetine caused only a slight reduction in the histochemical enzyme reaction. At a concentration of 10^{-9} M, perineurally applied vinpocetine did not cause any alteration.

At the electron microscope level, thousands of scallopped axon terminals, displaying FRAP and TMP enzyme reactions at their surface membranes, could be observed under normal conditions (Fig. 3a), which resulted in an enzyme active line in lamina II at the level of the light microscope. After vinpocetine treatment of the sciatic nerve, depletion of FRAP and TMP was accompanied by the formation of enzyme inactive labyrinthine structures (Fig. 3b).

The effect of vinpocetine upon SP and CGRP immunoreaction was evident but less conspicuous. These neuropeptides are known to be localized in lamina I and II but to a lesser degree also in lamina III of the dorsal horn. Perineural application of a Gelita tampon cuff containing 10^{-6} M vinpocetine induced a decrease in SP and CGRP immunoreactivity in the ipsilateral, segmentally related upper dorsal horn (Fig. 4). The effects of 10^{-7}, 10^{-8}, and 10^{-9} M vinpocetine were equivocal.

Densitometric measurements of the histochemical (TMP) and immunohistochemical (SP) reactions revealed that TMP was decreased significantly in the ipsilateral spinal dorsal horn at levels corresponding to vinpocetine treatment of the sciatic nerve ($p<0.01$). Contralaterally, and at irrelevant levels, no significant alterations could be seen. A decrease in ipsilateral SP immunoreactivity at the spinal cord level corresponding to vinpocetine treatment of the sciatic nerve was considerable ($p<0.1$). The corresponding values were as follows:

FRAP at level Th8: Mean 43.3, SEM: 0.71; SD: 2.26
FRAP at level L5, ipsilateral (vinpocetine treated): Mean 12.1, SEM: 0.71; SD: 1.45
FRAP at level L5, contralateral (saline treated): Mean 43.6, SEM: 0.56; SD: 1.78
SP at level Th8: Mean 34.9; SEM: 0.77; SD: 2.42
SP at level L5, ipsilateral (vinpocetine treated): Mean 12.6; SEM: 0.45; SD: 1.43

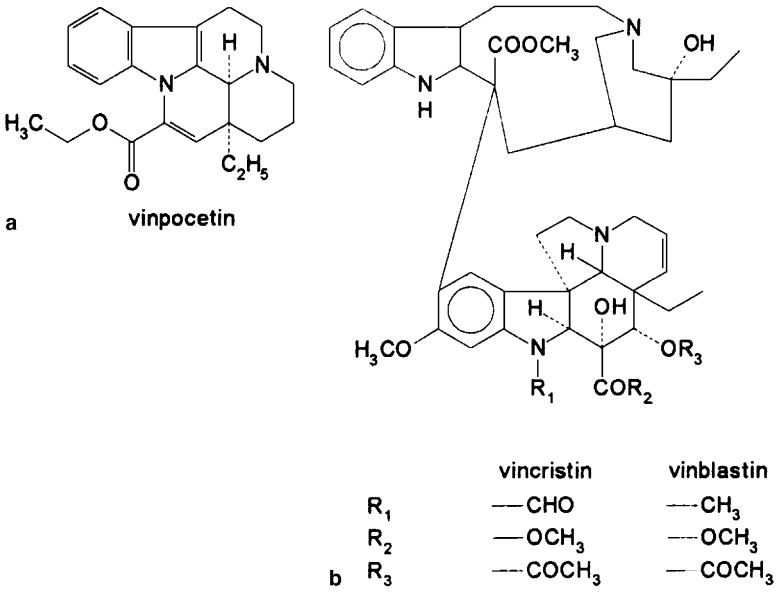

Figure 1. Structural formula of vinpocetine (a) and the Vinca alcaloids vincristin and vinblastin (b).

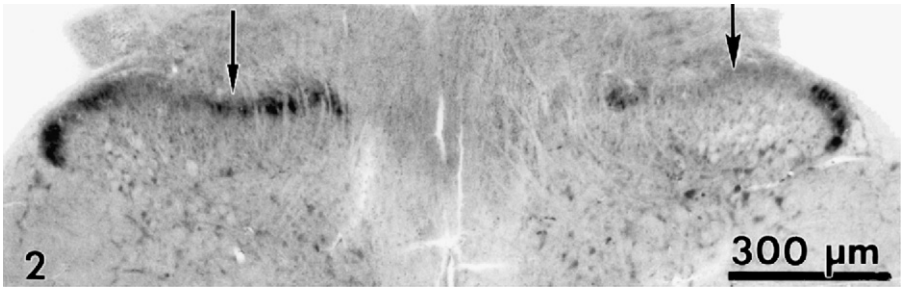

Figure 2. TMP activity of the Rolando substance at the spinal cord level L5 after unilateral perineural vinpocetine treatment (apparent right). Note virtual absence of TMP reaction at the projection of the cuff treatment (arrow) as contrasted with the intense and uninterrupted enzyme reaction outlining lamina II contralaterally.

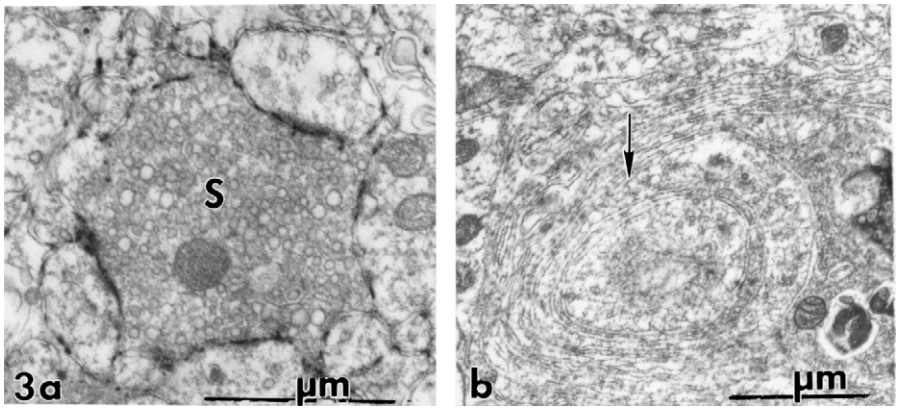

Figure 3. Electron microscopic appearance of FRAP under normal conditions in a scallopped axon terminal (S) in the Rolando substance (a) and formation of an axonal labyrinth (arrow) devoid of any marker enzyme activity, 17 days after application of a 10^{-6} M containing Gelita tampon cuff around the sciatic nerve (b).

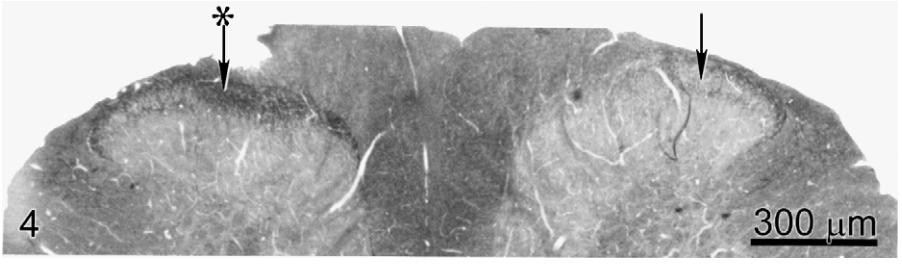

Figure 4. Substance P immunoreactivity of the upper dorsal horn at the spinal cord level L5 after unilateral perineural vinpocetine treatment (10^{-6} M) of the sciatic nerve (apparent right). Note decreased substance P immunoreaction at the site of projection of the cuff treatment (arrow), as contrasted to the intense immunoreaction outlining lamina and I and II contralaterally (arrow with asterisk).

SP at level L5, contralateral (saline treated): Mean 36.6; SEM: 0.48; SD: 1.50

SEM: standard error; SD: standard deviation.

Radiochemical measurements of [^{125}I] NGF in 0.5 cm long portions of the sciatic nerve revealed that in control animals, where the sciatic nerve was surrounded by a Gelita tampon cuff soaked in isotonic saline, radioactivity of [^{125}I] NGF was more or less evenly distributed throughout the entire length of the sciatic nerve. Concentration of radioactivity was seen in the related dorsal root ganglia (Fig. 5).

After *perineural treatment* of the sciatic nerve with 10^{-6} M vinpocetine, retrograde transport of ^{125}I-labeled NGF was virtually blocked at the site of the cuff. A conspicuous accumulation of ^{125}I was observed just distal to the cuff and in the region of

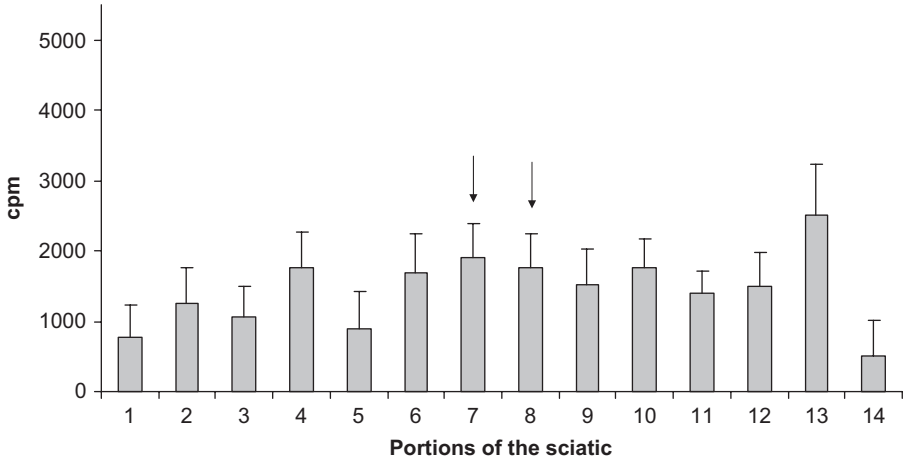

Figure 5. Distribution of radioactivity in the sciatic nerve, in dorsal root ganglion L5 and in the related dorsal root, 15 h after injection of 600 ng (50 μCi) of ^{125}I-labelled NGF-β under the skin of the hind paw, 72 h after perineural application of physiological saline in a Gelita tampon cuff. #1 is the most distal portion, #13 is the dorsal root ganglion and #14 is the dorsal root. The isotonic saline cuff was applied at portions #7 and #8 (arrows).

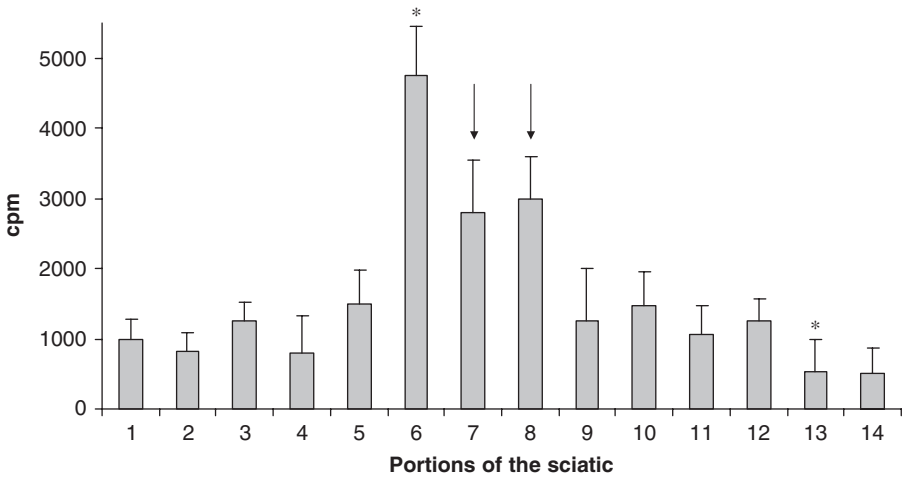

Figure 6. Distribution of radioactivity in the sciatic nerve, in dorsal root ganglion L5 and in the related dorsal root, 15 h after injection of 600 ng (50 μCi) of ^{125}I-labelled NGF-β under the skin of the hind paw, 72 h after perineural application of 10^{-6} M vinpocetine in a Gelita tampon cuff. #1 is the most distal portion; the vinpocetine cuff was applied at #7 and #8 (arrows); #13 is the dorsal root ganglion and #14 is the dorsal root. Asterisks indicate significant alterations (increase in #6; decrease in #13) as compared to control experiments.

the cuff itself. In portions proximal to the cuff, as well as in dorsal root ganglia and dorsal roots, radioactivity was significantly lower than in controls (Fig. 6). After perineural treatment of the sciatic nerve with 10^{-7} or 10^{-9} M vinpocetine containing cuffs, the retrograde transport of ^{125}I-labeled NGF was virtually unchanged.

Discussion

According to the enzyme histochemical, immunocytochemical and neurochemical studies described above, perineurally applied vinpocetine induces blockade of retrograde axoplasmic transport which consequently results in TDA in the ipsilateral, segmentally related upper dorsal horn. Vinpocetine, a derivate of vincamine, is proven to be devoid of any unwanted side effects. It is widely used in clinical pharmacotherapy of various cerebral circulatory diseases such as memory problems, motion disorders, climacterial complaints, vertigo and headache (Bonoczk et al., 2000). Vinpocetine is a drug very different from vincristine: principally, its effect is blockade of sodium channels (Sitges et al., 2005; Zhou et al., 2003) and of Ca^{2+} channels

(Sitges and Nekrassov, 1999) as well as of glutamate receptors (Kiss et al., 1991). The chemical structure of vinpocetine, a derivate of *Vinca minor* L., shows little resemblance, if any, to that of vincristine and vinblastine, obtained from *Vinca major* L.

The effects of vinpocetine upon retrograde axoplasmic transport are very similar to those exerted by vincristine and vinblastine, two semisynthetic derivates of *Vinca major* L. (Knyihar-Csillik and Csillik, 1981). TDA caused by vinpocetine is followed by the appearance of labyrinthine structures similar to those found earlier in the upper dorsal horn after perineural application of microtubule inhibitors vincristine and vinblastine (Knyihar and Csillik, 1976; Csillik et al., 1977).

FRAP has been suspected to be a genuine marker enzyme of nociception as early as more than three decades ago (Knyihar, 1971); yet this idea was supported by experimental facts only considerably later (Schoenen et al., 1985; Glykys et al., 2003). TMP, an isoenzyme of FRAP (O'Brien et al., 1989) undergoes alterations in the course of TDA similar to those of FRAP. SP and CGRP are regarded as pain-related neuropeptides (Snyder and McMahon, 1998). Disappearance of FRAP and TMP, and diminishment of SP and CGRP from the upper dorsal horn, can thus be suspected to be signs of a decreased nociception.

Vinpocetine was first synthesized by Lorincz et al. (1976) from the alkaloid vincamine, obtained from the leaves of *Vinca minor* L. (Bonoczk et al., 2000). According to the studies of the Marburg school of pharmacologists, vinpocetine prevents ischemic cell damage in the hippocampus (Sauer et al., 1988); it enhances cerebral blood flow and glucose utilization after forebrain ischemia (Rischke and Krieglstein, 1990). Vinpocetine was reported to protect against excitotoxic cell death of cerebrocortical neurons (Erdo et al., 1990); it increases the neuroprotective effect of adenosine in cell cultures (Krieglstein and Rischke, 1991). According to Bonoczk et al. (2000) positron emission tomography proves that vinpocetine is able to redistribute regional cerebral blood flow and enhance glucose supply of brain tissue in ischemic post-stroke patients. Lendvai et al. (2003) have shown that vinpocetine enhances morphological dynamics of dendritic spines of pyramidal cells in the neocortex.

Our studies reported here prove that perineurally applied vinpocetine is able to block retrograde axoplasmic transport of NGF in a peripheral nerve that induces TDA of primary sensory axon terminals in the segmentally related, ipsilateral upper dorsal horn, resulting in the depletion of FRAP and TMP, and partial depletion of SP and CGRP from the same area. Similar effects were observed earlier after perineural application of vinblastine and vincristine; also the blockade of retrograde axoplasmic transport of ^{125}I NGF after perineural application of vinpocetine is similar to that observed by us after perineural application of vinblastine and vincristine (Csillik et al., 1985). Since transcutaneous iontophoresis of vincristin and vinblastin alleviates chronic pain resulting from postherpetic, trigeminal and other neuralgias (Csillik et al., 1982), the possibility arises that vinpocetine, applied by transcutaneous iontophoresis, can also be used for alleviation of pain. Since iontophoretic administration of vinpocetine is feasible (Musaev et al., 1998) it can be assumed that iontophoretically applied vinpocetine may be effective in alleviation of chronic pain. Recent studies of Hua et al. (2004) revealed that an optimized microemulsion of vinpocetine represents a nonirritant transdermal delivery system. Accordingly, transdermal or transcutaneous administration of vinpocetine seems to be a promising possibility in the clinical treatment of neuropathic pain.

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