THESIS FOR THE DEGREE OF DOCTOR OF PHILOPSPHY (Ph.D.)

MOLECULAR PHARMACOLOGY OF VANILLOID RECEPTOR-1 IN ANIMAL MODELS IN VIVO AND IN CELLULAR SYSTEMS IN VITRO

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Debrecen
2006

INTRODUCTION

Capsaicin-sensitive neurons

A distinct subpopulation of primary sensory neurons, which cell-bodies are located in dorsal root (DRG) and trigeminal sensory ganglia – based on their marked sensitivity to capsaicin and to other vanilloids (e.g., resiniferatoxin (RTX) isolated from the latex of *Euphorbia resinifera*) – are generally referred to as "capsaicin-sensitive neurons". Vanilloid sensitive neurons are heterogeneous morphologically, neurochemically and functionally and they encompasses several subclasses of DRG neurons. In general, vanilloid-sensitive neurons are small diameter neurons, giving rise to thin, unmyelinated C-fibers. As their afferent function (via their central axons), cells transport sensory information to the central nervous system, whereas as their peripheral terminals are sites of release for a variety of proinflammatory neuropeptides (such as substance-P, calcitonin gene-related peptide) (efferent function). The released substances in turn act on other cells (such as keratinocytes, mast cells, macrophages or glia cells) and participate in the local trophic, vasoregulatory, and immunomodulatory processes which termed as neurogenic inflammation. Capsaicin-sensitive afferent fibers richly innervate the skin, mucous membranes, muscles, joints, and a host of visceral organs in the cardiovascular, respiratory and genitourinary systems. Visceral capsaicin-sensitive afferent neurons are involved in both reflex autonomic responses (blood flow, heart rate, micturation reflex etc.) to visceral stimuli and the conscious perception of visceral discomfort. Capsaicin-sensitive neurons have been detected in different areas of the brain.

The cellular action of capsaicin

The cellular effect of capsaicin and related compounds can be well characterized as three consecutive phenomena on sensory neurons. First

capsaicin evokes *excitation* (prompt depolarization after the application of capsaicin), during which the permeability of the cell membrane increases to (mostly) Ca^{2+} and Na^{+} ions leading to the depolarization of cells. The second characteristic process is called *desensitization,* which can be described as a decreased sensitivity to capsaicin and other (chemical, thermal or mechanical) nociceptive agents. Finally, capsaicin applied at high concentrations and/or for a long duration evokes *neurotoxicity,* which is mainly assigned to the increased intracellular calcium concentration ($[Ca^{2+}]_i$) and to subsequent activation of calcium-dependent proteases.

The vanilloid receptor (VR1)

The molecular entity that was believed to be responsible for the selective susceptibility of a subpopulation of primary neurons to vanilloids was first called the "capsaicin receptor". The recent cloning of the vanilloid-receptor type-1 (VR1) provided a means to investigate more fully the molecular and cell biological mechanisms of VR1-mediated pain signal transduction as well as processes underlying the susceptibility of VR1-expressing cells to impairment upon to vanilloid exposure. The rat VR1 is a 95 kDa protein consisting of 838 amino acids encoded by 2514 nucleotides. VR1 is a ligand-gated ion channel that integrates multiple noxious stimuli including vanilloids, protons and the heat. On the basis of its structural features, VR1 shows marked homology to the TRP (transient receptor potential) protein of the *Drosophila melanogaster*, it belongs to the TRP receptor family (TRPV1). The common features of these receptors that they possess 6 transmembrane domains and intracellular N- and C-terminals, and, most probably, they form tetramers in the membrane. It was also shown in various structural studies that both the extracellular-and intracellular sides of the VR1 contain various binding sites for regulatory molecules. Similarly to the native VR1 on sensory neurons, the cloned VR1 also functions as non-specific, chiefly Ca^{2+}-permeable cationic channel.

The central integratory role of VR1 in the development of pain sensation

VR1 can be activated not only by exogenous vanilloids but also by various endogenous compounds ("endovanilloids") such as the low-threshold heat (~43^{o}C), the decrease of pH (acidosis), and several inflammatory mediators (bradykinin, extracellular ATP, arachidonic acid derivatives, leukotriens, products of lipid-peroxidation). Some of these agents may directly activate the receptor such as heat and acidosis, while some others may indirectly regulate the function of VR1 via modification of intracellular signal transduction pathways (kinase systems, intracellular messengers). These effects may greatly decrease the heat threshold of the VR1 and thus contribute to the development of thermal hyperalgesia. Experiments carried out on VR1-knock out animals further strengthened that model.

Tissue distribution of VR1 and species-related differences

The tissue distribution of VR1 seemed to be restricted to the central nervous system, as VR1 mRNA transcripts were only detected in nociceptors located in peripheral sensory ganglia, trigeminal ganglia and in certain brain areas. However, others reported VR1 expression in non-neuronal cells such as kidney cells, lymphocytes and mast cells. Despite the mounting number of evidence for the expression of VR1 in the brain the existence of vanilloid receptors in the brain remained elusive. VR1 transcripts were detected by different methods (RT-PCR, Northern blot, Western blot) and biological responses to capsaicin microinjected into various brain areas were reported.

It has long been known that vanilloids show striking species-related differences in their biological actions. For example, birds do not respond to capsaicin, and among mammalians, rabbits are distinguished by their marginal sensitivity to capsaicin. Corresponding with that finding no [^{3}H]RTX binding was found in either birds or rabbits. In principles, these differences may reflect differences in VR1 expression, differences in neurotransmitter profile of VR1-

expressing neurons or simply species-related differences in the sequence of VR1. In birds, despite the vanilloid-insensitivity, low-threshold temperature-sensitivity is preserved. Analysis of the bird orthologue of VR1 revealed the lack of vanilloid-sensitive sequence. Based on these observations data from animal models could only be extrapolated to human with great caution.

Possible therapeutic potency of capsaicin and related vanilloid compounds

In principle, all the three characteristic actions of vanilloids (excitation, desensitization, and neurotoxicity) may have therapeutic value. Capsaicin is a standard ingredient in a variety of "over-the-counter" drugs used worldwide to relieve muscle ache. The beneficial capsaicin effect may be attributed to an increase in microcirculation in the treated area. Topical capsaicin has been tried as an adjuvant analgesic in a variety of neuropathic pain conditions such as postherpetic neuralgia, painful diabetic neuropathy and postmastectomy pain. Because of the important adverse effect of capsaicin, the initial burning sensation, the therapeutic value of capsaicin was found controversial in neuropathic pain conditions.

Vanilloid-sensitive nerves participate in various reflex responses such as micturation reflex. Capsaicin injected via a catheter into the urinary bladder is beneficial in conditions such as bladder hypersensitivity or detrusor hyperreflexia by decreasing the sensitivity of C-fibers subserving the micturation reflex. Complication of capsaicin installation into the urinary bladder is the initial hyperreflexic contractions and pain. Unsatisfactory clinical results with capsaicin are mostly due to the unfavorable ratio of irritation to desensitization of this drug. Consequently, resiniferatoxin (RTX) with improved desensitization to irritation ratio represents more promising therapeutic tool.
Indeed, RTX was found approximately 1000 times more potent than capsaicin for desensitizing urinary bladder afferents in the rat and caused dose-dependent down-regulation of specific RTX binding sites. The RTX-induced changes in

the bladder were long-lasting and reversible in both animal models and in human. Intravesical RTX fulfilled the expectations and caused lasting improvement in the life of patients with urge urinary incontinence.

The ability of vanilloid compounds to deplete or inhibit certain nociceptive neurons, while retaining all other sensory modalities and motor function, would represent a new therapeutic approach to control severe pain. Thus, by targeting one or many ganglia, using the intraganglionic, intratechal or epidural routes, the spatial extent of the therapeutic action may be adjusted to match varied clinical presentations.

RATIONALE AND AIMS OF THE STUDY

The major focus of our investigation was to understand better the process of desensitization *in vitro* at cellular level and *in vivo* in the rat.

In vivo we tested the effect of epidurally administered resiniferatoxin and compared its efficacy with that of given systemically. We investigated dose-response relationship for both routes of administration. Epidural administration of the drug enabled us to define an internal control in the experiments (front paws vs. hind paws). Loss of specific [^{3}H]RTX binding sites were in parallel measured to assess dose-dependency and efficacy in both routes of administration. This experimental setup made direct comparison possible. Time-course of both the initial hyperalgesic phase and the prolonged desensitization was accurately recorded.

In vitro experiments were designed to follow intracellular events triggered by receptor-ligand interaction. For that purpose, we labeled VR1 with a green fluorescent tag (eGFP). That construct enabled us to investigate ligand-induced cellular events “real time” employing confocal microscopy. Pharmacological comparison of tagged-VR1 constructs and the native variant were made. Using

confocal microscopy localization of VR1 and changes in cellular morphology was followed at real time, while in parallel intracellular calcium levels were also detected. Calcium-dependency of ligand-induced cellular events, as well as possible correlation between ligand-induced intracellular events and *in vivo* experimental findings were assessed.

Finally, we demonstrated specific, high affinity, saturable [^{3}H]RTX binding in various areas of monkey brain not known to be innervated by primary afferent neurons. We performed detailed pharmacological characterization of both the peripheral and centrally located receptor sites. We measured receptor density in selected regions of monkey brain. We described binding characteristics in both the peripheral and central VR sites. Direct comparison of binding parameters measured in monkey was made to similar values of other species.

MATERIALS AND METHODS

Assay of paw withdrawal latency

Catheter placement was performed as previously described by Durant. Briefly, 22-gauge polyurethane catheter was introduced under the cranial edge of L4 into the gap created by the spinal needle and was advanced 1-1.5 cm caudally into the epidural space. Assay for nociceptive response was performed in a Plantar Analgesia Instrument, which measures latency time for paw withdrawal after radiant thermal stimuli was applied to the paw. This approach permitted measurement of paw withdrawal latency both for the back paws as well as the front paws, thereby providing an internal control for regional change in response in the case of the epidurally administered RTX. Administration of RTX was performed under brief anesthesia using oxygen/isoflurane. For epidural treatment, 40 µl of graded doses of RTX solution or vehicle was

injected into the catheter. For the measurements, rats were placed into a plastic chamber with a glass floor. A radiant heat source was positioned under the paws and response of paw withdrawal was detected automatically to the nearest 0.1 sec. Baseline determination of the latency time was carried out earlier. Animals were examined to ascertain lack of neurological deficit. Time-course of desensitization was determined in both routes of administration as well as in control group. Statistical analysis of data was performed by using bidirectional analysis of variance (ANOVA) test followed by an unpaired t-test where indicated.

[^{3}H]RTX binding measurements on membrane preparations

Levels of vanilloid receptor in spinal cord membranes were assessed by [^{3}H]RTX. Membrane preparations from spinal cord or selected brain segments were prepared as followed. Briefly, samples were disrupted in ice-cold buffer. Homogenates were centrifuged at 1000 g for 10 min at 4^{o}C, pellets were resuspended and recentrifuged at 35000xg for 40 min at 4^{o}C. Pellets of the second centrifugation were used for binding assays. Membrane preparations were incubated with [^{3}H]RTX for 60 min at 37^{o}C in a total volume of 300 µl in Buffer A supplemented with bovine serum albumin. Bound and free [^{3}H]RTX were separeted by pelleting the membranes by centrifugation at 10000xg for 15 min at 4^{o}C. Nonspecific binding was determined in the presence of 100 nM nonradiactive RTX. Binding was expressed as fmol/mg protein. Data were analyzed by computer fit to the Hill equation.

[^{3}H]RTX binding measurements on transfected cells

48 h after transfection in 75-cm^2 T-flasks, cells were detached from the plastic surface by serum-free DMEM containing 1 mM EDTA and then washed and resuspended in 10 mM HEPES buffer (pH: 7.4). Intact cells were incubated

(10^5/well) in a filtration plate with 200 pM [^{3}H]RTX for 60 min at 37°C and then processed as above described.

Determination of ^{45}Ca uptake

Cells were transfected at 80% confluence in 75-cm^2 T-flasks with 20 µg of VR1eGFP or VR1ε plasmids. After 48 h, 5 x 10^4 cells were detached from the plastic surface by serum-free DMEM containing 1 mM EDTA and then washed two times and resuspended in medium without EDTA. Cell suspensions were incubated in serum-free DMEM containing 1 µCi/ml ^{45}Ca and ligands as indicated for 15 min at 35°C in 96-well filtration plates. ^{45}Ca uptake was terminated on ice and samples were processed for radiactive measurement.

RT-PCR cloning and epitop tagging of VR1

To obtain VR1-specific mRNA, total RNA were isolated from rat DRGs. A fragment, comprising the sequence between the *XbaI* and *AflIII* sites of rat VR1, was amplified first by access RT-PCR system (Promega), and then cloned into the BlueScript vector (Stratagene). The missing 5'-sequence was added likewise with the *SacI* and *XbaI* sites. At the 5' ends of the N-and C-terminal fragments, the *SacI* and *AflIII* sites were incorporated with forward primers AGATCTCGAGCTCAAATGGAACAACGGGCTAGCTTAGACTC and CTGTATTCCACATGTCTGGAGCTGTTCAAGTTC, respectively. As reverse primers ACTGAGTCCCGGGCGCTGATGT-CTGCAGGCT and CACACAGTCGACTTTCTCCCCT-GGGACCATGGAATCCTT were used, in which the *XbaI* and *SalI* sites were incorporated, respectively. The *SacI – AflIII* and the RT-PCR generated *AflIII-SalI* fragments were triple-ligated into a *SacI-SalI* cut pEGFP-N3 vector (Clontech). Employing similar strategy, rat VR1 with the short, 12-amino acid ε-tag (KGFSYFGEDLMP) was constructed in a vector, pεMTH, driven by the metallothionein promoter.

Western blotting

For the Western blot analysis of VR1 receptor and its tagged-variants, cells were washed with ice-cold phosphate-buffered saline (PBS), harvested in homogenization buffer, and disrupted by sonication on ice. Equal amounts of protein (20-30 μg per lane) were then subjected to SDS-PAGE (on 7.5 % gels) and transferred to nitrocellulose membranes. Membranes were then blocked with 5 % dry milk in PBS for 30 min and probed with the appropriate primary antibodies overnight. Peroxidase-conjugated secondary antibodies were used to visualize the immunoreactive bands by an ECL Western blotting detection kit on light sensitive films.

DRG culture

DRG neuron-enriched cultures were prepared from rats. Briefly, spinal cords were dissected, and DRG's were stripped off with the meninges. Cells were digested in 0.125% trypsin at 37^{o}C for 20 min. For plating, dissociated cells were changed into minimal essential medium containing 5% horse serum and 50 ng/ml nerve growth factor (NGF). Cells were seeded on 25-mm glass coverslips or on multiwell plates. Surfaces were coated with poly-D-lysine and laminin. DRG cultures were maintained in DMEM containing 20 mM HEPES, 7.5% fetal bovine serum, 7.5% horse serum, 5 mg/ml uridine supplemented with 2 mg/ml FUDR to inhibit cell division and 50 ng/ml NGF to promote neuronal survival and differentiation. Cultures were maintained in this medium for 1 week, at which point well differentiated neurons and non-dividing cells dominated the population. Primary DRG cultures in this stage were used for confocal microscopy.

Fluorescent confocal microscopy

COS7, NIH 3T3, and HEK293 cells were seeded on 25-mm coverslips and transfected with 1 μg each of the plasmid constructs, cultured for 24 h post-

transfection at 35°C, then mounted in a 1-ml chamber and examined with a MRC-1024 Bio-Rad confocal microscope. To study the two-and three-dimensional distribution of fluorescent chimeric proteins, each *x-y* plane was scanned over 1 s and at 0.2-µm increments in the *z* axis mode. To label different subcellular compartments of live cells fluorescently, the ER marker eGFP-KDEL (Clontech) was transiently transfected in COS7 and NIH 3T3 cells. To label mitochondria, MitoTracker (Molecular probes) dye was incubated for 30 min at a 250 nM concentration; the cells then were washed with Hanks' balanced salt solution supplemented with 1 mM $CaCl_2$ and 0.8 mM $MgCl_2$, buffered with 15 mM HEPES (pH 7.4) (HBSSH).

$[Ca^{2+}]_i$ Microfluorometry

For determination of $[Ca^{2+}]_i$, cells were cultured in glass bottom dishes and then transfected with 2 µg of VR1eGFP plasmid. After 24 h in culture, cells were loaded with Fura-2/AM for 30 min at 37°C. Single cells expressing the VR1eGFP construct were identified by eGFP fluorescence and were selected based on visual inspection of fluorescence intensity. In all experiments, cells exhibiting intermediate fluorescence in comparison to other cells in the same dish were picked for analysis. To determine the $[Ca^{2+}]_i$, the excitation ratio of Fura-2 at 340 and 380 nm was recorded photometrically in Krebs' buffer at a 10 Hz sampling rate and integrated over 0.5 s. $[Ca^{2+}]_i$ was calculated using a ratio based equation.

Ratiometric imaging of $[Ca^{2+}]_i$ employing confocal microscopy

Cells cultured on poly-D-lysine-coated coverslips were pre-loaded with 5 µM Indo-1 AM dye. After incubation for 30 min at 34°C, the cells were washed 3 times in HBSSH to remove excess dye and examined under the confocal microscope. To record in *zero* extracellular calcium cells were washed four times in HBSSH containing no $CaCl_2$ and 1 mM EGTA and imaged in the same

medium. Groups of cells expressing VR1eGFP and small size neurons were selected under the microscope. To quantitate the fluorescence ratio, perikarya of the cells were marked with the graphic tools of the LaserSharp software in the field of a x 40 objective of Bio-Rad confocal system. Ratiometric imaging was performed at 10-s intervals with an UV laser, and the ratio of fluorescence intensity emitted at 405 and 485 nm was calculated.

Electrophysiology

For patch clamp studies, VR1eGFP-expressing COS7 and HEK293 cells were voltage-clamped in Krebs' buffer containing (in mM) NaCl (124), KCl (4.9), KH_2PO_4 (1.2), $MgSO_4$ (2.4), $CaCl_2$ (2.5), $NaHCO_3$ (25.6), and glucose (10), using an Axopath 200B amplifier (Axon Instruments). Recordings were carried out with patch electrodes (2-10 MΩ) filled with 10 mM HEPES buffer (pH 7.4) containing (in mM) CsCl (120), tetraethylammonium chloride (20), $CaCl_2$ (1.0), $MgCl_2$ (2), EGTA (10), ATP (4), and GTP (0.5).

RESULTS

1. The *in vivo* analgesic effect of RTX an ultrapotent agonist of VR1

The objective of our *in vivo* study was to evaluate the ability of lumbar epidural RTX to produce prolonged regional desensitization in rats. For systemic administration, the paw withdrawal latency time – after an initial hyperalgesic period - increased as a function of time after administration, reaching a plateau at 4-5 h. Prolongation of the time of withdrawal latency represented the desensitization. The maximal increase in withdrawal latency was to 19.7 ± 0.6 s at 300 µg/kg RTX a dose that represented the highest tolerable dose of RTX. The ED_{50} value for systemically administered RTX, determined at

6 h, was 39.2 ± 3.9 μg/kg RTX. The slope of the curve was consistent with modest cooperativity, with a Hill coefficient of 1.38 ± 0.26.

For lumbar epidural RTX administration, the back paw latency time increased to its plateau value modestly more slowly than was the case for systemic administration. The plateau latency value was only achieved at approximately 6 h. Unlike for the systemic route of administration, for epidural RTX the onset of increased paw withdrawal latency was similar over the range of RTX doses examined. The maximal increase in withdrawal latency was to 33.4 ± 2.6 s at 100 μg/kg RTX. This increase in paw withdrawal latency was significantly greater than that achievable with the systemic route of administration ($p<0.001$). The ED_{50} value for epidurally administered RTX at 6 h, was 6.0 ± 0.3 μg/kg RTX. The Hill coefficient was 1.22 ± 0.10. Similar dose-response relation was found for the other time points once the plateau in level of desensitization had been achieved. The response of the front paws to lumbar epidural RTX treatment was monitored along with that of the back paws. The dose response curve for the front paws upon lumbar epidural RTX treatment was similar to or less than that observed for either the back or front paws upon systemic treatment. The systemic effect of the epidural RTX administration thus stands in marked contrast with its significantly enhanced localized effect as evident on the back paws. On the other hand, for systemically administered RTX, little difference was observed in the dose response curves for the front or back paws.

From previous reports it is known that desensitization to systemic RTX administration was associated with loss of vanilloid receptors, as quantitated by [^{3}H]RTX binding. To assess the localization of the effect of epidurally administered RTX, we measured the relative levels of [^{3}H]RTX binding to spinal cord samples removed from the treated animals. Markedly greater loss of binding was observed for the lumbar region of epidurally administered RTX. At the 100 μg/kg dose of epidurally administered RTX almost complete loss of

specific [^{3}H]RTX binding was observed in the lumbar region, whereas in the cervical region only 42% loss of binding was detected.

2. The *in vivo* effect of RTX in cells expressing VR1 – the mechanism of desensitization

The function and expression of VR1-expressing chimeric proteins

To investigate the effect of various vanilloid compounds we C-terminally epitop tagged VR1 with either a 27-kDa eGFP (enhanced green fluorescence protein) or with the ε-epitop (consists of 12 amino acids). VR1eGFP and VR1ε plasmid constructs were expressed in COS7, HEK 293, and NIH 3T3 cells. Western blot analysis with polyclonal eGFP and ε-tag-specific antibodies demonstrated that the VR1eGFP and VR1ε chimeric proteins expressed in cells were of the appropriate sizes, 120 and 93 kDa, respectively. Both capsaicin (CAP) and resiniferatoxin (RTX) evoked a large inward current promptly. In accordance with the electrophysiological data, RTX induced $^{45}Ca^{2+}$ uptake in VR1eGFP-expressing cells in a dose-dependent manner. ED_{50} values for ligand-induced $^{45}Ca^{2+}$ influx were 100 ± 50 pM for RTX. Similar value for CAP was 0.5 ± 0.15 μM. Similarly to the wild-type VR1, both tagged recombinants exhibited a high affinity, dose-dependent [^{3}H]RTX binding (K_d: 150 ± 10 pM). Hill coefficient were 1.5-2.0, indicating positive cooperativity among the receptor sites. Agonist-induced calcium mobilization and receptor binding were almost completely abolished by the competitive antagonist capsazepine (CZP) indicating VR1-specificity of actions. Our results clearly proved that C-terminal tag does not significantly change the pharmacological properties of VR1.

Intracellular distribution of VR1 – examination of RTX-induced cellular events and dynamic membrane changes with employing „real-time" confocal microscopy

Confocal fluorescence microscopy was employed to analyze the intracellular distribution of VR1eGFP. VR1eGFP signal was detected in the plasma membrane and in intracellular structures consistent with the ER. Dual-wavelength imaging studies demonstrated that the intracellular compartment containing VR1eGFP signal was distinct from the filamentous mitochondria. Our results were in good agreement with previous observations on native DRG neurons, where VR1-specific immunocytochemical staining of fixed DRG neurons showed high-density of staining throughout the neuronal cytoplasma. For testing the effect of $[Ca^{2+}]_i$ increase to intracellular morphology, ionomycin was added to cells expressing VR1eGFP or eGFP-KDEL. It was previously reported, that addition of ionomycin to cells induces intracellular membrane fragmentation (mainly ER) due to permeabilization of the plasma membrane to cations. This fragmentation (fragmentation of the ER and a rounding up of filamentous mitochondria) was identical that was occured with VR1eGFP-expressing cells upon RTX exposure. In controll cells expressing only eGFP the structure of the mitochondria and ER remained intact.

Calcium-dependency of cellular events in VR1-expressing cells

Based on our findings the following conclusions were drown: 1) VR1 is located in both the plasma membrane and in the intracellular compartments; 2) VR1-mediated cellular events are dependent on $[Ca^{2+}]_i$ increase. The preceding experiments focused on events occurring within the first few seconds following exposure to VR1 agonists. Addition of RTX induced a rapid (within 10 s) elevation of $[Ca^{2+}]_i$ in VR1eGFP-expressing cells which peaked 10-fold increase and, consistent with the concurrent ER and mitochondrial damage, did not returned to resting level. Similar response was not seen in control cells.

To follow in detail the time course of intracellular events "real-time" confocal microscopy was employed. The early events occurred as described within 30 s (*e.g.* transmembrane Ca^{2+} flux, intracellular remodeling), then the nuclear membrane was outlined with VR1eGFP fluorescence, and ER membrane vesicles were observed, then progressively growing blebs were noted in the nuclear membrane. Eventually, the membrane degradation concluded with bursting of the plasma membrane. In lower doses of RTX membrane fragmentation occurred in slower kinetics, but finally resulted in cell lysis within 3 h.

To explore potential activity of the ER-localized VR1 ratiometric confocal imaging was employed. VR1eGFP-expressing cells and DRG neurons were loaded with indo-1/AM and studied in the absence of external calcium. Upon RTX treatment rapid increase of $[Ca^{2+}]_i$ was seen in both cell types. Non-transfected control cells and a group of native sensory neurons (presumably VR1 negative neurons) did not respond to RTX treatment. In the absence of external calcium, RTX-induced $[Ca^{2+}]_i$ elevation was delayed in both types of VR1-expressing cells, and the time required for these changes was much longer, on the order of minutes not seconds. These data suggest that morphological changes occured upon RTX exposure are VR1-dependent, and the ER-localized VR1-receptor population is functional. Corresponding with our previous observations, RTX induced similar morphological changes (vesiculation of ER, disruption of mitochondria) even in the absence of external calcium however, the time course of changes was slower, usually occurred 3 min after RTX treatment. In primary cultures of native DRG neurons similar observations were noted. These data suggest, that ER-localized VR1 mobilizes calcium from intracellular stores, but that $[Ca^{2+}]_i$ accumulation occur more slowly.

To address the effects on DRG neurons of longer exposure to RTX, DRG cultures were subjected to dual wavelength fluorescent imaging. In repeated experiments, a group of specific neurons demonstrated instant increases in

$[Ca^{2+}]_i$ (within seconds) upon administration of RTX. Neurons exhibiting the RTX-induced elevation of $[Ca^{2+}]_i$ started to accumulate propidium iodide (PI) in the nucleus (indicating cell death) approximately 40 min after addition of RTX, time coincides with loss of plasma membrane integrity in VR1eGFP-expressing cells. In the same microscopic field there were neurons that neither responded to RTX with an elevation $[Ca^{2+}]_i$ nor accumulated PI even after 2 h of exposure. This result is consistent with the idea that RTX-induced cellular events are VR1-dependent in sensory neurons, similarly as it was described in recombinant VR1-expressing cells. This also suggests that vanilloid (RTX) application to sensory neurons may be an effective means for specific deletion of nociceptive neurons. To study this phenomenon, primary DRG cultures were treated with either resiniferatoxin (RTX) or olvanil (OLV, a long chain fatty acid modified synthetic vanilloid). Western blot of VR1 protein revealed that olvanil treatment almost completely eliminated (99% decrease) neurons expressing VR1 from cultures, similarly RTX, at much lower concentration, eliminated approximately 80% of VR1-expressing neurons. Other cell types present in the DRG culture were not affected by RTX treatment, as assessed by re-probing the same Western blots with an antibody recognizing the common tissue protein cytochrome-c.

3. Expression of VR1 in the central nervous system pharmacological characterization of vanilloid receptor located in the monkey brain

Specific [^{3}H]RTX binding in membrane preparations of spinal cord and dorsal root ganglion of the monkey

Levels of vanilloid receptor (VR1) in membrane preparations of spinal cord, dorsal root ganglia (DRG) and selected regions of the monkey brain were assessed by [^{3}H]RTX binding. We compared binding characteristics of the cenral (brain) and peripherial (spinal cord and DRG) receptor sites.

In control experiments high affinity, specific, saturable [^{3}H]RTX binding was detected in membrane preparations both from the dorsal horn of the spinal cord and from the dorsal root ganglia. The binding parameters for the dorsal horn and the dorsal root ganglia were determined as follows: the maximal binding capacities were 244.4±19.6 and 240±26.6 fmol/mg protein, respectively. K_d values representing half-maximal binding were 312±14 and 289±15 pM, respectively. The cooperativity indexes (P values) were 1.4±0.04 and 1.5±0.08, respectively, indicating positive cooperativity between the binding sites.

To further investigate specificity of [^{3}H]RTX binding competition experiments were performed. Non-radioactive RTX inhibited specific binding of 500 pM [^{3}H]RTX with K_i values of 349±25 pM for the dorsal horn of the spinal cord and 309±14 pM for the dorsal root ganglion membrane preparations, with P values similar to those obtained in the first set of experiments. Similarly, capsaicin inhibited specific [^{3}H]RTX binding with K_i value of 4.7±0.26 μM in membrane preparations from dorsal root ganglia; and 4.3±0.32 μM in membrane preparations of the dorsal horn of the spinal cord. Cooperativity was positive in both cases. Corresponding values for the competitive antagonist capsazepine were 3.1±0.23 μM in DRG and 3.73±0.35 μM in the spinal cord.

Specific [^{3}H]RTX binding on different regions of the monkey brain

Specific [^{3}H]RTX binding was detected in membrane preparations from different brain regions, including locus ceruleus (LC), preoptic area (AP), medial-basal hypothalamus (MBH), pontin reticular formation, ventral thalamus, cerebellum and both the somatomotor cortex and somatosensory cortex.

Bound values were 140±11, 129±17, 143,4±8,4, 66,6±4,8, 21,2±4,5, 48,4±6,2, 57,7±5,3 és 59,2±6,3 fmol/mg protein, respectively. For further characterization regions that represented a „high receptor density zone" (LC, AP, MBH) were chozen. The specific [^{3}H]RTX binding followed sigmoidal saturation kinetics with the following K_d values: 601±28 pM for LC /AP and 565±41 for MBH.

Capsaicin and the competitive antagonist capsazepine inhibited specific binding of the [^{3}H]RTX to membrane preparations of medial basal hypothalamus with K_i values of 0.95±0.1 μM and 0.86±0.11 μM.

DISCUSSION

Epidural RTX induced prolonged, regional analgesia on rats

Vanilloid compounds such as RTX and capsaicin have wide therapeutic potential as non-narcotic analgetics. Previous studies revealed significant quantitative and qualitative differences between *in vivo* and *in vitro* efficacy of different vanilloids. Clinical trials on human patients with bladder hyperreflexia revealed, that RTX, administered intravesically, desensitized C-fibers without inducing initial pain observed with capsaicin, thus urge incontinent patients experienced significant improvement. These data suggested that RTX has a more favorable therapeutic profile as compared to capsaicin.

To reduce side-effects associated with systemic desensitization of C-fibers by vanilloids, attention has focused on various routes for topical or otherwise localized application. For regional pain, intratechal or epidural administration represents a strategy for localizing the site of action. Well-defined pharmacological advantages of epidural administration include anatomical proximity to the targeted tissue and low systemic uptake. In the present study we demonstrated that epidural RTX caused long-lasting, regional thermal anesthesia with only minimal systemic effect. Our results confirm that epidurally administered RTX shows enhanced effectiveness relative to systemic RTX for blocking C-fiber mediated nociception. Comparison of the ED_{50} values suggests a 6.5-fold enhancement of potency via the epidural route. However, comparison

of doses causing comparable 3-fold increases in withdrawal latency indicates a 25-fold enhancement via the epidural route.

VR1-mediated selective cytotoxicity induced by vanilloid agonists

The loss of specific [^{3}H]RTX binding upon epidural RTX treatment suggested that vanilloid agonists can selectively deplete VR1-expressing nociceptive neurons. Thus, in the next set of experiments, VR1-dependent cellular events were investigated in both recombinant VR1-expressing cells and in „native" neurons. VR1-expressing heterolog cell systems displayed appropriate pharmacological and functional similarity to nociceptive neurons to serve as a model system. The expression of the fluorescent VR1eGFP chimeric protein enabled us to investigate „real-time" VR1-dependent cellular events and localization of VR1 within the cell. For the first time in the literature, we visualized VR1-signal at cellular level, and proved that VR1 localized not only in the plasma membrane but also in intracellular membrane compartments such as the endoplasmic reticulum and nuclear envelope. The intracellular location of VR1 was clearly separable from the mitochondrial compartment.

Addition of RTX to VR1-expressing cells induced approximately 10-fold accumulation of $[Ca^{2+}]_i$. This coincided with the rapid transformation of the normal lattice-like ER membrane structure into rounded vesicles, the earliest hallmark of Ca^{2+} cytotoxicity observed in living cells. In addition to ER, live cell imaging experiments revealed other membrane compartments that reacted to RTX-induced elevated $[Ca^{2+}]_i$. These include the following: 1) shedding of the plasma membranes; intracellular fragmentation of mitochondria; and later blebbing of the nuclear envelope occurred. These observations were consistent with RTX-induced cell death of VR1-expressing cells.

To further investigate the potential role of ER-localized VR1 we studied vanilloid-induced $[Ca^{2+}]_i$ changes in calcium-free medium. In accordance with previous reports, similar vanilloid-induced, VR1-specific remodeling of ER

occurred with Ca^{2+} mobilized from intracellular sources, although with a much slower time course. We concluded that in addition to localization of VR1 at the plasma membrane, a newly recognized, functionally active intracellular (ER) VR1-pool was demonstrated in both heterolog VR1-expressing cells and in a subgroup of DRG neurons.

Our findings and the following observations 1) RTX did not induce elevation of $[Ca^{2+}]_i$ in cells not-expressing VR1; 2) In DRG culture only a subgroup of neurons (presumably VR1-expressing sensory neurons) responded to RTX treatment, while glia cells and other neuronal elements remained unresponsive to RTX; 3) the competitive antagonist of VR1, capsazepine effectively inhibited RTX-induced cellular events; suggested that RTX-induced Ca^{2+}-mediated cytotoxicity, observed in heterolog expression systems is a specific, VR1-dependent process.

To verify that the rapid intracellular membrane changes observed in heterolog cells expressing VR1 also occurred in small-size DRG neurons, experiments were carried out on primary cultures from DRG. Upon RTX treatment similar morphological changes were observed in small-sized neurons than in heterolog cells expressing VR1. Vanilloids rapidly targeted intracellular structures (ER, mitochondria) of small-sized sensory neurons (VR1 positive neurons) within seconds. Although the primary steps of Ca^{2+} toxicity are similar to apoptosis, including nuclear blebbing and propidium iodide (PI) staining of the nucleus, chromatin fragmentation, apoptotic body formation, and caspase activation were not apparent within 24 h. The longer term dynamics visualized with VR1eGFP in the present study are consistent with those noted with end point observations in DRG neurons, including intracellular membrane fragmentation, mitochondrial swelling, and nuclear envelope segmentation.

It should be emphasized, that as a consequence of RTX treatment only a subgroup of DRG neurons (small-sized VR1-expressing nociceptive neurons) were eliminated, whereas glia cells or other medium-to large-diameter neurons

did not respond to vanilloid challenge. Obviously, VR1-mediated damage of vital cell organelles may be responsible for desensitization, and when changes are irreversible ("over the point of no return"), the targeted neuron will eventually die. The present data suggest a new potential therapeutic use of vanilloids, which is the targeted removal of nociceptive primary afferent neurons, without affecting the full spectra of DRG neurons, could be termed as "chemical microsurgery". Among wide variety of VR1 agonists, olvanil (OLV) may have several advantages for clinical applications. Our data convincingly showed, that OLV is nontoxic to cells devoid of VR1 up to 50 μM, yet this concentration almost completely and specifically removed VR1-expressing cells in DRG culture. That corresponds with our previous observation, that epidural RTX eliminated specific [^{3}H]RTX binding on spinal cord and caused prolonged regional analgesia. Clinical application of vanilloids could be made regionally selective to achieve appropriate pain control, without affecting other sensory modalities and motor function. Our hypothesis has already been proved in dogs with bone cancer, where tumor progression associated with neuropathic pain, locally administered RTX induced long-lasting, VR1-specific, effective, regional pain control. We believe that inhibition or removal of vanilloid-sensitive nociceptive neurons represents a promising therapeutic approach for the management of chronic tumor or neuropathic pain in patients, for example with tumor-related "pelvic pain" or bone pain, who experience poor pain control due to either tumor progression or to untoward side effects.

Pharmacology and tissue distribution of VR1 located in the brain

There are striking species-related differences in vanilloid sensitivity, thus making it difficult to extrapolate pharmacological data from animals to human. In our study, we demonstrated high-affinity, saturable [^{3}H]RTX binding on membrane preparations of the spinal cord (SC) and dorsal root ganglia (DRG) of the monkey. For comparison, receptor affinities determined in monkey DRG and

spinal cord samples were found approximately 3-fold higher than similar values in human, and 8-fold weaker than corresponding values from the rat. We detected specific [^{3}H]RTX binding in various regions of the monkey brain. The binding observed in in the brain areas displayed appropriate pharmacological specificity to represent the vanilloid receptor (VR1). The highest receptor-density was found in the locus ceruleus (LC), medial basal hypothalamus (MBH), and preoptic area of the hypothalamus (AP) in the brain. The pattern of receptor distribution was in a good agreement with results from previous reports obtained using other species. The specific [^{3}H]RTX binding observed in these regions followed sigmoidal saturation kinetics, indicating positive cooperativity of among the binding sites. Both their affinities and degrees of cooperativity were in general agreement with with values obtained from peripheral receptor sites (SC, DRG). However, modest differences were found in the structure-activity profiles between the peripheral (SC, DRG) and central receptor sites (LC, MBH, AP), which may correspond with slighly different modification of the receptors. Since none of the studied brain areas are known to receive primary sensory afferent innervation, our results suggest that those neurons autonomously express VR1.

Regions with the highest receptor density are involved in various sensory and autonomic functions, including pain perception and endogenous pain modulatory functions. Neurons of the locus ceruleus form part of the brainstem respiratory network as specific sites of CO_2- or pH-dependent chemoreception. It was reported, that capsaicin can activate LC neurons that can be blocked by capsaicin pretreatment (by selectively eliminating VR1 binding sites). A group of neurons in the preoptic-anterion hypothalamus responded to changes in local temperature and this brain area is thought to play important role in the central control of thermoregulation. Early studies reported that systemic and intrahypothalamic injections of capsaicin produce hypothermia and large doses

of capsaicin induce similar ultrastructural changes in hypothalamic and DRG neurons.

Acidosis and nociceptive thermal stimuli are known „activators" of the VR1, therefore vanilloid receptor expressing neurons in the hypothalamus and other brain areas may play a unique role in thermo-and chemosensation and nociception.

From mechanism to therapy: agonist, antagonist and partial agonist?

My thesis do not include already published results of structure-activity studies of more than 200 vanilloid compounds, that had been tested in our laboratory. But I am convinced, that thousands of vanilloid agents heve already been tested on a large scale in the pharmaceutical industry.

In my thesis, I only presented one possible therapeutic strategy, among many others, for the possible use of vanilloids. In our model we challenged the possibility to selectively deplete VR1-expressing DRG neurons, via targeting VR1 with vanilloid agonists. We emphasized that certain agonists, with more favorable pharmacological profile (RTX, OLV), can produce desensitization with minimal initial irritation, while others (CAP) provoke almost an intolerable initial pain before desensitization.

As another approach, blocking of VR1 by antagonists also seems like a sensible therapeutic strategy. Recent reports from the field of gastroenterology highlighted the importance of VR1-mediated processes in inflammatory bowel diseases. Painless, reversible inhibition of VR1 by antagonists, without an initial irritation, might be a useful approach to cure these conditions.

In fact, one could combine the advantage of agonist-evoked long-term desensitization with a presumably shorter-term antagonist action to avoid initial hyperalgesia, which is frequently accompanied with highly potent VR1 agonists. Partial agonists of VR1 may be excellent candidates for that, especially when their activation kinetics is slow.

In summary, I hope that series of vanilloid compounds, with excellent therapeutic profile, will be clinically trialed in the near future for the treatment of intractable pain conditions, which do not respond to convential drugs, as well as in certain inflammatory disorders.

SUMMARY

1. In *in vivo* experiments, we investigated the effect of epidurally administered resiniferatoxin (RTX). Epidural RTX induced dose-dependent, prolonged and regional anesthesia with minimal systemic effects. The analgesic efficacy of epidural RTX exceeded that of the systemic administration. Furthermore, depletion of vanilloid receptor-1 (VR1) sites were detected in the spinal cord. Elimination of VR1 binding corresponded with regional effect of epidural RTX treatment.
2. *In vitro* experiments were designed to test recombinant VR1-expressing cell systems, and to compare their pharmacological and functional properties with that of the nociceptive sensory neurons. Heterolog VR1-expressing cells displayed appropriate similarity to „native" neurons for modelling vanilloid-evoked electrophysiological and morphological changes.
3. VR1 was found in the internal membranes in both nativ neurons and in recombinant VR1-transfected cells.
4. VR1-dependent elevation of intracellular calcium occured even in the absence of external calcium, indicating the existence of functionally active VR1-pool in the (endoplasmic reticulum) ER.
5. VR1-specific neuron depletion can be achieved by the administration of RTX, an ultrapotent agonists of VR1. Selective ablation of VR1-expressing nociceptive neurons may be responsible for the analgesic effect of vanilloid agonists.

6. High-affinity, saturable, specific [^{3}H]RTX binding can be detected in various regions of the monkey brain. Pharmacological properties of the centrally located VR showed appropriate pharmacological similarity to the peripherial VR1 receptor. Tissue distribution of VR located int he brain was found to be similar to that of other species (rat, human).

7. RTX showed 7-fold weaker affinity to VR1 detected in monkey than to the rat VR1. Similar difference was only 3-fold for the favour of human.

8. Species related differences should be concerned to extrapolate data from animals to human.

PUBLICATIONS

THE THESIS WAS BUILT ON THE FOLLOWING *IN EXTENSO* PUBLICATIONS

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