THESES FOR THE DEGREE OF DOCTOR OF PHYLOSOPHY (Ph.D.)

INVESTIGATION OF FUNCTION AND REGULATION OF RECOMBINANT VANILLOID RECEPTOR (VR1) IN HETEROLOGOUS EXPRESSION SYSTEMS

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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". As their afferent function (via their central axons), the cells transport sensory information to the central nervous system, whereas their efferent role is to, upon activation, locally release certain neuropeptides (such as substance-P, calcitonin gene-related peptide) stored in the peripheral termini. 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, the abnormal, cascade-like enhancement of which may lead to neurogenic inflammation.

The cellular action of capsaicin

The cellular effect of capsaicin and related compounds can be well characterized as three consecutive phenomena on the sensory neurones. The first one is the *excitation* (developing right after the application of capsaicin), during which the permeability of the cell membrane increases to (mostly) Ca^{2+} and Na^{+} ions and, therefore, leads to the depolarization of cells. The second characteristic process is the *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)

In already 1968, Jancsó postulated the existence of a so called "pain receptor", which could “prove” the capsaicin selectivity of the sensory neurons. In 1997, the molecular description of the first capsaicin-sensitive specific molecule, the vanilloid (capsaicin) recepotor (VR1) was published using a rat cDNA library. The rat VR1 is a 95 kDa protein consisting of 838 aminoacids encoded by 2514 nucleotids. On the basis of its structural features, i.e., the 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, that they form mostly 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 VR on the sensory neurons, the cloned VR1 also functions as non-specific, chiefly Ca^{2+} permeable cation channel.

The calcium dependence of the VR1-mediated cell responses – the subcellular localization of VR1

During the investigation of the rather complex cellular phenomena induced by the activation of VR1, one of the most important aims was *to define the dependence of the VR1-mediated processes on extracellular calcium concentration ($[Ca^{2+}]_e$)*. It was shown that the vanilloid-induced membrane currents are only minimally affected by $[Ca^{2+}]_e$, whilts the desensitization/tachyphylaxis showed a marked dependence on the $[Ca^{2+}]_e$ in most cellular preparations.

The plethora of these trials, however, did not unambiguously show *how the increase in $[Ca^{2+}]_i$ is affected by the $[Ca^{2+}]_e$*. Several groups have reported that VR1 is exclusively expressed on the surface membrane of nociceptive

sensory neurons and that vanilloids, therefore, are capable of increasing $[Ca^{2+}]_i$ only in the presence of extracellular calcium. In contrast, other findings have suggested that VR1 could functionally be expressed not only in the plasma membrane but also in intracellular membranes (e.g., in the membrane of the endoplasmic reticulum, ER) of the neurons; hence, the VR1-mediated cellular responses may also be initiated in the absence of extracellular calcium.

Since the molecular characterization of VR1 in 1997, *various heterologous expression systems* have been used to describe molecular and pharmacological features of the receptor channel and cellular mechanisms initiated by the activation of VR1. Results obtained in these systems, however, did not resolve the issue of the calcium dependence of $[Ca^{2+}]_i$ responses and, moreover, provided conflicting data both on the localization of VR1 in cells and on the effect of various vanilloids. The ability of capsaicin to increase $[Ca^{2+}]_i$ was reported to be totally dependent on $[Ca^{2+}]_e$ in VR1 expressing CHO and in HEK293 cells. In contrast, using cos-7 cells expressing the enhanced green fluorescent protein (eGFP)-tagged VR1 (VR1eGFP), Oláh et al., have shown that the application of RTX initiate calcium rise and disruption of various intracellular organelles (ER and nuclear membranes). It was also described that VR1eGFP was localized in the surface membrane and, of great novelty, also in the membrane of the ER.

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). These compounds, on the one hand, may directly activate the receptor by binding to the VR1 (heat, acidosis), or, on the other, upon binding to their mostly metabotropic receptors, may indirectly

regulate the function of VR1 via the modification of intracellular signal transduction pathways (kinase systems, intracellular messengers). These effects may chiefly decrease the heat threshold of the VR1 and thus contribute to the development of thermal hyperalgesia.

Neurotrophins and their significance

The neurotrophin family consists of neuronal growth factor (NGF), brain-derived neurotrophic factor (BDNF), and the various neurotrophins (NT3, NT4). These factors are mostly produced by certain neurons but can also be synthesized in other non-neural cell types such as lymphoid cells, keratinocytes, glia cells, and mast cells. The neurotrophins bind to their specific membrane receptors with distinct affinities. Whereas all of the neurotrophins possess very similar sensitivities to the low-affinity p75 receptor, NGF binds mostly to TrkA, BDNF and NT4 to TrkB, and NT3 to the TrkC receptor. The activation of Trk receptors, initiates a tyrosine kinase receptor-coupled intracellular enzyme cascade, whilst the activation of the p75 receptor increases the intracellular ceramide level and hence may regulate the function of certain transcription factors (e.g., NFκB).

The presence of neurotrophins, acting as critical trophic factors, is indispensible for the embryonic development and neuronal differentiation of several neuron types. It was also shown that these agents play a central regulatory role in the postnatal neuronal survival, in regeneration of peripheral axons following injury, and in the development of numerous neuron-specific functional characteristics. It was also established that most of the neurotrophins are able to modify the morphogenesis and re-building of other non-neural organs (e.g., heart, kidney, skin), as well as the proliferation and apoptosis of cells in the given tissue.

Relationship between the neurotrophins and the VR1 expressing neurons

The presence of the neurotrophins is also crucial for the *in vivo* and *in vitro* development of the sensory neurons, hence for the capsaicin-sensitive nociceptive neurons as well. Very recent findings, however, also postulated the *acute effect* of the neurotrophins on sensory neurons. The tissue inflammation evokes the sensitization of the nociceptive afferents, which leads the development of the phenomenon called *inflammatory thermal hyperalgesia*. It was shown that NGF initiates hyperalgesia (which can be prevented by anti-NGF blocking antibodies) and that the levels of numerous neurotrophins (e.g., NGF, BDNF) locally increase in the inflammed area. Therefore, several research groups hypothesized the significance of neurotrophins in the above process. They claimed that certain neurotrophins, released from different cells (keratinocytes, fibroblasts) during inflammation of e.g., the skin, may either indirectly (e.g., via the degranulation of mast cells) or directly (via binding to their TrkA receptors expressed on sensory neurons) evoked the process of nociceptive sensitization. It appears, therefore, that there might be a close, intimate "connection" between the central integrator molecule of the pain sensation (i.e., the VR1) and acute (sensitizing) effects of the neurotrophins acting as potential inflammatory mediators.

RATIONALE AND AIMS OF THE STUDY

The molecular biological description of the VR1 – although it multiplied the efforts aiming to characterize VR1 as an ion channel – did not resolve the issue of the calcium dependence of $[Ca^{2+}]_i$ responses and, moreover, provided conflicting data both on the sensitivity of VR1 to various vanilloids and on the localization of VR1 in cells.

Therefore, in the first phase of our experiments, we constructed three different heterologous expression systems (transient, inducible, stable) to investigate the function of rat VR1. During the comparison of these systems:

– We intended to investigate the action of different vanilloid compounds (capsaicin, RTX) on the intracellular calcium homeostasis of the cells;

– Moreover, we analyzed the kinetic parameters and the $[Ca^{2+}]_e$ dependence of the specific VR1-mediated cellular responses;

– In addition, we studied the subcellular localization of the VR1 and the functional properties of receptors incorporated to different cellular compartments.

In the second phase of our work we characterized the effects of different neurotrophins (NGF, BDNF, NT-3, NT-4) on the function of VR1 in the VR1/C6 system:

– After determination of the neurotrophin receptor pattern of the cells, we investigated the effects (sensitization, activation) of neurotrophins on the capsaicin evoked $[Ca^{2+}]_i$ responses;

– Furthermore, we analyzed the role of potential signal transduction pathways that might mediate the action of neurotrophins.

MATERIALS AND METHODS

Generation of heterologous expression systems for rat VR1

For the transient expression system, cos-7 cells growing on glass coverslips were transfected with 1-2 μg VR1eGFP plasmid or with the control empty vector by the LipofectAMINE transfection reagent, using the protocol suggested by the manufacturer (*VR1eGFP/cos-7 cells*). Transfected cells,

cultured for 48 hours in supplemented DMEM solution, were then used for calcium imaging.

The inducible expression system (*VR1/CHO cells*) was generated as reported previously. In these cells, expression of the pUHG plasmid (hence VR1) is repressed in the presence of tetracycline and is expressed upon removal of the antibiotic. Therefore, cells were routinely cultured in supplemented Ham F-12 medium which contained 1 μg/ml tetracycline. 48 hours before calcium imaging, cells were seeded on glass coverslips and were switched to tetracycline-free Ham F-12 medium.

For the stable expression system, a previously constructed metallothionein promoter-based pεMTH vector encoding the cDNA of the rat VR1 was used. This vector (2-4 μg cDNA) was transfected into C6 rat glioma cells (*VR1/C6 cells*). Cells were then selected in supplemented DMEM containing 750 μg/ml G418; then single colonies were isolated which were cultured in supplemented DMEM containing 500 μg/ml G418.

Western blotting

For the Western blot analysis of VR1 and neurotrophin receptors, 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 apropriate 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.

Immunocytochemistry

Cells cultured on glass coverslips were washed with ice-cold PBS, fixed with 4 °C acetone for 5 minutes, and then were blocked and permeabilized with blocking solution for 30 minutes. Cells were then incubated with the appropriate anti-neurotrophin receptor antibodies (dissolved in blocking solution) for 2 hrs and then with FITC-conjugated secondary antibodies. Cells were eventually mounted with mounting medium containing DAPI and images were obtained using a fluorescence microscope.

Calcium imaging

Cells growing on glass coverslips were incubated with the calcium sensitive fura 2-AM dye (5 μM) for 1 hr, then the changes in $[Ca^{2+}]_i$ were measured by an inverted fluorescence microscope. For calcium imaging, excitation was altered between 340 and 380 nm using a dual wavelength monochromator. The emission was monitored at 510 nm with a photomultiplier at an acquisition rate of 10 Hz per ratio. $[Ca^{2+}]_i$ levels were calculated according to the method of Grynkiewicz et al. (1985). Cells were continuously washed by either normal or calcium-free Tyrode's solution using a slow background perfusion system, whereas the investigated agents were applied through a rapid perfusion system positioned in close proximity to the measured cell at room temperature.

Real-time confocal microscopy

Cos-7 cells were plated on glass coverslips and transfected with 1-2 μg VR1eGFP plasmid, and, after 48 hours in culture, were analyzed with a confocal microscope. To study the two- and three-dimensional distribution of fluorescent signals, each *x-y* plane was scanned over 1 s at 30-s intervals and at 0.2 μm increments in the *z* axis mode.

RESULTS

I. The comparison of functional properties of VR1 in different expression systems

A) The transient expression system – VR1eGFP/cos-7 cells

1. Using functional calcium imaging, we demonstrated that *capsaicin induced cellular responses more effectively in Ca^{2+}-containing than in Ca^{2+}-free solution* in the VR1eGFP/cos-7 transient expression system. We found that a higher portion of cells (79 %) responded with significant $[Ca^{2+}]_i$ increase to capsaicin in 1,8 mM $[Ca^{2+}]_e$, than under Ca^{2+}-free circumstances (46 %). In the Ca^{2+}-containing solution, the transients based on their various parameters, could be distributed to two groups. *Fast transient*s (69 % of the responding cells), belonging to the first group, possessed large amplitudes, steep slope, and fast kinetic features. In contrast, *slow transient*s (31 % of the cells) had less amplitudes and slower kinetic parameters. In Ca^{2+}-free solution, all of the capsaicin evoked $[Ca^{2+}]_i$ transients were slow ones and possessed much lower amplitudes.

2. In the cases of both types of transients, repeated applications of capsaicin resulted in a decrease in the amplitudes of the transients in both solutions; i.e., we could observe a *marked* (30-40%) *tachyphylaxis*.

3. In both solutions, *the effect of capsaicin was VR1 specific* since capsazepine (a competitive antagonist of VR1) was able to markedly yet reversibly inhibit the capsaicin evoked $[Ca^{2+}]_i$ responses.

4. These data, in good accordance with previous findings, suggested that the *VR1 was incorporated not only to the surface membrane but also to intracellular structures.* Therefore, by using the green fluorescence feature of the eGFP-tagged VR1, we investigated the actions of capsaicin on morphological characteristics of the cells by real-time confocal microscopy. We found that, in Ca^{2+}-containing solution, capsaicin induced the blebbing and

disruption of VR1 expressing intracellular membranes (ER, nuclear membrane) which effect could be inhibited by capsazepine. In contrast, capsaicin caused much less pronounced intracellular changes in Ca^{2+}-free solution.

5. However, by investigating the effect of the ultrapotent VR1 agonist RTX, we showed that *RTX evoked cellular responses were very similar in Ca^{2+}-containing and in Ca^{2+}-free solutions*. The RTX was able to evoke transients in similar percentage of the cells investigated in both solutions which, in Ca^{2+}-containing solution, exclusively whereas in Ca^{2+}-free solution chiefly (67 % of the cells) possessed the features of the *slow transient*s. These responses, similarly to those seen in the case of capsaicin, showed a marked tachyphylaxis upon repeated RTX applications, and could be reversibly inhibited by capsazepine.

6. Under Ca^{2+}-free circumstances, in order to clarify the functional role of the intracellularly incorporated VR1eGFP, we determined the effect of emptying the intracellular calcium stores using thapsigargin. We were able to show that *the emptying of intracellular calcium pools* with the inhibitor of the Ca-ATP-ase *markedly decreased the amplitude of the RTX evoked $[Ca^{2+}]_i$ transients*.

7. Finally, using confocal microscopy, we demonstrated that RTX induced the disruption of the intracellular membranes with similar efficiencies in Ca^{2+}-free and Ca^{2+}-containing solutions which effect could be remarkably prevented by the emptying of calcium stores.

B) The inducible expression system – VR1/CHO cells

1. Similarly to the findings in the transient expression system, in VR1/CHO cells, *capsaicin induced* fast and large $[Ca^{2+}]_i$ *transients more effectively* (on 66% of the cells) *in Ca^{2+}-containing than in Ca^{2+}-free solution* (25 % of the investigated cells responded with smaller and slower transients). In both cases, the effect of capsaicin was specific on VR1 since capsazepine succesfully inhibited the capsaicin evoked calcium responses.

2. However, as a marked constrast to our results obtained in the transient system, in the VR1/CHO cells, we *could not experience any tachyphylaxis in either solution* upon repeated capsaicin administration.

3. Of great importance, and of great difference from data obtained in the VR1eGFP/cos-7 cells, in the inducible system, the *RTX was completely ineffective in Ca^{2+}-free solution*. In addition, even in Ca^{2+}-containing medium, only a small faction of cells (16%) responded to RTX application with slow and minor $[Ca^{2+}]_i$ elevations.

C) The stable expression system – VR1/C6 cells

1. *In Ca^{2+}-containing solution, the effect of capsaicin* on VR1/C6 cells *was very similar to that seen in VR1/CHO cells.* Capsaicin evoked fast $[Ca^{2+}]_i$ transients with large amplitudes in all cells investigated, which elevations were VR1-specific (inhibited by capsazepine) and showed no tachyphylaxis upon repeated agonist application. However, in contrast to results obtained in VR1/CHO cells, we were unable to evoke any responses with capsaicin in Ca^{2+}-free solution on VR1/C6 cells.

2. Investigation of the effect of RTX provided almost identical results to those found on VR1/CHO cells. Namely, *the RTX was exclusively effective in Ca^{2+}-containing solution*, and even these slow and small $[Ca^{2+}]_i$ transients were recorded on a minor population (28 %) of cells investigated.

D) In VR1eGFP/cos-7 transient and VR1/CHO inducible expression systems, the surface membrane-incorporated VR1 may be partially opened in resting cells

1. During the investigation of different properties of the control and VR1 expressing cells, in the cases of the transient and inducible systems, we detected an interesting phenomenon in Ca^{2+}-containing solution. We found that *the resting $[Ca^{2+}]_i$ level of the VR1 transfected cells was remarkably greater* than those of the empty peGFP vector transfected cos-7 and the control CHO cells.

We have also shown that the application of capsazepine significantly (and reversibly) decreased the resting $[Ca^{2+}]_i$ in VR1eGFP/cos-7 and VR1/CHO cells. It should be noted that we have never experienced such phenomena in the stable VR1/C6 cells.

2. These data suggest that in the VR1eGFP/cos-7 (transient) and VR1/CHO (inducible) systems, but not in the VR1/C6 stable system, *the VR1-channel* localized to the surface membrane *may be partially opened*.

II. The regulation of the function of the VR1 by different neurotrophins

1. During the comparison of the various expression systems, the *VR1/C6 system proved to be the most ideal cell type* for studying the regulation of VR1, since, in these cells, capsaicin evoked uniform transients with high amplitudes and, most importantly, with the lack of tachyphylaxis upon repeated applications. Therefore, in our further experiments, we analyzed the effects of the neurotrophins on VR1 function on this system.

2. Results obtained with Western blot and immunocytochemistry techniques showed that *all*, so far described, *neurotrophin receptors* (namely, the TrkA, B and C, as well as the p75) *were expressed on VR1/C6 cells*.

3. Using functional calcium measurements, we found that all neurotrophins markedly, yet to different extent, increased the sensitivity of the VR1 to capsaicin; i.e., after the neurotrophin treatment, capsaicin evoked larger transients than before. After statistical analysis of data obtained on numerous cells, we were able to define a BDNF>>NGF>NT3>NT4 rank order of efficiency.

4. By analyzing the mechanism of action of neurotrophins, we showed that the K252a compound, an *inhibitor of the tyrosine-kinase pathway, fully inhibited the sensitizing effect of the neurotrophins.* Our findings, therefore, argue for that the effects of the neurotrophins to sensitize VR1 function are

specific; i.e., are most probably mediated by tyrosine kinase-coupled pathways via Trk receptor(s).

DISCUSSION

I. The recombinant rat VR1 expressed in various expression systems possesses different properties

In the current study, our goal was to compare the functional characteristics of recombinant rat VR1 expressed in different (transient, inducible, stable) systems. Our results clearly demonstrate that the vanilloid sensitivity and extracellular calcium dependence of VR1 mediated $[Ca^{2+}]_i$ responses are markedly different in the various systems.

Similar to previous results, here we showed that the GFP-tagged VR1 expressed in cos-7 cells was localized both to surface membrane and to intracellular (calcium store) structures. The intracellular VR1 was functional in that both capsaicin and RTX were able to increase $[Ca^{2+}]_i$ and initiate subcellular disorganization even in the absence of $[Ca^{2+}]_e$, and that emptying of intracellular calcium stores by thapsigargin interfered with these actions. There were, however, marked differences among the effectiveness of the two vanilloids to affect $[Ca^{2+}]_i$ depending on the calcium content of the medium. Namely, whereas the action of capsaicin strongly depended on the $[Ca^{2+}]_e$, RTX was equally effective in Ca^{2+}-containing and free solutions. These data were in good accordance with recent data on human recombinant VR1 expressed in HEK 293 cells, where RTX but not capsaicin was able to increase intracellular calcium in Ca^{2+}-free medium.

In the VR1eGFP/cos-7 cells, we observed a remarkable heterogeneity among the vanilloid-induced transients, i.e., both fast and slow transients were

recorded for capsaicin in high calcium and for RTX in Ca^{2+}-free solutions. Since these phenomena were exclusively seen in the VR1eGFP/cos-7 transient expression system, one explanation is that, due to the transient nature and variable degree of transfection of the VR1eGFP/cos-7 cells, the level of VR1 in the individual cells could be heterogenous, allowing the same vanilloid on different cells having different VR1 expression levels to cause different calcium responses. This hypothesis is consistent with previous molecular biological and functional data that VR1, both in sensory neurons and in recombinant systems, is expressed in various multimeric structures which may result in heterogeneous responses to the same vanilloid.

Among the three systems we studied, the most homogenous vanilloid induced responses were observed in the stable (VR1/C6) system. In these cells, similar to findings in sensory neurons, capsaicin and RTX were effective only in Ca^{2+}-containing extracellular medium. In addition, the capsaicin or RTX evoked responses were characteristically very similar in all of the cells examined, i.e., capsaicin evoked fast whereas RTX induced slow $[Ca^{2+}]_i$ elevations, also well reflecting the previously described difference in pharmacokinetics of the two vanilloids. These data suggest that the expression of VR1 in VR1/C6 cells is well controlled by the recombinant vector and uniform circumstances can be obtained for VR1 characterization.

The vanilloid evoked calcium responses in the inducible system (VR1/CHO cells) were generally very similar to those obtained in VR1/C6. In 1.8 mM $[Ca^{2+}]_e$ solution, both capsaicin and RTX induced uniform, fast and slow calcium responses, respectively. However, there were also differences between the inducible and stable expression systems. Namely, in VR1/CHO cells, capsaicin (but not RTX) was able to induce calcium transients even in Ca^{2+}-free solution. It should be noted, however, that, under such conditions, capsaicin was effective in only a much lower percentage of the cells examined and, furthermore, that the capsaicin evoked responses were much slower and

smaller than seen in Ca^{2+}-containing medium. Naturally, further experiments are needed to unambiguously clarify the putative localization of some portion of expressed VR1s to intracellular membrane structures (due to the nature of commercially available antibodies against VR1, i.e., they were developed to target intracellular domains of VR1, permeabilization of cells is required for immunocytochemistry and this may affect the localization). However, our data, consistent with recent findings by Tóth et al. that thapsigargin pretreatment of VR1/CHO cells completely abolished the capsaicin induced $[Ca^{2+}]_i$ elevations, strongly argue for additional (yet much less than seen in VR1eGF/cos-7 cells) intracellular incorporation of functional VR1 in this system.

A major "unexpected" result, both in the VR1/C6 and VR1/CHO cell systems when compared to previously described data in sensory neurons, was the complete lack of tachyphylaxis after repeated capsaicin applications under our conditions. Since the complex mechanism of tachyphylaxis in sensory neurons may also involve the decrease in VR1 density upon repeated or prolonged vanilloid administration, we suppose that the stable or relatively stable nature of these expression systems may contribute to the lack of tachyphylaxis. In addition, since the sophisticated calcium handling and signal transduction (kinase, phosphatase) systems described in the host cells are distinct from those of the sensory neurons, the regulation of VR1 by such systems could be markedly different. Nevertheless, the phenomenon that repeated applications of capsaicin result in almost identical calcium responses in VR1/C6 and VR1/CHO cells may even possess an attractive feature for pharmacological studies characterizing inhibitory or sensitizing actions of agents on VR1.

It has been extensively described that capsaicin and RTX, although acting qualitatively similarly, have distinct spectra of action resulting in differences in their relative potencies for different responses. RTX is generally regarded as an ultrapotent analog of capsaicin; however, there are some responses where it

shows only slightly greater potency or, in contrast to the action of capsaicin, ineffectiveness. This latter phenomenon seems to be true for the inducible and stable recombinant expression systems; namely, in VR1/CHO and VR1/C6 cells, RTX was able to evoke only small and slow transients and, of great importance, in a markedly lower percentage of cells than did capsaicin. These findings were in good accord with previously published data that the relative potency of capsaicin was much greater on VR1 ectopically expressed in HEK293 cells than on the native channel in sensory neurons, whereas RTX was more potent in activating VR1 in cultured neurons than in the recombinant expression system.

Naturally, it would be of greatest importance to identify those factors that may contribute to differences (subcellular localization, calcium dependence, vanilloid sensitivity) seen in the various expression systems. It appears, based on both our presented data and the literature, that the characteristics of the recombinant vector; the functional features (e.g., calcium-handling) of the host cells; the type of expression (i.e., transient or stable); the level of glycosylation and heterogenous stochiometry; and the sensitivities of the methods to record the calcium signals may all contribute to the distinct properties of the systems. In any case, although the relative contributions of different parameters to define VR1 functional characteristics in heterologous expression systems remain to be clearly established, our findings strongly argue for the influence of the heterologous expression system on the determination of VR1 cellular functions and suggest caution in extrapolating such findings to other systems such as primary sensory neurons.

II. All neurotrophins sensitize the stably transfected recombinant rat VR1 in C6 cells

In the second part of our experiments, we investigated the putative acute effects of different neurotrophins (NGF, BDNF, NT3, and NT4) – the presence

of which is crucial for the morphological and functional development of sensory neurons – on the function of the recombinant rat vanilloid receptor (VR1/C6 cells). We found that all neurotrophins were able to markedly senzitize the VR1; namely, they increased the sensitivity of the receptor to the action of capsaicin. Since neurotrophins may display their effect through different neurotrophin receptors, and, furthermore, since we have shown the existence all of the neurotrophin receptors in the system, our results strongly suggest that the sensitization process may occur through most of the neurotrophin receptors.

By investigating the effect of neurotrophins in the presence of K252a, an inhibitor of the tyrosine kinase (Trk) pathway, we found that the compound completely prevented the VR1 sensitizing action of all of the neurotrophins. Since we could not find any data in the literature about K252a affecting the p75 neurotrophin receptor-coupled signal transduction pathways, our findings strongly argue for that neurotrophins initiate their VR1 sensitizing action through the Trk receptors. Since in our hands all of the examined neurotrophins were proved to be effective to sensitize the VR1, the participation of all of the existing Trk receptors (i.e., TrkA, B and C) could be postulated in the process. However, we have also shown that the various neurotrophins differentially increased the sensitivity of VR1 to capsaicin; statistical analysis on numerous cells revealed a BDNF>>NGF>NT3>NT4 rank order of efficiency. Although, naturally, further experiments are needed to the exact pharmacological description of the effect of neurotrophins (e.g., dose-response curves), it appears that, on VR1/C6 cells, the activation TrkB receptor (most effectively by BDNF) migh exert the most potent sensitizing effect. This may be supported by our current normalized Western blot data that, among the Trk receptors, the expression levels of TrkB was the highest in VR1/C6 cells.

The nociceptive sensory neurons, bearing the native VR1, express mainly TrkA and B neurotrophin receptors but not the TrkC receptor. In good accordance with this, injection of NGF, BDNF, and NT4 to the skin induced

thermal hyperalgesia, whereas NT3 was ineffective in evoking the process. Comparing our experimental results to data obtained on sensory neurons, we can conclude that, in the heterologous expression system used in this study, the activation of both TrkA and TrkB receptor, similarly to that seen on the native VR1, caused significant sensitization of the receptor. In addition, TrkC receptor can also be found in our system (but not in DRG neurons), the activation of which has similarly positive effect on the sensitivity of VR1 to capsaicin. It appears, therefore, that if a given Trk receptor is expressed, after its activation, the process of VR1 sensitization may develop. This hypothesis can be supported by numerous data in the literature; namely signal transduction pathways activated by the different Trk receptors show marked convergence.

SUMMARY

1. In our experiments, we investigated the effects of capsaicin and RTX on cellular mechanisms in various heterologous systems expressing the rat recombinant VR1. We conclude that the VR1 functions as a Ca^{2+}-permeable channel in all three systems.
2. However, our results clearly show that the amplitudes and kinetic parameters of the VR1-mediated $[Ca^{2+}]_i$-responses, the sensitivity of VR1 to various vanilloid agonists, as well as the subcellular localization of the receptor differ markedly between the systems.
3. In the transient expression system (VR1eGFP/cos-7 cells), capsaicin induced cellular responses more effectively in Ca^{2+}-containing than in Ca^{2+}-free solution, whereas RTX showed similar efficiency. Upon repeated application of either agonist, a significant tachyphylaxis developed. We have also shown that, in this system, the VR1 is functionally expressed not only in the surface membrane but also in various intracellular structures serving as Ca^{2+} stores.

4. In the inducible (VR1/CHO cells) and in the stable (VR1/C6 cells) systems, in the presence of extracellular calcium, capsaicin evoked large $[Ca^{2+}]_i$-transients showing no tachyphylaxis, whereas RTX induced only minor responses. In Ca^{2+}-free solution, only capsaicin was effective to increase $[Ca^{2+}]_i$ (and only in VR1/CHO cells), whilst RTX had no effect.

5. In the VR1/C6 stable expression system, all of the investigated neurotrophins (NGF, BDNF, NT3, NT4) – although to different extent – significantly increased the sensitivity of VR1 to the action of capsaicin (sensitization), which effects were most probably mediated by tyrosine kinase (Trk) receptors.

IN EXTENSO PUBLICATIONS USED IN THE PREPARATION OF THE THESES:

1) ***Lázár J****., Szabó T., Kovács L., Blumberg P. M., and Bíró T.* (2003): Distinct features of recombinant rat vanilloid receptor-1 expressed in various heterologous expression systems. *Cell. Mol. Life Sci.* **60**:2228-2240. **IF: 5,259**

2) ***Lázár J****., Szabó T., Marincsák R., Kovács L., Blumberg P. M., and Bíró T.* (2003): Sensitization of recombinant vanilloid receptor-1 by various neurotrophic factors. *Life Sci. (in press)* **IF: 1,824**

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