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Regulation of TRPV1 ion channel activity on primary sensory neurons

by Agnes Jenes

Supervisors: István Nagy, László Csernoch

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DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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By Ágnes Jenes, MD

Supervisors:
István Nagy, MD, CSc
László Csernoch, DSc

Doctoral School of Molecular Medicine, University of Debrecen

Head of the **Examination Committee**:	János Szöllősi, DSc
Members of the Examination Committee:	Zita Puskár, PhD
	Tamás Szabó, MD, PhD

The examination takes place in Room 2.305-306, Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen, 13. 05. 2014, 11 am

Head of the **Defense Committee**:	János Szöllősi, DSc
Reviewers:	Gábor Jancsó, MD, DSc
	Attila Tóth, PhD
Members of the Defense Committee:	Zita Puskár, PhD
	Tamás Szabó, MD, PhD

The PhD defense takes place at the Lecture Hall of the In Vitro Diagnostics, Faculty of Medicine, University of Debrecen, 13. 05. 2014, 1 pm

Introduction

Primary sensory neurons (PSN), which establish connections between peripheral tissues and the central nervous system, form two major sub-populations, namely the non-nociceptive and nociceptive cells. While non-nociceptive neurons are activated by innocuous stimuli, such as touch or mild temperatures, nociceptive cells respond to noxious mechanical and thermal stimuli that have the potential to induce tissue injury. Hence, acute activation of nociceptive PSN evokes a response, which is characterised by escape, avoidance, pain-related behaviour and pain. A great proportion of nociceptive PSN express the transient receptor potential vanilloid 1 (TRPV1) receptor and the cannabinoid 1 (CB1) receptor. While CB1 activation induces a significant inhibitory effect on the activity of nociceptive PSN and produces a significant reduction in pain, activation of TRPV1 increases neuronal excitability.

As a polymodal nocisensor, TRPV1 is responsive to different exogenous activators including various toxins and other painful agents, heat above 43 ºC, protons and membrane depolarisation. In addition to some physiological functions, TRPV1 also plays a pivotal role in the development of various pathological processes, most prominently, in the development of inflammatory pain. Of TRPV1's activating agents, the prototypical activator is capsaicin, the main pungent ingredient of the hot chilli pepper, and one of its major endogenous agonists is anandamide.

N-arachidonoylethanolamine (anandamide) is an endogenous lipid agent implicated in a variety of physiological functions and pathological processes both within, and outside, the nervous system. Although anandamide interacts with a variety of proteins, the majority of its actions are mediated via the CB1 receptor and the TRPV1 ion channel. One of the most prominent functions of anandamide resides in its TRPV1- and CB1 receptor-mediated regulatory action in nociception in primary sensory neurons. Several cell types in various tissues, including PSN, produce anandamide, the synthesis of which is believed to occur either in a Ca^{2+}-sensitive, or Ca^{2+}-insensitive manner and through several enzymatic pathways. The molecular identities and biochemical activities of six enzymes implicated in

the synthesis of various N-acylethanolamines (NAEA) including anandamide from corresponding N-acylphosphatidyl-ethanolamines (NAPE) have now been established. One of these six enzymes, N-acylphosphatidylethanolamine-selective phospholipase D (NAPE-PLD), which is the only known Ca^{2+}-sensitive enzyme involved in anandamide synthesis, is expressed by a sub-population of PSN. However, the ability of PSN to produce anandamide in the absence of Ca^{2+} indicates that, in addition to the Ca^{2+}-sensitive pathway, one or more Ca^{2+}-insensitive pathways must also be present in these cells.

Transient receptor potential vanilloid type 1 molecules, either on their own, or together with TRPV1 splice variants, or other members of the transient receptor potential vanilloid sub-family, assemble to form the non-selective cationic channel known as the capsaicin receptor (TRPV1 ion channel). When one of the splice variants of TRPV1, TRPV1b is co-expressed with TRPV1 in heterologous systems, an inhibitory effect on channel activity, as evoked by various TRPV1 activators, including capsaicin, is seen. Nociceptive primary sensory neurons constitute the archetypical cell type that expresses TRPV1. In these neurons, the TRPV1ion channel, through its increased responsiveness, is essential in signalling peripheral inflammatory events to the central nervous system, and subsequently it plays a pivotal role in the development and maintenance of inflammatory heat hyperalgesia and visceral hyper-reflexia. The co-expression of TRPV1 and TRPV1b molecules in PSN, and the negative dominant effect of TRPV1b on the channel's responsiveness found in heterologous systems suggest that alterations in the composition of the TRPV1 ion channel might contribute to the inflammation associated increase in the responsiveness of this receptor. Such an alteration, in a long term, should be reflected by altered ratio in the expression of TRPV1 and TRPV1b transcripts and proteins.

Aims

Anandamide synthesised in a Ca^{2+}-sensitive manner induces excitation of PSN through the activation of TRPV1. However, the effect of anandamide synthesised by Ca^{2+}-insensitive enzymes acting on NAPE in PSN is not known. Therefore, we examined the expression and function of putative Ca^{2+}-insensitive NAPE-converting enzymes in cultured rat PSN.

Next we studied the effect of anandamide produced by these enzymes in the TRPV1-expressing primary sensory neurons, since activated CB1 receptor has inhibitory effect on TRPV1, indicating a possible role of this endocannabinoid in alleviating pain. The direct effect of anandamide produced by the PSN on the activity of TRPV1 – other than the CB1 receptor-mediated inhibition – has not yet fully established, leaving the question of overall effect on the activity and on the responsiveness of polymodal nociceptors unanswered.

When the TRPV1 and its splice variants are co-expressed, a modified receptor sensitivity, usually a reduced channel activity can be detected, compared to the homotetramer TRPV1 ion channel. In our experiments, we assessed the effect of inflammatory mediators (BK and PGE_2) on the responsiveness of the TRPV1 ion channel, while we were measuring TRPV1 and TRPV1b mRNA and protein expressions. We hypothesised that culturing primary sensory neurons in an “inflammation-associated milieu” results in changes in the ratio of TRPV1 and TRPV1b mRNA and protein expressed by those cells, leading to altered responsiveness of the channel.

Materials and methods

Animals, drugs. Altogether, ninety four 80–200 g male Sprague-Dawley rats, five wild type (C57BL/6 x129SvJ; WT) and six TRPV1$^{-/-}$ mice (with C57BL/6 x129SvJ background; KO) were used. NAPE, capsaicin, anandamide, rimonabant, mustard oil and ionomycin were dissolved in ethanol, Tocrisolve, DMSO or in the mixture of these solvents. The maximum final concentration of Tocrisolve and DMSO were 0.05%, for ethanol it was 0.1%.

Cultures of dorsal root ganglia. DRG from the first cervical to the sixth lumbar segments were collected in culture medium. Following incubation in collagenase, DRG were triturated and the cells were plated on poly-DL–ornithine-coated glass coverslips. Cells were grown for 24-48 hours. Some cultures were grown in the presence of inflammatory mediators (prostaglandin E_2 and bradykinin).

Isolation of total RNA, reverse transcriptase polymerase chain reaction (RT-PCR). The total RNAwas isolated using QIA shredder and RNeasy Mini or RNeasy Plus Mini Kits according to the manufacturer's instructions. Extracted RNA was reverse-transcribed using Super-Script II cDNA synthesis reagents. Primers designed to amplify rat glycerophosphodiester phosphodiesterase 1 (GDE1), α/β-hydrolase 4 (ABHd4), protein tyrosine phosphatase, non-receptor type 22 (PTPn22), group 1b secretory phospholipase A 2 (sPLA2G1b), inositol 5′-phosphatase (Inpp5) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used, then the products were separated and visualized on agarose gels.

Sequencing of rat TRPV1b. The RT-PCR products were purified from the agarose gels following electrophoresis using the QIAquick Gel extraction kit and the microcentrifuge protocol. Sequencing was carried out using the ABI Prism automated sequencing system using the primers used previously for RT-PCR.

Immunofluorescence staining. Cells attached to the coverslips were washed with PBS and fixed by 4% paraformaldehyde. Permeabilisation and blocking were followed by overnight incubation with primary antibodies, and visualisation with secondary antibodies. Cells were examined using a Leica DMR Fluorescence microscope.

Western blotting. Following preparation of whole cell extracts, or membrane proteins, of neurons, proteins were denatured, then fractionated by Bis-Tris gel and blotted onto PVDF membrane. After blocking, the membrane was incubated with primary antibody, then horseradish peroxidase-conjugated secondary antibody was applied to the membrane, followed by detection using Western blotting luminal reagent.

Anandamide release and anandamide measurement. Cultures were washed twice with HBSS–HEPES buffer then incubated for 5 min in HBSS–HEPES buffer containing the drugs. The superfusate was collected on ice and processed immediately for lipid extraction using ethyl acetate. The amount of anandamide synthesized was measured using three slightly different methods by three independent laboratories (protocol „A", „B" and „C").

Cobalt uptake. A cells attached to the coverslips were washed then incubated in the presence of cobalt and the drugs. The cobalt taken up by the cells was precipitated by 2.5% β-mercaptoethanol. Cells were then fixed and the mean gray value of cells was established by a Leica light microscope attached to a PC, and analysed with the ImageJ software package.

Ca^{2+}-imaging. To assess the calcium homeostasis of the neurons two fluorescent Ca^{2+}-imaging techniques were used. The experiments were performed either on Fura2-AM loaded cells with a Peltier element-cooled slow scan charge-coupled device camera system, or on Fluo4-AM loaded cells using Zeiss LSM 5 LIVE laser scanning microscope.

Whole-cell voltage-clamp recordings. Whole-cell currents were recorded at 37 °C on 15-30 µm diameter cultured PSN. In the voltage-clamp mode, the holding potential was –60 mV. Cells were regarded as anandamide- or capsaicin-responsive if the drug application-associated change in the current exceeded 50 pA.

Statistics. Data of repeated measurements were averaged. Differences between the averaged values were analysed by Student's t-test, one-way analysis of variance (ANOVA), repeated measure multivariate ANOVA or Fisher's exact test as appropriate. Data are shown as mean±standard error of mean.

Results

Several Ca^{2+}-insensitive enzymes, which are implicated in anandamide synthesis, are expressed in rat cultured primary sensory neurons. RT-PCR analysis showed gene expression for all five Ca^{2+}-insensitive enzymes that are known to be implicated in the synthesis of NAEA, including anandamide, from the corresponding NAPEs in cultures prepared from rat DRG. In order to study the expression of the enzymes at a cellular level, immunostaining was performed on cultured rat PSN using antibodies raised against the enzymes together with an anti-NeuN-antibody, which identifies neurons. By analysing the staining in at least 100 neurons in each culture, we found that sub-populations of PSN express ABHd4, GDE1, Inpp5, and PTPn22. The analysis also revealed that while only neurons express ABHd4, Inpp5 and PTPn22, neurons and some non-neuronal cells express GDE1. The antibody raised against sPLA2G1b did not produce staining in the cultures. However, the same antibody produced staining in the pancreas. The anti-ABHd4-, anti-Inpp5-, anti-PTPn22- and anti-GDE1 antibodies also produced staining in tissues in which the expression of these enzymes have been reported previously. When the primary antibodies were replaced with normal serum, no staining was seen either in cultured PSN or in any tissues which we processed.

Application of 20:4-NAPE induces anandamide production in cultured PSN. 20:4-NAPE is the substrate for anandamide synthesis used by all the known enzymatic pathways. Therefore, to ascertain whether the enzymes found to be expressed in cultured rat PSN form any functional anandamide-synthesising pathways, the level of 20:4-NAPE application-induced anandamide synthesis was measured. Exposure of the cells to 100 μM 20:4-NAPE significantly increased the anandamide content of the buffer from a level below the quantitation threshold in the control to 8.9±3 pmol/ml (n=4; Protocol B) at 37°C. After normalisation to the protein content, the anandamide concentration was 13.25±4.39 ng/mg protein (n=4). Incubation of the cells in the vehicle resulted in an anandamide content of 0.01±0.01 ng/mg (n=3) protein in the superfusate. This value was not significantly different from that measured in the control of this experiment (0.04±0.04 ng/mg protein, n=3). Incubation of the cells in 100 μM 20:4-NAPE at room temperature also significantly increased the anandamide concentration in the buffer (from 0.06±0.03 ng/mg protein to

1.17±0.03 ng/mg protein, n=3). However, the 20:4-NAPE application-induced increase in the anandamide content of the buffer at room temperature was significantly less than that measured at 37°C indicating that the production of anandamide depends on enzyme activity. To make sure that no 20:4-NAPE hydrolysing activity in the buffer contributed to the increase in the anandamide levels, we also quantified the anandamide content of cell-free superfusate containing 100 μM 20:4-NAPE. The buffer was kept either at room temperature or 37°C up to 4 h. We measured the lowest and highest anandamide concentration at 2.5 h at 37°C (0.023 ng/ml) and at 5 min at 37°C (0.15 ng/ml), respectively. We found no apparent relationship between the anandamide content and the time or temperature of incubation. These data indicated that cultured rat PSN has the ability to convert 20:4-NAPE into anandamide. The 20:4-NAPE application-evoked increase in the anandamide content of the supernatant at 37°C was concentration-dependent (Protocol A). The lowest 20:4-NAPE concentration at which the anandamide content of the superfusate was significantly different from that of the control was 10 μM. Among the known anandamide-synthesising enzymes, NAPE-PLD activity is significantly enhanced in the presence of Ca^{2+}. To ascertain whether or not during 20:4-NAPE application, any Ca^{2+} influx increases the $[Ca^{2+}]_i$ to an extent which is enough to contribute NAPE-PLD to anandamide synthesis, we applied 20:4-NAPE to cultured PSN either with Ca^{2+} present or absent in the buffer. In the presence of Ca^{2+}, the anandamide content of the superfusate of cells incubated with vehicle was 0.6±0.4 ng/mg protein (n=3), whereas in the absence of Ca^{2+}, it was 1±0.1 ng/mg protein (n=3; Protocol A). These values did not differ significantly from each other. Application of 20:4-NAPE (100 μM) resulted in a significant increase in the anandamide content of the superfusate in both the presence and absence of Ca^{2+} (9.57±2.58 ng/mg protein and 11.47±0.92 ng/mg protein). Thus, the anandamide content of the Ca 2+ -containing, and Ca 2+ -free, superfusates did not differ significantly from each other after the application of this substrate. We also compared the anandamide content of the superfusate to that of 90% of the cells together with the superfusate (the other 10% of the cells was used for protein measurements). The anandamide content of the cells and superfusate together under control conditions (incubation with vehicle) was 0.09 ng/mg protein (n=2; Protocol C). Following 100 μM 20:4-NAPE application for 5 min, the anandamide content of the cells and superfusate increased to 28.03 ng/mg protein (n=2). Together, these data indicate that the 20:4-NAPE

application-evoked anandamide synthesis in cultured rat PSN is not affected by the removal of Ca^{2+} from the extracellular buffer. These data also show that about 2/3 of anandamide produced in PSN following 20:4-NAPE application, similar to anandamide produced by increasing the $[Ca^{2+}]_i$ in central neurons, is retained in the cells.

20:4-NAPE application induces cobalt influx into a sub-population of cultured rat PSN. Anandamide synthesised in PSN by increasing the $[Ca^{2+}]_i$ induces TRPV1-mediated excitation. TRPV1 activation by anandamide results in cobalt accumulation in a sub-population of PSN. Therefore, to test whether endogenous anandamide synthesised following the application of 20:4-NAPE has similar effect to that synthesised in a Ca^{2+}-sensitive manner, we studied cobalt accumulation during 20:4-NAPE application. The efficacy of this technique for assessing TRPV1-activity has been consistently demonstrated. While application of the vehicle did not, application of 20:4-NAPE significantly increased the proportion of labelled neurons and this effect was concentration-dependent. The lowest concentration of 20:4-NAPE which produced a significant increase in the proportion of labelled cells was 0.1 μM (6.5±1%, n=4 cultures). The EC_{50} of 20:4-NAPE was 7.4±1 μM. The majority of the cobalt-labelled cells were small diameter neurons. These observations suggest that the cobalt influx occurred in nociceptive neurons, the great majority of which express TRPV1.

20:4-NAPE application induces inward currents in a sub-population of cultured rat PSN. To confirm the excitatory effect of 20:4-NAPE application, we recorded whole cell currents from cultured rat PSN. In total, 22 cells were found that responded with inward currents to 50 μM 20:4-NAPE which is the near maximal concentration for this agent to induce cobalt uptake. The peak amplitude of the response was –0.41±0.1 nA (n=22). All nine of the 20:4-NAPE-responsive neurons that we tested also responded to 500 nM capsaicin. The average amplitude of the 20:4-NAPE-evoked currents (–0.35±0.1 nA, n=9) was significantly smaller than that of the capsaicin-evoked currents (–2.36±0.6, n=9). However, in addition to the double-responsive cells, 27 neurons which responded to capsaicin but not to 20:4-NAPE application were also found. In the control experiments, no neurons produced any noticible responses to the vehicle (n=11).

20:4-NAPE application increases the $[Ca^{2+}]_i$ in a capsazepine-and rimonabant-sensitive manner. In order to study the pharmacological properties of the 20:4-NAPE application-evoked excitatory responses, we assessed changes in $[Ca^{2+}]_i$ produced by the application of 50 µM 20:4-NAPE. Application of the vehicle for a minute did not produce any increase in the $[Ca^{2+}]_i$ in any of the 384 KCl-responding neurons, whereas 234 of these 384 responded to 1 µM capsaicin application. Application of 20:4-NAPE, however, increased $[Ca^{2+}]_i$ in 189 of 546 KCl-responding neurons (35%). All the neurons that responded to 20:4-NAPE application were sensitive to capsaicin. In addition to the double-responsive neurons, 273 of the 546 neurons (50%) responded only to capsaicin. Hence, the total number of capsaicin-responsive neurons was 462 of 546 (85%), which was significantly higher than the proportion of capsaicin-sensitive neurons measured when only the vehicle or the buffer (224 of 343 cells) was applied before capsaicin application. The 20:4-NAPE application-evoked changes in $[Ca^{2+}]_i$ activated more slowly than those evoked by capsaicin. Furthermore, the amplitude of the 20:4-NAPE application-evoked responses were significantly smaller than those evoked by capsaicin when normalised to the KCl-evoked responses (20:4-NAPE/KCl=0.15±0.01, n=189; capsaicin/KCl=0.78±0.02, n=462). Interestingly, the great majority of neurons, which generated responses to 20:4-NAPE application, exhibited sustained/very slowly inactivating Ca 2+ transients to capsaicin application. On the other hand, the great majority of capsaicin-responsive neurons, which did not respond to 20:4-NAPE application, exhibited fast inactivation of responses during capsaicin application. In the presence of capsazepine (5 µM), 403 KCl-responding neurons exhibited a stable baseline before 20:4-NAPE application. Of these, 26 responded to 20:4-NAPE and capsaicin (6%). In addition to these neurons, 16 cells responded only to 20:4-NAPE application, and 21 cells responded only to capsaicin. Thus, the overall proportions of both the 20:4-NAPE- (42 of 403, 10%) and capsaicin-responding neurons (47 of 403, 12%) in the presence of capsazepine were significantly smaller than that measured in the absence of capsazepine. In the presence of rimonabant, 20:4-NAPE application increased $[Ca^{2+}]_i$ in 105 of 498 KCl-responding neurons with a stable base line before 20:4-NAPE application (21%). This proportion was significantly smaller from that measured in the control. Rimonabant, however, had no significant effect on the overall number of capsaicin-responding neurons

(421 of 498 cells, 85%). Rimonabant significantly reduced the amplitude of both the 20:4-NAPE application-evoked (to 20:4-NAPE/KCl=0.12±0.009), and the amplitude of the capsaicin-induced responses (to capsaicin/KCl=0.61±0.018). These data suggest that the 20:4-NAPE application-evoked excitatory effect is mediated by TRPV1, and that 20:4-NAPE application, when assessed average responses, does not result in a CB1 receptor mediated inhibitory effect.

The 20:4-NAPE application-evoked increase in $[Ca^{2+}]_i$ in mouse PSN is mediated by TRPV1. In order to confirm the role of TRPV1 in 20:4-NAPE application-evoked responses, we also studied the effect of this agent on PSN isolated from WT and TRPV1$^{-/-}$ (KO) mice. Ten of 68 KCl-responsive neurons (15%) from WT mice were regarded as responsive to 20:4-NAPE (50 μM) application. As in experiments using rat PSN cultures, all the 20:4-NAPE-responsive neurons were responsive to capsaicin (1 μM) and the 20:4-NAPE application-evoked responses activated more slowly than the capsaicin-evoked responses. As with cultures prepared from rat DRG, cultures prepared from WT mouse DRG also had neurons, which responded only to capsaicin (32 of 68, 49%). In contrast, none of the KCl-responsive neurons (n=59) responded to 20:4-NAPE application in cultures prepared from DRG of KO mice. As expected, none of the neurons in the cultures prepared from DRG of KO mice responded to capsaicin. However, 22 of the 59 KCl-responding cells responded to mustard oil, showing that the cells were healthy and responsive to agents for which they expressed receptors. Therefore, our findings on neurons of TRPV1$^{-/-}$ mice confirm that the 20:4-NAPE application-evoked excitatory effects are mediated through TRPV1.

Sub-populations of cultured rat primary sensory neurons co-express TRPV1 and enzymes implicated in Ca^{2+}-insensitive anandamide synthesis. The concentration of anandamide found in the superfusate (~10 nM) is significantly lower than the concentration of exogenous anandamide needed to induce TRPV1-mediated excitatory effects. Therefore, we hypothesised that the excitatory effect of anandamide of PSN origin is mediated through autocrine signalling. To find out whether there is an anatomical basis for such autocrine signalling, we used combined immunostaining for studying the co-expression of TRPV1 with the Ca^{2+}-insensitive anandamide-synthesising enzymes. Analysis of this double immunofluorescent staining showed that a significant proportion of cultured rat PSN exhibit

co-expression for TRPV1 and the putative Ca^{2+}-insensitive anandamide-synthesising enzymes.

Both TRPV1 and TRPV1b are expressed in cultured PSN. Rat TRPV1b lacks exon 7 either completely or in part. Hence, as expected, our primers flanking exon 7 amplified two products. The larger product had a size close to that predicted for the full length rat TRPV1 sequence, whereas the smaller product size was ~325 base pairs. DNA sequencing of the RT-PCR products confirmed that the respective sequence of the larger and smaller products corresponded to the relevant section of rat TRPV1 transcript with differences reflecting alternative splicing of 180 bases of sequence, encompassing exon 7.

Exposure of cultured rat PSN to BK and PGE_2 for two days increases the responsiveness of the cells to capsaicin. In cultures, incubated in capsaicin-free cobalt uptake buffer, only few neurons exhibited labelling above the detection threshold both in the naive and BK- and PGE_2-exposed cultures. The proportions of labelled cells in the control experiment between the two conditions were not significantly different from each other. Addition of 30 nM capsaicin to the cobalt uptake buffer resulted in a significant increase in the number of labelled cells both in the naive (11±2%; n = 4) and BK- and PGE_2–exposed (23±6%; n = 4) cultures. The relative number of labelled cells in the BK- and PGE_2-exposed cultures was significantly higher than that in the naive cultures.

Exposure of cultured PSN to BK and PGE_2 for two days increases TRPV1 mRNA, but does not alter TRPV1b mRNA or TRPV1 and TRPV1b protein expression. We assessed TRPV1 and TRPV1b mRNA expression in cultures kept in control medium or in a medium containing BK and PGE_2 by semi-quantitative RT-PCR. Both the size of the RT-PCR products and the sequences of both products were identical to the respective products and sequences in naive and BK- and PGE_2-exposed cultures. By measuring intensity of the products we found that while the expression of TRPV1 mRNA was significantly increased (362±80%, n = 4) that of TRPV1b mRNA was not (188±18%, n = 4). These changes meant that the TRPV1/TRPV1b mRNA expression ratio was significantly increased from 1.1±0.2 (n = 4) in the naive cultures to 1.9±0.2 (n = 4) in the BK- and PGE_2 –exposed cultures. The anti-TRPV1 antibody recognised two easily distinguishable (at ~110 kDa and ~88 kDa), and one faintly visible (~95 kDa), proteins both in the naive and BK- and PGE_2-exposed cultures

in the whole cell lysates. In the membrane fractions, a fourth easily distinguishable protein (~75 kDa) has also been identified in both conditions. The ~110 kDa and ~95 kDa proteins correspond to the predicted molecular weight of the fully glycosylated full length TRPV1 and the non-glycosylated full length TRPV1, respectively. Given that the TRPV1b mRNA contains 180 less bases than the TRPV1 mRNA (current study), and that Vos and colleagues identified rat TRPV1b as an ~88 kD protein in rat DRG, it is feasible to assume that the ~88 kDa protein revealed in our Western blots by the TRPV1 antibody is the TRPV1b protein. The ~75 kDa protein found in the membrane fraction may be a product formed during the preparation of the fractions, as it could not be seen in whole cell lysates. Due to its faint appearance, the expression of the ~95 kDa protein was not quantified. Nevertheless, the non-glycosylated form of TRPV1, due to its low level of expression, might contribute little toTRPV1-mediated responses in cultured primary sensory neurons. We found a marked difference between TRPV1 and TRPV1b expression in the whole cell lysates and membrane fractions. We also found that BK and PGE_2 did not induce significant change in the expression of either the TRPV1 or TRPV1b protein in the whole cell lysates. Similarly, the inflammatory mediators did not induce significant changes either in TRPV1, or in TRPV1b protein expression in the membrane fractions.

Discussion

Endogenous anandamide

We have shown in the present study that four of the five enzymes (ABHd4, GDE1, Inpp5 and PTPn22) which have been implicated previously in Ca^{2+}-insensitive anandamide synthesis from 20:4-NAPE, are found at transcript level in cultures prepared from rat DRG, and at protein level in cultured rat (and mouse) PSN. The GDE1 protein is also expressed by non-neuronal cells as well as by PSN. The anti-sPLA2G1b antibody, which we used in this study, does not produce any staining in rat PSN cultures, although it labels cells in acini in the pancreas. RT-PCR shows weak expression of sPLA2G1b mRNA in PSN cultures. Hence, one cannot exclude the possibility that this enzyme may be expressed under the detection threshold of the immunostaining technique in some neurons or non-neuronal cells. 20:4-NAPE application to PSN cultures increases the anandamide concentration in the cells and the superfusate. The finding that application of 20:4-NAPE to cell-free superfusate does not increase the anandamide content of the buffer, together with the finding that the anandamide content of the cells and superfusate is greater than that of the superfusate alone indicates that 20:4-NAPE passes the cells membrane and anandamide is synthesised intracellularly. Indeed, all NAPE-converting enzymes are intracellular and transiently associated with membranes to act on NAPEs embedded in those. Therefore, the conversion of 20:4-NAPE to anandamide in an enzymatic way, as we have shown here, can be only through the rapid incorporation of this phospholipid into the membranes and its hydrolysis by some of the enzymes we investigated here. The rate of anandamide production induced by 100 μM 20:4-NAPE application (~38 pmol/5 min/mg protein) is well within the range of 20:4-NAPE hydrolysis (up to ~40 pmol/min/mg protein) measured in brain homogenates. The distribution of enzymes across PSN and non-neuronal cells indicates that, while non-neuronal cells may produce some anandamide, the greatest amount of this endocannabinoid/endovanilloid is likely to be synthesised in PSN. Calcium regulates the synthesis of anandamide mostly through the NAPE-synthesising enzyme known as Ca^{2+}-dependent N-acyltransferase, which catalyses the formation of 20:4-NAPE. However, when this rate-limiting step in anandamide and NAEA biosynthesis is bypassed, as in our case, the

only way for anandamide to be produced in a Ca^{2+}-sensitive manner is via NAPE-PLD action on 20:4-NAPE. Indeed, in the absence of Ca^{2+}, NAPE-PLD activity is negligible even in the presence of the substrate, whereas increasing the $[Ca^{2+}]_i$ alone results in anandamide production. Although, application of 20:4-NAPE induces some increase in the $[Ca^{2+}]_i$, the finding that removal of Ca^{2+} from the extracellular buffer does not change the anandamide concentration in the superfusate suggests that Ca^{2+} influx during 20:4-NAPE application is not sufficiently high to induce NAPE-PLD activity. Hence, following 20:4-NAPE application, at least the great majority of anandamide is synthesised by PSN by Ca^{2+}-insensitive pathways. Consequently, some of the Ca^{2+}-insensitive anandamide-synthesising enzymes present in the cultures prepared from rat DRG form functional pathways, which may also include the Ca^{2+}-independent NAPE biosynthesising enzyme previously identified by Jin and colleagues. However, the exact identity of the pathway(s) is not known and attempts to dissect them would produce ambiguous results at present because of the unavailability of selective and specific blockers of the enzymes. Application of 20:4-NAPE results in cobalt-influx in a sub-population of small diameter cultured PSN, the majority of which express TRPV1. While the lowest concentration of 20:4-NAPE which induces a significant increase in the number of cobalt-labelled neurons is 0.1 μM, the lowest concentration of 20:4-NAPE which results in a significant increase in the anandamide content of the superfusate is 10 μM. We have shown here, however, that PSN – similar to central neurons – retain the majority of anandamide. Hence, the concentration of anandamide at the intracellular anandamide-binding site of TRPV1, is probably considerably higher than that in the superfusate. This difference renders any direct comparison between minimally effective concentrations of 20:4-NAPE at increasing the anandamide concentration in the superfusate and at evoking Co^{2+} uptake very difficult. In addition to inducing cobalt accumulation, application of 20:4-NAPE also increases the $[Ca^{2+}]_i$, and induces whole-cell currents. All the cells which respond to 20:4-NAPE application with increased $[Ca^{2+}]_i$, or inward currents, in control conditions, also respond to the archetypical TRPV1 agonist, capsaicin. Taken together, these data suggest that the 20:4-NAPE application-evoked excitatory effect is mediated through TRPV1 in PSN. Indeed, the TRPV1 antagonist capsazepine, which we applied at a concentration known to have little effect on other molecules than TRPV1, significantly reduces the proportion of neurons responding to 20:4-

NAPE application. However, the inhibitory effect of capsazepine might be different in species other than rat. Nevertheless, the finding that cultured PSN prepared from TRPV1$^{-/-}$ mouse DRG do not, whereas a sub-population of cultured PSN prepared from WT mouse DRG does, respond to 20:4-NAPE application provides evidence that, in PSN, the 20:4-NAPE application-evoked excitatory effect is indeed mediated through TRPV1. Several findings indicate that the 20:4-NAPE application-induced excitatory effects are exerted indirectly, through anandamide production. First, both the anandamide production, and the 20:4-NAPE application-induced cobalt accumulation, depend on the concentration of the substrate. Second, if 20:4- NAPE activated TRPV1 directly all capsaicin-sensitive neurons would be expected to respond. However, we found that only ~40% of capsaicin-responsive neurons exhibit responses to 20:4-NAPE application. Third, if 20:4-NAPE activated TRPV1 directly, a similar pattern of temperature-dependent change in the responsiveness of neurons to 20:4-NAPE and capsaicin is expected to occur. However, while application of 20:4-NAPE, which induces responses in about a third of the neurons at 37 °C, fails to produce responses in any neurons at room temperature. At the same time, the proportion of capsaicin-responsive neurons is the same at 37 °C and room temperature. Fourth, the lack of responsiveness to 20:4-NAPE at room temperature co-occurs with a 10-fold reduction in the anandamide content of the superfusate following the application of 20:4-NAPE to the cells. Fifth, direct TRPV1 activators, such as capsaicin and anandamide, activate TRPV1 with fast kinetics. However, the kinetics of 20:4-NAPE-induced responses are significantly slower than those of the capsaicin-evoked responses. Sixth, the amplitude of whole-cell currents produced by 20:4-NAPE application, which results in about 30 nM anandamide when the concentration is measured in the cells and superfusate together, is compatible with the amplitude of whole-cell currents evoked by 100 nM anandamide included into the recording electrode. These findings collectively provide evidence that the 20:4-NAPE-evoked effects depend on enzyme activity and at least the majority, if not all, of the 20:4-NAPE application-induced TRPV1-mediated excitation is produced indirectly, via the conversion of 20:4-NAPE to anandamide. The involvement of 20:4-NAPE-derived metabolites other than anandamide is highly unlikely because thin layer chromatography shows the presence of no other lipids than anandamide and 20:4-NAPE in the superfusate. The limited responsiveness of capsaicin-sensitive neurons to 20:4-NAPE may appear in contrast with the

co-expression pattern of TRPV1 and the four enzymes we examined in this study. However, each of these four enzymes constitutes only a member of specific enzymatic pathways, none of which is selective for the synthesis of anandamide. Due to the unknown molecular identity of some enzymes, at present, it is impossible to establish the proportion of neurons which express (or co-express) a complete anandamide-synthesising pathway(s). Nevertheless, our findings suggest that less than half of TRPV1-expressing neurons may express pathways, which are able to produce anandamide in a Ca^{2+}-insensitive manner. These findings, together with the concentration of anandamide in the superfusate, following 20:4-NAPE application, being well below the minimum concentration of exogenous anandamide needed for TRPV1 activation also suggest that anandamide synthesized in a Ca^{2+}-insensitive manner, similar to anandamide produced through a Ca^{2+}-sensitive pathway, activates TRPV1 through autocrine signalling. Notably, the overall proportion of capsaicin-responsive cells is increased from ~60 % to above 80 % when 20:4-NAPE is applied before capsaicin. Furthermore, more dual-responsive than capsaicin-only-responsive neurons exhibit sustained/slowly inactivating capsaicin-induced increase in the $[Ca^{2+}]_i$. Hence, the autocrine signalling by anandamide synthesised in a Ca^{2+}-insensitive manner by PSN seems to have some sensitising effect on TRPV1-mediated responses, which results in exhibiting such responses above the detection threshold in neurons which do not produce detectable responses to capsaicin in control conditions. While exogenous anandamide induces a CB1 receptor-mediated inhibition on TRPV1-mediated responses in PSN, the selective and specific CB1 receptor antagonist, rimonabant, reduces the proportion of neurons responding to, and the amplitude of responses induced by, 20:4-NAPE application. These data may suggest that exogenous anandamide, and anandamide produced from 20:4-NAPE via Ca^{2+}-insensitive pathways may exert their actions through not completely overlapping mechanisms (i.e., while exogenous anandamide induces a CB1 receptor-mediated inhibitory effect, endogenous anandamide does not induce such effect). However, the effect of exogenous anandamide on TRPV1 activity depends on its concentration and the availability of the CB1 receptor. Hence, exogenous anandamide above 1 μM induces larger responses in TRPV1-CB1 receptor co-expressing human embryonic kidney 293 cells than in such cells when they express TRPV1 alone. Consistently, the exogenous anandamide-evoked excitatory effect is reduced by rimonabant in cultured rat PSN. Furthermore, like the effect

of anandamide on TRPV1, the effect of capsaicin is also reduced when the CB1 receptor is not available either due to the blocking CB1 receptor activity with rimonabant or to the deletion of the CB1 receptor. In agreement with findings in $CB1^{-/-}$ mice, we found here that rimonabant reduces the amplitude of capsaicin-evoked responses, but not the proportion of neurons responding to capsaicin. Therefore, the inhibitory effect of rimonabant on the proportion of neuron responding to 20:4-NAPE by increased $[Ca^{2+}]_i$ is consistent with the proposed constitutive sensitising action of the CB1 receptor on TRPV1, which may occur under certain conditions. Hence, the effects of exogenous anandamide above 1 µM and the effects of endogenous anandamide synthesised from 50 µM 20:4-NAPE in a Ca^{2+}-insensitive manner, at least as far as activating TRPV1 and not producing CB1 receptor-mediated inhibitory effects are concerned, are similar in PSN. Together these findings indicate that anandamide that is synthesised in a Ca^{2+}-insensitive manner from 50 µM 20:4-NAPE has an excitatory rather than an inhibitory effect in PSN.

In conclusion, results of the present study show that: (a) a sub-population of PSN has at least one enzymatic pathway which synthesises anandamide from 20:4-NAPE in a Ca^{2+}-insensitive manner; (b) the synthesis of anandamide from exogenous 20:4-NAPE primarily, if not exclusively, occurs via Ca^{2+}-insensitive pathways; and (c) anandamide of PSN origin synthesised from 20:4-NAPE in a Ca^{2+}-insensitive manner produces an autocrine TRPV1-mediated excitation, which may complement the previously reported excitatory effects on TRPV1 of anandamide synthesised in a Ca^{2+}-sensitive manner in PSN. The effect of anandamide of PSN origin together with the effects of exogenous anandamide on PSN emphasises the high degree of flexibility of action of this important lipid mediator, and its multi-faceted role in controlling nociceptive processing. Therefore, a better understanding of the expression and function of the anandamide-synthesising enzymes in PSN may allow us to control the activity of those cells, and hence develop more effective treatments of somato- and viscerosensory disturbances outside the central nervous system.

The rat TRPV1b splice variant

Sequencing the two RT-PCR products generated by primers flanking exon 7 revealed that while the larger product corresponds perfectly to the appropriate section of rat TRPV1 mRNA, the smaller transcript lacks 180 bases of this mRNA. Those 180 bases represent the entire exon seven. We regard the smaller transcript amplified from rat DRG in our samples

the rat homologue of TRPV1b, which reduces the responsiveness of the ion channel in a ratio-dependent manner when co-expressed with the full length TRPV1. Our findings indicate that both the TRPV1 and TRPV1b transcripts are translated into proteins in cultured PSN.

Although, inflammation is associated with the production, release and accumulation of a myriad of agents, among them, regarding the development and maintenance of pain, BK and PGE_2 are believed to have the highest importance. Both BK and PGE_2 induce rapid TRPV1 phosphorylation which results in a significant reduction in the heat threshold of the ion channel. Due to the integration of effects on the channel's gating apparatus produced by various TRPV1 activators, that reduction results in increased TRPV1-mediated responses including capsaicin-induced cobalt accumulation. We found that, in addition to increasing capsaicin-responsiveness, BK and PGE_2, during prolonged application, also up-regulates *trpv1* transcription, which results in uneven increase in TRPV1 and TRPV1b mRNA expression. In contrast to this finding, no evidence for increased *trpv1* transcription was found in a recent *in vivo* experiment. The discrepancy in gene transcription between the *in vivo* and *in vitro* studies could be due to the differences in the mediators found in inflamed tissues and used to create the inflammation-associated milieu in the present study, as well as in the proportion of the sampled PSN which were exposed to the mediators *in vitro* and *in vivo*. In spite of the BK- and PGE_2-induced up-regulation in TRPV1 mRNA expression, TRPV1 protein expression was not changed either in whole cell lysates or membrane fractions. This mismatch between changes in TRPV1 mRNA and protein expression could be due to activity-dependent increase in the turnover of the TRPV1 protein. While TRPV1 and TRPV1b protein expression was not altered, the capsaicin-induced, hence TRPV1 ion channel-mediated cationic influx was significantly enhanced by exposing neurons to BK and PGE_2. Therefore, the most parsimonious explanation for the development of the sensitised state of TRPV1 is that it is produced by BK- and PGE_2-induced post-translational modifications of TRPV1 alone. However, it is also possible that BK- and PGE_2 exposure induces, without changing TRPV1 and TRPV1b protein expression, re-arrangement of TRPV1 and TRPV1b subunits. Such a re-arrangement might result in the formation of TRPV1 and TRPV1b homotetramers in inflamed conditions from TRPV1/TRPV1b heterotetramers found in naive conditions. Hence, the higher number of fully responsive

TRPV1 homotetramers, without increased TRPV1 protein expression, could mediate the increased capsaicin-evoked responses. Clearly, providing evidence for such a re-arrangement requires further studies, which, at present, are hindered, by the lack of a selective and specific anti-TRPV1b antibody. Nevertheless, the high level of expression of the negative dominant TRPV1b splice variants in the membrane fraction suggests that TRPV1b could indeed have an important role in regulating the responsiveness of the channel, and supports the view that changes in the composition of the ion channel could contribute to increased responsiveness of the capsaicin receptor in inflammatory conditions.

Abstract

In this work, we investigated the regulatory effects of endogenous anandamide and exogenous inflammatory mediators (bradykinin (BK) and prostaglandin E_2 (PGE_2)) on the activity of primary sensory neurons.

The endogenous lipid agent anandamide, among other effects, has been shown to be involved in nociceptive processing both in the central and peripheral nervous systems. Anandamide is thought to be synthesised by several enzymatic pathways both in a Ca^{2+}-sensitive and Ca^{2+}-insensitive manner, and rat primary sensory neurons produce anandamide. Here, we show that cultured rat primary sensory neurons express Ca^{2+}-insensitive enzymes implicated in the synthesis of anandamide, and that application of NAPE, the common substrate of the anandamide-synthesising pathways, results in anandamide production. We also show that anandamide, which has been synthesised in primary sensory neurons following the application of NAPE induces transient receptor potential vanilloid type 1 ion channel (TRPV1)-mediated excitatory effect that is not inhibited by concomitant activation of the cannabinoid type 1 receptor. We show that subpopulations of TRPV1-expressing primary sensory neurons also express some of the putative Ca^{2+}-insensitive anandamide-synthesising enzymes. Together, these findings indicate that anandamide synthesised by primary sensory neurons via a Ca^{2+}-insensitive manner has an excitatory rather than an inhibitory role in primary sensory neurons and that excitation is mediated predominantly through autocrine signalling.

TRPV1 plays a pivotal role in the development of inflammatory heat hyperalgesia. The splice variant of the TRPV1 molecule, TRPV1b has been suggested to be a naturally-occurring inhibitory subunit of the TRPV1 ion channel. We assessed TRPV1 and TRPV1b mRNA and protein expression, as well as capsaicin-induced TRPV1 activity in cultured rat PSN grown for 2 days in control or in an inflammation-associated milieu created by adding BK and PGE_2 to the medium to find whether alterations in TRPV1 and TRPV1b expression could contribute to the increased responsiveness of the TRPV1 ion channel in inflammation. We found that both TRPV1 and TRPV1b mRNA and protein are expressed in PSN and that TRPV1b mRNA lacks the entire exon 7. Culturing neurons in the presence of BK and PGE_2 significantly increases TRPV1 responsiveness and TRPV1 mRNA expression. However, the presence of BK and PGE_2 does not alter TRPV1b mRNA, and TRPV1 and TRPV1b protein expression.

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List of publications related to the dissertation

1. Mistry, S., Paule, C.C., Varga, A., Photiou, A., **Jenes, Á.**, Avelino, A., Buluwela, L., Nagy, I.: Prolonged exposure to bradykinin and prostaglandin E2 increases TRPV1 mRNA but does not alter TRPV1 and TRPV1b protein expression in cultured rat primary sensory neurons. *Neurosci. Lett. Epub ahead of print (2014)*
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