Examination of the effect of lipid raft disruption on the activation of Transient Receptor Potential ion channels

Doctoral (PhD) thesis

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Pécs

2022.

1. RESEACRH CONCEPT

Pain relief and the discovery of analgesic drugs is still a very important area of biomedical research, as neuropathic pain based on central and/or peripheral nervous system dysfunction is one of the most important economic and health problems of the modern era. The treatment of this condition is a major challenge, as classical drug therapies such as non-steroidal anti-inflammatory drugs (NSAIDs), most commonly e.g. diclofenac, ibuprofen, or opioids such as the weak opioid tramadol or the stronger morphine or fentanyl, do not have a satisfactory therapeutic effect and/or cannot be used in long term due to side effects (e.g. NSAID-induced gastric ulcer, opioid dependence). Therefore, currently accepted therapeutic protocols try to alleviate patients' pain with other indicated drugs, so-called adjuvant analgesics (e.g. antiepileptics, antidepressants), but in many cases these do not achieve adequate therapeutic effect (1).

Therefore, there is a need to investigate new therapeutic approaches that differ from classical pharmacological receptor antagonism, as several attempts to target Transient Receptor Potential (TRP) ion channels, which play a major role in the development of neuropathic pain, have failed in recent decades. The new approach of our research group is based on an alternative manipulation of the activation mechanisms of TRP Vanilloid 1 (TRPV1) and TRP Ankyrin 1 (TRPA1) receptors on capsaicin-sensitive sensory nerve endings, which involves disrupting the hydrophobic connections between cell membrane microdomains, so-called lipid rafts, and TRP proteins.

2. INTRODUCTION, BACKGROUND

2.1. A brief history of pain relief

Pain is perhaps one of the most difficult human emotions to define, so it is not surprising that its alleviation has been a preoccupation of mankind since the beginning of time. The latest definition of the International Association for the Study of Pain's latest is "an unpleasant sensory and emotional experience associated with, or resembling, actual or potential tissue damage". Records documenting the history of pain relief date back to antiquity, and one of the most famous is the Egyptian *Ebers Papyrus*, which contains descriptions of numerous ancient diseases, cures and religious elements that permeate the healing process. For example, there are several ancient records and collections from the Far East that describe the foundations (e.g. Yin and Yang theory) and methods (e.g. spitting, acupuncture) of contemporary Traditional Chinese Medicine (TCM), but it should be noted that some of the teachings and methods of TCM are integral to the practice of alternative medicine today.

The *Codex Ayasofya*, written in the 14th century, summarised the medical knowledge of the time, information on medicinal plants and the substances used in their treatment. The next big step was the work of Friedrich Sertürner, who in 1817 was the first to isolate morphine from the opium poppy (*Papaver somniferum*), which later opened the way for the development of the major analgesics still in use today. The development of NSAIDs was also triggered by new discoveries in the 19th century, when in 1897 Felix Hoffmann of Bayer Pharmaceuticals chemically synthesised acetylsalicylic acid. This breakthrough was followed by the development of further NSAIDs from the 1950s onwards. Today, a number of new drug candidates with new mechanisms of action are being developed, some of which follow classical pharmacological considerations, but there is also a growing interest in alternative ways of targeting receptors and sensory nerve endings involved in the development of pain sensation.

2.2. Physiology of pain

The integrity of tissues can be damaged by mechanical, chemical and thermal stimuli that activate specialised high threshold free nerve endings called nociceptors. This name comes from Sherrington and is derived from the Latin *nocere* (to harm, to damage). Nociceptors can be found in the skin, subcutaneous tissue, bones, muscles, dental pulp, acid membranes and many other sites in the body. Based on their sensitivity, we distinguish between nociceptors that can be activated by a single stimulus (unimodal) and nociceptors that can be activated by multiple stimuli (polymodal). On the basis of their myelinisation, we distinguish between Aδ and C-fibres. The former has a thin myelin sheath and are slow conducting (on average about 15 m/s) fibres, whose terminals are activated mainly by intense (painful) mechanical stimuli and hot temperatures. The latter are thin fibres with no myelin sheath and even slower conduction (average 1 m/s). A subpopulation of C-fibres is formed by polymodal nociceptors, which can be activated by all three types of stimuli and in some of which proinflammatory neuropeptides are produced, such as substance P or calcitonin gene-related peptide (CGRP), which are released from the nerve endings following activation and cause local so-called neurogenic inflammation. The stimulus from activated nociceptors enters the dorsal root ganglia (DRG) (neuron I) via the primary pain fibres. From there, the central extensions enter the grey matter of the spinal cord, where a part of them is switched to projection neurons (neuron II) and transport the stimulus in the *lateral tractus spinothalamicus* to the *thalamus*, from where, after switching to neuron III, the pain sensation is consciously transmitted to the *cortex* via *thalamocortical* neural connections (2,3).

2.3. History of capsaicin research

The alcoholic extracts of Capsicum species have been an integral part of the medicinal treasure of ethnomedicine for centuries, as empirical information shows that they have appetite-enhancing, skin-reddening and analgesic effects and are still used in the preparation of magisterial medicines as an official material in the VIII. edition of the Hungarian Pharmacopoeia (*Capsici tinctura normata Ph. Hg. VIII.*). In 1878, Endre Hőgyes demonstrated that the extract of capsaicin (CAPS) of partial purity can activate sensory nerve endings relatively selectively (4). Following Hőgyes' observation, pharmacological research on CAPS was overshadowed by the experiments on autonomic nervous system agents of the 19th and 20th centuries, and it was only about seventy years later that Miklós Jancsó returned to CAPS and described that higher doses could induce a long-lasting desensitization to chemical irritants (5). Ten years later, after the death of Miklós Jancsó, his wife Aranka Jancsó-Gábor and his colleague János Szolcsányi published the results in which they described that plasma protein extravasation following orthodromic or antidromic stimulation could be inhibited by neural denervation or CAPS desensitization. The results of these experiments led to the conclusion that proinflammatory substances are released from capsaicin-sensitive sensory nerve endings (6,7). Further experiments led to the first suggestion of the existence of a specific CAPS receptor, the schematic structure of which was reported in two papers (8,9). Over the next few decades, a series of papers on CAPS were published, describing the involvement of non-cholinergic non-adrenergic mechanisms and the concept of a capsaicin-sensitive nervous system, which was confirmed in relation to pro-inflammatory neuropeptides such as CGRP, substance P (10,11). A new chapter has been opened with the synthesis of the first CAPS antagonist compounds and the study of the effects of another vanilloid structured substance, resiniferatoxin (RTX) (12,13). At that time, it was assumed that there were two vanilloid receptors, but in 1997 Michael Jerry Caterina and co-workers cloned the CAPS receptor (14), which was first named Vanilloid 1 receptor and later classified as the first member of the TRP ion channel family, within the vanilloid subfamily, based on the structure, and thus named TRPV1.

2.4. Function of capsaicin-sensitive nerve endings

Capsaicin-sensitive sensory nerve endings have a triple function. The first important function is that, when activated, the classical afferent mechanism transmits the stimuli to the central nervous system, where nociception occurs. The second function is a local efferent effect, the activation leads to an increase in intracellular Ca^{2+} levels, which leads to the release of stored peptide mediators (CGRP, substance P), which in turn induce neurogenic inflammation in the innervated area by plasma protein extravasation, vasodilation, activation of inflammatory cells. The third, a systemic efferent function, is the release of somatostatin from the nerve endings after activation, which, by entering the systemic circulation, exerts systemic anti-inflammatory and analgesic effects at distant sites in the body. This triple function of sensory nerve endings has been defined as sensory neuronal function (15).

2.5. The Transient Receptor Potential ion channels

TRP proteins are one of the largest ion channel families, with 28 members identified in mammals (27 in humans). The cation channels can be classified into seven subfamilies based on structural and sequence homology (16): TRPC ("Canonical"), TRPV ("Vanilloid"), TRPM ("Melastatin"), TRPP ("Polycystin"), TRPML ("MucoLipin"), TRPA ("Ankyrin"), and TRPN ("NO-Mechano-Potential C"). In 1969, Cosens and Manning described, that photoreceptors in a mutant strain of *Drosophila melangoster* exhibit transient activity, and later named these photoreceptors TRP channels based on electroretinography studies. Subsequently, the receptor in question was discovered to belong to the TRPL subfamily. Later, Michael Jerry Caterina and colleagues cloned and characterized the TRPV1 receptor (14). Members of the TRP family are mostly non-selective cation channels, but certain subtypes show a high selectivity mainly for Ca^{2+} and to a lesser extent for Mg^{2+} ions. Ion channels have six transmembrane (TM) domains (S1-S6), usually form a functional tetrameric unit, and the pore for ion passage is located in the loop region between the fifth and sixth domains. The N-terminal and C-terminal ends of membrane proteins are located intracellularly and have well-known molecular structures with characteristic functions. These include, among others, the Ankyrin Repeated Domain (ARD), the TRP domain, EF-hand structures, and other characteristic formations (16).

2.6. Structure and function of TRPV1

The protein, also known as the CAPS receptor, has six TM regions similar to the other members of the TRP family, with an ion-passable pore formed between the S5 and S6 segments, which is connected to the S1-4 TM segment. It contains the TRP domain of 23-25 amino acids, located downstream of the S6 TM region. The N- and C-terminal ends are also located intracellularly, and the N-terminal is an important part of the subfamily-specific ARD, which has a functional role. TRPV1 is expressed primarily on primary sensory neurons, but is also found in many extra neuronal tissues such as keratinocytes, mast cells and the gastrointestinal system (16). TRPV1 receptor function is regulated by a number of endogenous and exogenous substances. Among its exogenous agonists, the most important are molecules containing a vanilloid structural element - CAPS and RTX, respectively (13,17) - which bind to the so-called "vanilloid pockets" (S505-T550) between the S3 and S4 transmembrane regions. Other amino acids of importance for CAPS are T511, S512 and W549, and for RTX M547. Allicin from another plant family, garlic of the *Allium* species, also activates TRPV1 through its N-terminal C157 position. In addition to plant pungents, a number of animal toxins (e.g. scorpion and spider venoms) also induce TRPV1 activation. In addition to exogenous compounds, many endogenous substances and physicochemical changes can activate the channel. The endocannabinoids anandamide, N-arachidonoyl-dopamine and the non-cannabinoid N-oleoyl-dopamine activate the receptor, as do arachidonic acid and other fatty acid metabolites. The most important of the physicochemical parameters are high temperature (>43 ºC) and low pH (<6) (14). Changes in both parameters activate the receptor in the extracellular part of the S5-S6 pore-forming TM region. The critical amino acids involved in temperature sensing are N628, N652 and Y653, while E600 and E648 are involved in proton sensing (18). In addition to agonists, TRPV1 antagonists also play an important role, notable among which are ruthenium red, a non-specific inhibitor, pellitorine isolated from *Tetradium daniellii*, and the specific inhibitor capsazepine, synthesized on the basis of the CAPS structure (19,20). Other important antagonists are the endogenous lipid mediators resolvins (21).

2.7. Structure and function of TRPA1

The TRPA1 receptor is found primarily in the DRG and trigeminal ganglion (TG) (22), but is also found extra neuronally in keratinocytes (23) and on enterochromaffin cells (24). TRPA1 and TRPV1 are co-expressed in approximately 30% of capsaicin-sensitive sensory nerves and are primarily involved in pain perception (25). Research on the receptor structure and the compounds that influence its function is challenging, becuase TRPA1 structure varies to some extent between species (26). A characteristic structural element is the 14-18 element ARD, which provides several binding sites important for channel activation (27). Like TRPV1, exogenous and endogenous ligands can activate TRPA1, with a significant proportion of exogenous agonists being chemical irritants, e.g. many of the endogenous agonists are formed as a result of inflammatory processes, e.g. methylglyoxal, hydrogen peroxide or 4-hydroxy-nonenal (30). In addition to these ligands, TRPA1 activation is also triggered by low temperature (<17 ºC) and mechanical stimuli, on the basis of which TRPA1 is also commonly referred to as a mechanosensitive receptor (22,31). Among the compounds with antagonistic activity, mention should be made of HC-030031, compound SZV-1287, a compound with a complex mechanism of action, TRPA1/TPRV1 antagonist and semicarbazide-sensitive amine oxidase activity (32,33).

2.8. Cell membrane structure and the lipid raft model

The classical fluid-mosaic model of cell membranes was published by Singer and Nicolson in 1972 (34). According to this theory, membrane components such as phospholipids, glycolipids and cholesterol are uniformly distributed in the membrane according to the laws of thermodynamics, while proteins are randomly distributed. The skeleton is formed by the phospholipid bilayer, the hydrophilic head part of whose molecules is oriented towards the aqueous phases and the hydrophobic tail part towards the inside of the membrane, i.e. the other lipophilic layer, thus forming an impermeable barrier for water-soluble compounds. Membrane proteins are located on the outer or inner surface, but in some cases, they may be more or less immersed in the membrane (integral membrane proteins) or may completely penetrate it (transmembrane proteins) (35).

This model has been confirmed by several structural and biochemical studies, but it cannot explain the fact that some proteins are unevenly distributed on the cell membrane surface. To explain this, Simons and Ikonen developed the so-called lipid raft theory (36), according to which membranes contain microdomains with a specific structure and arrangement, called lipid rafts. These raft regions are rich in cholesterol, sphingolipids and gangliosides, and their location within the membrane varies. The extracellular side is rich in sphingomyelins and glycosphingolipids, while the intracellular side is more glycerophospholipids. Cholesterol, as the major component responsible for the integrity of the structure, is evenly distributed (36). This high degree of structural organization results in raft regions having different physicochemical properties compared to other regions of the membrane. One such characteristic property is their insolubility in non-ionic detergents (e.g. Triton-X100 detergent at 4 ºC). However, this property can be eliminated by depletion of the major constituent cholesterol, leading to the disintegration of lipid rafts (37).

2.9. Physiological and pathological role of lipid rafts

Lipid rafts have many functions in the body, e.g. regulating membrane transport processes, influencing the proper function of the immune, cardiovascular and nervous systems (38). In addition, they also play a regulatory role in many inflammatory processes, hence the concept of "inflammaraft" introduced by Miller et al. (39). There is also evidence that a number of ion channels, including several TRP receptors (40-42), as well as a number of proteins associated with malignant tumour types, are located in raft regions (38) A special subtype of lipid rafts are caveolae, which are membrane elements with a characteristic morphology and caveolin content. Caveolae play a major role in the process of endocytosis, which has been described to be involved, among other things, in the penetration of many pathogens into cells. However, in addition to pathogen entry, caveolae also have therapeutic potential, as they can be used to target large molecule drugs (43). Another special subtype is the ceramide-rich regions, which are mainly produced by the enzyme sphingomyelinase (SMase) and vary in size from a few nanometres to micrometres (44). Their function is involved in the clustering of membrane receptors, e.g. they regulate the junctional processes of CD40/CD40 ligand and CD95/CD95 ligand (44). Another special group is the ganglioside-rich rafts, which are involved in the cellular entry of various toxins and in the molecular pathomechanism of Alzheimer's disease (45)

2.10. Pharmacological study of lipid rafts

The pharmacological investigation of lipid rafts has been a major focus of basic research over the last decade, with a focus on disrupting the integrity of raft regions to investigate the activation of a number of signalling pathways and receptors. Two main mechanisms can be distinguished for the disruption of raft regions, one is the degradation of the constituents and the other is the inhibition of their assembly. The most studied mechanism in lipid raft research is the deplete cholesterol, usually using methyl-β-cyclodextrin (MCD), which forms a complex with cholesterol. The sphingomyelin content of rafts can also be degraded enzymatically by SMase, which converts sphingomyelin to phosphocholine and ceramide. Among the agents that inhibit the synthesis of the constituents, those that inhibit the synthesis of sphingolipids are used experimentally. These include myriocin (Myr), which inhibits the enzyme serine palmitoyl transferase, the rate-determining step in sphingolipid synthesis. Much of the research on lipid rafts has been carried out using *in vitro* systems and has mostly investigated the effect of MCD, but the results are controversial in several cases. Liu et al. described that MCD treatment does not alter heat-induced TRPV1 activation in transfected human embryonic kidney 293 (HEK293) cells (46), but inhibits proton and CAPS-induced currents in DRG neurons (40). Binding of other ligands, e.g. 3[H]RTX to the TRPV1 receptor, was also investigated, but was not affected by cholesterol depletion. The effect of MCD on the activation of other TRP ion channels has also been investigated, Startek et al. demonstrated that MCD treatment decreases the activation of the TRPA1 receptor by cold stimuli or bacterial endotoxin, but its expression within the membrane remains unchanged (47,48). Similar effects were later described with the SMase enzyme (49). Our research group has also described that under *in vitro* conditions, MCD, SMase or Myr pretreatment can inhibit CAPS and RXT-induced TRPV1 activation in Chinese Hamster Ovary (CHO) cells stably expressing the receptor, as well as in primary sensory neuron cultures (41,42). Also, our research group described that a carboxamido-steroid compound (C1), synthesized for us at the Institute of Organic Chemistry, University of Pannonia, has an inhibitory effect on TRPV1 activation similar to that of MCD, but at a 1000-fold lower concentration (50,51).

Beyond *in vitro* studies, there are only a few animal data on the role of lipid rafts in pain and these are mostly concerned with different cyclodextrin derivatives. Sauer et al. have described that local or systemic pretreatment with random methylated-β-cyclodextrin (RAMEB) reduces complete Freund's adjuvant-induced thermal allodynia and mechanical hyperalgesia in rats (52). MCD is effective in attenuating hyperalgesia in a mouse model of RTX-induced neuropathy (53) and after administration of prostagalndin E2 in rats (54). The role of gangliosides has also been investigated *in vivo*, with intraplantar injection of the ganglioside GT1b eliciting nociceptive responses and enhancing the formalin-induced pain response, but reducing the pain response following administration of the enzyme sialidase, which degrades sialyl residues from gangliosides (55).

3. AIMS

The treatment of neuropathic pain is still unsatisfactory, as most of the drugs used cause serious side effects and/or do not provide adequate clinical efficacy when used over long periods of time. This is because the pharmacological manipulation of capsaicin-sensitive sensory nerve endings has encountered many difficulties, inter alia, because the classical receptor antagonist approach has not yet been able to develop an agent with a risk/benefit ratio (e.g. hyperthermia, thermosensory disturbances) associated with adequate clinical efficacy. This is why there is a need to explore new alternative pharmacological options, such as the manipulation of TRP receptors through lipid rafts, which could open a new direction in the development of analgesics.

In my work I have therefore set the following general objectives:

1. **Investigation of the effect of lipid raft disruption on cell membrane polarity and fluidity**
2. **To study the effect of sphingolipid depletion on the activation of TRPV1 and TRPA1 ion channels *in vivo***
3. **Examination of the effect of cholesterol depletion on the activation of TRPV1 and TRPA1 ion channels *in vivo***

4. EXPERIMENTAL MODELS AND TEST METHODS

4.1. Fluorescence spectroscopy

6-dodecanoyl-N,N-dimethylnaphthylamine (Laurdan) is a widely accepted and used fluorescent dye suitable for the study of both biological and model membranes (56). It can be used to study both the polarizability and fluidity of membranes. For sample preparation, native CHO cells were incubated with lipid raft damaging agents (30 mU SMase, 100 nM Myr, 10 mM MCD, 100 μM C1 - dissolved in extracellular solution (ECS)) or ECS for 45 min or 24 h for Myr at 37 ºC. The cells were then washed three times with sterile phosphate-buffered saline (PBS) and incubated with 40 μM Laurdan (in ECS) for 40 min at 37 ºC. Cells were then washed three times with sterile PBS and plated in 1 ml sterile PBS. Measurements were performed with a FL3-2iHR spectrofluorimeter (HORIBA Jobin-Yvon NanoLog) in 4 mm path length quartz cells (Hellma 104F-QS) at a fixed temperature of 20 ºC.

4.1.1. Membrane polarity test

Membrane polarity, which in contrast to cell biological polarity in fluorescence spectroscopy depends on the proportion of polar water molecules incorporated in the phospholipid bilayer, can be inferred from the variation of the Laurdan excitation and emission spectra, which are related to the current hydration level of the membrane, i.e. the ratio of liquid ordered (lower water content) and liquid disordered (higher water content) phases (57). The wavelengths varied between 300 and 420 nm for excitation spectra and between 380 and 600 nm for emission spectra.

4.1.2. Membrane fluidity test

To study the membrane fluidity, 14 decay curves (λ = 10 nm) in the 410-540 nm range of the emission spectrum were recorded, from which lifetime and pre-exponential values were obtained by 4 exponential fits. The obtained pure decays were used as a basis for the determination of further parameters. The first parameter investigated is the generalized polarization (GP), which provides information on the spectral (blue and red) shift related to the proportion of water molecules incorporated in the membrane (58).

The second parameter studied is the velocity component (Center of Gravity - CoG), which characterizes the change in GP center of gravity and provides information on solvent relaxation, i.e. the rotational mobility of water molecules in the membrane around Laurdan, often identified with membrane viscosity. The third parameter studied is the rotation lifetime (τ_"rot") derived from the anisotropy decay determined by time-resolved polarization measurements at the spectral maximum of the emission (450 nm), which provides information on the mobility of Laurdan molecules in the phospholipid bilayer, i.e. their confinement in the membrane (59,60). Interpreting these parameters separately, they characterize the behaviour of Laurdan molecules in the membrane and, taken together, provide information on the fluidity of the membrane. It should be noted, however, that fluorescence spectroscopy is not suitable for the selective study of the plasma membrane, since the inner and outer membrane systems cannot be separated, but Laurdan molecules are present in both membrane systems (61). Therefore, the results obtained were evaluated qualitatively, with relative conclusions drawn from the direction of change.

4.2. CAPS-induced acute chemonociceptive reaction model

In our experiments, CAPS solution (20 μL, 30 μg/mL) was instilled into the right eye of the animals and the number of eye wipes was counted for 1 min in a glass cage (62). Only wipes with one paw were considered for the evaluation, wash or two paw movements were excluded. CAPS solution administration was repeated at the second and third hours of the experiment.

4.3. RTX-induced thermal allodynia and mechanical hyperalgesia model

Injection of the ultrapotent, selective TRPV1 agonist RTX (20 μL, 0.1 μg/mL) into the right hindpaw induced an acute neurogenic inflammatory response, in which thermal allodynia develops through peripheral mechanisms and mechanical hyperalgesia through complex peripheral and central mechanisms (63). Prior to the measurement, conditioning tests were performed on two consecutive days to determine the thermal threshold using a increasing temperature Hot Plate (Hot Plate, IITC Life Science, Woodland Hills, CA, USA) and the mechanical pain threshold using a Dynamic Plantar Aesthesiometer (DPA, Ugo Basile, Comerio, Italy). In this study, after RTX injection, a thermal threshold test was performed at 10, 20, and 30 min, followed by mechanical pain threshold determination at 30, 60, and 90 min (64).

4.3.1. Increasing temperature Hot Plate (Hot Plate)

The animals to be tested were placed on a heatable metal plate on which a plastic cage was placed. For the control tests, the temperature was increased at a rate of 12 ºC/min starting at 35 ºC (up to a maximum of 53 ºC) until the animals showed nocifensive behaviour (licking, lifting, shaking the hind leg), at which point the apparatus was stopped and the temperature corresponding to the thermal threshold was recorded. After the RTX was administered, the measurement was performed at 12ºC/min starting from 25ºC.

4.3.2. Dynamic Plantar Aesthesiometer (DPA)

In the study, the animals were separated from each other and placed in plastic cages, opaque to each other but transparent to the experimenter, on a grid where they could move freely. The DPA is a mirror manipulator equipped with a blunt-ended needle, which is used to stimulate precisely the hindpaw of the animal, thus exerting a mechanical force (max. 10 g, with a needle rise of 2.5 g/s). When the mechanonociceptive threshold is reached, i.e. when the animal shows a deflective reaction - i.e. it moves its leg away - the digital counter stops and the value is read on the display in grams. In all measurements, the right leg of the animal was tested, the left leg served as a self-control.

4.4. Formalin-induced acute inflammatory pain model

In the model, TRPA1-activating formalin (20 μL, 2.5%) was injected into the right hindpaw of mice, and the time of the pain response (chewing, licking, holding, shaking) was measured in two phases, 0-5 min and 20-45 min. The first phase is associated with pain mediated by direct chemical stimulation of TRPA1 receptors, followed by a pain-free period of 10-15 min, and then peripheral neurogenic inflammatory processes maintain the pain in the second phase (65,66).

4.5. Ethical aspects

For the study of CAPS-induced acute chemonociceptive response, 12-16 week-old male C57BL/6 mice were used, whereas for the RTX-induced thermal allodynia and mechanical hyperalgesia and formalin-induced acute inflammatory pain models, NMRI mice of the same age and sex were used. The animals were bred and kept in the animal house of the Department of Pharmacology and Pharmacotherapy, Medical School, University of Pécs. Our experimental methods and procedures were in full compliance with the requirements of the Government Decree No. 1998/XXVIII on the performance of animal experiments and the European Parliament Directive 2010/63. The Ethics Committee for Animal Experiments of the University of Pécs approved the experimental protocols (Permit No. BA02/2000-45/2020). In all experiments, 4 groups were used, two of which (treated twice with physiological saline/dimethyl sulfoxide (DMSO) and with substance followed by physiological saline) served as controls to study the possible pain induced by the puncture and the possible pain induced by the substance alone. Statistical analyses were performed on the two groups of animals that received either physiological saline or substance as pretreatment followed by irritant administration as treatment. In the case of Myr, another group was used where single and double administration of DMSO were compared to see if DMSO pretreatment causes any significant desensitizing effect in the animals.

4.6. Statistical analysis

In all cases, data are presented as mean ± SEM, calculated from animal experiments with at least 6 elements. For the evaluation, we used repeated measures Two-way analysis of variance (ANOVA) with Bonferroni post hoc test for each study. All results above $p<0.05$ were considered significant. GraphPad Prism 8 software was used for statistical analysis of animal experiments. Fluorescence spectroscopy was performed with an element count of 2, all samples were pooled according to the measurement specifications and data were analysed using OriginPro 8.5 software.

5. RESULTS

5.1. Investigation of the effect of lipid raft disruption on cell membrane polarity and fluidity

5.1.1. Effect of SMase and Myr on membrane polarity

The excitation (between 300-420 nm) and emission (between 380-600 nm) spectra recorded during the membrane polarity studies were only minimally changed by the 30 mU SMase treatment. However, the 100 nM Myr treatment significantly altered the excitation and emission spectra over the whole wavelength range, suggesting that the membrane polarity and microenvironment - a more closed membrane - were altered compared to the untreated (control) sample.

5.1.2. Effect of lipid raft disruption on membrane fluidity

For the determination of the overall polarization, the intensity values obtained by integrating the fluorescence decay from treated (30 mU SMase, 100 nM Myr, 10 mM MCD and 100 μM C1) and untreated samples were used, comparing the results obtained in each case with the control. In all cases, except for Myr, it was found that the GP values decreased as a result of the treatment, which implies a shift of the spectrum towards the red direction. The shift towards the red region is clear evidence of a transition from the liquid ordered to the liquid disordered phase of the membrane, i.e. the substances alter the microenvironment of the membranes to a greater or lesser extent. For SMase, Myr and C1, there is an increase in τCoG values compared to untreated samples, characterised by a slowing of the spectral shift, which suggests that there is some increase in membrane microviscosity. In contrast, for MCD, a significant decrease in τCoG is seen, which clearly implies faster solvent relaxation, i.e. reduced microviscosity. In addition to solvent relaxation, we also investigated the mobility property of Laurdan molecules, the characteristic parameter of which is τrot, calculated using time-resolved anisotropy spectra. The variation of τrot values provides information about the rotational properties of Laurdan in the membrane, i.e., how much the molecule is confined in the membrane. For SMase, MCD and C1, the values decrease compared to the control, indicating faster rotation and thus less confinement of Laurdan molecules, but the opposite is true for Myr, where there is an increase, suggesting slower rotation and thus more confined Laurdan molecules in the membrane.

5.2. Examination of the effect of sphingolipid depletion on the activation of TRPV1 and TRPA1 ion channels *in vivo*

5.2.1. Effects of SMase and Myr on CAPS-induced acute chemonociceptive response

Pre-treatment with 50 mU SMase significantly reduced the number of CAPS-induced eye wipings in the first hour of the study compared to the control group pretreated with physiological saline. The 1 mM Myr pretreatment resulted in a significant reduction in CAPS-induced eye wipings in the DMSO pretreated group at both the first and second hours of the study. A single eye instillation of DMSO did not cause a desensitizing effect.

5.2.2. Effects of SMase and Myr on RTX-induced acute thermal allodynia and mechanical hyperalgesia

The RTX-induced thermal allodynia was significantly attenuated by 50 mU SMase pretreatment compared to physiological saline at 10, 20 and 30 min of the study. Furthermore, the developing mechanical hyperalgesia was significantly affected by SMase at 30 min of the study. Pretreatment with 1 mM Myr significantly inhibited the RTX-induced decrease in heat threshold at 10 and 20 min compared to the DMSO-pretreated group, but had no effect at 30 min and did not affect the developing mechanical hyperalgesia at either time point. A single application of DMSO did not cause a desensitising effect.

5.2.3. Effect of SMase and Myr on formalin-induced acute inflammatory pain

Pretreatment with 50 mU SMase did not affect the duration of phase I of formalin-induced acute inflammatory pain, but significantly reduced the duration of phase II compared to pretreatment with physiological saline. In contrast, 1 mM Myr pretreatment did not affect either phase duration compared to DMSO pretreatment. A single application of DMSO did not cause a desensitizing effect.

5.3. Examination of the effect of cholesterol depletion on the activation of TRPV1 and TRPA1 ion channels *in vivo*

5.3.1. Effects of MCD and C1 on CAPS-induced acute chemonociceptive response

Pretreatment with 15 mM MCD significantly reduced the number of CAPS-induced eye wipings in the first hour of the study compared to the control group pretreated with physiological saline. The 100 μM C1 pretreatment also showed a significant reduction in the number of eye wipings following CAPS instillation in the eye compared to the physiological saline pretreated group during the first and second hours of the study.

5.3.2. Effects of MCD and C1 on RTX-induced acute thermal allodynia and mechanical hyperalgesia

The 15 mM MCD pretreatment did not significantly affect the RTX-induced thermal allodynia nor the mechanical hyperalgesia compared to the physiological control group. As with MCD, 100 μM C1 had no effect on RTX-induced thermal threshold reduction, but significantly attenuated mechanical hyperalgesia at 60 and 90 min of the study. In this model, we investigated whether there was a dose-dependent effect of C1 on thermal allodynia and therefore also used a concentration of 500 μM. However, 500 μM C1 also had no effect on the change in thermal threshold, but significantly inhibited the development of mechanical hyperalgesia at all time points.

5.3.3. Effect of MCD and C1 on formalin-induced acute inflammatory pain

Pretreatment with 15 mM MCD did not affect the duration of phase I of formalin-induced acute inflammatory pain, but significantly reduced the duration of phase II compared to pretreatment with physiological saline. Similar to MCD, pretreatment with 100 μM C1 did not affect the duration of phase I but significantly reduced the duration of phase II.

6. DISCUSSION, CONCLUSIONS

The TRPV1 and TRPA1 ion channels are of major importance in many physiological and pathophysiological processes (16), but their role in neuropathic pain is one of the major importance for drug discovery. Due to their multimodal receptor properties, a large number of substances can affect their function, but the development of classical antagonist compounds has encountered many problems due to side effects (67). The only formulation used therapeutically for the treatment of neuropathy after shingles and in diabetic neuropathy is the 8% capsaicin patch (Qutenza®) (68). The mechanism of action of this formulation is based on the sustained desensitisation of the TRPV1 receptor (69). Although much is known about the function of ion channels, relatively little is known about their relationship with the surrounding cell membrane.

A new approach to the organization of membranes and the inhomogeneous location of some proteins is called lipid raft theory (36). Lipid rafts are defined microdomains that are rich in cholesterol, sphingolipids and gangliosides (36) and have been shown to form functional units with several ion channels, e.g. AMPA glutamate receptor, GABA receptor, nicotinic acetylcholine receptor, TRP ion channels including TRPV1 and TRPA1 receptors, which are the focus of my PhD thesis (41,42,51). The manipulation of lipid rafts has opened an alternative way in pharmacological studies to investigate both the microenvironment of membranes and the function of proteins located within them. An experimental approach to the environment and fluidity of membranes is the Laurdan fluorescent dye, whose excitation and emission spectra can be used to infer the current hydration level of the membrane, i.e. the ratio of liquid-ordered to liquid-disordered phases (56). In addition, the characteristic parameters of the decay curve of the time-resolved emission spectra - GP, CoG and τrot - can be used to characterize fluidity (58-60).

Our research group has previously demonstrated that cholesterol-depleting MCD and C1 alter cell membrane polarity (42,51). In our current studies, we investigated the effects of SMase, which hydrolyses sphingomyelins, and Myr, which inhibits *de novo* synthesis of sphingolipids, on membrane polarity changes. In the case of SMase, negligible changes were seen in the excitation and emission spectra of Laurdan, but in the case of Myr, significant changes were observed for both spectra over the full wavelength range. The spectral shift in the positive direction observed for the Myr treatment indicates that the Laurdan molecules were placed in a more confined environment, i.e. the membrane environment was significantly altered.

In the fluidity assay, the change in GP values for SMase, MCD and C1 showed a red shift, indicating that the membrane structure has changed in a more disordered direction. This finding is in agreement with membrane assays performed by other methods (48). For Myr, the GP change showed a different blue direction from the others, which, in agreement with the previous measurement, suggests that the cell membrane has moved into a more ordered phase. This observation is in contrast to recently reported results where the effects of Myr were investigated by two-photon microscopy (70). A significant decrease in Laurdan GP was observed after 5 min of 2.5 μM Myr treatment, but the authors note that other lipidomic analyses measured elevated glycerophospholipid levels that compensated to some extent for the effect of Myr (70,71). The difference in the results is presumably due to the difference in concentration (100 nM vs. 2.5 μM in our study), the duration of treatment (24 h vs. 5 min in our study) and the difference in method, since both measurements were performed using CHO cells, but two-photon microscopy selectively examines the plasma membrane, whereas fluorescence spectroscopy provides information on the whole membrane system of the cell.

From the rate of change of CoG values over time, we can infer the relaxation of the solvent surrounding the Laurdan, which is related to microviscosity. The decrease in MCD is a clear sign of faster solvent relaxation, indicating lower microviscosity. However, for SMase, Myr and C1, there was a smaller to larger increase in τCoG values as a result of treatment, indicating a slowing of the spectral shift, i.e. increased microviscosity. In the case of SMase, our observation may be explained by the fact that phosphocholine and ceramide formed during the hydrolysis of sphingomyelin slow down solvent relaxation processes by remaining in the membrane (72,73). In the case of Myr, the aforementioned glycerophospholipid formation processes may be involved (70,71), whereas in the case of C1, a structure similar to cholesterol may explain the slowing of solvent relaxation, as it may substitute cholesterol in the membrane due to some structural similarities, thus causing a slowing of the spectral shift.

For τrot, we found that SMase, MCD and C1 treatment resulted in reduced values compared to controls, indicating a less confined and therefore faster spinning Laurdan molecule. For Myr, we also obtained the opposite result here, indicating a more confined presence of Laurdan. The explanation for SMase could be the structural differences between sphingomyelins and phosphocholine and ceramide, for C1 the explanation is also structural, for MCD the inclusion of cholesterol in the inclusion complex is a clear explanation, while for Myr the formation of glycerophospholipids could explain the observations.

In addition to structural and integrity studies, the study of the pharmacological manipulation of lipid rafts and thus the activation of different receptors and ion channels, such as TRP receptors, is of great importance. Although many research groups are working on this topic, experiments are mostly performed *in vitro* and the results are often controversial. Our research group has described that SMase, Myr, MCD and C1 have inhibitory effects on TRPV1 and TRPA1 in *in vitro* TG cultures as well as on the CHO cell line stably expressing the receptors (41,42,51). In my thesis, I investigated this inhibitory effect *in vivo* in different acute pain mouse models. First, we described that degradation of sphingomyelin and inhibition of *de novo* synthesis of sphingolipids mediate TRPV1 and TRPA1 activation, respectively, and thus antinociceptive effects. SMase pretreatment reduced the number of eye wipings in CAPS-induced acute chemonociceptive response by 37% and Myr pretreatment by 41% in the first hour. Furthermore, Myr was also shown to be effective in the second hour (32% reduction), which may be due to its prolonged and complex mechanism of action (74). In our experiments, we also demonstrated that SMase pretreatment significantly reduced RTX-induced thermal allodynia, mainly based on peripheral mechanisms, and mechanical hyperalgesia involving both peripheral and central mechanisms (63). In contrast, Myr was only able to reduce the decrease in thermal threshold. In the case of formalin activating TRPA1, in the typical two-phase reaction (65), the duration of the first phase, which results from direct chemical stimulation of the receptors, was not affected by either substance, but the duration of the second phase, which is maintained by peripheral neurogenic inflammatory mechanisms, was shortened by 64 % by SMase compared to the control group, whereas Myr showed only a weak biological effect, probably due to the irritant effect of the solvent used, DMSO.

Our research group demonstrated that MCD pretreatment reduced the number of eye wipings to CAPS eye drops by 32% and shortened the pain response to formalin-induced TRPA1 activation in the second phase by 51% compared to the control group. In the RTX-induced complex mechanism pain model, MCD did not exert a significant inhibitory effect on the reduction of any parameter. MCD is a widely used compound in lipid raft research, but almost all studies have been conducted in *in vitro* systems (40,41). There is some animal evidence for the effects of MCD and other cyclodextrins (e.g. RAMEB) (52,54). Of particular relevance to my own research is the work of Lin et al. describing the effects of MCD in a mouse model of RTX-induced mononeuropathy (53). The authors point out that phosphatidylinositol-4,5-bisphosphate and prostate-specific acid phosphatase are also thought to play a role in TRPV1 inhibition (53).

Based on our previous *in vitro* results (42,50,51), we have demonstrated that C1 can also inhibit the activation of TRPV1 and TRPA1 receptors *in vivo*, resulting in an antinociceptive effect. C1 pretreatment reduces the number of CAPS-induced eye wipings by 45% in the first hour and 26% in the second hour compared to the control group. Although C1 was unable to affect RTX-induced thermal allodynia, it significantly inhibited the decrease in mechanical pain threshold. In the acute inflammatory pain response to formalin, which activates TRPA1, only the second phase duration was reduced by 36% compared to the control group, similar to the other agents. Several compounds with steroidal structures have been described to be able to affect the function of TRP ion channels. Dehydroepiandrosterone reduces CAPS-induced currents in primary sensory neurons (75), but the exact mechanism is not known, so the authors hypothesize that it may also affect lipid rafts. Pregnenolone sulphate, pregnanolone, pregnanolone sulphate, progesterone and dihydrotestosterone inhibited TRP canonical 5 (TRPC5) ion channel activation with a latency of 1-2 min, but 17-β-estradiol and dehydroepiandrosterone sulphate had weak inhibitory effects. From this study, it can be clearly concluded that stereoselectivity is of paramount importance for some steroidal compounds (76). Our research group has demonstrated that estradiol sensitizes TRPV1 through a tropomyosin receptor kinase A mediated pathway (77).

Our results provide evidence that both sphingolipids and cholesterol depletion from lipid rafts effectively inhibit TRPV1 and TRPA1 ion channels *in vivo* in animal studies. Although the exact mechanism of action is currently unknown, it is hypothesised that disruption of the integrity of lipid rafts is associated with a change in the position of the receptors in the membrane, which causes occlusion of certain binding sites thereby inhibiting the activation of ion channels. This hypothesis is supported by previous studies of our research group (41), which described that CAPS and RTX-induced activation can be differentially inhibited by disruption of lipid rafts in CHO cells expressing the TRPV1 receptor and in primary sensory neuron cell culture, respectively, which is also due to the positions of amino acids critical for binding sites and the level of receptor expression.

Another potential mechanism for C1 may be that its structure is similar to cholesterol in some elements, and cholesterol has been described to be able to directly inhibit TRPV1 CAPS, heat and voltage-induced currents through the cholesterol recognition amino-acid consensus motif (CRAC) (78).

7. SUMMARY OF NEW RESULTS

In my PhD work, I aimed to investigate the effects of lipid raft disruptor SMase, Myr, MCD and C1 on cell membrane parameters *in vitro* and to study the potential antinociceptive effect in acute pain models activating TRPV1 and TRPA1 in mice. In our experiments, we have described the following new results:

1. All four lipid raft disruptor agents alter membrane characteristic parameters - membrane polarity, GP, τCoG and τrot. MCD clearly decreased the values of all parameters, suggesting that membrane fluidity was increased. For SMase, Myr and C1, the change in values is more indicative of a decrease in membrane fluidity.
2. Depletion of sphingolipids with 50 mU SMase or 1 mM Myr has antinociceptive effects *in vivo* via inhibition of TRPV1 and TRPA1 activation.
3. Depletion of cholesterol with 15 mM MCD or 100 μM or 500 μM C1 inhibits the activation of TRPV1 and TRPA1 *in vivo*, thereby mediating analgesic effects.

Compound	CAPS-induced acute chemonociception	RTX-induced thermal allodynia	RTX-induced mechanical hyperalgesia	Formalin-induced acute inflammatory pain
50 mU SMase	1. hour ****	10. min. **** 20. min. **** 30. min. ***	30. min. ***	II. phase ****
1 mM Myr	1. hour *** 2. hour *	10. min. *** 20. min. **	ns	ns
15 mM MCD	1. hour *	ns	ns	II. phase ****
100 μM C1	1. hour **** 2. hour *	ns	60. min. ** 90. min. *	II. phase **
500 μM C1	ni	ns	30. min. **** 60. min. **** 90. min. *	ni

***Summary of the in vivo* results**. Ns: non-significant, ni: not investigated. Level of significance: *p<0,05; **p<0,01; ***p<0,001; ****p<0,0001

Our studies confirm the hypothesis that the failure to develop antagonists that affect TRP receptor function, particularly TRPV1, has led to the need to explore alternative approaches to affect ion channel function. Our research group considers the pharmacological investigation of hydrophobic interactions between lipid rafts and TRP receptors important, as they may contribute to the development of new drugs with new mechanisms of action, especially for topical application, as potential drug targets.

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9. PUBLICATION LIST

9.1. Publications related to the thesis

Horváth Ádám*, Biró-Sütő Tünde*, Kántás Boglárka, Payrits Maja, Skodáné-Földes Rita, Szánti-Pintér Eszter, Helyes Zsuzsanna, Szőke Éva. Antinociceptive effects of lipid raft disruptors, a novel carboxamido-steroid and methyl β-cyclodextrin, in mice by inhibiting Transient Receptor Potential Vanilloid 1 and Ankyrin 1 ion channel activation. Frontiers in Physiology. 11, 559109 (2020) DOI: 10.3389/fphys.2020.559109; *co-first author; IF: 4,14

Horváth Ádám, Payrits Maja, Steib Anita, Kántás Boglárka, Biró-Sütő Tünde, Erostyák János, Makkai Géza, Sághy Éva, Helyes Zsuzsanna, Szőke Éva. Analgesic effects of lipid raft disruption by sphingomyelainse and myriocin via Transient Receptor Potential Vanilloid 1 and Transient Receptor Potential Ankyrin 1 ion channel modulation. Frontiers in Pharmacology. 11, 593319 (2021) DOI: 10.3389/fphar.2020.593319; IF: 5,51

9.2. Other original publications

Kántás Boglárka, Börzsei Rita, Szőke Éva, Bánhegyi Péter, **Horváth Ádám**, Hunyady Ágnes, Borbély Éva, Hetényi Csaba, Pintér Erika, Helyes Zsuzsanna. Novel Drug Like Somastostatin Receptor 4 Agonists are Potential Analgesics for Neuropathic Pain. Internation Journal of Molecular Sciences. 20 (24): 6245 (2019). DOI: 10.3390/ijms20246245; IF: 4,56

Payrits Maja, **Horváth Ádám**, Biró-Sütő Tünde, Erostyák János, Makkai Géza, Sághy Éva, Pohóczky Krisztina, Kecskés Angéla, Kecskés Miklós, Szolcsányi János, Helyes Zsuzsanna, Szőke Éva. Resolvin D1 and D2 inhibit Transient Receptor Potential Vanilloid 1 and Ankyrin 1 ion Channel Activation on sensory neurons via lipid raft modification. International Journal of Molecular Sciences. 21 (14): 5019 (2020). DOI: 10.3390/ijms21145019; IF:5,54

Kántás Boglárka, Szőke Éva, Börzsei Rita, Bánhegyi Péter, Junaid Ashgar, Lina Hudhud, Steib Anita, Hunyady Ágnes, **Horváth Ádám**, Kecskés Angéla, Borbély Éva, Hetényi Csaba, Pethő Gábor, Pintér Erika, Helyes Zsuzsanna. In silico, in vitro and in vivo pharmacodynamic characterization of novel analgesic drug candidate somatostatin sst4 receptor agonists. Frontiers in Pharmacology. 11 601887 (2021) https://doi.org/10.3389/fphar.2020.601887; IF: 5,51

10. ACKNOWLEDGEMENTS

I would like to thank my thesis supervisor, Éva Szőke, who put her trust in me from the very first time we met, and who has helped me with her human and professional dedication and advice over the past four years. I am grateful to Erika Pintér, Head of the Doctoral School of Pharmaceutical Sciences and the Department of Pharmacology and Pharmacotherapy for supporting my work, and to Zsuzsanna Helyes, who supported my work with her professional knowledge, ideas and unconditional dedication to research. I would like to thank Rita Skoda Földes and her team for the synthesis of the carboxamido-steroid compound. Special thanks to János Erostyák for his help with fluorescence spectroscopy measurements, to my fellow PhD student Boglárka Kántás, with whom we worked closely during all our experiments, and to Tünde Biró-Sütő, Dóra Ömböli and Cecília Disztl, who contributed to the success of our experiments with their work as assistants. I am grateful to the Richter Gedeon Talentum Foundation for supporting my PhD studies. Furthermore, I would like to thank all the staff of the Department of Pharmacology and Pharmacotherapy for their support and good humour every day. Finally, I would like to thank my family and my partner for their support and I would like to dedicate my PhD thesis to the memory of my late grandmother.