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# Evidence for the role of lipid rafts and sphingomyelin in $Ca^{2+}$-gating of Transient Receptor Potential channels in trigeminal sensory neurons and peripheral nerve terminals

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ABSTRACT

Transient Receptor Potential (TRP) cation channels, such as TRP Vanilloid 1 and TRP Ankyrin repeat domain 1 (TRPV1 and TRPA1) are nocisensors playing important role to signal pain. Two "melastatin" TRP receptors, like TRPM8 and TRPM3 are also expressed in a subgroup of primary sensory neurons. These channels serve as thermosensors with unique thermal sensitivity ranges and are activated also by several exogenous and endogenous chemical ligands inducing conformational changes from various allosteric ("multisteric") sites. We analysed the role of plasma membrane microdomains of lipid rafts on isolated trigeminal (TRG) neurons and TRPV1-expressing CHO cell line by measuring agonist-induced $Ca^{2+}$ transients with ratiometric technique. Stimulation-evoked calcitonin gene related peptide (CGRP) release from sensory nerve endings of the isolated rat trachea by radioimmunoassay was also measured. Lipid rafts were disrupted by cleaving sphingomyelin (SM) with sphingomyelinase (SMase), cholesterol depletion with methyl β-cyclodextrin (MCD) and ganglioside breakdown with myriocin. It has been revealed that intracellular $Ca^{2+}$ increase responses evoked by the TRPV1 agonist capsaicin, the TRPA1 agonsits allyl isothiocyanate (AITC) and formaldehyde as well as the TRPM8 activator icilin were inhibited after SMase, MCD and myriocin incubation but the response to the TRPM3 agonist pregnenolon sulphate was not altered. Extracellular SMase treatment did not influence the thapsigargin-evoked $Ca^{2+}$-release from intracellular stores. Besides the cell bodies, SMase also inhibited capsaicin- or AITC-evoked CGRP release from peripheral sensory nerve terminals, this provides the first evidence for the importance of lipid raft integrity in TRPV1 and TRPA1 gating on capsaicin-sensitive nerve terminals. SM metabolites, ceramide and sphingosine, did not influence TRPA1 and TRPV1 activation on TRG neurons, TRPV1-expressing CHO cell line, and nerve terminals. We suggest, that the hydrophobic interactions between TRP receptors and membrane lipid raft interfaces modulate the opening properties of these channels and therefore, targeting this interaction might be a promising tool for drug developmental purposes.



*Abbreviations:* AITC, allyl isothiocyanate; $[Ca^{2+}]_i$, intracellular free calcium concentration; CGRP, calcitonin gene-related peptide; D-MEM, Dulbecco's- Modified Eagle Medium; DMSO, dimethyl sulfoxide; ECS, extracellular solution; GP, generalized polarization; laurdan, 6-dodecanoyl-*N*,*N*-dimethyl-naphthylamine; MCD, methyl β-cyclodextrin; NGF, nerve growth factor; PBS, phosphate-buffered saline; PS, pregnenolone sulphate; SM, sphingomyelin; SMase, sphingomyelinase; TM, transmembrane; TRG, trigeminal ganglion; TRPV1, Transient Receptor Potential vanilloid 1; TRPA1, Transient Receptor Potential Ankyrin repeat domain 1; TRPM8, Transient Receptor Potential melastatin 8 (TRPM8); TRPM3, Transient Receptor Potential melastatin 3 (TRPM3).

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## 1. Introduction

The members of the Transient Receptor Potential (TRP) superfamily composed of six subfamilies are nonselective cation channels that open to ligand binding, changes in temperature and other alterations of the channel protein [1,2]. Alterations in TRP channel functions cause several diseases, such as inherited pain syndrome, multiple kidney diseases and skeletal muscle disorders [3,4]. Despite the great effort to find potential drug targets, there are only some candidates acting at few TRP channels (Vanilloid 1: TRPV1, Ankyrin repeat domain 1: TRPA1, Melastatin: TRPM8 and Vanilloid 3: TRPV3) that reached the clinical stages of drug development [4–6].

TRPV1 is a nocisensor plasma membrane protein expressed in the large population of polymodal-type nociceptors gated by painful chemical agents, noxious heat (>43 °C) and protons (pH < 6.0) [7–11]. Besides the two classical vanilloid-type agonists capsaicin and resiniferatoxin (RTX), TRPV1 is also gated by several highly lipophylic compounds, such as endogenous arachidonic acid or other fatty acid metabolites [12–16]. The TRPA1 receptor present in the TRPV1-expressing subset of sensory neurons was identified as a cold-gated [17] and mechanical transduction channel [18,19]. A variety of exogenous and endogenous ligands, such as mustard oil (allyil isothiocianate: AITC), formaldehyde, 4-hydroxynonenal, allicin and [20–26] can activate the TRPA1 receptor. Several studies suggest that TRPV1 and TRPA1 play an integrative role in regulating nociceptor function [27,28]. Pro-inflammatory neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP) released from capsaicin-sensitive sensory nerves in response to the activation of TRPA1 and TRPV1 induce neurogenic inflammation in the innervated area [29–31]. Both channels are sensitized by several inflammatory mediators, e.g., bradykinin and prostaglandins, therefore, they play an important role in inflammatory processes and related pain.

TRPM8 is activated by temperatures below 26 °C and chemical agonists, such as menthol and the supercooling agent icilin [32–35]. TRPM8 has 6 transmembrane (TM) domains with cytoplasmic C and N-termini but unlike TRPC and TRPV channels, it does not contain any ankyrin-repeat domains in the N-terminal cytoplasmic region. Residues in the TM2 region and the TRP domain are involved in sensitivity to menthol and icilin, and specific structural elements in the TM2-TM3 linker is needed for icilin activation [36,37].

TRPM3 is a member of the melastatin subfamily of TRP channels which is expressed in a variety of neuronal and non-neuronal tissues [38–40]. The neurosteroid pregnenolone sulphate (PS) derived from cholesterol is its most potent activator [41]. The presence of the sulphate group and its stereochemical orientation is important for TRPM3 activation [42]. PS is the precursor for a wide range of steroid hormones and its role has been described in acute heat sensing and inflammatory heat hyperalgesia [43]. Other steroids, such as epipregnenalone sulphate, dihydro-D-erythro-sphingosine and *N*,*N*-dimethyl-D-erythro-sphingosine also activate this ion channel. It is suggested that a specific steroid binding site is located in TRPM3 [42,44,45] and a new permeation pathway was proposed for TRPM3 [46].

Previous discoveries on TRP channels described important properties of these proteins, but little is known about the potential function of the lipid rafts surrounding them in the plasma membrane. Lipid rafts are microdomains rich in cholesterol but sphingomyelin (SM) and gangliosides are also important constituens of these structures around some membrane receptors, like the nicotinic acetylcholine channel [47–51]. Nevertheless, clarification of their role in the function of TRP channels resulted in controversial observations. Depletion of cholesterol with methyl β-cyclodextrin (MCD) incubation resulted in impaired TRPC1 and TRPC3 signalling processes [52–54]. Meanwhile, it had no effect on heat-activation currents of TRPV1-transfected Xenopus laevis oocytes [55] but significantly reduced the amplitude of capsaicin-activated currents in dorsal root ganglion neurons [56]. Others reported previously that $^{3}$[H]RTX binding to TRPV1 receptors was not influenced by cholesterol depletion in rat C6 glioma cells [57]. The involvement of lipid rafts in TRPM8 receptor activation was also supported by MCD treatment, it shifted the threshold for TRPM8 activation to a warmer temperature [58]. Our previous results suggested that disrupting lipid rafts by pharmacologically depleting their various constituents, such as SM, cholesterol or gangliosides inhibited the capsaicin- and RTX-induced opening properties of TRPV1 both on native sensory neurons and a TRPV1-transfected cell line [59].

Sphingomyelinase (SMase) decreases membrane SM by hydrolizing SM to phosphocholine and ceramide [60,61]. Like all sphingolipids, SM has a ceramide frame, which is composed of sphingosine and a fatty acid via an amide linkage. It contains one polar head group, which is either phosphoethanolamine or phosphocholine. The lipid bilayer in rafts is asymmetric, the exoplasmic leaflet is rich in SM [62]. During SMase treatment three kinetic processes were described: (1) release of SM by the dissolution of SM-enriched domains within the raft membrane, (2) diffusion of SM from the dissolution sites to the reaction site, and (3) after an enzymatic reaction, consumption of SM by the enzymatic reaction at this reaction site. This change in cell membrane influences the signal transduction through the cell membrane [63]. Besides MCD and SMase, the third mechanism to disrupt the lipid rafts, is to inhibit the glycosphingolipid synthesis by myriocin treatment [64].

The aim of the present study was to investigate whether lipid raft disruption alters the chemical activation of four thermosensor TRP channels in cultured trigeminal neurons. Furthermore, in case of the two nocisensor channels of TRPV1 and TRPA1 the effect of SMase and SM metabolites on peripheral nerve terminals was also investigated by measuring agonist-induced release of CGRP.

## 2. Materials and methods

### 2.1. Primary cultures of trigeminal ganglion (TRG) neurons

Cell cultures were made from the trigeminal ganglia of 1–4 days old Wistar rat pups. We cut the ganglia and placed into ice cold phosphate-buffered saline (PBS), incubated for 35 min at 37 °C in PBS containing collagenase (Type XI, 1 mg/mL) and then in PBS with deoxyribonuclease I (1000 units/mL) for 8 min. The ganglia were then rinsed with PBS and dissociated by trituration. Cell cultures were plated on poly-D-lysin-coated glass coverslips and grown in a nutrient-supplemented medium that contained 180 mL Dulbecco's-Modified Eagle Medium (D-MEM), 20 mL horse serum, 20 mL bovine albumin, 2 mL insulin-transferrin-selenium-S, 3,2 mL putrescin dihydrocloride (100 μg/mL), 20 μL triiodo-thyronine (0.2 mg/mL), 1,24 mL progesterone (0.5 mg/mL), 100 μL penicillin, 100 μL streptomycin. Cultures were maintained at 37 °C in a humidified atmosphere with 5% $CO_2$, and nerve growth factor (NGF, 200 ng/mL) was added every second day, as described earlier [65].

### 2.2. Ratiometric technique of intracellular free calcium concentration ($[Ca^{2+}]_i$) measurement with the fluorescent indicator fura-2 AM

One-three days old cell cultures were stained for 30 min at 37 °C with 1 μM of fluorescent $Ca^{2+}$ indicator dye, fura-2AM (Molecular Probes) in a solution containing (in mM): NaCl, 122; KCl, 3.3; $CaCl_2$, 1.3; $MgSO_4$, 0.4; $KH_2PO_4$ 1.2; HEPES, 25; glucose, 10; (pH 7.3). Dye loading was followed by at least 5 min washing in