COMPLEX REGULATION OF TRPM8 COLD RECEPTOR-CHANNEL: ROLE OF ARACHIDONIC ACID RELEASE FOLLOWING M3 MUSCARINIC RECEPTOR STIMULATION

Alexis Bavencoffe1,4, Artem Kondratskyi2,4, Dimitra Gkika1, Brigitte Mauroy1,3, Yaroslav Shuba2, Natalia Prevarskaya1,§ and Roman Skryma1,§

From 1 Inserm U1003, Equipe Labellisée par la Ligue Nationale Contre le Cancer, Université des Sciences et Technologies de Lille (USTL), F59656 Villeneuve d’Ascq, France; 2 Bogomoletz Institute of Physiology and International Center of Molecular Physiology of the National Academy of Sciences of Ukraine, Kyiv, Ukraine; 3 Université Catholique de Lille, France;

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4 These authors contributed equally to this work
§ Share senior authorship

Address correspondence to: Natalia Prevarskaya, Inserm U1003, USTL, F59656 Villeneuve d’Ascq, France. Tel.: (33) 3 20 33 60 18, fax: (33) 3 20 43 40 66; E-mail: natacha.prevarskaya@univ-lille1.fr and Roman Skryma, Inserm U1003, USTL, F59656 Villeneuve d’Ascq, France. Tel.: (33) 3 20 33 60 18, fax: (33) 3 20 43 40 66; E-mail: roman.skryma@univ-lille1.fr

Cold/menthol-activated transient receptor potential channel melastatin member 8 (TRPM8) is primarily expressed in sensory neurons, where it constitutes the principal receptor of environmental innocuous cold. TRPM8 has been shown to be regulated by multiple influences such as phosphorylation, pH, Ca2+, and lipid messengers. One of such messengers is arachidonic acid (AA), which has been shown to inhibit TRPM8 channel activity. However, the physiological pathways mediating AA’s inhibitory effect on TRPM8 remain still unknown. Here we demonstrate that TRPM8 is regulated via M3 muscarinic acetylcholine receptor-coupled signaling cascade based on the activation of cytosolic phospholipase A2 (cPLA2) and cPLA2-catalyzed derivation of AA. Stimulation of M3 receptors heterologously co-expressed with TRPM8 in HEK-293 cells by non-selective muscarinic agonist, oxotremorine methiodide (Oxo-M), caused inhibition of TRPM8-mediated membrane current, which could be mimicked by AA and antagonized by pharmacological or siRNA-mediated cPLA2 silencing. Our results demonstrate the intracellular functional link between M3 receptor and TRPM8 channel via cPLA2/AA, and suggest a novel physiological mechanism of arachidonate-mediated regulation of TRPM8 channel activity through muscarinic receptors. We also summarize the existing TRPM8 regulations and discuss their physiological and pathological significance.

The members of Transient Receptor Potential (TRP) superfamily of cationic channels display extraordinary diverse activation mechanisms and participate in the plethora of physiological and pathological processes (1), which made them the focus of intense research over the last decades. A number of TRPs, dubbed thermo-TRPs, from TRPV (Vanilloid), TRPM (Melastatin) and TRPA (Ankyrin) subfamilies can be activated by various ambient temperatures ranging from noxious cold to noxious heat. They also respond to the chemical imitators of temperatures of various modalities and to the number of chemical and environmental irritants (2). Among them TRPM8, which is activated by innocuous cold (<25° C) (3) and a well-known cooling agent, peppermint oil component, menthol, represents a cold receptor. Except for the innocuous cold and menthol, TRPM8 can also be activated by some other cooling agents such as icilin and eucalyptol as well as by non-cooling compounds hydroxy-citronellal, geraniol and linalool (4). It has been shown that the mechanism of TRPM8 activation by cold and menthol involves negative shift in the channel’s voltage-dependent opening from very positive unphysiological membrane potentials toward physiological values (5,6).

Despite being proven as the principal detector of environmental cold (7-9), TRPM8 expression is by far not limited to the subset of cold-sensitive dorsal root ganglion (DRG) and trigeminal sensory neurons in which it functions as cold-activated receptor-channel. In fact, sizable TRPM8 expression was found in prostate (10), in sperm (11) some epithelial (12,13) and smooth muscle (14) cells as well as in cancer tissues (10). Consistent with its quite broad expression, TRPM8 function appears to be
regulated not only by cooling temperatures and exogenous chemical imitators of cold, but also by a number of second messengers, which are generated during activation of surface receptor-coupled signaling pathways. Among them is the substrate of phospholipase C (PLC) catalytic activity, phosphatidylinositol bisphosphate (PIP2) (15,16), the products of catalytic activity of Ca\(^{2+}\)-independent subtype of phospholipase A2 (iPLA2), lysophospholipids (LPL) (17,18), the factors affecting cAMP/protein kinase A (PKA)-dependent phosphorylation (19,20) as well as intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) acting either via Ca\(^{2+}\)-sensitive PLC\(\delta\) (16) or protein kinase C (PKC) (21,22). It was also shown that TRPM8 can be directly inhibited by polyunsaturated fatty acids (PUFA) including the important lipid second messenger, arachidonic acid (AA) (17).

AA is generated for signaling purposes by the action of PLA2 subtypes including less substrate-specific iPLA2, which is classified as type VI PLA2, and phosphatidylycholine-specific cytosolic, Ca\(^{2+}\)-dependent cPLA2, known as type IV PLA2 (23,24). These phospholipases are differentially regulated in cells and mediate AA release in response to stimulation of various surface receptors. Despite AA being well established as a TRPM8 inhibitor, so far the surface receptors that utilize AA as second messenger to target TRPM8 as well as the type of PLA2 involved in AA derivation for this purpose remain unknown.

One way of agonist-induced derivation of AA was previously shown to be associated with the stimulation of M3-type muscarinic acetylcholine receptors (mAChR) (25). M3 receptors are most commonly linked via Gq protein to the PLC-catalyzed inositol phospholipids breakdown pathway that generates two important messengers, diacylglycerol (26), regulating protein kinase C (PKC)-dependent phosphorylation as well as some TRP channels directly, and endoplasmic reticulum (ER) Ca\(^{2+}\) store-mobilizing agent, inositol trisphosphate (IP\(_3\)), providing for the increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) (27). However, M3 receptors stimulation can also take an alternative path dubbed “non-classical” consisting in Gq- and Ca\(^{2+}\)-independent activation of the cPLA2 via not as yet fully understood mechanism and production of AA (25,28). In particular, such mechanism was implicated in the M3 receptor-stimulated activation of the store-independent, arachidonate-regulated Ca\(^{2+}\) (ARC) entry channels (25,28).

In this paper we studied how stimulation of M3 receptors would influence TRPM8 function and what principal signaling pathway(s) would be involved. Our results show that application of non-selective muscarinic agonist, oxotremorine methiodide (Oxo-M), produces inhibition of TRPM8 heterologously expressed in HEK-293 cells and that this effect results from stimulation of cPLA2 and cPLA2-catalyzed derivation of AA, which directly inhibits TRPM8 channel. Thus, in this work we propose a novel physiological mechanism regulating TRPM8 channel activity through muscarinic receptors. Furthermore, by summarizing ours and others data we compile a general scheme of TRPM8 regulation by the cohort of intracellular stimuli.

**EXPERIMENTAL PROCEDURES**

**Cells and electrophysiology** — HEK-293 cells stably transfected with the human M3 muscarinic receptor (HEK\(_{\text{M3}}\)) (kind gift from T. J. Shuttleworth (Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, USA)) (29) were cultured as described previously (30) and transiently transfected with human TRPM8 encoding vector using Nucleofector as recommended by the manufacturer (Amaxa).

PC-3 (Prostate Carcinoma; ATCC) cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% FCS, L-glutamine (5 mM), and kanamycin (100 \(\mu\)g/ml). PC-3 cells were transfected with human TRPM8 encoding vector using Nucleofector as recommended by the manufacturer (Amaxa).

The composition of the normal extracellular solution used for electrophysiological recordings was (in mM): 140 NaCl, 5 KCl, 2 CaCl\(_2\), 1 MgCl\(_2\), 0.3 Na\(_2\)HPO\(_4\), 0.4 KH\(_2\)PO\(_4\), 4 NaHCO\(_3\), 5 glucose, 10 HEPES (pH adjusted to 7.4 with NaOH). Patch-clamp pipettes were filled with an intracellular solution containing (in mM): 140 KCl, 1 MgCl\(_2\), 2.5 CaCl\(_2\), 4 EGTA, 10 HEPES (calculated free Ca\(^{2+}\) concentration: 150 nM, pH adjusted to 7.2 with KOH).

Whole-cell patch-clamp experiments were performed using Axopatch 200B amplifier and pClamp 9.0 software (Molecular Devices, Union City, CA) for data acquisition and analysis. Patch pipettes for the whole-cell recordings were fabricated from borosilicate glass capillaries (World Precision Instr., Inc., Sarasota, FL) on horizontal puller (Sutter Instruments Co.,...
Novato, CA) and had resistance of 3–5 MΩ when filled with intracellular solutions.

In the course of patch-clamp recording drugs and solutions were applied to the cells using temperature-controlled microperfusion system (Cell MicroControls, Norfolk, VA) with common outflow of the multiple solution lines, which was placed in the close proximity (~200 μm) to the studied cell. Membrane currents through TRPM8 channels (I_{TRPM8}) were activated by temperature drop from 33º C to 20º C (cold), icilin (10 μM) or menthol (500 μM) and monitored by applying every 3 s voltage-clamp pulses that consisted of initial 200 ms depolarization to +100 mV enabling full current activation followed by descending ramp (rate 0.4 mV/ms) to -100 mV. The ramp portion of the current served to construct I-V relationship.

**Ca²⁺ measurements** – [Ca²⁺]i was measured using the ratiometric dye Fura-2, as previously described (30,31). During measurements, the cells were bathed in the same normal extracellular solution used for electrophysiological recording. To produce Ca²⁺-free conditions, CaCl₂ was removed from this solution, and EGTA (0.1 mM) was added.

**Plasmid and siRNA transfection** – HEK_{M3} cells were co-transfected with 2 μg of TRPM8 construct and 0.4 μg of pmax GFP or 50 nM of siRNA against cPLA2 (against the sequence 5’aatctagggacagcaacatt 3’, (32)) using a Nucleofector™ (Amaxa, Gaithersburg, Maryland, USA). Control experiments were performed by transfecting the empty vector or siRNA against Luciferase (siLuc) (supplemental Fig. S1B).

**Drugs and chemicals** – All chemicals were purchased from Sigma Aldrich except for icilin which was from Tocris. The final concentration of ethanol and DMSO in the experimental solution did not exceed 0.1%.

**Data analysis** – Data were analyzed with Clampfit 9.0 and Origin 5.0 (Microcal Software Inc., Northampton, MA). Data are expressed as mean±s.e.m. Overall statistical significance was determined by analysis of variance (ANOVA). In case of significance, differences between the means of two groups were analyzed by unpaired t-test, while multiple comparisons between groups were performed by ANOVA tests followed by Dunnett tests unless otherwise indicated. P<0.05 was considered significant. The statistical analyses were performed using the InStat v3.06 (GraphPad Software, Inc., San Diego, CA).

### RESULTS

Consistent with the presence of Gq-coupled M3 receptor, exposure of HEK_{TRPM8/M3} cells to the non-specific muscarinic agonist, oxotremorine (Oxo-M, 10 μM), caused an increase in [Ca²⁺], which could be inhibited by pre-application before the Oxo-M of the muscarinic receptor antagonist, atropine (1 μM, Fig. 1A). Moreover, pretreatment of HEK_{TRPM8/M3} cells with 10 μM Oxo-M for 10-20 min also resulted in an approximately three-fold decrease of I_{TRPM8} density activated by menthol (500 μM), which could be prevented by atropine (1 μM, Fig. 1B), suggesting that it is a direct consequence of muscarinic receptor stimulation.

The inhibitory effect of Oxo-M on I_{TRPM8} was not dependent on the TRPM8 channel activating stimuli, as a similar extent of inhibition was detected for the currents evoked by the three major TRPM8 agonists: menthol (500 μM), cold (temperature drop from 33º C to 20º C) and icilin (10 μM) (Fig. 1C).

To test whether PLC is involved in the signal transduction from muscarinic receptors to TRPM8 channel, we used the PLC inhibitor, U73122. As shown in Fig. 2A, pretreatment of HEK_{TRPM8/M3} cells with U73122 (1 μM) for 5 min did not influence the inhibitory action of Oxo-M (10 μM) on I_{TRPM8}, activated by cold, icilin or menthol, indicating that G₀-stimulated PLC catalytic activity and the concomitant reduction of PIP₂ levels is not involved in the M3 receptor-mediated inhibition of TRPM8 channel. Since activation of Gq/PLC pathway also brings about the ER Ca²⁺ store depletion followed by iPLA2-catalyzed generation of LPLs and AA (33,34), which were shown to influence TRPM8 channel gating (17,18), experiments with U73122 also exclude such a scenario in the M3 receptor-mediated modulation of TRPM8 channels.

It has been previously demonstrated that stimulation of M3 receptors, overexpressed in HEK-293 cells, leads to the activation of cPLA2 and increase in AA production (25,28). Moreover, polyunsaturated fatty acids, such as AA, were reported to inhibit the TRPM8 activity (17). To determine whether cPLA2/AA pathway is involved in the Oxo-M-induced suppression of TRPM8 activity we first conducted experiments to confirm the inhibitory action of AA per se on I_{TRPM8} in our experimental model. Fig. 2B shows that consistent with previous studies (17), pre-incubation of HEK_{TRPM8/M3} cells with the AA (10
µM, 20 min) indeed led to the decrease of menthol-activated I_{TRPM8} density by 55±8% at +100 mV. Because cPLA2 directly cleaves AA from phospholipids, we next examined whether inhibiting cPLA2 activity minimizes the effect of Oxo-M on I_{TRPM8}. Inclusion of cPLA2 inhibitor, arachidonyl trifluoromethyl ketone (AACOCF3) (50 µM) in the intracellular pipette solution totally abrogated inhibitory effect of Oxo-M on menthol- and icilin-activated I_{TRPM8} (Fig. 2C). The same result was also attained with non-pharmacological, siRNA-mediated, cPLA2 knockdown: two days after transfection of HEKTRPM8/M3 cells with anti-cPLA2 siRNA the inhibitory effect of Oxo-M on the I_{TRPM8} was completely eliminated, whilst in HEKTRPM8/M3 cells transfected with control siLuc it remained unchanged (Fig. 2D).

Thus, the Oxo-M-induced inhibition of TRPM8 is primarily mediated by the cPLA2/AA pathway, which is activated in response to muscarinic receptors stimulation. However, since suppression of PLC by U73122 did not influence Oxo-M-induced inhibition of TRPM8, the cPLA2-catalyzed generation of AA is not a downstream event of PLC activation, but most likely occurs in Ca2+-independent manner.

Following the establishment of the functional coupling between M3 receptors and TRPM8 in HEKTRPM8/M3 cells, we next tested whether the muscarinic receptor agonist, oxotremorine-M, can modulate TRPM8-mediated membrane current (I_{TRPM8}) in prostate carcinoma PC3 cell line, expressing endogenous M3 receptors. As PC-3 cells express very low levels of endogenous TRPM8 mRNAs (31) we transfected them with human TRPM8. As expected, pretreatment of PC3TRPM8 cells with 10 µM Oxo-M for 10-20 min caused a decrease in I_{TRPM8} activated by cold (temperature drop from 33º C to 20º C), icilin (10 µM) or menthol (500 µM) (Fig. 3 A, B).

DISCUSSION

In this study, we established a functional link between M3 muscarinic receptors and TRPM8 channels. Our results demonstrate, that stimulation of M3 muscarinic receptors with the nonspecific agonist oxotremorine-M causes suppression of TRPM8 via a signaling pathway that involves Gq- and Ca2+-independent stimulation of cPLA2 and the generation of AA, which in turn acts as a direct channel inhibitor (Fig. 4, panel 5). In the event of M3 receptors mediated TRPM8 inhibition, the involvement of the Gq/PLC pathway would be most anticipated, as the substrate of PLC activity, PIP2, is a well known co-factor in TRPM8 activation (15,16), whose depletion during PLC activity causes I_{TRPM8} suppression (35). However, our experiments with PLC inhibitor, U73122, did not support the notion on Gq/PLC involvement, as this compound failed to impair in any essential way the inhibitory effects of Oxo-M on TRPM8 in HEKTRPM8/M3 cells. Although LPLs which are produced during IP3-dependent Ca2+ store depletion and concomitant stimulation of iPLA2 are known as positive TRPM8 modulators (17,18,36), experiments with U73122 also provided additional strong evidence against their role in the Oxo-M effect observed.

In our hands the inhibitory effect of Oxo-M on TRPM8 could be mimicked by AA and eliminated by pharmacological or siRNA-mediated suppression of the cPLA2. The possibility of the M3 receptor-activated generation of AA in HEK-293 cells has been previously documented (25,28), although this mechanism still remains poorly understood. The type IV cPLA2 is Ca2+-dependent enzyme, whose “classical” surface receptor-stimulated activation involves two distinct steps. First, a Ca2+-dependent translocation to the membrane that enables interaction with its phospholipid substrate and second a phosphorylation-induced enhancement of activity that is usually mediated via MAPK and/or PKC (23). However, the experiments with PLC blocker, U73122, which failed to impair the effects of Oxo-M on TRPM8, indicated that neither an increase in [Ca2+]i nor the activation of PKC was required for the observed Oxo-M-induced, AA-mediated TRPM8 inhibition. Thus, our data are consistent with the “non-classical”, [Ca2+]i-independent cPLA2 activation mechanism, which was proposed to explain muscarinic receptor-stimulated activation of the store-independent, arachidonate-regulated Ca2+ entry channels (ARC) (25,28). This mechanism postulates the existence of a discrete pool of the total cellular cPLA2 that is already localized within the plasma membrane at resting levels of [Ca2+], and is more directly coupled to the receptor, whose stimulation by low concentrations of muscarinic agonists involves an increase in the phosphorylation state of the enzyme by yet unknown mechanism.

Thus, it seems that despite the existence of several receptor-coupled signaling systems in the cell, only certain ones can establish preferred
functional link with TRPM8 channel under given conditions. We believe that this is possible only if the TRPM8 channel and signaling complexes that regulate its function are organized in spatial structural microdomains. In the case of muscarinic receptors this complex must include at least the M3 receptor subtype, the pool of the PM-localized cPLA2 and the TRPM8 channel.

Our present data together with the previously obtained ones indicate that TRPM8 cold-receptor is the subject of complex regulation via multiple surface receptor-coupled signaling systems (Fig. 4) resulting in the fine-tuning of TRPM8-mediated cold sensitivity under various conditions. The requirement of the PLC substrate, PIP2, for sustaining TRPM8 function indicates that any receptor causing a PIP2 depletion may cause both the inactivation and the desensitization of TRPM8 (Fig. 4, panel 1). A negative impact on channel function and cold perception can also be achieved via the receptor-mediated recruitment of Gi/AC/cAMP/PKA or cPLA2/AA pathway, as a result of α2-adrenergic and M3 muscarinic receptors stimulation, respectively (Fig. 4, panels 3 and 5). Downregulation of TRPM8 by α2-adrenoreceptor-coupled Gi/AC/cAMP/PKA cascade may underlie the analgesic effect of α2-AR agonists in TRPM8 overexpression-evoked cold allodynia (37). Ca2+-induced PKC-dependent phosphorylation has been shown to be indirectly involved in Ca2+-mediated TRPM8 desensitization (21,22). The latter was also attributed to the depletion of positive TRPM8 modulator, PIP2, by the activity of Ca2+-sensitive PLCs (16). In addition, extracellular Ca2+ as well as protons are able to inhibit TRPM8 by altering voltage-dependency of its activation via membrane surface charge screening (38).

On the contrary, positive regulation of TRPM8 channel activity is accomplished by the main products of iPLA2 catalytic activity, LPLs, regarded as endogenous agonists of the channel inducing cold hypersensitivity (36). Interestingly, LPLs activate the channel in the tissues not exposed to the environmental cold (18) (Fig. 4, panel 2). Catalytic activity of iPLA2 and consequently LPLs production can be stimulated by the ER Ca2+-store depletion (33,34), which potentially provides for the possibility of TRPM8 activation in the internal tissues via Gq-coupled surface receptors stimulating PLC-catalyzed derivation of store-mobilizing IP3. The resultant effect on TRPM8 function in such cases will apparently depend on the balance of the contributions of decreased PIP2 and increased LPL levels.

Positive modulation of TRPM8 can also be achieved via the Gs/AC/cAMP/PKA phosphorylation pathway, which can be recruited in response to β-adrenergic receptors stimulation (19) (Fig. 4, panel 3). Activation of AC, accumulation of cAMP and enhancement of PKA-dependent phosphorylation may also result from the recently discovered process of store-operated cAMP signaling (SOcAMPS) (39), which may, thus, operate via the receptors coupled to the IP3-mediated mobilization of Ca2+. However, these mechanisms would be able to enhance TRPM8 activation only if its basal cAMP/PKA-dependent phosphorylation is compromised. All modulatory influences primarily work by shifting voltage-dependence of TRPM8 activation either towards or opposite most physiological membrane potentials.

Interestingly, TRPM8 activity can also be increased by the Prostate Specific Antigen (PSA), which was recently identified as a physiological TRPM8 agonist. PSA activates TRPM8-mediated current via the bradykinin 2 receptor (B2R) signaling and induces the accumulation of functional channels in the plasma membrane (Fig. 4, panel 4). PSA/B2R-mediated regulation of TRPM8 required PKC activity and resulted in a slower prostate cancer cell migration (40). Since migratory behavior is an important target of anti-cancer agent development and serves as an indicator of malignancy, TRPM8 regulation by PSA is of interest in exploring new potential alternative treatments.

In conclusion, the above mentioned mechanisms of TRPM8 regulation may be important for modulating normal and pathologic cold sensitivity as well as regulation of such processes, as cancer cells migration (40-42) and viability (43), regulation of vascular tone (14), respiration (13), thermotaxis or chemotaxis (11) and bladder function (44).
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The abbreviation used are: AC, adenylate cyclase; AA, arachidonic acid; B2R, bradykinin 2 receptor; [Ca^{2+}]-intracellular Ca^{2+} concentration; cPLA2, Ca^{2+}-dependent phospholipase A2; DRG, dorsal root ganglion; GPCR, G protein-coupled receptor; I_{TRPM8}, current through TRPM8 channel; LPL, lysophospholipid; mAChR, muscarinic acetylcholine receptor; Oxo-M, oxotremorine methiodide; PSA, Prostate Specific Antigen; PIP2, phosphatidylinositol bisphosphate; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PM, plasma membrane; TRP, Transient Receptor Potential; TRPM8, TRP melastatin 8.

**FIGURE LEGENDS**

![Fig. 1. Agonist-induced stimulation of muscarinic receptors causes inhibition of TRPM8. A: Effect of Oxo-M (10 µM) on [Ca^{2+}]_i in HEK_{TRPM8/M3} cells in Ca^{2+}-free EGTA-containing extracellular medium. Incubation with atropine (1 µM) antagonized responses to Oxo-M, confirming that responses were mediated by activation of muscarinic receptors (mean±S.E., n=25-50). B: Averaged I-V relationships (mean±S.E., n=5) of menthol (500 µM)-activated I_{TRPM8} density under control conditions (black symbols), in the presence of Oxo-M (10 µM, open circles) alone and Oxo-M plus atropine (1 µM, grey triangles); inset shows quantification of the respective I_{TRPM8} densities at +100 mV. C: Quantification of I_{TRPM8} density (at +100 mV) activated by cold, icilin (10 µM) and menthol (500 µM) in HEK_{TRPM8/M3} cells under control conditions (black columns) and in the presence of Oxo-M (10 µM, white columns) (mean±S.E., n=6). On all graphs (**) denotes statistically significant differences to control with p < 0.02.](attachment:fig1.png)

![Fig. 2. Functional link between muscarinic receptors and TRPM8 is mediated via cPLA2/AA pathway. A: Quantification of I_{TRPM8} density (at +100 mV) activated by cold, icilin and menthol in HEK_{TRPM8/M3} cells under control conditions (black columns), in the presence of Oxo-M (white columns) and Oxo-M plus PLC inhibitor U73122 (1 µM, grey columns) (mean±S.E., n=4-6). B: Averaged I-V relationships (mean±S.E., n=6) of menthol-activated I_{TRPM8} density under control conditions (black symbols) and in the presence of arachidonic acid (AA, 10 µM, open circles); inset shows quantification of the respective I_{TRPM8} densities at +100 mV. C: Quantification of I_{TRPM8} density (at +100 mV) activated by icilin and menthol in HEK_{TRPM8/M3} cells under control conditions (black columns) and in the presence of Oxo-M during cells’ dialysis with control intracellular solution (white columns) and the one supplemented with cPLA2 inhibitor AACOCF3 (50 µM, grey columns) (mean±S.E., n=4-6).](attachment:fig2.png)
6). **D:** Quantification of $I_{\text{TRPM8}}$ density (at +100 mV) activated by cold, icilin and menthol in the control HEKTRPM8/M3 cells (i.e. transfected with siLuc) under control conditions (black columns) and following application of Oxo-M (white columns) as well as in HEKTRPM8/M3 cells transfected with anti-cPLA2 siRNA (sicPLA2) under the action of Oxo-M (grey columns) (mean±S.E., n=4-6). For all data presented $I_{\text{TRPM8}}$ was activated by temperature drop from 33°C to 20°C (cold), icilin (10 µM) or menthol (500 µM) and Oxo-M was used at 10 µM. On all graphs (*) and (**) denote statistically significant differences to control with p < 0.05 and p < 0.02, respectively.

**Fig. 3.** Functional link between muscarinic receptors and TRPM8 in PC3 cells. **A:** Averaged time course of $I_{\text{TRPM8}}$ (measured as current density at +100 mV) in response to TRPM8-activating stimuli (shown by horizontal bars): temperature drop from 33 to 20 °C (cold), icilin (10µM), and menthol (500µM) in PC3 cells transiently transfected with the human TRPM8 under control conditions (black circles) or following treatment with Oxo-M (10 µM, open circles). **B:** Quantification of $I_{\text{TRPM8}}$ density (at +100 mV) activated by cold, icilin and menthol in PC3 cells transiently transfected with the human TRPM8 under control conditions (black columns) or in the presence of Oxo-M (white columns) (mean±S.E., n=5). (**) denotes statistically significant differences to control with p < 0.02.

**Fig. 4.** Schematic diagram of principal signaling pathways involved in plasmalemmal TRPM8 regulation. Each numbered box depicts molecular interconnection related to the specific signaling pathway. 1 – PIP₂ depleton pathway: phosphatidylinositol bisphosphate (PIP₂) is a key lipid co-factors in TRPM8 activation, which depletion as a result of Gq-coupled receptor-mediated stimulation (PDGF receptor depicted) of phospholipase C (PLC) reduces TRPM8 functionality. 2 – iPLA2/LPL pathway: the products of iPLA2 catalytic activity, lysophospholipids (LPLs), act as TRPM8 activators; stimulation of iPLA2 and consequently enhancement of LPLs production can be achieved by endoplasmic reticulum (ER) Ca²⁺-store depletion. 3 – cAMP/PKA-pathway: decrease of the basal level of cAMP/PKA-dependent phosphorylation of TRPM8 via recruitment of α2-adrenoreceptor(α2AR)-coupled Gi/AC/cAMP/PKA pathway inhibits channel function; the level of cAMP/PKA-dependent phosphorylation and consequently TRPM8 activation can be restored via Gs/AC/cAMP/PKA pathway coupled to β-adrenoreceptor (βAR). 4 – PLC/PKC pathway: the PSA can activate TRPM8 via the bradykinin receptor (B2R) signaling pathway involving protein kinase C (PKC); Ca²⁺-dependent activation of PKC may also indirectly contribute to the Ca²⁺-mediated TRPM8 channel desensitization. 5 – M3 muscarinic receptor-stimulated cPLA2/AA pathway identified in this study: arachidonic acid (AA) derived as a result of “non classical” M3 muscarinic receptor-mediated stimulation of cPLA2 (marked by “?”) acts as TRPM8 channel inhibitor. Abbreviations: AC – adenylate cyclase (AC), ATP – adenosine triphosphate, cAMP – cyclic adenosine monophosphate, cPLA2 – Ca²⁺-dependent phospholipase A2, DAG – diacylglycerol, IP₃ – inositol trisphosphate, iPLA2 – Ca²⁺-independent phospholipase A2, ISO – isoproterenol, Oxo-M – oxotremorine, PDGF – platelet-derived growth factor, PDGFR –PDGF receptor, PKA – protein kinase A, PSA – prostate-specific antigene. Green circles with “+” and green arrows indicate stimulatory action, red circles with “−” and red arrows – inhibitory action; downward red arrow near PIP₂ indicates decreasing of its content.
Figure 1
Figure 2
Figure 3
Figure 4
Complex regulation of TRPM8 cold receptor-channel: role of arachidonic acid release following M3 muscarinic receptor stimulation

Alexis Bavencoffe, Artem Kondratskyi, Dimitra Gkika, Brigitte Mauroy, Yaroslav Shuba, Natalia Prevarskaya and Roman Skryma

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