Complex Regulation of the TRPM8 Cold Receptor Channel

ROLE OF ARACHIDONIC ACID RELEASE FOLLOWING M3 MUSCARINIC RECEPTOR STIMULATION

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Cold/menthol-activated TRPM8 (transient receptor potential channel melastatin member 8) 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, Ca\(^{2+}\), and lipid messengers. One such messenger is arachidonic acid (AA), which has been shown to inhibit TRPM8 channel activity. However, the physiological pathways mediating the inhibitory effect of AA on TRPM8 still remain 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 the 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 noncooling 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 voltage-dependent opening of the channel 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 and trigeminal sensory neurons in which it functions as cold-activated receptor channel. In fact, sizable TRPM8 expression was found in prostate (10), 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 (PIP\(_2\)) (15, 16), the products of catalytic activation of Ca\(^{2+}\)-sensitive PLC (16) or PKC (21, 22). It was also shown that TRPM8 is directly inhibited by polyunsaturated fatty acids, including the important lipid second messenger arachidonic acid (AA) (17).

prostate-specific antigen; PIP\(_2\), phosphatidylinositol bisphosphate; PKA, protein kinase A; PLC, phospholipase C; IP\(_3\), inositol trisphosphate; Luc, luciferase.

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TRPM8 Regulation via M3 Receptors

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 phosphatidylcholine-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 a 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 (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 PKC-dependent phosphorylation as well as some TRP channels directly, and endoplasmic reticulum 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 “nonclassical” 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 a mechanism was implicated in the M3 receptor-stimulated activation of the store-independent, arachidonate-regulated Ca\(^{2+}\) 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 the nonspecific 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 our data as well as other’s 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\(_{M3}\)) (kind gift from T. J. Shuttleworth (Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY)) (29) were cultured as described previously (30) and transiently transfected with human TRPM8 encoding vector using Nucleofector (Amaxa, Gaithersburg, Maryland). Control experiments were performed by transfecting the empty vector or siRNA against luciferase (siLuc) (supplemental Fig. S1A) using a NucleofectorTM (Amaxa, Gaithersburg, Maryland). 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 dimethyl sulfoxide 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. Overall statistical significance was determined by analysis of variance. In case of significance, differences between the means of two groups were analyzed by unpaired t test, whereas multiple comparisons between groups were performed by analysis of variance tests followed by Dunnett tests unless otherwise indicated. p < 0.05 was considered significant. The statistical analyses were performed using the Instat (version 3.06, GraphPad Software, Inc., San Diego, CA).

RESULTS

Consistent with the presence of the Gq-coupled M3 receptor, exposure of HEK\(_{TRPM8/M3}\) cells to the nonspecific musca-
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Inhibitory effect of Oxo-M on $I_{\text{TRPM8}}$ 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 33 to 20 °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 HEKTRPM8/M3 cells with U73122 (1 μM) for 5 min did not influence the inhibitory action of Oxo-M (10 μM) on $I_{\text{TRPM8}}$ mediated by cold, icilin or menthol, indicating that Gq-stimulated PLC catalytic activity and the concomitant reduction of PIP2 levels is not involved in the M3 receptor-mediated inhibition of TRPM8 channel. Because activation of the Gq/PLC pathway also brings about the endoplasmic reticulum 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 TRPM8 activity (17). To determine whether the 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 $I_{\text{TRPM8}}$ in our experimental model. Fig. 2B shows that consistent with previous studies (17), preincubation of HEKTRPM8/M3 cells with the AA (10 μM for 20 min) indeed led to the decrease of menthol-activated $I_{\text{TRPM8}}$ 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 $I_{\text{TRPM8}}$. 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 $I_{\text{TRPM8}}$ (Fig. 2C). The same result was also attained with nonpharmacological, siRNA-mediated, cPLA2 knockdown; 2 days after transfection of HEKTRPM8/M3 cells with anti-cPLA2 siRNA, the inhibitory effect of Oxo-M on the $I_{\text{TRPM8}}$ was completely eliminated, whereas 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, as 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, Oxo-M, can modulate the TRPM8-mediated membrane current ($I_{\text{TRPM8}}$) in prostate carcinoma PC-3 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 PC-3TRPM8 cells with Oxo-M (10 μM) caused an increase in [Ca2+]i, which could be inhibited by preapplication before the Oxo-M of the muscarinic receptor antagonist, atropine (1 μM, Fig. 1A). Moreover, pretreatment of HEKTRPM8/M3 cells with 10 μM Oxo-M for 10–20 min also resulted in an ~3-fold decrease of $I_{\text{TRPM8}}$ 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
with 10 μM Oxo-M for 10–20 min caused a decrease in $I_{\text{TRPM8}}$ activated by cold (temperature drop from 33 to 20 °C), icilin (10 μM), or menthol (500 μM) (Fig. 3, A and 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 Oxo-M causes suppression of TRPM8 via a signaling pathway that involves Gq- and Ca$^{2+}$-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 receptor-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 $I_{\text{TRPM8}}$ 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 HEK$\text{TRPM8/M3}$ cells. Although LPLs which are produced during IP3-dependent Ca$^{2+}$ 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 study, 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 documented previously (25, 28), although this mechanism still remains poorly understood. The type IV cPLA2 is Ca$^{2+}$-dependent enzyme, whose “classical” surface receptor-stimulated activation involves two distinct steps. First, a Ca$^{2+}$-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 exper-
the store-independent, arachidonate-regulated Ca$^{2+}$-mobilizing mechanism (16). In addition, extracellular Ca$^{2+}$, as well as protons are able to inhibit TRPM8 by altering voltage dependence of its activation via membrane surface charge screening (38).

Our present data, together with the previously obtained data, indicate that the 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, PIP$_2$, for sustaining TRPM8 function indicates that any receptor causing a PIP$_2$ depletion may cause both the inactivation and the desensitization of TRPM8 (21, 22). The latter was also attributed to the depletion of positive TRPM8 modulator, PIP$_2$, by the activity of Ca$^{2+}$-sensitive PLCδ (16). In addition, extracellular Ca$^{2+}$ as well as protons are able to inhibit TRPM8 by altering voltage dependence of its activation via membrane surface charge screening (38).

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 mobilization of Ca$^{2+}$ (39), which potentially provides for the possibility of TRPM8 activation in the internal tissues via Gq-coupled surface receptors stimulating PLC-catalyzed degradation of store-mobilizing IP$_3$. The resultant effect on TRPM8 function in such cases will apparently depend on the balance of the contributions of decreased PIP$_2$ and increased LPL levels.

Figure 3: Functional link between muscarinic receptors and TRPM8 in PC-3 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 lines): temperature drop from 33 to 20 °C (cold), icilin (10 μM), and menthol (500 μM) in PC-3 cells transiently transfected with human TRPM8 under control conditions (filled 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 PC-3 cells transiently transfected with human TRPM8 under control conditions (black bars) or in the presence of Oxo-M (white bars) (mean ± S.E., n = 5). On all graphs, single and double asterisks denote statistically significant differences to control with p < 0.05 and p < 0.02, respectively.
either toward 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 signaling and induces the accumulation of functional channels in the plasma membrane (Box 4, iPLA2/LPL pathway). The products of iPLA2 catalytic activity, LPLs, act as TRPM8 activators; stimulation of iPLA2 and consequently enhancement of LPL production can be achieved by endoplasmic reticulum (ER) Ca\(^{2+}\) store depletion. Box 3, the cAMP/PKA pathway. Decrease of the basal level of cAMP/PKA-dependent phosphorylation of TRPM8 via recruitment of the 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 the Gs/AC/cAMP/PKA pathway coupled to β-adreno-receptor (βAR). Box 4, PLC/PKC pathway. The PSA can activate TRPM8 via the bradykinin receptor (B2R) signaling pathway involving protein kinase C (PKC) Ca\(^{2+}\)-dependent activation of PKC may also indirectly contribute to the Ca\(^{2+}\)-mediated TRPM8 channel desensitization. Box 5, M3 muscarinic receptor-stimulated cPLA2/AA pathway identified in this study. AA derived as a result of nonclassical M3 muscarinic receptor-mediated activation of cPLA2 (marked by ?) acts as a TRPM8 channel inhibitor. DAG, diacylglycerol; ISO, isoproterenol; PDGF, PDGF receptor. Green circles with + and green arrows indicate stimulatory action, red circles with − and red arrows indicate inhibitory action; and a downward red arrow near PIP\(_2\) indicates decreasing of its content.

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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