Activation of gene transcription via CIM0216, a synthetic ligand of transient receptor potential melastatin-3 (TRPM3) channels

Sandra Rubil and Gerald Thiel
Department of Medical Biochemistry and Molecular Biology, Saarland University Medical Faculty, Homburg, Germany

ABSTRACT
Several compounds have been proposed to stimulate TRPM3 Ca\(^{2+}\) channels. We recently showed that stimulation of TRPM3 channels with pregnenolone sulfate activated the transcription factor AP-1, while other proposed TRPM3 ligands (nifedipine, D-erythro-sphingosine) exhibited either no or TRPM3-independent effects on gene transcription. Here, we have analyzed the transcriptional activity of CIM0216, a synthetic TRPM3 ligand proposed to have a higher potency and affinity for TRPM3 than pregnenolone sulfate. The results show that CIM0216 treatment of HEK293 cells expressing TRPM3 channels activated AP-1 and stimulated the transcriptional activation potential of c-Jun and c-Fos, 2 basic region leucine zipper transcription factors that constitute AP-1. CIM0216-induced gene transcription was attenuated by knock-down of TRPM3 or treatment with mefenamic acid, a TRPM3 inhibitor. CIM0216 was similarly or less capable in activating TRPM3-mediated gene transcription, suggesting that pregnenolone sulfate is still the ligand of choice for changing the gene expression pattern via TRPM3.

KEYWORDS
c-Fos; c-Jun; gene expression; lentivirus; TRPM3

Introduction
Transient receptor potential M3 (TRPM3) is a Ca\(^{2+}\) permeable cation channel and stimulation of TRPM3 triggers a rise in the intracellular Ca\(^{2+}\) concentration. We discovered that TRPM3 stimulation induces an intracellular signaling cascade that results in the activation of the stimulus-responsive transcription factors AP-1, c-Fos, c-Jun, CREB, Egr-1, and Elk-1.\(^{1-5}\) The natural ligands for TRPM3 channels are still unknown, but several compounds have been suggested to function as TRPM3-specific agonists. In most of these studies, the influx of Ca\(^{2+}\) ions into the cells and the subsequent rise in the intracellular Ca\(^{2+}\) concentration was used as an indication of activated TRPM3 channels. However, experiments with cultured neurons and neuronal cell lines revealed that the influx of Ca\(^{2+}\) ions into the cells is not always sufficient to induce an intracellular signaling cascade that leads to changes in the gene expression pattern.\(^{6-8}\) Recently, we compared the ability of putative activators of TRPM3 to induce gene transcription.\(^{3}\) The result revealed that pregnenolone sulfate is a powerful activator of TRPM3 mediated gene transcription, while other proposed TRPM3 ligands (nifedipine, D-erythro-sphingosine) exhibited either no or TRPM3-independent effects on gene transcription. These compounds induce a rise in intracellular Ca\(^{2+}\), but the Ca\(^{2+}\) signal is insufficient to induce an intracellular signaling cascade that changes the gene expression pattern of the cells. Recently, a synthetic compound termed CIM0216 has been described whose potency in generating a Ca\(^{2+}\) influx via TRPM3 channels greatly exceeded that of pregnenolone sulfate.\(^{9}\) We therefore asked whether CIM0216 is a potent activator of gene transcription as well, mediated by the stimulation of TRPM3 channels.

Results and discussion
As a sensor to measure AP-1 regulated transcription we used lentiviral gene transfer to implant a collagenase promoter/luciferase reporter gene into the chromatin of HEK293 cells that contain a tetracycline-inducible TRPM3 transcription unit (Fig. 1A). We tested several concentrations of CIM0216 (Fig. 1B) for induction of AP-1-responsive reporter gene transcription. As a control, we stimulated the cells with...
pregnenolone sulfate (Fig. 1C). The results show that CIM0216 significantly stimulated transcription from the Coll.luc reporter gene. However, pregnenolone sulfate...
sulfate was more than twice as potent as CIM0216 in upregulating AP-1 via TRPM3 channels (Fig. 1D).

Originally, the transcription factor AP-1 was identified as a dimer of the basic region leucine zipper transcription factors c-Fos and c-Jun. To measure the transcriptional activation potentials of c-Fos and c-Jun, fusion proteins were expressed consisting of the DNA binding domain of the yeast transcription factor GAL4 and the activation domains of c-Fos and c-Jun that are regulated via phosphorylation (Fig. 2A, 2B). To measure the transcriptional response, we integrated a reporter gene into the genome that had GAL4 binding sites (termed UAS, upstream activating sequence) in its regulatory region (Fig. 2C). The results show that the transcriptional activation potential of c-Fos and c-Jun were significantly increased in TRPM3-expressing HEK293 cells that had been stimulated with either CIM0216 or pregnenolone sulfate (Fig. 2D, E).

Recently, we showed that the upregulation of AP-1 in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 was almost completely blocked by the preincubation of the cells with mefen acid.3

Figure 3. CIM0216 and pregnenolone sulfate are TRPM3 ligands. (A) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding the collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with either CIM0216 (20 μM) or pregnenolone sulfate (PregS, 20 μM) for 24 hours as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of 3 experiments performed in quadruplicate; (★★★★, P < 0.001).

Figure 2. (see previous page) Stimulation of TRPM3 channels with CIM0216 or pregnenolone sulfate upregulates the transcriptional activation potentials of c-Fos and c-Jun. (A, B) Schematic representation of the modular structure of c-Fos and GAL4-c-Fos (A) and c-Jun and GAL4-c-Jun (B). The bZIP DNA binding and the dimerization domains are depicted. The GAL4 fusion proteins lack the bZIP domain, but retain the C-terminal activation domain of c-Fos (GAL4-cFos), or the N-terminal activation domain of c-Jun (GAL4-cJun). The truncated c-Fos and c-Jun proteins are expressed as fusion proteins together with the N-terminal DNA binding domain of GAL4. (C) Schematic representation of the integrated provirus encoding a luciferase reporter gene under the control of the minimal promoter, consisting of 5 GAL4 binding sites (UAS, upstream activating sequence), 2 Sp1 binding sites, a TATA box and an initiator element. (D, E) HEK293 cells containing a tetracycline-inducible TRPM3 expression unit were double-infected with a lentivirus containing the GAL4-responsive luciferase reporter gene and a lentivirus encoding either GAL4-c-Fos (D) or GAL4-c-Jun (E). The cells were serum-starved for 24 hours in the presence of tetracycline (1 μg/ml) and then stimulated with either CIM0216 (20 μM) or pregnenolone sulfate (PregS, 20 μM) for 24 hours as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean +/- SD of 3 experiments performed in quadruplicate (★★★★, P < 0.001).
channels as well. Furthermore, expression of a TRPM3-specific shRNA significantly reduced AP-1 activity in TRPM3-expressing HEK293 cells following stimulation of the cells with either CIM0216 or pregnenolone sulfate (Fig. 3B). Thus, both CIM0216 and pregnenolone sulfate-induced gene transcription relies on the activation of TRPM3 channels.

**Conclusion**

The experiments show that CIM0216 and pregnenolone sulfate are powerful activators of AP-1-regulated gene transcription via TRPM3. Moreover, both compounds increased the transcriptional activation potential of c-Jun and c-Fos. A comparison of the efficiency of CIM0216 and pregnenolone sulfate to induce gene transcription revealed that pregnenolone sulfate is stronger in activating AP-1 and exhibits similar activity to CIM0216 in stimulating the transcriptional activation potential of c-Jun and c-Fos.

**Materials and methods**

**Cell culture**

HEK293 cells containing the human TRPM3 coding region under the control of a tetracycline-regulated promoter were kindly provided by David Beech and Yasser Majeed, University of Leeds, UK and cultured and stimulated as described.3,10 Stimulation with pregnenolone sulfate (20 μM, Sigma-Aldrich # P162, dissolved in DMSO) or CIM0216 (1-20 μM, Tocris # 5521, dissolved in DMSO) was performed for 24 hours.

**Lentiviral gene transfer**

The lentiviral transfer vector pFUW-GAL4-c-Fos has been described previously.11 The GAL4-c-Jun expression plasmid pGAL4-c-Jun, expressing the amino acids 1-246 of c-Jun, was a kind gift of Michael Karin, University of California San Diego. To generate the lentiviral transfer vector pFUW-GAL4-c-Jun, plasmid pGAL4-c-Jun was cut with BamHI, filled in with the Klenow fragment of DNA polymerase I, and recut with Hpal. The fragment was cloned into plasmid pFUW-GAL4-NK10,12 replacing the repression domain of NK10 with the phosphorylation-dependent activation domain of c-Jun. The lentiviral transfer vectors pLL.TRPM3, encoding a TRPM3-specific shRNA, has been described.1,4 The viral particles were produced as previously described by triple transfection of 293T/17 cells with the gag-pol-rev packaging plasmid, the env plasmid encoding VSV glycoprotein, and the transfer vector.

**Reporter assays**

The lentiviral transfer vectors pFWColl.luc and pFW-UAS5Sp12luc have been described elsewhere.12,15 Cell extracts were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. Each experiment was performed at least 3 times in quadruplicate giving consistent results.

**Statistics**

Statistical analysis were done by using the 2-tailed student’s t-test. Data shown are mean +/- SD from 3 to 4 independent experiments performed in quadruplicate. Statistical probability is expressed as P < 0.001. Values were considered significant when P < 0.05.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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