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RESEARCH PAPER

Activation and inhibition of transient receptor potential TRPM3-induced gene transcription

Andrea Lesch*, Sandra Rubil* and Gerald Thiel

Department of Medical Biochemistry and Molecular Biology, University of Saarland Medical Center, Homburg D-66421, Germany

Correspondence
Dr Gerald Thiel, Department of Medical Biochemistry and Molecular Biology, University of Saarland Medical Center, Building 44, Homburg D-66421, Germany.
E-mail: gerald.thiel@uks.eu

*These authors contributed equally

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BACKGROUND AND PURPOSE
Transient receptor potential-3 (TRPM3) channels function as Ca^{2+} permeable cation channels. While the natural ligands for these channels are still unknown, several compounds have been described that either activate or inhibit TRPM3 channel activity.

EXPERIMENTAL APPROACH
We assessed TRPM3-mediated gene transcription, which relies on the induction of intracellular signalling to the nucleus following activation of TRPM3 channels. Activator protein-1 (AP-1) and Egr-1-responsive reporter genes were integrated into the chromatin of the cells. This strategy enabled us to analyse gene transcription of the AP-1 and Egr-1-responsive reporter genes that were packed into an ordered chromatin structure.

KEY RESULTS
The neurosteroid pregnenolone sulfate strikingly up-regulated AP-1 and Egr-1 transcriptional activity, while nifedipine and D-erythro-sphingosine, also putative activators of TRPM3 channels, exhibited either no or TRPM3-independent effects on gene transcription. In addition, pregnenolone sulfate robustly enhanced the transcriptional activation potential of the ternary complex factor Elk-1. Pregnenolone sulfate-induced activation of gene transcription was blocked by treatment with mefenamic acid and, to a lesser extent, by the polyphenol naringenin. In contrast, progesterone, pregnenolone and rosiglitazone reduced AP-1 activity in the cells, but had no inhibitory effect on Egr-1 activity in pregnenolone sulfate-stimulated cells.

CONCLUSION AND IMPLICATIONS
Pregnenolone sulfate is a powerful activator of TRPM3-mediated gene transcription, while transcription is completely inhibited by mefenamic acid in cells expressing activated TRPM3 channels. Both compounds are valuable tools for further investigating the biological functions of TRPM3 channels.

LINKED ARTICLES
This article is part of a themed section on the pharmacology of TRP channels. To view the other articles in this section visit http://dx.doi.org/10.1111/bph.2014.171.issue-10

Abbreviations
bZIP, basic region leucine zipper; CNO, clozapine-N-oxide; HDAC1, histone deacetylase 1; SRE, serum response element; TCF, ternary complex factor; TRE, phorbol 12-O-tetradecanoylphorbol-13-acetate (TPA)-responsive element; TRP, transient receptor potential

Introduction

The transient receptor potential melastatin-3 (TRPM3) cation channel is a typical TRP cationic channel (receptor nomenclature follows Alexander *et al.*, 2013), containing six transmembrane domains and a pore domain between the fifth and sixth transmembrane domain. Both amino and carboxy termini are located in the cytosol (Thiel *et al.*, 2013). Initially, constitutive activity was attributed to TRPM3 channels (Grimm *et al.*, 2003; 2005; Oberwinkler *et al.* 2005, Naylor *et al.*, 2010). Later, several endogenous and exogenous compounds were described as activators of TRPM3 channels, including the neurosteroid pregnenolone sulfate, the lipid D-erythro-sphingosine and the L-type voltage-gated Ca^{2+} channel inhibitor nifedipine (Grimm *et al.*, 2005; Wagner *et al.*, 2008; Naylor *et al.*, 2010; Islam, 2011). Additionally, progesterone, mefenamic acid, the antidiabetic PPAR-γ agonist rosiglitazone and the polyphenol naringenin have been proposed to prevent TRMP3 channel activation (Naylor *et al.*, 2010; Klose *et al.*, 2011; Majeed *et al.*, 2011; 2012; Straub *et al.*, 2013).

Activation of TRPM3 channels is often assessed by measuring either intracellular Ca^{2+} concentrations with appropriate indicators and/or cationic membrane current with whole-cell patch-clamp techniques. Thus, the influx of Ca^{2+} ions into the cells and the subsequent rise in the intracellular Ca^{2+} concentration is used to demonstrate the activation of TRPM3 channels. Recently, we showed that stimulation of endogenous TRPM3 channels in insulinoma cells by pregnenolone sulfate activates a signal cascade that leads to an activation of gene transcription (Mayer *et al.*, 2011; Müller *et al.*, 2011). The rise of intracellular Ca^{2+} is necessary for TRPM3-mediated transcriptional induction, as intracellular signalling and transcription via TRPM3 channels is impaired by chelating extracellular or intracellular Ca^{2+} (Mayer *et al.*, 2011).

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 signalling cascade leading to changes in the gene expression pattern. In particular, the mode of entry of Ca^{2+} determined whether gene transcription is induced or not (Gallin and Greenberg, 1995). Ca^{2+} influx via L-type voltage-gated Ca^{2+} channels, for instance, triggered phosphorylation of the transcription factor cAMP responsive element binding (CREB), while Ca^{2+} influx via N- and P/Q-type Ca^{2+} channels did not induce CREB-dependent gene transcription (Deisseroth *et al.*, 1998; West *et al.*, 2001).

Here, we have investigated the effect of putative activators and inhibitors of TRPM3 on changes in gene transcription regulated by the transcription factors, AP-1 and Egr-1. This approach implies that the initial rise of intracellular Ca^{2+} is sufficient to induce an intracellular signalling cascade that translocates the Ca^{2+} signal through the cytoplasm to the nucleus to change the gene expression pattern. We used reporters that specifically detect AP-1 and Egr-1 activity. AP-1 and Egr-1 transcription factors respond to multiple extracellular signalling molecules and are an important connection between cellular stimulation and the gene expression pattern of the cells. Often, reporter genes are introduced into cultured cells via transient transfection of plasmids. However, this approach has the disadvantage that the structure of these plasmids may be incompletely organized in comparison with cellular chromatin, and may thus resemble a prokaryotic gene organization including a non-restrictive transcriptional ground state. In our study, we implanted the reporter genes into the chromatin using lentiviral gene transcription to ensure that these genes were packed into an ordered nucleosomal structure.

We have investigated a range of putative activators and inhibitors of TRPM3 channels and the results of this study show that pregnenolone sulfate is a powerful activator of TRPM3-mediated gene transcription, while its activity is attenuated by mefenamic acid.

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 as described (Naylor *et al.*, 2008). Expression of TRPM3 channels was induced by adding tetracycline [1 μg mL^{-1}, Sigma-Aldrich (Deisenhofen, Germany) # T7680, dissolved in water] to the culture medium containing 0.05 % FBS for 24 h prior to the stimulation with putative TRPM3 activators. As a control, cells were analysed that had not received tetracycline. Stimulation with pregnenolone sulfate (20 μM, Sigma # P162, dissolved in DMSO), nifedipine (20 μM, Sigma # N7634, dissolved in DMSO) and D-erythro-sphingosine (20 μM, BioMol # 10007907, dissolved in ethanol) was performed for 24 h. To test putative TRPM3 channel inhibitors, cells were pre-incubated for 2 h with either pregnenolone (10 μM, Sigma # P9129, dissolved in DMSO), progesterone (10 μM, Sigma # P8783, dissolved in DMSO), rosiglitazone [10 μM, Axxora, Enzo Life Sciences (Lörrach, Germany) # ALX-205-125, dissolved in ethanol], mefenamic acid (30 μM, Santa Cruz, Heidelberg, Germany, # sc-205380, dissolved in DMSO), naringenin (10 μM, Sigma # N5893, dissolved in DMSO), quercetin (10 μM, Sigma # Q4951, dissolved in DMSO) and BD1047 dihydrobomide [100 μM, Tocris Bioscience (R&D GmbH, Wiesbaden, Germany) # 0956, dissolved in DMSO]. Stimulation with pregnenolone sulfate or D-erythro-sphingosine was performed in the presence of the putative inhibitors in medium containing 0.05 % FBS. Stimulation with either 1 μM clozapine-*N*-oxide (CNO, 1 μM, Enzo Life sciences, # NS-105-0005, dissolved in ethanol) or phorbol 12-*O*-tetradecanoylphorbol-13-acetate [TPA, 10 ng mL^{-1}, Calbiochem (via VWR International GmbH, Darmstadt, Germany) # 524400-1, dissolved in DMSO] was performed in medium containing 0.05 % FBS that lacked tetracycline.

Lentiviral gene transfer

The lentiviral transfer vectors pFUW-GAL4-Sp1, pFUW-GAL4-Elk-1 and pFUW-Rαq have been described previously (Ekici *et al.*, 2012; Thiel and Rössler, 2011; Thiel *et al.*, 2012; Kaufmann *et al.*, 2013). The viral particles were produced as previously described (Keim *et al.*, 2012) by triple transfection of HEK293T/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, pFWEBS2[4].luc, pFWStREluc and pFWUAS[5]Sp1[2]luc have been described elsewhere (Rössler *et al.*, 2008; Müller *et al.*, 2009; Ekici *et al.*, 2012; Thiel and Rössler, 2011; Thiel *et al.*, 2012; Kaufmann *et al.*, 2013). Cell extracts were prepared using reporter lysis buffer (Promega, Mannheim, Germany) and analysed for luciferase activities as described (Thiel *et al.*, 2000). Luciferase activity was normalized to the protein concentration.

RT-PCR

RT-PCR was performed as previously described (Bauer *et al.*, 2007). The primers used to detect TRPM3 and GAPDH transcripts were described recently (Naylor *et al.*, 2010; Ekici *et al.*, 2012). RNA samples were reverse transcribed into cDNA with RevertAid M-MuLV RT (Fermentas GmbH, St. Leon-Rot, Germany) in the presence of RNase Inhibitor (Fermentas). The PCR conditions were: 95°C for 10 min, 27 amplification cycles, each cycle consisting of denaturation at 95°C for 30 s, primer annealing at 54°C for 30 s, and extension at 68°C for 30 s.

Western blots

Nuclear extracts were prepared as described (Kaufmann and Thiel, 2002). Thirty micrograms of nuclear proteins were separated by SDS-PAGE and the blots were incubated with antibodies directed against Egr-1 (Santa Cruz, # sc-189), c-Fos (Santa Cruz, # sc-52), or c-Jun (Santa Cruz, # sc-1694). An antibody directed against histone deacetylase 1(HDAC1; Santa Cruz, # sc-81598) was used as a loading control as described recently (Spohn *et al.*, 2010; Mayer *et al.*, 2011). Immunoreactive bands were detected via enhanced chemiluminescence as described (Spohn *et al.*, 2010; Mayer *et al.*, 2011).

Data analysis

Data shown are mean ± SD from four independent experiments. Data were analysed with the two-tailed Student's *t*-test. Differences between means were considered significant when $P < 0.05$.

Results

Experiments performed with insulinoma cells revealed that both TRPM3 channels and L-type voltage-gated Ca^{2+} channels are involved in the regulation of pregnenolone sulfate-induced gene expression (Mayer *et al.*, 2011). In contrast, it has been shown that Ca^{2+} influx following TRPM3 stimulation by pregnenolone sulfate is independent of L-type voltage-gated Ca^{2+} channels in HEK293 cells (Wagner *et al.*, 2008; Majeed *et al.*, 2010). These observations indicate that TRPM3 channels function in a heterologous system as a ligand-activated ionotropic receptor in the absence of L-type voltage-gated Ca^{2+} channels, leading to an influx of Ca^{2+} into the cells following activation. Therefore, we employed the HEK293 expression system to avoid interference of TRPM3 signalling by L-type voltage-gated Ca^{2+} channel activation. We used an engineered HEK293 cell line, in which expression of TRPM3 channels was induced by adding tetracycline to the culture medium (Figure 1A).

Chromatin-integrated reporter genes to measure stimulus-induced up-regulation of AP-1 and Egr-1 activity

AP-1 is a dimeric transcription factor complex composed of members of the Jun, Fos and ATF families of basic region leucine zipper transcription factors. To measure AP-1-regulated transcription, a collagenase promoter/luciferase reporter gene was implanted into the chromatin of HEK293 cells that contain a tetracycline-inducible TRPM3 transcription unit. The collagenase promoter contains an AP-1 binding site in the proximal promoter region and therefore frequently has been used to monitor AP-1 activity (Steinmüller *et al.*, 2001; Thiel and Rössler, 2011; Thiel *et al.*, 2012; Kaufmann *et al.*, 2013). The AP-1 binding site encompasses the sequence 5′-TGAGTCA-3′, also known as TPA-responsive element (TRE). To detect Egr-1 activity in the cells, a chromosomally embedded Egr-1-responsive luciferase reporter gene termed EBS2[4].luc was used. The implanted transcription unit encoded the luciferase reporter gene, controlled by a minimal promoter consisting of four binding sites for Egr-1, derived from the synapsin I gene, a TATA box and an initiator element. A schematic depiction of the integrated proviruses is shown in Figure 1B.

AP-1 and Egr-1 activities are regulated by many extracellular signalling molecules. Figure 1C shows that stimulation of Rαq-coupled designer receptors leads to a striking up-regulation of AP-1 and Egr-1 transcriptional activity in HEK293 cells following stimulation with clozapine-*N*-oxide (CNO). Likewise, high AP-1 and Egr-1 activities were measured in HEK293 cells that had been stimulated with the phorbol ester TPA (Figure 1D).

Stimulation of TRPM3 channels up-regulates AP-1 and Egr-1 activity and induces the biosynthesis of c-Fos, c-Jun, and Egr-1

The neurosteroid pregnenolone sulfate, the sulfated form of pregnenolone (Figure 2A), stimulates Ca^{2+} influx into the cells via TRPM3 channels (Wagner *et al.*, 2008; Ciurtin *et al.*, 2010; Majeed *et al.*, 2010; Naylor *et al.*, 2010; Klose *et al.*, 2011). In addition, we have shown that stimulation of endogenous TRPM3 channels expressed in insulinoma cells with pregnenolone sulfate led to changes in gene transcription (Mayer *et al.*, 2011; Müller *et al.*, 2011). We therefore tested several concentrations of pregnenolone sulfate for induction of AP-1- and Egr-1-responsive reporter genes in HEK293 cells that contain a tetracycline-regulated TRPM3 transcription unit. Cells were infected with lentivirus encoding luciferase under the control of AP-1 (Coll.luc) or Egr-1 (EBS2[4].luc). Cells were treated with tetracycline to induce TRPM3 expression and stimulated with pregnenolone sulfate for 24 h. The results show that pregnenolone sulfate stimulated transcription from both reporter genes in a robust and concentration-dependent manner, leading to higher AP-1 and Egr-1 activities in the cells (Figures 2B,D). Despite some experimental variations in the fold induction of AP-1- and Egr-1-mediated transcription, these results indicate that stimulation of TRPM3 channels alters the genetic programme of the cells by activating genes controlled by AP-1 and/or Egr-1. The AP-1 transcription factor was originally described as a heterodimer of the basic region leucine zipper transcription factors c-Jun

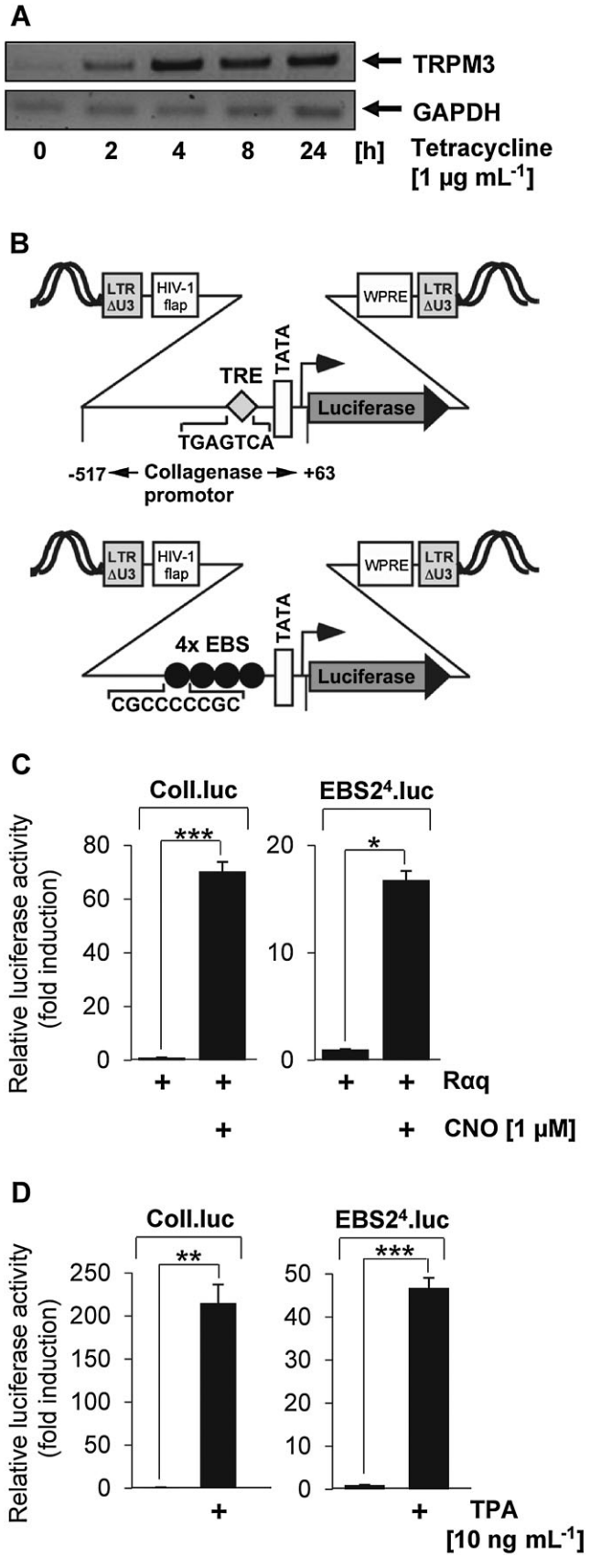

Figure 1

Regulation of AP-1 and Egr-1 activity in HEK293 cells. (A) Tetracycline-regulated expression of TRPM3 in HEK293/TRPM3 cells. The cells were cultured for 24 h in medium containing 0.05% serum. Stimulation with tetracycline (1 µg mL^{-1}) was performed with medium containing 0.05% serum. RNA was prepared at the indicated time points and analysed by PCR using TRPM3 and GAPDH-specific primers. (B) Schematic representation of the integrated proviruses encoding either an AP-1-responsive collagenase promoter/luciferase reporter gene (Coll.luc) or an Egr-1-responsive reporter gene (EBS2^4.luc). The location and sequence of the phorbol TRE within the collagenase promoter is shown, as well as the sequence of the Egr-1 binding site within the regulatory region of the EBS2^4.luc reporter gene. The location of the woodchuck hepatitis virus post-transcriptional regulatory element (WPRE) and the HIV flap element are shown. (C) HEK293 cells containing a tetracycline-inducible TRPM3 expression unit were infected with a recombinant lentivirus expressing an Rαq-coupled designer receptor. In addition, the cells were infected with lentiviruses encoding either the collagenase promoter/luciferase reporter gene (Coll.luc), or the Egr-1-sensitive reporter gene EBS2^4.luc. The cells were cultured for 24 h in medium containing 0.05% serum in the absence of tetracycline. Stimulation with CNO (1 µM) was performed with medium containing 0.05% serum. (D) HEK293 cells containing a tetracycline-inducible TRPM3 expression unit were infected with lentiviruses encoding either the collagenase promoter/luciferase reporter gene (Coll.luc), or the Egr-1-sensitive reporter gene EBS2^4.luc. The infected cells were stimulated with TPA (10 ng mL^{-1}) for 24 h. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean ± SD, *, $P < 0.05$; **$P < 0.01$; ***, $P < 0.001$, significantly different as indicated; $n = 4$.

and c-Fos. Figure 2C shows that the biosynthesis of both proteins is up-regulated in pregnenolone sulfate-treated HEK293 cells that expressed TRPM3 channels. Likewise, the biosynthesis of Egr-1 was induced under these circumstances as well (Figure 2E). Based on these results, we chose a concentration of 20 µM of pregnenolone sulfate for further experiments. Stimulation of HEK293 cells with pregnenolone sulfate in the absence of tetracycline did not induce an up-regulation of reporter gene transcription (Figure 2F), indicating that TRPM3 expression is essential for connecting pregnenolone sulfate stimulation with enhanced transcription of both AP-1 or Egr-1-responsive genes. Stimulation of TRPM3 channels with pregnenolone sulfate did not induce transcription from a Nrf2-regulated reporter gene (StRE.luc), which is typically activated by antioxidative or electrophilic compounds (Figure 2 G,H).

Role of nifedipine and D-erythro-sphingosine on TRPM3-regulated gene transcription

The dihydropyridine nifedipine (Figure 3A), an inhibitor of voltage-gated Ca^{2+} channels, triggers a rise of intracellular Ca^{2+} concentration via TRPM3 channels (Wagner *et al.*, 2008; Majeed *et al.*, 2011). In addition, D-erythro-sphingosine (Figure 3E) has been proposed to act as a TRPM3 ligand, although the activity of this compound towards TRPM3 channels is a matter of debate and may depend on the experimental design (Grimm *et al.*, 2005; Wagner *et al.*, 2008;

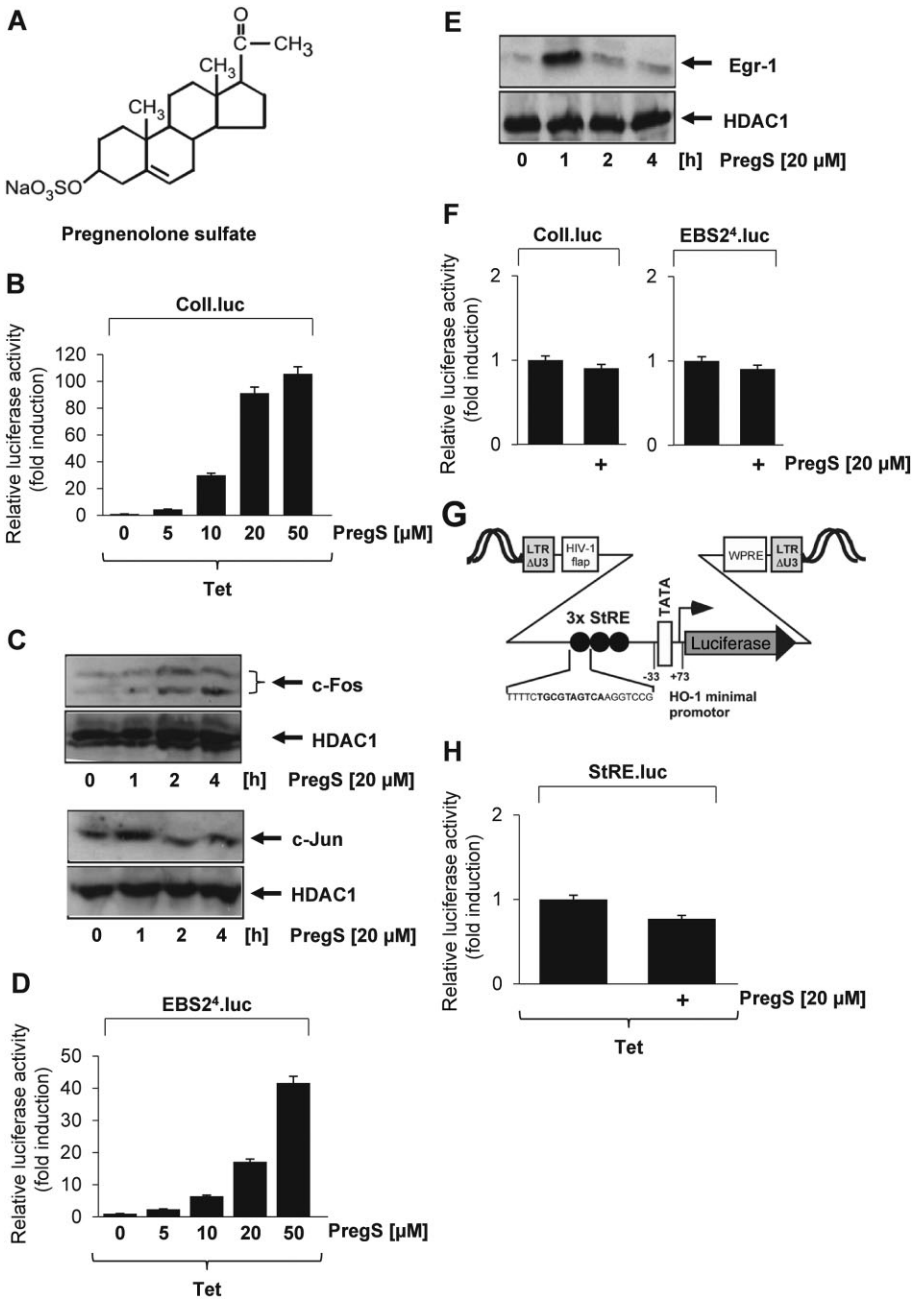

Figure 2

Up-regulation of AP-1 and Egr-1 activities and Egr-1, c-Jun and c-Fos expression in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 channels. (A) Pregnenolone sulfate. (B, D) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene or the Egr-1-responsive EBS2^4.luc reporter gene. The cells were serum-starved for 24 h in the presence of tetracycline (1 μg mL^{-1}) and then stimulated with pregnenolone sulfate (PregS, 20 μM) for 24 h. (C, E) HEK293 cells expressing TRPM3 channels were stimulated with pregnenolone sulfate (20 μM) in medium containing 0.05% serum. Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against either c-Fos, c-Jun (C), or Egr-1 (E). The antibody directed against HDAC1 was used as a loading control. (F) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene or the Egr-1-responsive EBS2^4.luc reporter gene. The cells were serum-starved for 24 h in the absence of tetracycline and then stimulated with pregnenolone sulfate (20 μM) for 24 h. (G) Schematic representation of the integrated provirus encoding a Nrf2-responsive luciferase reporter gene (StRE.luc) The sequence of the stress–response element is in bold. The regulatory region contains three Nrf2 binding sites, derived from the haem oxygenase gene, upstream of a minimal haem oxygenase promoter. (H) HEK293 cells containing a tetracycline-inducible TRPM3 expression unit were infected with a recombinant lentivirus encoding the Nrf2-regulated reporter gene StRE.luc. The cells were serum-starved for 24 h in the presence of tetracycline (1 μg mL^{-1}) and then stimulated with pregnenolone sulfate (20 μM) for 24 h. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean ± SD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant, as indicated; $n = 4$.

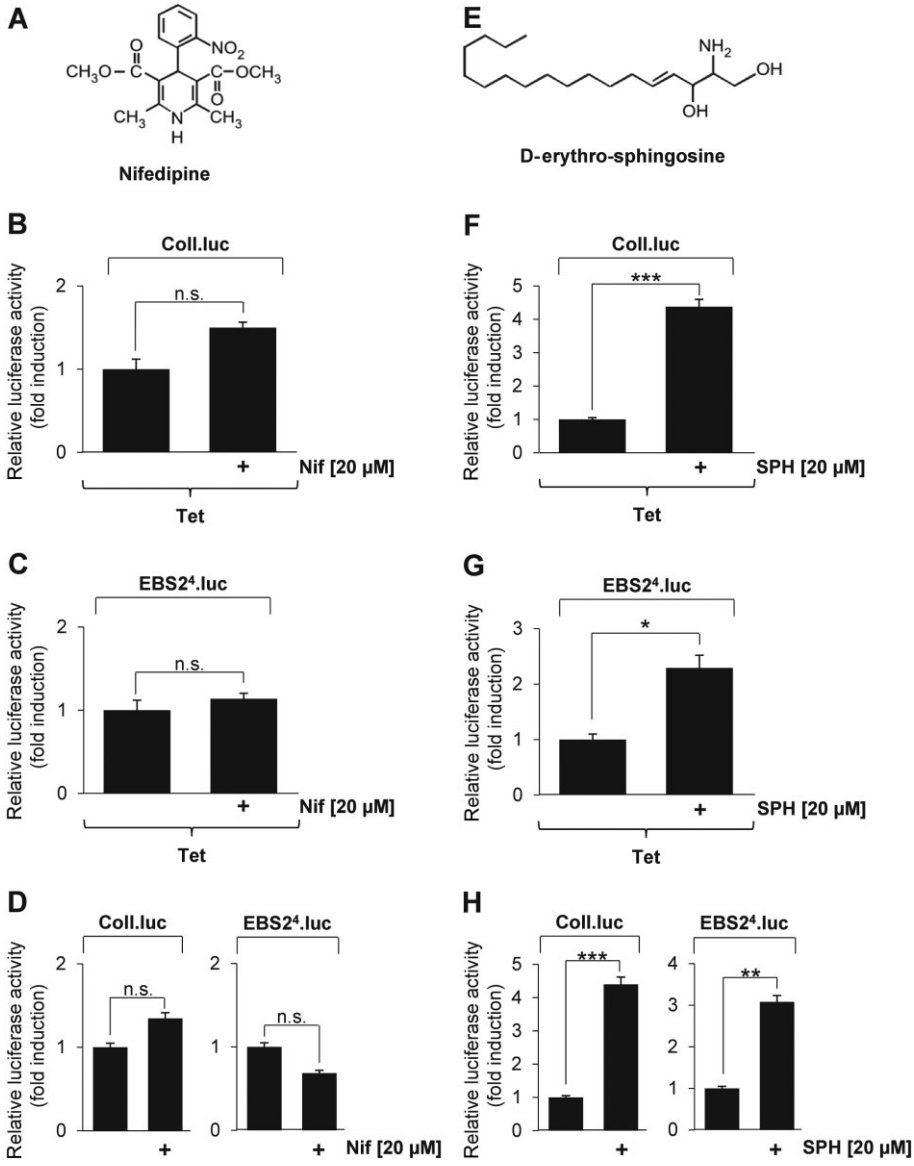

Figure 3

Nifedipine and D-erythro-sphingosine as activators of TRPM3-mediated gene transcription. (A, E) Nifedipine (Nif) and D-erythro-sphingosine (SPH). (B, C, F, G) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene (B, F) or the EBS2⁴.luc reporter gene (C, G). The cells were serum-starved for 24 h in the presence of tetracycline (1 μg mL^{-1}) and then stimulated with either nifedipine (20 μM) (B, C), or D-erythro-sphingosine (20 μM) (F, G) for 24 h. (D, H) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene or the EBS2⁴.luc reporter gene. The cells were serum-starved for 24 h and then stimulated with either nifedipine (20 μM) (D), or D-erythro-sphingosine (20 μM) (H) for 24 h. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean ± SD, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant, as indicated; $n = 4$.

Naylor *et al.*, 2010). We tested both nifedipine and D-erythro-sphingosine for their effect on the intracellular AP-1 and Egr-1 activity. Figures 3B and 3C show that nifedipine stimulation of HEK293 cells expressing TRPM3 channels had no effect on the AP-1 and Egr-1 activity of the cells. In addition, nifedipine treatment did not change the AP-1 and Egr-1 activity in HEK293 cells in the absence of TRPM3 channel expression (Figure 3D). Stimulation of the cells with D-erythro-sphingosine (Figure 3E) triggered an up-regulation of AP-1 activity (≈ fourfold) and Egr-1 activity (≈ twofold) (Figures 3F,G). D-erythro-sphingosine already increased AP-1 and Egr-1 activity in the absence of tetracycline (Figure 3H), indicating that this compound can activate transcription in a TRPM3-independent manner.

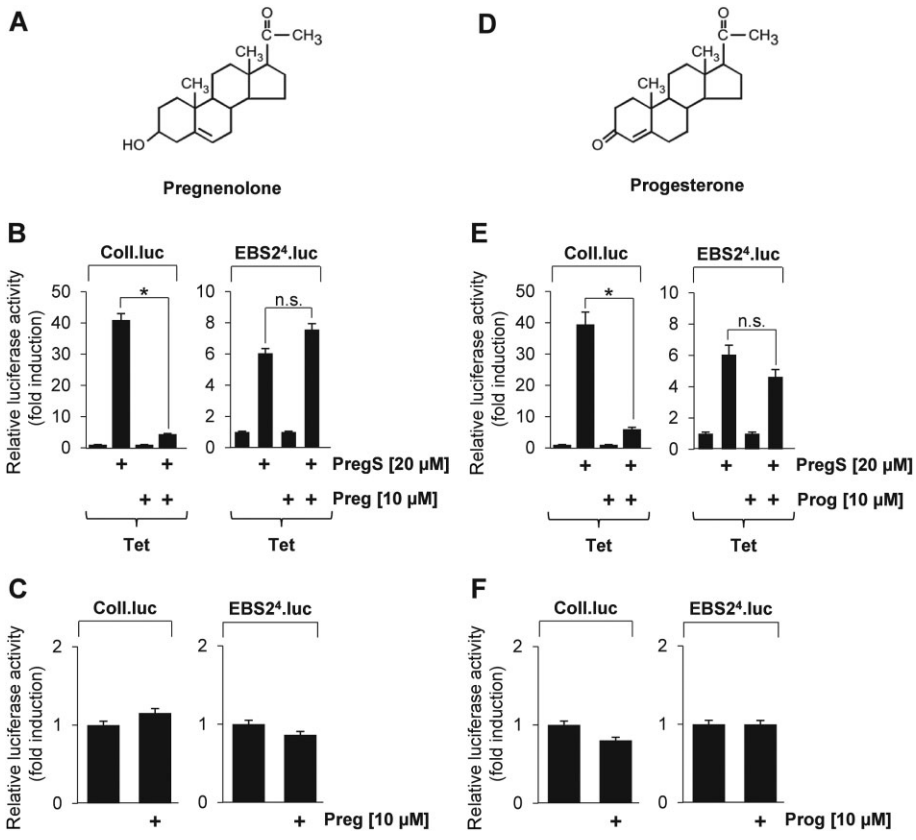

Figure 4

Pregnenolone and progesterone inhibit pregnenolone sulfate-induced up-regulation of AP-1 activity. (A, D) Pregnenolone, progesterone. (B, E) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene or the EBS2⁴.luc reporter gene. The cells were serum-starved for 24 h in the presence of tetracycline (1 μg mL^{-1}) and then stimulated with pregnenolone sulfate (PregS, 20 μM) in the presence or absence of either pregnenolone (Preg, 10 μM) (B) or progesterone (Prog, 10 μM; E) for 24 h. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. (C, F) The experiments were repeated in the absence of tetracycline. Data shown are mean ± SD, *, $P < 0.05$; n.s., not significant, as indicated; $n = 4$.

Regulation of TRPM3-regulated gene transcription by pregnenolone and progesterone

Recently, we showed that pregnenolone or progesterone did not induce Egr-1 expression in insulinoma cells, in contrast to pregnenolone sulfate (Mayer *et al.*, 2011). Progesterone also did not stimulate Ca^{2+} influx via TRPM3 channels (Majeed *et al.*, 2012). Furthermore, progesterone inhibited the Ca^{2+} influx and the rise in intracellular Ca^{2+} following activation of TRPM3 channels by pregnenolone sulfate (Majeed *et al.*, 2012). Based on these experiments, it had been suggested that the TRPM3 channel contains an extracellular steroid binding site that accepts a limited number of steroids, including pregnenolone sulfate, pregnenolone, and progesterone (Majeed *et al.*, 2012). This hypothesis is further substantiated by similar chemical structures (Figure 4A, in comparison with Figure 2A). We assessed the impact of pregnenolone and progesterone on the AP-1 and Egr-1 transcriptional activity in pregnenolone sulfate-stimulated cells expressing TRPM3 channels. Both pregnenolone and progesterone interfered with the up-regulation of AP-1 activity (Figure 4B,E, left panels). Surprisingly, both steroids did not significantly change the Egr-1 activity of the cells (Figure 4 B,E, right panels). Pregnenolone and progesterone did not change the AP-1 and Egr-1 activity in HEK293 cells in the absence of TRPM3 channels (Figures 4C,F).

Mefenamic acid, an inhibitor of TRPM3-regulated gene transcription

Mefenamic acid is a fenamate type of non-steroid anti-inflammatory drug and its structure is shown in Figure 5A. This compound also selectively inhibited TRPM3-mediated Ca^{2+} entry in HEK293 cells expressing TRPM3 channels (Klose *et al.*, 2011; Straub *et al.*, 2013). In the present experiments we found that the up-regulation of AP-1 and Egr-1 activities in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 channels was almost completely blocked by preincubation of the cells with mefenamic acid (Figure 5B,C). Mefenamic acid did not change the AP-1 and Egr-1 activity in HEK293 cells in the absence of TRPM3 expression (Figure 5D).

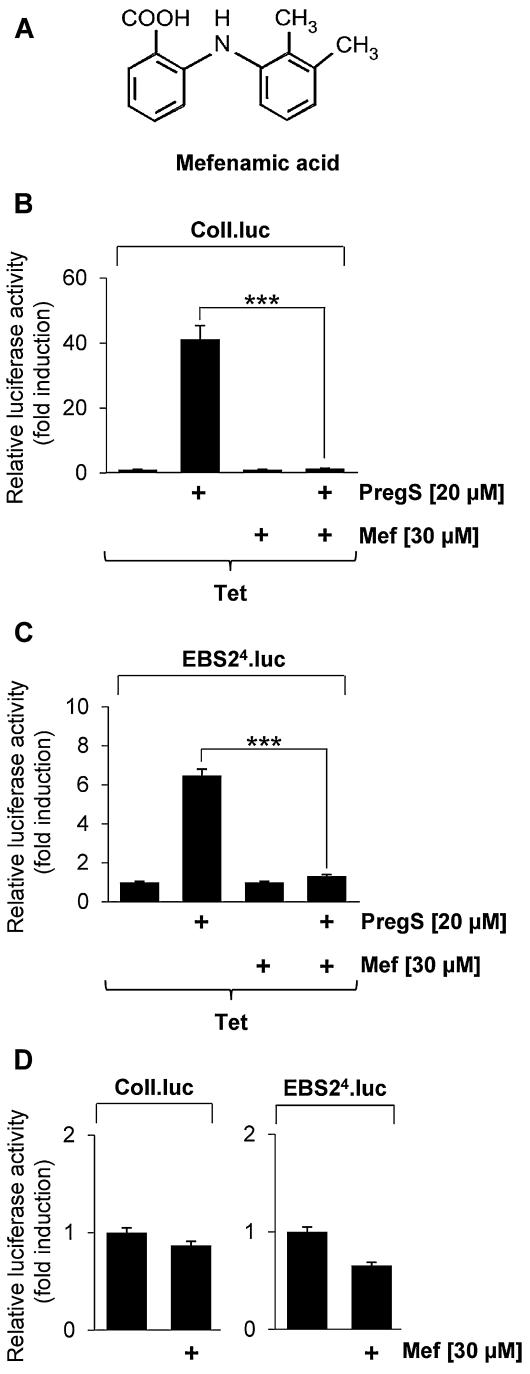

Figure 5

Mefenamic acid inhibits pregnenolone sulfate (PregS)-induced up-regulation of AP-1 and Egr-1 activity. (A) Mefenamic acid (Mef). (B, C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene or the EBS2^4.luc reporter gene. The cells were serum-starved for 24 h in the presence of tetracycline (1 µg mL^{-1}) and then stimulated with pregnenolone sulfate (20 µM) in the presence or absence of mefenamic acid (30 µM) for 24 h. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. (D) The experiments were repeated in the absence of TRPM3 expression. Data shown are mean ± SD, ***, $P < 0.001$ significantly different as indicated; $n = 4$.

Rosiglitazone affects AP-1 activity following TRPM3 stimulation

Rosiglitazone, a PPAR-γ agonist (Figure 6A), inhibits pregnenolone sulfate-induced Ca^{2+} influx and a rise in intracellular Ca^{2+} concentration in HEK293 cells expressing TRPM3 channels (Majeed *et al.*, 2011). Treatment of these cells with rosiglitazone (Figures 6B, 6D) reduced the AP-1 activity of the cells following stimulation with pregnenolone sulfate (Figure 6B). Rosiglitazone did not reduce Egr-1 activity in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 channels (Figure 6C). In addition, rosiglitazone did not affect the AP-1 and Egr-1 activity in HEK293 cells in the absence of TRPM3 channels.

Role of the polyphenols naringenin and quercetin on AP-1 and Egr-1 activities in HEK293 cells expressing TRPM3 channels

The citrus fruit polyphenol naringenin (Figure 7A) was identified as an inhibitor of pregnenolone sulfate-induced Ca^{2+} entry in HEK293 cells expressing TRPM3 channels by Straub *et al.*, (2013). In the present series of experiments, naringenin also reduced the AP-1 and Egr-1 activity in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 channels (Figure 7B). While incubation of the cells with naringenin had been described to completely block the Ca^{2+} influx into the cells via TRPM3 channels, pregnenolone sulfate-induced gene transcription was only partially impaired by this compound. As naringenin is structurally similar to the dietary polyphenol quercetin (Figure 7A), we also assessed the effects of quercetin in our system and found that quercetin treatment reduced AP-1 activity but slightly induced Egr-1 activity in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 channels (Figure 7C, right panel). Both polyphenols did not change the AP-1 or Egr-1 activity in the absence of TRPM3 channels (Figure 7D).

Pregnenolone sulfate stimulates the transcriptional activation potential of the ternary complex factor (TCF) Elk-1 in HEK293 cells expressing TRPM3 channels

The up-regulation of Egr-1 activity in pregnenolone sulfate-stimulated cells expressing TRPM3 channels requires the biosynthesis of Egr-1. The most important genetic elements within the Egr-1 promoter are five serum response elements (SRE) that are binding sites for the serum response factor (SRF) and TCFs. TCFs are proteins such as Elk-1 that contact DNA and additionally bind to a SRF dimer, resulting in the generation of a ternary complex, which is required to stimulate gene transcription of those genes containing SREs in their regulatory regions. In addition, TCFs are regulators of AP-1 via the control of c-Fos expression (Müller *et al.*, 2010; 2011; Thiel *et al.*, 2012; Kaufmann *et al.*, 2013). Therefore, the transcriptional activation potential of Elk-1 as a result of TRPM3 activation was assessed via expression of a GAL4-Elk-1 fusion protein (Figure 8A). The DNA-binding domain of Elk-1 is localized on the N-terminus while the transcriptional activation domain is localized on the C-terminus. A regulatory domain lies within this transcriptional activation domain encompassing the key phosphoacceptor sites S383 and S389.

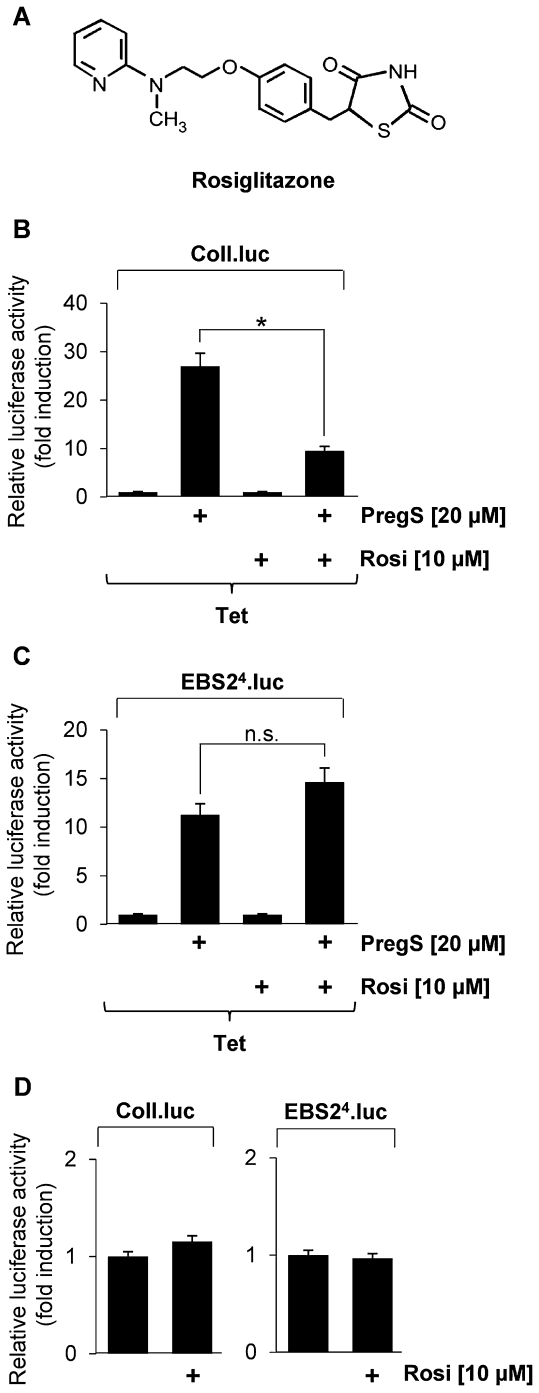

Figure 6

Effect of rosiglitazone (Rosi) on AP-1 and Egr-1 activities following activation of TRPM3. (A) Rosiglitazone (B, C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene or the EBS2^4.luc reporter gene. The cells were serum-starved for 24 h in the presence of tetracycline (1 µg mL^{-1}) and then stimulated with pregnenolone sulfate (PregS, 20 µM) in the presence or absence of rosiglitazone (10 µM) for 24 h. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. (D) The experiments were repeated in the absence of TRPM3 expression. Data shown are mean ± SD, *, $P < 0.05$; n.s., not significant, as indicated; $n = 4$.

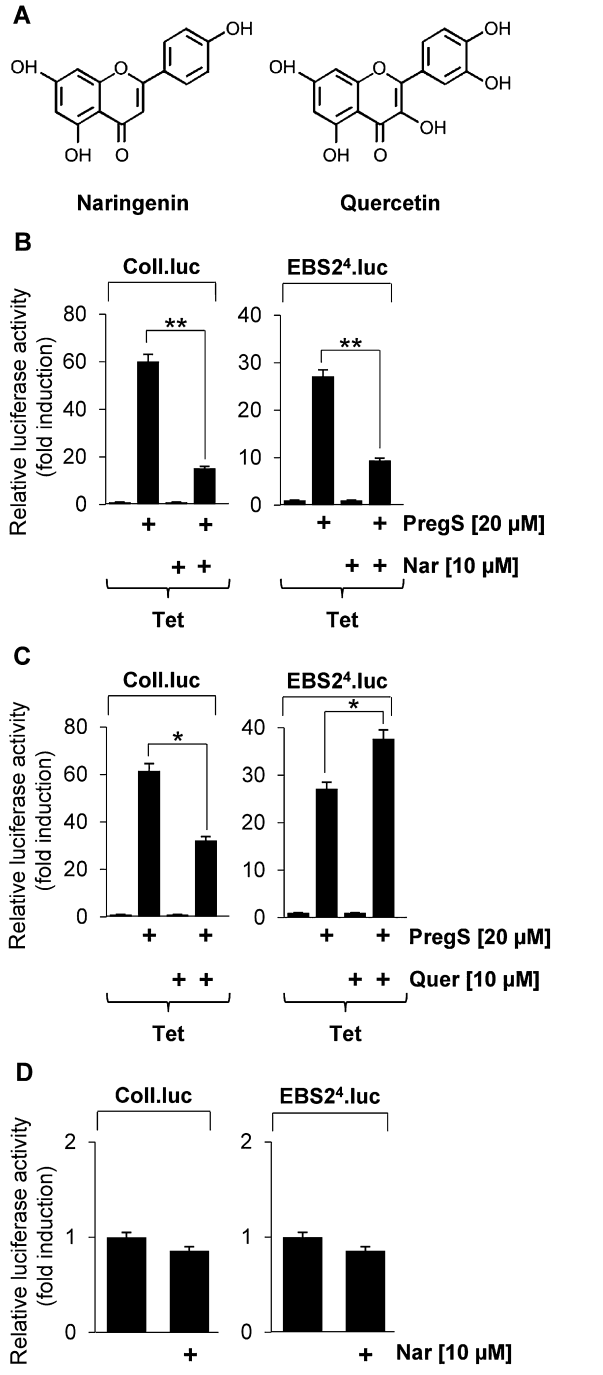

Figure 7

Regulation of AP-1 and Egr-1 activities by naringenin (Nar) and quercetin (Quer) following activation of TRPM3. (A) Naringenin, quercetin (B, C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene or the EBS2^4.luc reporter gene. The cells were serum-starved for 24 h in the presence of tetracycline (1 µg mL^{-1}) and then stimulated with pregnenolone sulfate (PregS, 20 µM) in the presence or absence of either naringenin (10 µM) or quercetin (10 µM) for 24 h. (D) The experiments were repeated in the absence of TRPM3 expression. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean ± SD, *, $P < 0.05$; **, $P < 0.01$, significantly different as indicated; $n = 4$.

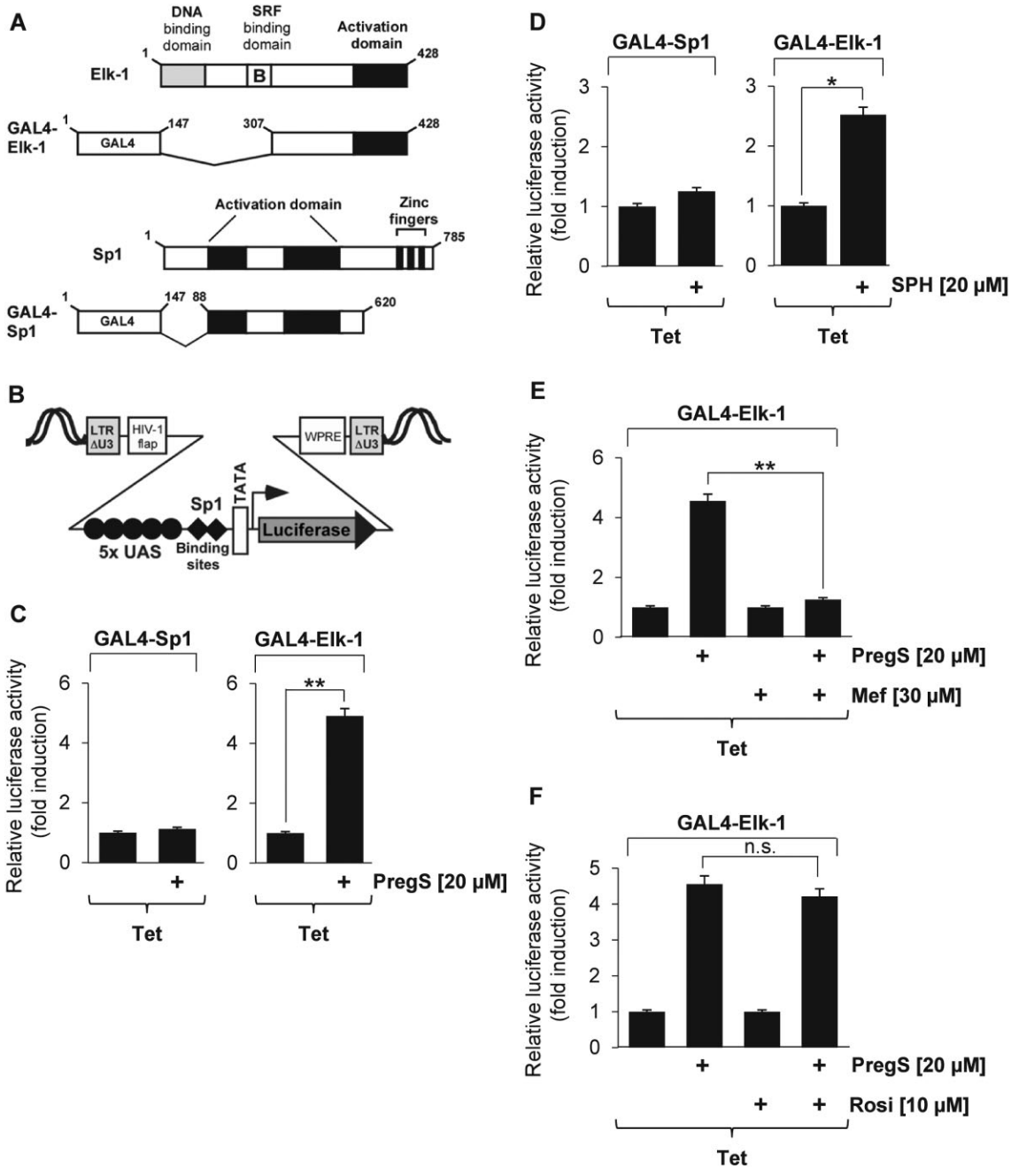

Figure 8

Up-regulation of the transcriptional activation potential of Elk-1 in pregnenolone sulfate (PregS)-stimulated HEK293 cells expressing TRPM3 channels. (A) Schematic representation of the modular structure of Elk-1, GAL4-Elk-1, Sp1, and GAL4-Sp1. (B) Schematic representation of the GAL4-responsive transcription unit. (C, D) HEK293 cells containing a tetracycline regulated transcription unit to express TRPM3 channels were infected with a lentivirus encoding a GAL4-responsive luciferase reporter gene. In addition, cells were infected with a lentivirus encoding either GAL4-Elk-1 or GAL4-Sp1. The infected cells were stimulated with tetracycline for 24h to induce TRPM3 expression. Then, cells were treated with either pregnenolone sulfate (20 μM) (C) or D-erythro-sphingosine (SPH, 20 μM) (D). (E, F) The experiment was repeated in the presence of either mefenamic acid (E) or rosiglitazone (F) as indicated. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean ± SD, *, $P < 0.05$; **, $P < 0.01$; n.s., not significant, as indicated; $n = 4$.

Elk-1 binds with its B-domain to SRF, allowing the formation of the ternary Elk-1-SRF complex. The B-domain also couples the C-terminal phosphorylation of Elk-1 with enhanced DNA binding via the Ets domain. The GAL4-Elk-1 fusion protein lacks the DNA and SRF binding domains, but retains the phosphorylation-regulated C-terminal activation domain of Elk-1. The truncated Elk-1 is expressed as a fusion protein together with the N-terminal DNA-binding domain of GAL4.

As a control, we expressed a GAL4-Sp1 fusion protein consisting of the N-terminal activation domain of Sp1 (amino acids 88–620), fused to the GAL4 DNA-binding domain (Figure 8A). The zinc finger domain of Sp1 has been deleted. Because GAL4 does not bind to any known mammalian gene promoter element, interference by other transcriptional regulatory proteins was avoided. To measure the biological activities of the GAL4-Elk-1 and GAL4-Sp1 fusion proteins we implanted a GAL4 responsive reporter gene into the chromatin of HEK293 cells expressing TRPM3 to ensure that the reporter gene is packed into an ordered nucleosomal structure. Figure 8B shows a schematic depiction of transcription unit, consisting of the luciferase reporter gene and a minimal promoter that included two Sp1 binding sites, a TATA box and an initiator element. Upstream of the minimal promoter, five GAL4 binding sites (UAS, upstream activating sequence) were inserted. HEK293 cells containing the tetracycline controlled TRPM3 transcription unit were infected with a lentivirus encoding the reporter gene together with a lentivirus that encoded either the GAL4-Elk-1 or the GAL4-Sp1 fusion protein. The results (Figure 8C, right panel) reveal that the transcriptional activation potential of Elk-1 was significantly elevated in cells expressing activated TRPM3 channels. In contrast, the transcriptional activation potential of the GAL4-Sp1 fusion protein was not changed in TRPM3 expressing cells that were stimulated with pregnenolone sulfate (Figure 8C, left panel). Treatment of the cells with D-erythro-sphingosine also activated transcription via GAL4-Elk-1 (Figure 8D), while transcription mediated by GAL4-Sp1 was not changed by the treatment of the cells with D-erythro-sphingosine (Figure 8D). The up-regulation of the transcriptional activation potential of Elk-1 in pregnenolone sulfate-stimulated TRPM3 expressing cells was almost completely inhibited by mefenamic acid, while rosiglitazone did not interfere with the activation of Elk-1 (Figures 8E,F).

The signal receptor antagonists BD1047 blockes TRPM3-regulated gene transcription

Recently, it has been shown that the sigma-1 receptor antagonist BD1047 blocked the Ca^{2+} influx via TRPM3. Whole-patch-clamp recording confirmed the inhibitory effect of BD1047 following stimulation of TRPM3 with pregnenolone sulfate (Amer *et al.*, 2012). We therefore assessed the effect of BD1047 on TRPM3-mediated gene transcription. Figure 9 shows that the up-regulation of both AP-1 and Egr-1 activities in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 was almost completely prevented.

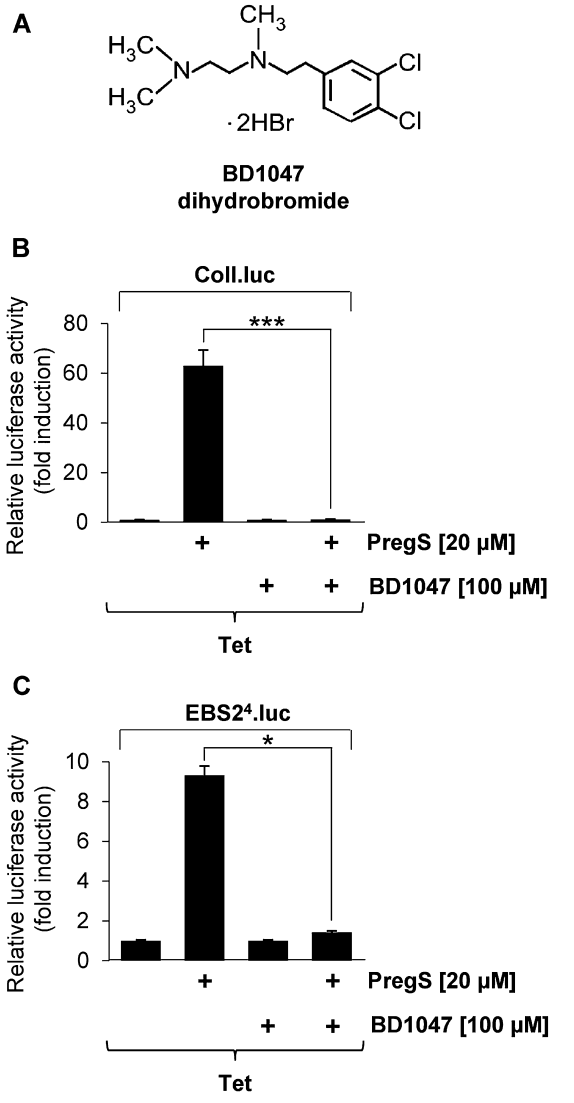

Figure 9

A sigma receptor antagonist (BD1047) interferes with TRPM3-induced gene transcription. (A) Structure of BD1047. (B, C) HEK293 cells containing a tetracycline-inducible TRPM3 transcription unit were infected with recombinant lentiviruses encoding either the collagenase promoter/luciferase reporter gene (B) or the Egr-1-responsive EBS24.luc reporter gene (C). The cells were serum-starved for 24 h in the presence of tetracycline (1 µg mL^{-1}) and then stimulated with pregnenolone sulfate (PregS, 20 µM) in the presence or absence of BD1047 (100 µM) for 24 h. Cell extracts were prepared and analysed for luciferase activities. Luciferase activity was normalized to the protein concentration. Data shown are mean ± SD, *, $P < 0.05$; ***, $P < 0.001$, significantly different as indicated; $n = 4$.

Discussion

TRPM3 channels are prominently expressed in various tissues, including kidney, liver, ovary, brain, spinal cord, pituitary, vascular smooth muscle, and testis (Grimm *et al.*, 2003; Lee *et al.*, 2003; Naylor *et al.*, 2010). TRPM3 transcripts have also been detected in beta cells of the pancreas and sensory neurons (Mayer *et al.*, 2011; Vriens *et al.*, 2011). Several biological functions have been attributed to TRPM3 channels. The analysis of TRPM3-deficient mice revealed a role for these channels in the perception of noxious heat (Vriens *et al.*, 2011), indicating their functions as thermosensitive nociceptor channels. TRPM3 channels have also been connected to the regulation of vascular smooth muscle cell contraction (Naylor *et al.*, 2010) and glutamatergic transmission of cerebellar Purkinje cells (Zamudio-Bulcock *et al.*, 2011). In addition, TRPM3 channels may function as ionotropic steroid receptors in pancreatic beta cells, regulating insulin biosynthesis and secretion (Thiel *et al.*, 2013). We

recently showed that pregnenolone sulfate stimulation of insulinoma cells is accompanied by the biosynthesis and activation of Egr-1, a transcription factor that plays an important role in controlling insulin biosynthesis, glucose homeostasis, and proliferation/cell death of pancreatic beta cells *in vivo* (Müller *et al.*, 2012b). Egr-1 induces insulin gene transcription via activation of the transcription factor Pdx-1 (Eto *et al.*, 2006; 2007; Mayer *et al.*, 2011; Müller *et al.*, 2011; 2012b), providing a link between glucose sensing and transcription of the insulin gene. In addition, the regulation of insulin secretion by TRPM3 channels has been proposed (Wagner *et al.*, 2008; Klose *et al.*, 2011). However, the fact that TRPM3-deficient mice did not show alterations in resting blood glucose levels (Vriens *et al.*, 2011) indicates that these channels play no or only a marginal role in the regulation of insulin secretion by beta cells (Thiel *et al.*, 2013) – in contrast to findings in TRPM2- and TRPM5-deficient mice who exhibited a pre-diabetic phenotype (Colsoul *et al.*, 2010; Uchida *et al.*, 2011).

The availability of TRPM3-specific pharmacological agonists and antagonists will certainly help to elucidate the biological functions of TRPM3 channels in different cell types. Using either Ca^{2+} indicators and/or whole-cell patch-clamp as an indicator for activation of TRPM3 channels, several compounds have been described to either activate or inhibit TRPM3-regulated Ca^{2+} influx. These assays reflect the activity of TRPM3 as a cation channel following activation, leading to an influx of Ca^{2+} into the cells and a rise in the intracellular Ca^{2+} concentration. Studies performed with neurons revealed that a Ca^{2+} influx is not necessarily connected with a subsequent Ca^{2+}-dependent activation of gene transcription (Deisseroth *et al.*, 1998). The regulation of noxious heat as well as a regulation of insulin biosynthesis and secretion requires, at some point, a change in the gene expression pattern of the cells. Thus, we propose that the regulation of TRPM3 channel function includes TRPM3-regulated activation of a particular set of genes, based on the activation of certain transcription factors as described (Mayer *et al.*, 2011; Müller *et al.*, 2011). Therefore, we assessed putative TRPM3 activators and inhibitors for their role in TRPM3-regulated gene transcription. We used as sensors AP-1 and Egr-1-responsive reporter genes. Both AP-1 and Egr-1 activities are up-regulated following stimulation of the cells with many extracellular signalling molecules (Al-Sarraj and Thiel, 2002, 2004; Bauer *et al.*, 2005; Stefano *et al.*, 2007; Mayer *et al.*, 2008; Rössler *et al.*, 2008; Mayer and Thiel, 2009; Rössler and Thiel, 2009; Müller *et al.*, 2010; 2012a; Thiel and Rössler, 2011; Thiel *et al.*, 2012; Kaufmann *et al.*, 2013).

Experiments involving expression of a TRPM3-specific short hairpin RNA in insulinoma cells revealed that TRPM3 channels are required to induce a Ca^{2+}-dependent gene transcription cascade in pregnenolone sulfate-stimulated insulinoma cells that were maintained in medium containing low glucose concentrations (2 mM). However, the initial Ca^{2+} influx into the cells, mediated by activation of TRPM3 channels was shown to be insufficient for induction of an intracellular signalling cascade, leading to changes in gene transcription. Rather, activation of voltage-gated Ca^{2+} channels was additionally required, as incubation of the cells with verapamil, a voltage-gated Ca^{2+} channel blocker, inhibited the signalling cascade induced by pregnenolone sulfate under these conditions (Mayer *et al.*, 2011). Based on these results, it has been proposed that stimulation of TRPM3 channels with pregnenolone sulfate induces a depolarization of the plasma membrane of insulinoma cells, leading to the activation of L-type voltage-gated Ca^{2+} channels, a further influx of Ca^{2+} into the cells and the initiation of a signalling cascade that triggers changes in the gene expression pattern of the cells.

In the present study, we employed a heterologous expression system, based on engineered HEK293 cells, which allowed us to specifically induce expression of TRPM3 channels by adding tetracycline to the culture medium. The results highlight the potent activity of pregnenolone sulfate in activation of TRPM3-mediated gene transcription. The fact that HEK293 cells are devoid of L-type voltage-gated Ca^{2+} channels (Wagner *et al.*, 2008; Majeed *et al.*, 2010) indicates that the pregnenolone sulfate-induced influx of Ca^{2+} through TRPM3 channels is sufficient to induce an intracellular signalling cascade that triggers changes of the gene expression programme of the cells in a L-type voltage-gated Ca^{2+} channel-independent manner.

The stimulation of HEK293 cells expressing TRPM3 channels with nifedipine did not activate AP-1 or Egr-1-controlled gene transcription. This observation indicates that a Ca^{2+} influx into the cells, as has been reported for cells expressing TRPM3 channels (Wagner *et al.*, 2008; Majeed *et al.*, 2011), does not necessarily imply a Ca^{2+}-dependent change in gene transcription. Treatment with D-erythro-sphingosine increased the AP-1 and Egr-1 activities in HEK293 cells, but in the absence of TRPM3 channels. Together, these data indicate that pregnenolone sulfate is the compound of choice to activate TRPM3-mediated Ca^{2+} influx and gene transcription. Accordingly, pregnenolone sulfate has already been successfully used in the analysis of TRPM3 channel function in pancreatic beta cells and sensory neurons (Wagner *et al.*, 2008; Vriens *et al.*, 2011).

The compounds progesterone, mefenamic acid, rosiglitazone and naringenin inhibit TRPM3-mediated influx of Ca^{2+} ions into the cells (Naylor *et al.*, 2010; Klose *et al.*, 2011; Majeed *et al.*, 2011; 2012; Straub *et al.*, 2013). Our analysis showed that mefenamic acid is a powerful inhibitor of TRPM3-mediated gene transcription. The use of mefenamic acid may supplement experiments involving TRPM3-specific shRNAs blocking expression of TRPM3 channels (Wagner *et al.*, 2008; Mayer *et al.*, 2011). The other tested agents, progesterone, rosiglitazone or naringenin, reduced AP-1 activity in pregnenolone sulfate-stimulated HEK293 cells expressing TRPM3 channels, but the inhibitory effect on AP-1 activity was much less pronouced than obtained in mefenamic acid treated cells. Moreover, the pregnenolone sulfate-induced up-regulation of Egr-1 activity was not impaired by the treatment of the cells with either progesterone, or rosiglitazone. In contrast to pregnenolone sulfate which activates TRPM3 channels only from the extracellular side (Wagner *et al.*, 2008), progesterone, rosiglitazone and naringenin may have profound effects in the cell that may interfere with the signalling cascade that connects activation of TRPM3 channels with enhanced AP-1 or Egr-1-regulated gene transcription. In this context, it would be of interest to elucidate the molecular mechanism used by mefenamic acid to inhibit TRPM3 channels.

This study showed that the transcriptional activation potential of the transcription factor Elk-1 is enhanced in HEK293 cells on stimulation of TRPM3 channels. Elk-1 belongs to the TCF family of transcription factors that play a key role in stimulus-transcription coupling. The TCF proteins are substrates for many protein kinases and function as a molecular convergence point of cellular signalling cascades to convert the stimulus-induced signalling cascades into a change in gene transcription (Shaw and Saxton, 2003). The fact that stimulation of TRPM3 channels increases the transcriptional activity of Elk-1 suggests that these channels interfere with key signalling pathways in the cells.

Additionally, we have shown in this study that the sigma-1 receptor antagonist BD1047 prevented the up-regulation of AP-1 and Egr-1 following stimulation of TRPM3 channels. This compound was as powerful as mefenamic acid in inhibiting TRPM3-mediated gene transcription. However, BD1047 is not a specific TRPM3 inhibitor, as this compound also inhibits histamine-induced Ca^{2+} entry in endothelial cells and it has been suggested that it acts directly on Ca^{2+} channels (Amer *et al.*, 2012). The molecular mechanism of how BD1047 interferes with different types of Ca^{2+} channels needs to be elucidated.

In summary, we present here the first comparative analysis of putative activators or inhibitors of TRPM3 channels on TRPM3-regulated gene transcription. Using transcriptional assays as an indication for TRPM3 activation or inhibition, we infer that the initial Ca^{2+} signal, induced by activation of TRPM3 channels, is sufficient to trigger an intracellular signalling cascade to the nucleus and, eventually, to change the transcription of a particular set of genes. Our results also revealed that a rise in intracellular Ca^{2+}, by itself, is not enough to alter gene transcription. The experiments show that pregnenolone sulfate is a powerful activator of AP-1 and Egr-1-regulated gene transcription via TRPM3. In contrast, mefenamic acid is the inhibitor of choice for TRPM3 channels, because this compound efficiently inhibits both Ca^{2+} influx and gene transcription following activation of TRPM3 channels.

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Conflict of interest

None declared

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