Pregnenolone Sulfate Activates Basic Region Leucine Zipper Transcription Factors in Insulinoma Cells: Role of Voltage-Gated Ca\(^{2+}\) Channels and Transient Receptor Potential Melastatin 3 Channels

Isabelle Müller, Oliver G. Rössler, and Gerald Thiel

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

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ABSTRACT

The neurosteroid pregnenolone sulfate activates a signaling cascade in insulinoma cells involving activation of extracellular signal-regulated protein kinase and enhanced expression of the transcription factor Egr-1. Here, we show that pregnenolone sulfate stimulation leads to a significant elevation of activator protein-1 (AP-1) activity in insulinoma cells. Expression of the basic region leucine zipper (bZIP) transcription factors c-Jun and c-Fos is up-regulated in insulinoma cells and pancreatic β-cells in primary culture after pregnenolone sulfate stimulation. Up-regulation of a chromatin-embedded c-Jun promoter/luciferase reporter gene transcription in pregnenolone sulfate-stimulated insulinoma cells was impaired when the AP-1 binding sites were mutated, indicating that these motifs function as pregnenolone sulfate response elements. In addition, phosphorylation of cAMP response element (CRE)-binding protein is induced and transcription of a CRE-controlled reporter gene is stimulated after pregnenolone sulfate treatment, indicating that the CRE functions as a pregnenolone sulfate response element as well. Pharmacological and genetic experiments revealed that both L-type Ca\(^{2+}\) channels and transient receptor potential melastatin 3 (TRPM3) channels are essential for connecting pregnenolone sulfate stimulation with enhanced AP-1 activity and bZIP-mediated transcription in insulinoma cells. In contrast, pregnenolone sulfate stimulation did not enhance AP-1 activity or c-Jun and c-Fos expression in pituitary corticotrophs that express functional L-type Ca\(^{2+}\) channels but only trace amounts of TRPM3. We conclude that expression of L-type Ca\(^{2+}\) channels is not sufficient to activate bZIP-mediated gene transcription by pregnenolone sulfate. Rather, additional expression of TRPM3 or depolarization of the cells is required to connect pregnenolone sulfate stimulation with enhanced gene transcription.

Introduction

Steroids, including progesterone, pregnenolone, pregnenolone sulfate, and dehydroepiandrosterone, that are synthesized in the central and peripheral nervous system, independent, at least in part, of steroidogenic gland secretion, are termed neurosteroids. In the nervous system, neurotransmission, receptor function, and the strength of synaptic transmission is modified by pregnenolone sulfate stimulation (Baulieu, 1998). Intracerebral infusion of pregnenolone sulfate was shown to influence cognitive processes, neuronal survival, and neurogenesis (Mayo et al., 2001; Charalampopoulos et al., 2008). In pancreatic β cells and insulinoma cells, pregnenolone sulfate triggers a rapid Ca\(^{2+}\) influx into the cells, leading to enhanced insulin secretion (Wagner et al., 2008). A comprehensive analysis of the signaling pathway induced by pregnenolone sulfate in insulinoma cells revealed that influx of Ca\(^{2+}\) ions via TRPM3 and/or L-type voltage-gated Ca\(^{2+}\) channels, elevation of the cytosolic Ca\(^{2+}\) level and activation of ERK leads to enhanced biosynthesis of the transcription factor Egr-1 and the transcription of Egr-1 target genes (Mayer et al., 2011). These data showed that pregnenolone sulfate induces a signaling cascade in insulinoma cells that is very similar to the signaling cascade induced by glucose in β cells.

ABBREVIATIONS: TRPM3, transient receptor potential melastatin 3; ERK, extracellular-signal-regulated kinase; AP-1, activator protein 1; bZIP, basic region leucine zipper; TRE, 12-O-tetradecanoylphorbol-13-acetate–responsive element; CRE, cAMP response element; DMSO, dimethyl sulfoxide; FPL64178, 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-1H-pyrrole-3-carboxylic acid methyl ester; HDAC1, histone deacetylase-1; RT-PCR, reverse transcription-polymerase chain reaction; rRNA, ribosomal RNA; SRE, serum response element; CREB, cyclic AMP response element binding protein; LTP, long-term potentiation; SRF, serum response factor.
Elevated levels of glucose have a profound effect on gene transcription. In particular, the activity of the transcription factor activator protein-1 (AP-1) is up-regulated in glucose-stimulated insulinoma cells (Glauser et al., 2007; Müller et al., 2010). We sought to determine whether pregnenolone sulfate stimulation also increases AP-1 activity and basic region leucine zipper (bZIP)-mediated transcription in insulinoma cells and β cells of the pancreas. Here, we describe the first analysis of pregnenolone sulfate-induced activation of bZIP transcription factors. We show that pregnenolone sulfate stimulation up-regulates the biosynthesis of c-Jun and c-Fos in insulinoma cells and β cells of the pancreas, leading to enhanced AP-1 activity. We identified the phorbol 12-O-tetradecanoylphorbol-13-acetate-responsive element (TRE) and the cAMP response element (CRE) as pregnenolone sulfate response elements. We further addressed the role of L-type Ca²⁺ channels and TRPM3 channels within the signaling cascade that connects pregnenolone stimulation with enhanced bZIP activity and bZIP-regulated transcription.

Lentiviral Expression of Short Hairpin RNAs. The lentiviral vector pLentiLox3.7 (pL3.7) was purchased from the American Type Culture Collection (Manassas, VA). The lentiviral transfer vector pLLTRPM3, used to knock down TRPM3 expression, has been described previously (Mayer et al., 2011).

Reporter Assays. The lentiviral transfer vectors pFWColl-luc and pFWColl-lucΔTRE have been described elsewhere (Rössler et al., 2008; Müller et al., 2010). Plasmids −1600/+1700JUNCAT5 and −1600/+1702TREAPICAT-5 were kind gifts of P. Angel, (Deutsches Krebsforschungszentrum, Heidelberg, Germany) (Angel et al., 1988). The wild-type and mutated c-Jun promoter was cloned as BglII fragments into a lentiviral transfer vector, generating plasmid pFWc-Jun luc and pFWc-JunlucΔTRE. Plasmid phfos-luc, containing c-Fos gene promoter sequences from −711 to +42, was obtained from Klaus Roemer (José Carreras Forschungszentrum der Universitätskliniken des Saarlandes, Homburg, Germany). The promoter was cloned into a lentiviral transfer vector upstream of the luciferase gene. Plasmid pcFosCREluc, which contains four copies of the CRE derived from the c-Fos gene upstream of the HIV LTR TATA box and the adenovirus late promoter initiator element, has been described elsewhere (Thiel et al., 2005). The regulatory region, including the CREs, the TATA box, and the initiator sequence, was cut out and cloned into a lentiviral transfer vector, generating plasmid pFWc-FosCreΔTREluc. The lentiviral transfer vector pFWU6luc, encoding luciferase under the control of the human ubiquitin C promoter, was constructed by inserting the coding region for luciferase into the plasmid pcDNA3.1+ U6. The vector pCMVglovLacZΔ1 was kindly provided by Klaus Roemer (Jose Carreras Forschungszentrum der Universitätskliniken des Saarlandes, Homburg, Germany) and analyzed for luciferase activities as described previously (Thiel et al., 2000). Luciferase activity was normalized to the protein concentration.

Western Blot Analysis. Whole-cell extracts, nuclear extracts, and crude membranes were prepared as described previously (Kaufmann and Thiel, 2002). Proteins were separated by SDS-polyacrylamide gel electrophoresis, blotted, and incubated with antibodies directed against either c-Jun (Santa Cruz Biotechnology, Heidelberg, Germany), the phosphorylated form of c-Jun (Millipore, Billerica, MA), c-Fos (Santa Cruz Biotechnology), CREB (Millipore), or the phosphorylated form of CREB (Millipore). The antibody directed against histone deacetylase-1 (HDAC1) was used as a loading control as described previously (Spohn et al., 2010; Mayer et al., 2011). To detect FLAG-tagged proteins, we used the M2 monoclonal antibody directed against the FLAG epitope (Sigma-Aldrich) at 1:3000 dilution. Immunoreactive bands were detected via enhanced chemiluminescence as described previously (Spohn et al., 2010; Mayer et al., 2011). Values are expressed as the mean ± S.D. from three independent experiments.

RT-PCR. RT-PCR was performed as described previously (Bauer et al., 2007). Primers specific for detection of TRPM3 mRNA, termed 9-12, have been published (Wagner et al., 2008). To monitor Cav1.2 expression, we used the primer pair 5′-GGA GCT GGA CAA GGC TAT GA-3 and 5′-GAC CTA GAG AGG CAG AGC GA-3′ in the PCR. Cav1.3 expression was detected in RT-PCR experiments using the primers 5′-GAC TCA GAT TAC AAC CCA GG-3′ and 5′-GTT GTG TTC TTC GCA GGG TA-3′. Quantitative real-time PCR was performed using SYBR Green chemistry and gene-specific primers on a Stratagene MX3000P system. The primers 5′-AGA GGC GTG CCT ACG GCT ACA GTA A-3′ and 5′-CAA GAA CTC GGA CCT TCT CAC CTC G-3′ were used to detect c-Jun mRNA. The primers 5′-ACC ATG ATG TTC TCG GGT TCT AA-3′ and 5′-GCT GGT GGA GAT GGC TGT CAC-3′ were used to detect c-Fos mRNA. The primers used to detect 18S rRNA transcripts were described recently (Mayer et al., 2011). Total RNA isolated from islet cells was purified with the RNeasy Plus Micro Kit (QIAGEN, Valencia, CA). RNA samples were...
reverse transcribed into cDNA with RevertAid Moloney murine leukemia virus RT (Fermentas GmbH, St. Leon-Rot, Germany) in the presence of RNase Inhibitor (Fermentas GmbH). The PCR conditions were as follows: one cycle at 95°C for 10 min followed by 40 amplification cycles, each with denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, and extension at 72°C for 30 s. Gene expression values were calculated based on the comparative threshold cycle (Cq) method as described in Applied Biosystems User Bulletin 2 (http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf), normalized to the expression of 18S rRNA, and displayed as fold induction relative to unstimulated cells.

**Statistics.** Statistical analysis were done by using the two-tailed Student’s t test. Data shown are mean ± S.D. from four independent experiments. Statistical probability is expressed as P < 0.05, P < 0.01, and P < 0.001. Values were considered significant when P < 0.05.

**Results**

**Up-Regulation of AP-1 Activity in Pregnenolone Sulfate-Stimulated Insulinoma Cells.** Glucose stimulation preferentially activates gene transcription of those genes that are regulated by the transcription factor AP-1 (Glauser et al., 2007). We recently confirmed that glucose stimulation triggers an increase in AP-1 activity in insulinoma cells (Müller et al., 2010). To measure AP-1 regulated transcription in pregnenolone sulfate-stimulated cells, we implanted a collagenase promoter/luciferase reporter gene into the chromatin of INS-1 insulinoma cells. The human collagenase promoter contains an AP-1 binding site in the proximal promoter region and therefore frequently has been used to monitor AP-1 activity (Angel et al., 1987; Steinmüller et al., 2001). A schematic depiction of the integrated provirus encoding the collagenase promoter/luciferase reporter gene is seen in Fig. 1A, including the sequence of the wild-type TRE and the mutated TRE. INS-1 cells that had been infected with a lentivirus encoding the collagenase promoter/luciferase reporter gene were serum-starved for 24 h in medium containing 2 mM glucose. Cells were stimulated with either pregnenolone sulfate, pregnenolone, or progesterone for 24 h. Figure 1B shows that pregnenolone sulfate stimulation enhanced transcription mediated by AP-1, whereas treatment of the cells with either pregnenolone or progesterone had no or only marginal effect upon transcription of the collagenase promoter/luciferase reporter gene. To show the importance of the TRE within the collagenase promoter, we mutated the TRE sequence 5'-TGAGTCA-3' to 5'-TGATAGT-3'. Figure 1C shows that stimulation of reporter gene transcription by preg-

**Fig. 1.** Up-regulation of AP-1 activity in pregnenolone sulfate-stimulated insulinoma cells. A, schematic representation of the integrated provirus encoding a collagenase promoter/luciferase reporter gene. The promoter fragment was inserted upstream of the luciferase reporter gene. The location and sequence of the wild-type and mutated TRE within the collagenase promoter is depicted. LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. B, INS-1 insulinoma cells cultured in medium containing 2 mM glucose were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for 24 h and then stimulated with either pregnenolone sulfate (PregS; 50 μM), pregnenolone (Preg; 50 μM), or progesterone (Prog; 50 μM) for 24 h. C, INS-1 cells were infected with recombinant lentiviruses encoding a collagenase promoter/luciferase reporter gene with an intact or a mutated TRE. The cells were serum-starved for 24 h and then stimulated with PregS (50 μM) for 24 h. D, INS-1 cells were infected with recombinant lentiviruses encoding an ubiquitin C promoter/luciferase reporter gene. The cells were serum-starved for 24 h and then stimulated with PregS (50 μM) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration (***, P < 0.001).
Pregnenolone sulfate was strikingly reduced when the TRE was mutated. These experiments reveal that the TRE within the collagenase promoter is of major importance for responsiveness to pregnenolone sulfate. Despite some experimental variations in the fold-induction of AP-1 mediated transcription, these results indicate that pregnenolone sulfate stimulation alters the genetic program of INS-1 cells by activating genes controlled by the transcription factor AP-1. In contrast, transcription of a reporter gene under the control of the ubiquitin C promoter was not enhanced in pregnenolone sulfate-stimulated INS-1 cells (Fig. 1D). Moreover, we show that serum in general and EGF in particular activated transcription of an integrated collagenase promoter/luciferase reporter gene (Supplemental Fig. 1). Thus, cells had to be cultured in serum-free medium to measure the effect of pregnenolone sulfate stimulation upon gene transcription.

**Up-Regulation of c-Jun and c-Fos Expression in Pregnenolone Sulfate-Stimulated Insulinoma Cells and Pancreatic β Cells.** The AP-1 transcription factor was originally described as a heterodimer of c-Jun and c-Fos (Chiu et al., 1988). These bZIP transcription factors dimerize via their leucine zipper domains, which in turn bring together their basic domains to bind DNA in a sequence-specific manner. We therefore analyzed expression and biological activity of c-Jun and c-Fos in pregnenolone sulfate-stimulated insulinoma cells. Figure 2A shows that stimulation with pregnenolone sulfate increased the biosynthesis of c-Fos and c-Jun. c-Jun is a substrate for c-Jun N-terminal protein kinases and phosphorylation of c-Jun is required for activation of the transcriptional activation potential of c-Jun. Figure 2A shows that stimulation of insulinoma cells with pregnenolone sulfate induced the phosphorylation of c-Jun, indicating that c-Jun was activated as a result of stimulation.

To show that pregnenolone sulfate also activates c-Jun and c-Fos expression in primary cultured cells, we isolated pancreatic islets and kept the cells in short-term culture. Cells were stimulated with pregnenolone sulfate (50 μM) for 1 h. RNA was isolated and reverse-transcribed, and gene expression was monitored using quantitative real-time PCR. Figure 2B shows that stimulation with pregnenolone sulfate increased the c-Jun and c-Fos mRNA concentrations.

**Enhanced c-Jun and c-Fos Promoter Activity in Pregnenolone Sulfate-Stimulated Insulinoma Cells.** The previous results were corroborated by an analysis of a chromosomally embedded c-Jun promoter luciferase reporter gene. A schematic depiction of the integrated provirus encoding the c-Jun promoter/luciferase reporter gene is seen in Fig. 3A. The c-Jun promoter contains two TRE-like motifs termed jun1TRE and jun2TRE. Figure 3B shows that stimulation of insulinoma cells with pregnenolone sulfate significantly increased c-Jun promoter controlled reporter gene transcription. However, up-regulation of an integrated c-Jun promoter/luciferase reporter, where both TRE-like motifs had been inactivated by mutation, was completely abolished, indicating that the TREs within the c-Jun promoter function as pregnenolone sulfate response elements.

Next, we inserted a c-Fos promoter/luciferase reporter gene into the chromatin of insulinoma cells using lentiviral gene transfer (Fig. 3C). Stimulation with pregnenolone sulfate significantly increased transcription of a chromosomally embedded c-Fos promoter/reporter gene, as shown in Fig. 3D.

**The SRE and the CRE Function As Pregnenolone Sulfate Response Elements within the Human c-Fos Promoter.** The c-Fos promoter contains a CRE and a serum response element (SRE) as depicted in Fig. 3C. We expressed dominant-negative mutants of Elk-1 and CREB to clarify the impact of the CRE and the SRE for the pregnenolone sulfate-responsiveness of the c-fos gene. We assessed the impact of the SRE on the regulation of c-Fos promoter activity in INS-1 cells using a dominant-negative mutant of the ternary complex factor Elk-1 termed REST/Elk-1C (Fig. 3E). This mutant retains the DNA-binding and SRF interaction domains but lacks the C-terminal activation domain of Elk-1. REST/Elk-1C additionally contains the N-terminal repression domain of the transcriptional repressor REST, a FLAG epitope for immunological detection and a nuclear localization signal. The role of CREB in the regulation of c-Fos expression was assessed using a dominant-negative mutant of CREB termed REST/CREB (Fig. 3E). This mutant retains the bZIP domain
of CREB but lacks the activation domains. REST/CREB additionally contains the N-terminal repression domain of REST, a FLAG epitope and a nuclear localization signal. Nuclear proteins of mock-infected INS-1 cells or INS-1 cells infected with either a REST/Elk-1ΔC- or a REST/CREB-encoding lentivirus were fractionated by SDS-PAGE and the fusion protein was identified by Western blot analysis using antibodies targeting the FLAG epitope (Fig. 3F). Next, we assessed the functional effects of REST/Elk-1ΔC and REST/CREB expression upon the pregnenolone sulfate-induced activity of the c-Fos promoter. Fig. 3, G and H, shows that expression of both REST/Elk-1ΔC and REST/CREB abolished the up-regulation of reporter gene transcription in pregnenolone sulfate-stimulated insulinoma cells, indicating that both the CRE and the SRE contribute to the pregnenolone sulfate responsiveness of the c-fos gene.

Phosphorylation of CREB and Stimulation of CRE-Regulated Transcription in Pregnenolone Sulfate-Stimulated Insulinoma Cells. Pregnenolone sulfate induces a signaling cascade in insulinoma cells that is very similar to that induced by glucose in β cells (Mayer et al., 2011). We have shown that the bZIP protein CREB is phosphorylated in glucose-stimulated insulinoma cells (Mayer and Thiel, 2009). We therefore assessed whether CREB is also phosphorylated in insulinoma cells that had been stimulated with pregnenolone sulfate. Figure 4A shows that treatment of the cells with pregnenolone sulfate leads to a significant phosphorylation of CREB. However, CREB phosphorylation is not a reliable predictor of CREB target gene activation (Zhang et al., 2005). To directly measure CREB-mediated gene transcription, we implanted a model reporter gene into the chromatin of insulinoma cells that contained four copies of the CRE derived from the c-fos gene upstream of a minimal promoter (Fig. 4B). In this transcription unit, only the CREs regulate activation of transcription. Transcription of this CRE-controlled reporter gene was significantly stimulated in pregnenolone sulfate-treated insulinoma cells (Fig. 4C).

TRPM3 Is Required for Pregnenolone Sulfate-Stimulated Up-Regulation of c-Jun and c-Fos Expression in Insulinoma Cells. We recently showed that stimulation of TRPM3 channels is required for pregnenolone sulfate-induced Egr-1 expression in insulinoma cells cultured in low-glucose medium (2 mM) (Mayer et al., 2011). In contrast, when the cells were cultured in medium containing 11 mM glucose, TRPM3 activation was not involved in the signaling cascade that leads to the biosynthesis of Egr-1 as a result of pregnenolone sulfate stimulation. Here, we assessed the role of TRPM3 in the regulation of bZIP-mediated gene transcription in insulinoma cells cultured in medium that contained 2 mM glucose. To assess the involvement of TRPM3 channel activation, a TRPM3-specific shRNA was expressed using lentiviral gene transfer. The high expression rate of a TRPM3-specific shRNA in insulinoma cells after lentiviral infection has recently been demonstrated (Mayer et al., 2011), allowing biochemical analysis of the cells that expressed the TRPM3-specific shRNA. Figure 5A shows that pregnenolone sulfate-stimulated c-Jun promoter/luciferase reporter gene transcription was completely blocked in insulinoma cells expressing an shRNA directed against TRPM3. These data were corroborated by Western blot analysis showing that the up-regulation of c-Jun expression was impaired in cells that expressed the TRPM3-specific shRNA (Fig. 5B). Likewise, transcription of a c-Fos promoter/luciferase reporter gene and enhanced c-Fos expression was impaired in pregnenolone sulfate-treated insulinoma cells that expressed a TRPM3-specific shRNA (Fig. 5, C and D). In contrast, the knock-down of TRPM3 did not influence the up-regulation of AP-1 activity, measured as fold activation of a collagenase promoter/luciferase reporter gene, in insulinoma cells that have been stimulated with either glucose or KCl/FPL64176 (Fig. 5, E and F).

TRPM3 Is Required for Pregnenolone Sulfate-Induced Transcription of a CRE-Controlled Reporter Gene in Insulinoma Cells. Figure 5G shows that the up-regulation of CRE-mediated gene transcription as a result of pregnenolone sulfate-stimulation was similarly blocked in insulinoma cells expressing a TRPM3-specific shRNA. We conclude that TRPM3 expression is required for the up-regulation of c-Jun and c-Fos promoter activity, the enhanced transcription of a CRE-controlled reporter gene, as well as for the stimulation of c-Fos and c-Jun expression in pregnenolone sulfate-treated INS-1 insulinoma cells cultured in medium containing low glucose concentrations.

Role of L-Type Voltage-Gated Ca2+ Channels in the Transcriptional Response to Pregnenolone Sulfate Stimulation. Activation of L-type voltage-dependent Ca2+ channels is necessary for glucose signaling in pancreatic β cells. In neurons, pregnenolone sulfate activates L-type voltage-gated Ca2+ channels (Chen and Sokabe, 2005; Hige et al., 2006; Chen et al., 2010). We showed recently that L-type voltage-gated Ca2+ channels are required for the pregnenolone sulfate-triggered up-regulation of Egr-1 biosynthesis in insulinoma cells cultured in medium containing 11 mM glucose (Mayer et al., 2011). Here, we used a pharmacological approach to assess the involvement of L-type voltage-gated Ca2+ channels in pregnenolone sulfate-stimulated transcription in insulinoma cells cultured in low glucose medium. Incubation of the cells with the L-type voltage-gated Ca2+ channel blocker verapamil completely blocked the transcriptional up-regulation of reporter gene transcription controlled by the collagenase, c-Jun, or c-Fos promoters, respectively (Fig. 6, A–C). In addition, pregnenolone sulfate-induced up-regulation of a CRE-controlled reporter gene was completely blocked by preincubation of the cells with verapamil (Fig. 6D). As a control, we show that verapamil treatment naturally blocked the transcription of a collagenase promoter/luciferase reporter gene in insulinoma cells that had been stimulated with KCl and FPL64176 (Fig. 6E). Together, these data indicate that in INS-1 insulinoma cells cultured in medium containing 2 mM glucose, L-type voltage-gated Ca2+ channels are required for the pregnenolone sulfate-triggered up-regulation of reporter gene transcription controlled by either the collagenase promoter, the c-Jun promoter, the c-Fos promoter, or multiple CRE motifs. Thus, both TRPM3 and L-type voltage-gated Ca2+ channels are necessary to connect pregnenolone sulfate stimulation with enhanced transcription.

Expression of TRPM3 and L-Type Voltage-Gated Ca2+ Channels in Islets, Insulinoma Cells, and Corticotrophs. To define the role of either TRPM3 or L-type voltage-gated Ca2+ channels, we searched for a cell line that expresses L-type voltage-gated Ca2+ channels but lacks expression of TRPM3 Ca2+ channels. RT-PCR experiments us-
Stimulation with pregnenolone sulfate up-regulates c-Jun and c-Fos promoter activity in insulinoma cells. A and B, transcriptional up-regulation of c-Jun promoter/luciferase reporter genes in pregnenolone sulfate-stimulated INS-1 cells. A, schematic representation of the integrated provirus encoding a c-Jun promoter/luciferase reporter gene. The location of the two TRE-like motifs is depicted. Both TREs are mutated in the c-Jun promoter ΔTRE/luciferase reporter gene. LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. B, INS-1 cells were infected with a recombinant lentivirus encoding c-Jun promoter/luciferase reporter genes containing intact or mutated TREs. The infected cells were treated with pregnenolone sulfate (PregS; 50 μM) for 24 h as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. C and D, transcriptional up-regulation of a c-Fos promoter/luciferase reporter gene in pregnenolone sulfate-stimulated INS-1 cells. C, schematic representation of integrated provirus encoding a c-Fos promoter/luciferase reporter gene. The CRE and the SRE are depicted. D, INS-1 cells were infected with a recombinant lentivirus encoding a c-Fos promoter/luciferase reporter gene. The infected cells were treated with PregS (50 μM) for 24 h as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. E, schematic representation of wild-type Elk-1 and CREB and the dominant-negative mutants REST/Elk-1 and REST/CREB. The DNA binding domain of Elk-1 is located on the N terminus. The transcriptional activation domain is located on the C terminus. A regulatory domain lies within this transcriptional activation domain encompassing the key phosphoacceptor sites Ser383 and Ser389. 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 dominant-negative mutant REST/Elk-1 lacks the phosphorylation-regulated activation domain, but retains the DNA and SRF binding domains. The phosphorylation-dependent transcriptional activation domain of CREB [KID (kinase-inducible domain)] is depicted. The bZIP domain is located on the C terminus. The dominant-negative mutant REST/CREB lacks the KID domain but retains the DNA and dimerization domains. Both mutants are expressed as a fusion protein together with a transcriptional repression domain derived from the transcriptional repressor REST. NLS, nuclear localization signal.
ing specific primers showed that TRPM3 and the L-type voltage-gated Ca\(^{2+}\) channel subtypes Cav1.2 and Cav1.3 are expressed in islets and in INS-1 insulinoma cells (Fig. 7, A and B). In contrast, AtT20 corticotrophs expressed Cav1.2 and Cav1.3 but did not express TRPM3 under these conditions (Fig. 7C). A faint band was visible in a PCR involving 35 amplification cycles, indicating that AtT20 cells express trace amounts of TRPM3. We used AtT20 cells to answer the question of whether expression of Cav1.2 and Cav1.3 is sufficient to activate transcription after pregnenolone sulfate-stimulation. Figure 8A shows that AtT20 cells expressed functional L-type voltage-gated Ca\(^{2+}\) channels. Stimulation of the cells with KCl/FPL64176 increased transcription of a chromatin-embedded collagenase promoter/luciferase reporter gene, indicating that the cells contained higher AP-1 activity under these conditions. The up-regulation of AP-1 activity was blocked by preincubation of the cells with verapamil, confirming that stimulation of L-type voltage-gated Ca\(^{2+}\) channels was responsible for this effect. In contrast, stimulation of the cells with pregnenolone sulfate did not significantly increase AP-1 activity. Thus, expression of only L-type voltage-gated Ca\(^{2+}\) channels is not sufficient to obtain a transcriptional response after pregnenolone sulfate stimulation. These results were corroborated by a Western blot analysis. Figure 8B shows that pregnenolone sulfate stimulation fails to induce c-Fos and c-Jun expression in AtT20 corticotrophs, whereas activation of L-type voltage-gated Ca\(^{2+}\) channels by KCl/FPL64176-stimulation induced the biosynthesis of c-Fos and c-Jun.

**Discussion**

The neurosteroid pregnenolone sulfate acts on the nervous system by regulating neurotransmission and receptor function, leading to changes in synaptic strength, neuronal survival, and neurogenesis. We showed recently that pregnenolone sulfate regulates gene transcription in insulinoma cells and pancreatic islets. In particular, pregnenolone sulfate stimulation triggers the expression of the zinc finger transcription factor Egr-1 and the transcription of Egr-1-responsive target genes (Mayer et al., 2011). In addition, pregnenolone sulfate induces a signaling cascade in insulinoma cells that is very similar to the signaling cascade induced by glucose in \(\beta\) cells. Elevated levels of glucose have a profound effect on gene transcription and several transcription factors have been identified to be activated in glucose-stimulated \(\beta\) cells, including Egr-1 (Frödin et al., 1995; Jøsefsen et al., 1999; Bernal-Mizrachi et al., 2000; Mayer and Thiel, 2009), CREB (Wang et al., 2008; Mayer and Thiel, 2009), Elk-1 (Mayer and Thiel, 2009; Bernal-Mizrachi et al., 2001), and c-Fos (Glauser and Schlegel, 2007). An important role for the transcription factor AP-1 in insulinoma cells has been proposed, based on the fact that a microarray analysis revealed a significant over-representation of genes containing AP-1 binding sites in their regulatory regions (Glauser et al., 2007). We showed recently that AP-1 activity is significantly elevated in glucose-treated INS-1 cells (Müller et al., 2010). The objective of this study was to investigate the regulation of AP-1 and bZIP-controlled transcription in pregnenolone sulfate-stimulated insulinoma cells and pancreatic islets.

To measure AP-1 transcriptional activity, we used an AP-1-responsive collagenase promoter/luciferase reporter gene. Reporter genes are often introduced into cultured cells via transient transfection of plasmids. This approach has the disadvantage that the structure of these plasmids may be incompletely organized compared with cellular chromatin (Smith and Hager, 1997) and may thus resemble a prokaryotic gene organization, including a nonrestrictive transcriptional ground state. In contrast, the chromatin structure in eukaryotes causes a restrictive ground state, occluding proteins such as RNA polymerases and transcriptional regulators from binding to DNA. Hence, promoter/reporter genes should be integrated into the chromatin to investigate transcriptional regulatory mechanisms. We therefore used a lentivirus-based technique to implant the reporter gene into the chromatin of insulinoma cells or corticotrophs. This strategy enabled us to analyze AP-1-mediated gene transcription of the collagenase promoter/luciferase reporter gene that was packed into an ordered chromatin structure. Using this strategy, we demonstrated that pregnenolone sulfate stimulation triggered an up-regulation of AP-1 activity in INS-1 insulinoma cells. Initially, AP-1 was described as a heterodimer of c-Jun and c-Fos, and we could show that expression of c-Jun and c-Fos is up-regulated in insulinoma cells and pancreatic islets after pregnenolone sulfate stimulation. Likewise, enhanced transcription of chromatin-embedded c-Jun promoter/luciferase and c-Fos promoter/luciferase reporter genes were monitored as a result of pregnenolone sulfate stimulation. Mutation of the two AP-1 like elements within the c-Jun promoter abolished pregnenolone sulfate responsiveness, indicating that these genetic elements function as pregnenolone sulfate response elements.

Stimulation of insulinoma cells with glucose leads to the phosphorylation of CREB (Wang et al., 2008; Mayer and Thiel, 2009), making it likely that pregnenolone sulfate stimulation has a similar effect. Moreover, phosphorylation of CREB has been observed in pregnenolone sulfate-treated slices from the hippocampal dentate gyrus (Chen et al., 2007). This study showed that CREB is phosphorylated in insulinoma cells that had been treated with pregnenolone sulfate. However, phosphorylation of CREB does not automatically indicate an increased transcription of CREB-regulated target genes (Zhang et al., 2005). We therefore went a step further and showed that pregnenolone sulfate stimulation leads to an up-regulation of reporter gene transcription controlled solely by CRE motifs.

In our previously published study, we showed that influx of Ca\(^{2+}\) ions via TRPM3 and L-type voltage-gated Ca\(^{2+}\) channels are integral parts of the signaling cascade connecting pregnenolone sulfate stimulation with enhanced Egr-1 gene

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F. Western blot analysis of INS-1 cells either mock infected or infected with a recombinant lentiviruses encoding REST/Elk-1ΔC or REST/CREB. Western blots were probed with an antibody against the FLAG-tag. Molecular mass markers (in kilodaltons) are shown on the left. G and H, INS-1 cells were double-infected with a lentivirus encoding a c-Fos promoter-controlled luciferase reporter gene and with a lentivirus encoding either REST/Elk-1ΔC or REST/CREB as control cells were infected with lentiviral stocks prepared with the lentiviral transfer vector pFUW (mock). The infected cells were treated with PregS (50 μM) for 24 h as indicated. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.
transcription (Mayer et al., 2011). In the nervous system, pregnenolone sulfate-induced Ca\(^{2+}\)/H\(^{+}\) influx into neurons may occur by activating neurotransmitter receptors (i.e., N-methyl-D-aspartate receptors) or by stimulating L-type voltage-gated Ca\(^{2+}\)/H\(^{+}\) channels. The analysis of perforant path-granule cell

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Fig. 4. Phosphorylation of CREB and stimulation of CRE-controlled transcription in pregnenolone sulfate-treated insulinoma cells. A, phosphorylation of CREB in pregnenolone sulfate-stimulated INS-1 insulinoma cells. Cells were serum-starved for 24 h and then stimulated with pregnenolone sulfate (PregS; 50 \(\mu\)M) as indicated. Nuclear extracts were prepared and subjected to Western blot analysis using an antibodies directed against either CREB, or the phosphorylated form of CREB. The antibody directed against HDAC1 was used as a loading control. B, schematic representation of the integrated provirus encoding a c-Fos CRE/luciferase reporter gene. The regulatory region of the reporter gene contains a minimal promoter consisting of the human immunodeficiency virus TATA box, the adenovirus major late promoter initiator element, and four copies of CRE derived from the c-Fos promoter. LTR, long terminal repeat; WPRE, woodchuck hepatitis virus post-transcriptional regulatory element. C, INS-1 insulinoma cells were infected with a recombinant lentivirus encoding the c-Fos CRE/luciferase reporter gene. The cells were cultured in medium containing 2 mM glucose were either mock-infected or infected with lentiviruses that encoded for a TRPM3-specific shRNA. Cells were serum-starved for 24 h and then stimulated with pregrenolone sulfate (PregS; 50 \(\mu\)M) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration.

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Fig. 5. Expression of TRPM3 is required for pregnenolone sulfate-induced c-Jun and c-Fos expression and CRE-controlled transcription in insulinoma cells. A and C, INS-1 insulinoma cells cultured in medium containing 2 mM glucose were either mock-infected or infected with lentiviruses that encoded for a TRPM3-specific shRNA. In addition, cells were infected with a lentivirus encoding either a c-Jun promoter/luciferase reporter gene (A) or a c-Fos promoter/luciferase reporter gene (C). Cells were serum-starved for 24 h and then stimulated with pregnenolone sulfate (PregS; 50 \(\mu\)M) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration. B and D, INS-1 insulinoma cells cultured in medium containing 2 mM glucose were either mock-infected or infected with lentiviruses that encoded for a TRPM3-specific shRNA. Cells were serum-starved for 24 h and then stimulated with PregS (50 \(\mu\)M) for 1 h. Nuclear extracts were prepared and subjected to Western blot analysis using antibodies directed against either c-Jun or c-Fos. The antibody directed against HDAC1 was used as a loading control. E and F, INS-1 insulinoma cells cultured in medium containing 2 mM glucose were infected with a lentivirus encoding a collagenase promoter/luciferase reporter gene. In addition, cells were either mock-infected or infected with lentiviruses that encoded for a TRPM3-specific shRNA. Cells were serum-starved for 24 h and then stimulated with either glucose (11 mM) (E) for 48 h or with KCl (25 mM)/FPL64176 (2.5 \(\mu\)M) (F) for 24 h. Cell extracts were prepared and analyzed for luciferase activities. G, INS-1 insulinoma cells cultured in medium containing 2 mM glucose were infected with a lentivirus encoding a CRE-controlled reporter gene. The cells were additionally either mock-infected or infected with lentiviruses that encoded for a TRPM3 specific shRNA. Cells were serum-starved for 24 h and then stimulated with PregS (50 \(\mu\)M) for 24 h. Cell extracts were prepared and analyzed for luciferase activities.
synaptic transmission revealed that the presynaptic effect of pregnenolone sulfate was attenuated—at least in part—by the L-type voltage-gated Ca\(^{2+}\) channel blocker nifedipine (Chen and Sokabe, 2005). Likewise, pregnenolone sulfate facilitates glutamate release from calyx synapses via the direct modulation of presynaptic voltage-dependent Ca\(^{2+}\) channels (Hige et al., 2006). A recent study revealed that LTP induced by conditioning electric stimuli at 20 Hz was dependent on L-type voltage-gated Ca\(^{2+}\) channels (Chen et al., 2010). In the previous study performed with insulinoma cells, we showed that pregnenolone sulfate-induced up-regulation of Egr-1 required L-type voltage-gated Ca\(^{2+}\) channels when the cells were cultured in a medium containing 11 mM glucose. This concentration of glucose is sufficient to induce an elevation of the intracellular Ca\(^{2+}\) concentration (Kindmark et al., 1991; Gromada et al., 1996). Under these conditions, TRPM3 did not play a significant role. However, when the cells were cultured in medium containing 2 mM glucose, TRPM3 expression was necessary to connect pregnenolone sulfate stimulation with transcriptional up-regulation (Mayer et al., 2011). The role of L-type voltage-gated Ca\(^{2+}\) channels was not assessed under these conditions. Here, we...
cells, showing a requirement of L-type voltage-gated Ca$^{2+}$ channels. In contrast, activation of gene transcription by a direct activation of L-type voltage-gated Ca$^{2+}$ channels, via stimulation of the cells with either glucose or KCl/FPL64176, does not require TRPM3. In addition, the requirement of TRPM3 can be abolished by culturing the cells in medium containing 11 mM glucose. Under these conditions, the cytoplasmic Ca$^{2+}$ concentration is increased (Kindmark et al., 1991; Gromada et al., 1996), and Ca$^{2+}$ ions are able to bind to calmodulin that is tethered to the intracellular carboxy-terminal tail domain of Cav1.2.

We further tested the role of pregnenolone sulfate on L-type voltage-gated Ca$^{2+}$ channel activation in pituitary corticotrophs that had been used in the past to analyze the transcriptional activation potential of c-Jun after KCl/FPL64176 stimulation (Cruzalegui et al., 1999). AtT20 corticotrophs express L-type voltage-gated Ca$^{2+}$ channel subunits Cav1.2 and Cav1.3, but they express only trace amounts of TRPM3. The results clearly show that expression of L-type voltage-gated Ca$^{2+}$ channels is insufficient to obtain a transcriptional response after pregnenolone sulfate stimulation. We propose that L-type voltage-gated Ca$^{2+}$ channel expression must be accompanied by expression of a second Ca$^{2+}$ channel, e.g., N-methyl-D-aspartate receptors in neurons or TRPM3 in insulinoma cells to allow pregnenolone sulfate-regulated gene transcription. We speculate that the interaction of pregnenolone sulfate with L-type Ca$^{2+}$ channels in INS-1 cells cultured in low-glucose medium may be too weak to directly activate these channels. In contrast, L-type voltage-gated Ca$^{2+}$ channels mediate pregnenolone sulfate signaling in INS-1 cells cultured at higher glucose levels with no need of TRPM3 channels (Mayer et al., 2011). The sites attributed to pregnenolone sulfate binding to L-type voltage-gated Ca$^{2+}$ channel have yet to be identified.

In summary, we present here the first analysis of pregnenolone sulfate-induced gene transcription of bZIP proteins, including c-Jun, c-Fos, and CREB. We identified the TRE and CRE as pregnenolone sulfate response elements that connect pregnenolone sulfate stimulation with enhanced transcription of bZIP-regulated genes. Furthermore, we showed that activation of both TRPM3 and L-type voltage-gated Ca$^{2+}$ channels are necessary to induce bZIP-mediated gene transcription in pregnenolone sulfate-stimulated insulinoma cells.

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Authorship Contributions

Participated in research design: Thiel.
Conducted experiments: Müller and Rössler.
Contributed new reagents or analytic tools: Thiel.
Performed data analysis: Müller, Rössler, and Thiel.
Wrote or contributed to the writing of the manuscript: Thiel.

References


Supplementary FIGURE S1:

Role of EGF and serum on collagenase promoter activity in INS-1 cells. 
(A) INS-1 insulinoma cells cultured in medium containing 2 mM glucose were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were either serum-starved for twenty-four hours or maintained in medium containing 10% serum. Cells were stimulated with pregnenolone sulfate (PregS, 50 µM) for twenty-four hours. 
(B) INS-1 insulinoma cells cultured in medium containing 2 mM glucose were infected with a recombinant lentivirus encoding a collagenase promoter/luciferase reporter gene. The cells were serum-starved for twenty-four hours and then stimulated with epidermal growth factor (EGF, 10 ng/ml) for twenty-four hours. Cell extracts were prepared and analyzed for luciferase activities. Luciferase activity was normalized to the protein concentration (***, P < 0.001).